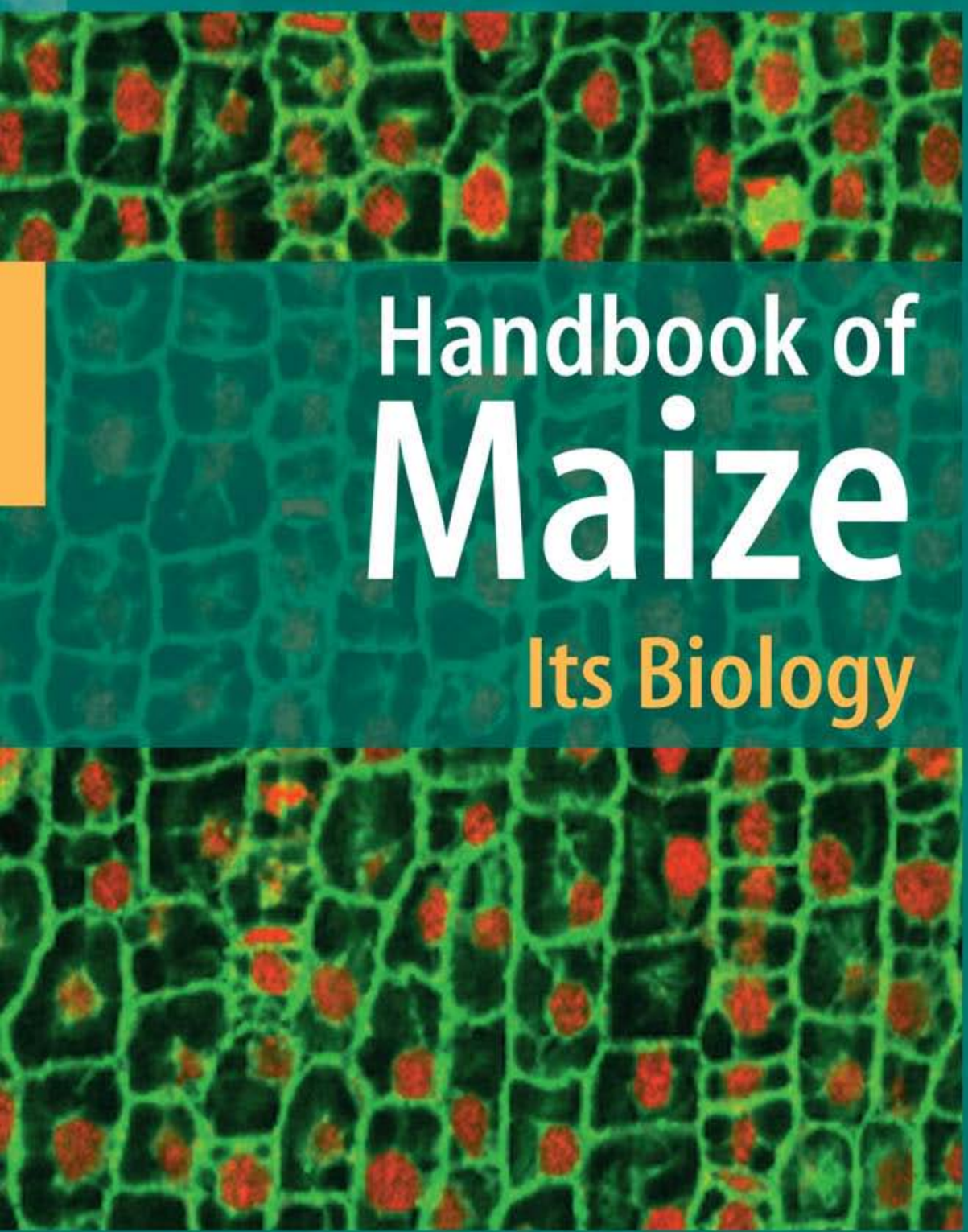
Jeff Bennetzen • Sarah Hake



Handbook of Maize

Its Biology

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Jeff L. Bennetzen • Sarah C. Hake
Editors

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Editors
Jeff L. Bennetzen
University of Georgia
Department of Genetics
Athens GA
USA

Sarah C. Hake
University of California
Berkeley
USDA Plant Gene Expression Center
800 Buchanan Street
Albany CA
USA

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Preface

Soon after the rediscovery of Gregor Mendel's work about a century ago, maize became the first plant to undergo detailed genetic analysis, resulting in the first evidence of linkage in plants and the first fragmentary genetic map. The great ease of maize genetics stems largely from the vigor of the plant, its numerous morphological types, its abundant seed set from easily separated gametophytic lineages, and the collections of fascinating mutants detected and used by Native American and immigrant farmers over the last several thousand years. For these reasons - and for its role as one of the world's most productive crops, maize remains a model for the genetic analysis of plant biology. As genetics has become the universal tool for biological study in fields as wide-ranging as biochemistry, developmental biology, ecology, evolutionary biology, pathology, physiology and taxonomy, understanding maize has become even more important. In the last decade, comparative genomics has uncovered the many genetic commonalities between plant taxa, demonstrating that model plants like maize will become the foundation species for understanding the shared common biology within any plant lineage, and a site of examination for rarer changes that make each species unique.

In *Handbook of Maize*, we attempt to capture a significant portion of the great diversity of high quality research in the maize scientific community. Given the history of maize research, it is not surprising that many of these studies have a strong underpinning of genetics and are conducted by scientists who view themselves as maize geneticists. The field has matured to a point, however, where the pursuit of purely genetic questions like recombination or mutation constitutes a significantly smaller portion of the maize genetics portfolio than do investigation into development biology or physiology, for instance. *Handbook of Maize* covers much of the breadth of research within the Maize community, but cannot possibly capture the entire depth of skill and achievement among this group of researchers. To provide even a minimal sampling of the best work and major achievements in this field would require a much larger opus. Having said this, it should be noted that no other book, monograph series or other publication format has succeeded in capturing the state-of-the-art in maize research in a single resource. With the great renaissance in plant science initiated by the genomics era, and the near-completion of a maize genome reference sequence, now is an apt time to assemble this first comprehensive treatise on the biology of maize.

Volume I addresses the basics of maize biology, starting with development and covering a great span of study leading to the final applied goal of crop research, namely, understanding and improving economic traits. The first ten chapters focus on the plant and its parts, with an emphasis on genetic mutants that are informative for growth and development. Chapter 1 examines the vegetative meristem and establishment of patterning in the maize plant. Chapter 2 focuses on inflorescence meristems and elaboration of ears and tassels, while Chapter 3 focuses on genes that regulate flowering time. Chapters 4 and 5 examine the male and female gametophytes, respectively. The activity of the haploid gametophytic stage of development is crucial for double fertilization, leading to embryo and endosperm. Next, Chapter 6 looks at patterns of gene expression in the embryo with a discussion of the differences between maize and another important model plant, *Arabidopsis thaliana*. Chapter 7 concerns development of the kernel including the endosperm, embryo and the maternally derived outer tissue. An enormous number of mutations affecting kernel development has been discovered, thanks, no doubt, to the large and easily observed kernels held together on an ear. Chapter 8 discusses the anatomy and morphology of maize roots, and chapter 9 looks at leaf development and mutants that inform us about patterning in the leaf. Chapter 10 focuses on cell biology, in particular, division, expansion and differentiation of epidermal cells in the leaf.

The following ten chapters examine plant responses to the environment and the utilization of quantitative trait loci in maize improvement. Chapter 11 addresses photobiology in maize, the phytochromes in particular. Chapters 12 through 14 focus on the genetics of the resistance of maize to fungal and bacterial disease, virus infection and insect damage, respectively. Chapters 15-17 examine maize responses to key abiotic stresses, cold, drought and submergence. Chapters 18, 19 and 20 inspect breeding efforts to produce corn that tolerates aluminum or prospers with fewer inputs of phospate and nitrogen. Chapter 21 looks at seed phosphate composition and chemistry while Chapter 22 discusses the regulation of seed starch biosynthesis. Much of the current understanding of the enzymatic steps required for starch synthesis in all plants has been acquired using maize as a model organism. The next five chapters describe the employment of plant breeding to improve corn. Chapters 23 and 24 discuss breeding for yield and heterosis, while Chapter 25 focuses on a specific experiment that demonstrates the long-term effects of breeding. Chapter 26 discusses QTL for a number of important agronomic traits such as lodging and architecture, and Chapter 27 outlines cultural practices and breeding efforts of maize in China. Finally, Chapter 28 describes the diversity of maize in its originating home, Mexico, with implications for how the Mexican landraces can be mined for further improvement of maize.

In its entirety, Volume I of *Handbook of Maize* describes what we now know, what we will soon know, and where we are headed for a great variety of questions concerning plant form, development, growth and responses to the environment. The last few chapters illustrate how the exceptional genetic diversity and genetic tools available in maize have been and are being used to improve this crop. They also help provide an excellent transition to Volume II, which details the history of maize as a crop and genetic model in the context of the great range of modern genetic tools currently available to the maize research community.

Contents

Vegetative Shoot Meristems

Dave Jackson

Abstract The shoot apical meristem (SAM) is responsible for the indeterminate growth of the maize shoot. Formed during embryogenesis, the SAM consists of a pool of stem cells that divide to give rise to daughter cells that either maintain stem cell fate or are incorporated into leaf primordia, axillary shoot meristems or the growing stem tissues. Classical studies over the past century have revealed the cellular organization of the SAM, its ability to respond to systemic signals from distant organs, and behavior and fates of cells in this small but essential structure. More recently, we have started to understand molecular mechanisms of SAM function, an insight that has only been possible through forward genetic analysis. Several pathways for meristem maintenance and proliferation control are now known, however an integrated model of how the meristem functions awaits further genetic and genomic analysis. As the SAM is formed during embryogenesis and persists through the inflorescence phase, and its activity is intimately integrated with leaf initiation, readers are encouraged to also consult the chapters on Maize Embryogenesis by Wolfgang Werr, Axial patterning of the maize leaf by Toshi Foster and Marja Timmermans, Floral Transition in Maize by Joe Colasanti and Mike Muszynski and Inflorescences by Robert Schmidt and Erik Vollbrecht.

1 Introduction

The establishment of distinct pools of stem cells, or "meristems" at the shoot and root poles of the immature maize embryo is essential for continued growth and development of the plant. The shoot apical meristem (SAM) is initiated at about 10 days after pollination, depending on genetic background and growth conditions (Randolph, 1936), and is maintained throughout the life of the plant by stem cell divisions. The SAM is generally defined as the region above the most recently initiated leaf primordium. In maize, the SAM is a domed structure, consisting of about two thousand cells in the embryonic stage (Bommineni et al., 1995). It is a dynamic structure that produces a new leaf primordium every 1–2 days. This cycle, measured as the time between leaf initiation events, is called the plastochron. The most recently initiated leaf is referred to as plastochron 1, or P1, and the position where

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the next leaf will form is P zero, or P0. The SAM gradually expands between leaf initiation events and becomes smaller as cells are incorporated into new primordia. Therefore to accurately assess SAM size, it is necessary to measure several samples and take a mean value (Abbe et al., 1951). The average size of the SAM also increases with each plastochron, as the plant matures, from about 80 μm in diameter in the embryo to around 150 μm as the 12th leaf is being initiated (Ledin, 1954). SAM size is also dependent on genetic background (Vollbrecht et al., 2000).

Maize leaves are initiated one at a time, with consecutive leaves being initiated from opposite flanks of the SAM, resulting in an alternating or distichous phyllotaxy (Jackson and Hake, 1999). As each leaf is initiated, cells are also set aside to form an associated axillary shoot meristem opposite the midrib of the initiating leaf, and a section of stem, called the internode. Together these three structures make up the basic repeating unit of shoot development, known as the phytomer (Sharman, 1942; Poethig et al., 1986). The maize stem expands little during the first few weeks of growth, and the SAM can be found just above the shoot-root junction (Fig. 1a). At around the time of the floral transition the stem internodes expand rapidly and the SAM is elevated, becoming converted to an inflorescence apical

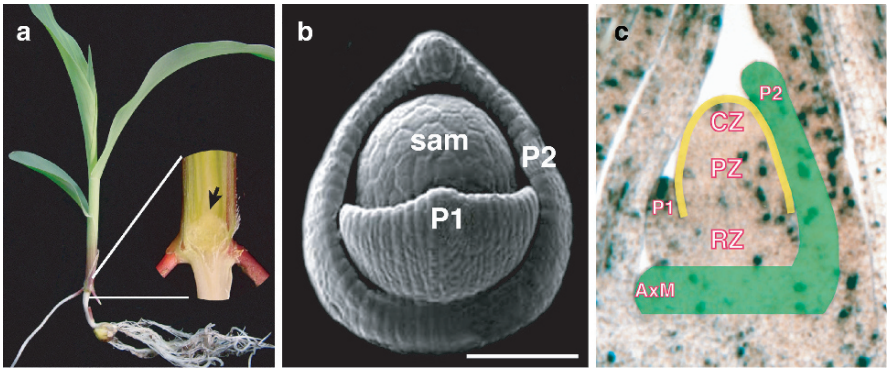

Fig. 1 Location, morphology and histology of the maize seedling SAM. (**a**) A maize seedling at about 10 days post germination, with four visible leaves. Inside the whorl several additional leaves are tightly wrapped around the SAM. To the right is shown an enlarged median section through the shoot, at a position corresponding to the white lines, with the position of the SAM arrowed. At this stage of development the SAM is at the summit of a small region of unexpanded stem, at about 2–3 mm above the root-shoot junction. (**b**) Further dissection and careful removal of most of the leaf primordia reveals the SAM and the most recently initiated leaf primordia, P1 and P2, viewed here in the scanning electron microscope. Scale bar = 100 μm (figure adapted from Jackson and Hake, 1999). (**c**) A longitudinal section through the SAM, showing the cellular organization of the meristem (L1 layer of SAM is shaded yellow, the P2 "phytomer" in green) AxM = axillary meristem of the P2 phytomer. In the figure, the distribution of cell divisions is marked by dark punctate in situ staining of a cyclin mRNA (figure adapted from Jackson and Hake, 1999). The SAM zones are also labeled, central zone (*CZ*), peripheral zone (*PZ*) and rib zone (*RZ*). Note the relatively low rate of cell division in the SAM compared to the leaf primordia, except for the region on the right flank of the SAM, which corresponds to the P0 site of leaf initiation opposite the P1

meristem that will generate the tassel. The axillary shoot meristems often remain dormant during seedling development, but in some lines or under certain growth conditions they grow out to form branches, or tillers, that reiterate the pattern of the main shoot axis. The suppression of axillary branches was selected during the domestication of maize from teosinte, and is regulated by the *teosinte branched1* and *barren inflorescence2* genes (see chapter on Inflorescences, and Hubbard et al., 2002; McSteen et al., 2007). Upon the floral transition, some axillary meristems develop into ear shoots.

The central importance of the SAM in plant development has made it a topic of study for centuries. However it is only recently, and through genetic analysis, that we have started to understand the molecular basis of SAM development. Several genes that regulate its function have been cloned in maize and in other species. It is likely that many of the genes and functions are conserved throughout angiosperm species, though the complexities of gene duplications and redundancy mean that such homologies are not always easy to determine. This chapter focuses on the state of knowledge of the SAM, primarily in maize. For recent reviews on other species, especially *Arabidopsis*, see Evans and Barton, 1997, and Williams and Fletcher, 2005).

2 SAM Organization and Classical Studies

The internal organization of the SAM can be described in several different ways. A simple observation of longitudinal tissue sections reveals a layered pattern of cellular organization, where the cells in the outer, epidermal layer divide within the plane of the layer. This layer of anticlinally dividing cells is referred to as the L1, or tunica layer (Fig. 1c). Periclinal divisions are rarely observed in the L1, and usually only at the site of leaf initiation. Underlying the L1 layer, the cells divide in all directions. This inner mass of cells is referred to as the L2, or corpus (Abbe et al., 1951; Ledin, 1954). The SAM organization in maize differs from that of other plants, such as *Arabidopsis*, where two outer layers of anticlinally dividing cells make up the tunica, resulting in an L1, L2, L3 organization (Evans and Barton, 1997). The SAM can also be subdivided into radial zones of cellular activity (Steeves and Sussex, 1989). In the central zone (CZ) at the apex of the SAM, cells divide relatively infrequently and are more highly vacuolate; this zone also contains the semi-permanent stem cells, also known as initials. Below and surrounding the CZ, the peripheral zone contains more actively dividing cells that are soon to be incorporated into leaf primordia. In the lower, central region, the rib zone is another region of actively dividing cells that give rise to the stem tissues (Fig. 1c).

Our understanding of the regulation of the shoot apical meristem first came from experimental manipulations. These studies were mostly carried out in other plants, but have recently been performed in maize. If the maize embryonic SAM is bisected with a sharp blade, the remaining half is able reorganize, and a new SAM regenerates within about 6 days in culture (Bommineni et al., 1995). Similar studies

in other plants reveal the SAM's ability to regenerate itself even after the vast majority of the cells are surgically removed (Sussex, 1989). These experiments reveal the self-organizing properties of the SAM. Maize SAMs with one or 2 associated leaf primordia can also be cultured, and these explants are able to regenerate whole plants (Irish and Nelson, 1988). Culturing of maize apices from adult plants leads to "re-setting" of the meristem, such that it initiates leaves with juvenile characteristics (Irish and Karlen 1998). These studies indicate that the SAM is not intrinsically programmed, but that it responds to signals from the rest of the plant.

A second class of manipulations used to characterize the SAM and shoot development is clonal analysis, where cells are genetically marked with cell autonomous visible markers such as anthocyanin pigments or albinism (lack of chlorophyll). If a cell in the SAM is marked, all tissues derived from divisions of that cell will carry the marker. Thus, marked sectors in leaves and internodes can be used to trace the fate of a single meristematic cell. Such studies provided evidence for the organization of the shoot into clonally related phytomers (Poethig et al., 1986). They also indicate that the developmental fate of specific cells or groups of cells in the SAM is not fixed (McDaniel and Poethig, 1988) suggesting that these cells develop according to their position, rather than lineage, and cell-cell communication is likely to be critical for cell specification during shoot development.

In summary, the shoot apical meristem is a compact, highly organized group of cells that serve as a stem cell population to enable leaf, axillary meristem and stem initiation throughout vegetative development. Although the SAM has a predictable cellular organization, in layers and zones, the fates of specific cells in the SAM is not determined, and cells are incorporated into shoot structures stochastically, presumably as a consequence of their position.

3 Mutants and Genes in Maize SAM Development

The SAM is a fascinating structure; it is a self-organizing stem cell system that regulates its own size, by balancing stem cell proliferation and incorporation of cells into new primordia, it can regenerate itself, and it initiates all of the organs present in the developing shoot system. Perhaps because of its central importance, genetic insights were initially slow to emerge, because mutants impaired in SAM formation are usually early seedling or embryonic lethal. In maize, genetic screens are often done in the field, and hence SAM mutants can easily be lost in the dirt, or scored as reduced germination. Many of the meristem mutants described in the following paragraphs were discovered either because they have weaker alleles with phenotypes later in development, or because they have more easily visible phenotypes, such as phyllotaxy.

In *Arabidopsis*, many SAM mutants have been described and characterized over the past 15 years (reviewed in (Evans and Barton, 1997; Williams and Fletcher, 2005). Two parallel pathways appear to regulate the basic function of stem cell maintenance. In the first, the *clavata* (*clv*) and *wuschel* (*wus*) genes form a negative feedback loop that ensures a balance in cell proliferation and meristem size. The *clavata*

genes encode a leucine rich repeat (LRR) receptor kinase, CLV1, an LRR receptor like protein, CLV2 and their predicted ligand, CLV3, whereas *WUS* encodes a homeodomain transcription factor (Clark et al., 1997; Fletcher et al., 1999; Jeong et al., 1999; Mayer et al., 1998, reviewed in Williams and Fletcher, 2005). The second stem cell maintenance pathway involves members of the *knox* (*knotted1*-related homeobox) transcription factor genes (Kerstetter et al., 1997; Long et al., 1996; Vollbrecht et al., 2000), which function in part through regulation of hormone signaling.

Mutants in *knox* and *clv* genes have been isolated in maize using forward genetic approaches, as described in the following sections. However the role of *WUS* orthologs in the maize SAM has not been established. Maize contains two putative WUS orthologs, *Zmwus1* and *2*, that have distinct expression patterns (Nardmann and Werr, 2006). *Zmwus1* has the most similar expression to *Arabidopsis WUS*, which is confined to a small group of cells in the inner region of the SAM, called the organizing center (OC) (Mayer et al., 1998). *ZmWUS1* expression marks cells in an equivalent position in the maize SAM, at least during early embryo development (Fig. 2f, g) and in spikelet pair meristems. However its expression appears to be transient, as if it perhaps varies during the leaf initiation cycle, and it appears to be weak or absent in the vegetative SAM and in the inflorescence apical meristem (Nardmann and Werr, 2006). The role of *wus* genes in maize SAM development awaits further analysis and identification of loss of function mutants.

Several other maize homologs of genes with SAM phenotypes or expression patterns in *Arabidopsis* have also been studied, and are described in the chapter on Maize Embryogenesis.

3.1 Mutants Defective in SAM Initiation and Maintenance

knotted1 (*kn1*) was originally described as a dominant mutation affecting maize leaf development (Vollbrecht et al., 1991). However in wild type plants, *kn1* is expressed not in leaves, but in the SAM (Fig. 2h, Jackson et al., 1994; Smith et al., 1992). Its identification as a homeobox gene, a member of a class of developmental master regulators first described in *Drosophila* homeotic genes, suggested the role of *kn1* was in SAM specification and/ or maintenance. Indeed, loss of function mutants of *kn1* have smaller inflorescence and vegetative meristems (Kerstetter et al., 1997; Vollbrecht et al., 2000). The most severe *kn1* loss of function phenotype is a complete loss of all seedling leaves, and the mutants produce only a coleoptile and root, then arrest in their development (Fig. 2a-c). Interestingly, the severity of *kn1* loss of function phenotypes is strongly modified by genetic background, and maize inbreds that naturally have bigger (taller) meristems suppress the vegetative SAM defects (Vollbrecht et al., 2000). Nonetheless, the strong loss of function phenotype indicates that *kn1* is required for SAM initiation, and/ or maintenance. The specific interpretation depends on whether one believes that the coleoptile (monocots) or cotyledons (dicots) are made by the SAM. This issue is still under debate; in one point of view the SAM forms very early in embryogenesis, and all leaf like structures, including the

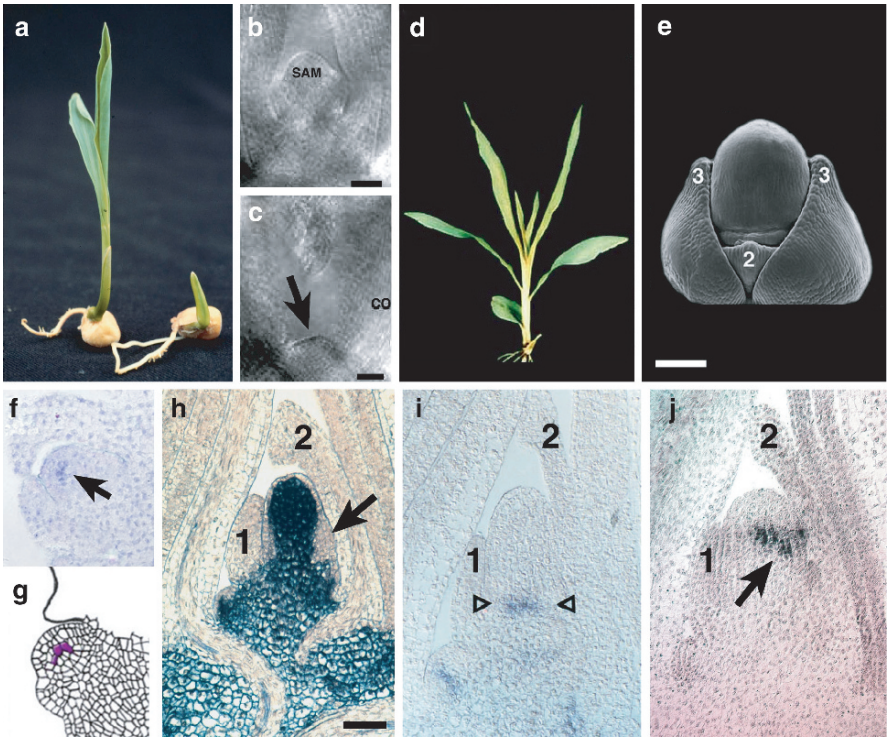

Fig. 2 Maize SAM mutants and gene expression patterns. (**a**) A normal maize seedling at about 5 days post germination, with 2 leaves visible. On the right is a *kn1* loss of function mutant seedling of the same age. The seed has germinated with a normal root, but the shoot has made only a coleoptile, and then arrested. (**b**) A histologically cleared normal maize embryo reveals the shoot apical meristem (*SAM*) and surrounding leaf primordia, in (**c**) a *kn1* loss of function embryo has a relatively flat structure in place of the SAM (arrow), and the only shoot organ visible is the coleoptile (*co*) (from Vollbrecht et al., 2000). (**d**) The *abphyl1* mutant of maize makes leaves in opposite pairs, in a decussate phyllotaxy (compare to Fig. 1a. Here, the first pair of leaves is fused, but the 2nd, 3rd and 4th leaves are symmetrically arranged in opposite pairs. In (**e**) a scanning electron micrograph of an *abph1* mutant shoot apex shows that the leaf primordia are initiated in opposite pairs by the SAM, scale bar = 100 μm (from Jackson and Hake, 1999). (**f**–**j**) Maize SAM gene expression patterns revealed by in situ hybridization. (**f**), expression of *Zmwus1* transiently marks an organizing center-like group of cells in the embryonic SAM (arrowed); the staining is weak and is represented in purple in the sketch below, (**g**) (from Nardmann and Werr, 2006). (**h**) Expression of *kn1* mRNA in the seedling SAM, and developing stem. Note that the mRNA does not accumulate in the L1 layer, nor in leaf primordia (1, 2) or in the site of initiation of the next leaf, the P0 (arrowed), scale bar = 100 μm. (**i**) Expression of the *knox* gene *roughsheath1* in a stripe of cells at the base of the SAM, arrowed. (**j**) Expression of *abphyl1* in the P0 region of the seedling SAM, arrowed. (**h**, **i**, from Jackson et al., 1994, and j from Giulini et al., 2004)

coleoptile, or cotyledons, are its products (Kaplan and Cooke, 1997). In another view, based on observations in *Arabidopsis*, the SAM is initiated later, after the cotyledons form, and they are not considered products of the SAM (McConnell and Barton, 1998). As the coleoptile and cotyledons are equivalent structures in monocots and

dicots, respectively, the difference is unlikely to be species specific, but depends on an interpretation of when each structure is determined in relation to the SAM.

kn1 is expressed in a localized pattern throughout the SAM and developing stem, but its expression is excluded from leaves, and even from the position on the flank of the SAM where the next leaf will form, also known as the P0 (Fig. 2h, Jackson et al., 1994; Smith et al., 1992). This expression pattern supports the role of *kn1* in SAM maintenance, and suggests that down-regulation of its expression is necessary for leaf initiation. For further discussion of the genes that regulate *kn1* down-regulation during leaf initiation, see the chapter by Foster and Timmermans.

Remarkably, when the expression patterns of KN1 protein and mRNA were compared, the protein was detected in cells that did not accumulate the corresponding mRNA (Jackson et al., 1994; Lucas et al., 1995). These observations were particularly interesting, because the dominant *Kn1* allele acts non cell-autonomously to signal aberrant divisions during knotted leaf development (Hake and Freeling, 1986). It was later discovered that the KN1 protein is able to traffic cell-to-cell through plasmodesmata (Lucas et al., 1995; Kim et al., 2002), making it the founding member of a growing class of proteins that use this novel mode of intercellular signaling in plants (Ruiz-Medrano et al., 2004; Benitez Alfonso et al., 2007). Cell-to-cell trafficking of KN1 protein explains the non-cell autonomy of *Kn1* mutants (Kim et al., 2002), and it may also be necessary for the normal function of *kn1* in development of the SAM (Kim et al., 2003).

kn1 related maize homeobox ("*knox*") genes are also expressed in specific meristem domains, suggesting they might regulate specific developmental properties of the SAM. For example, the *knox3* and *roughsheath1* genes are expressed in stripes in the SAM that appear to mark the basal part of each phytomer (Fig 2i, Jackson et al., 1994). Evidence that this domain may include cells that are determined to become tissues of the stem comes from analysis of loss of function mutants in a rice homolog, *OSH15*, which have a dwarf phenotype caused by abnormal stem and vascular development (Sato et al., 1999). Studies carried out mostly in plants other than maize indicate that *knox* genes control the SAM through regulation of hormonal signaling by cytokinins and giberellins (Ori et al., 1999; Sakamoto et al., 2001; Jasinski et al., 2005; Yanai et al., 2005; Sakamoto et al., 2006).

3.2 Mutants with Enlarged Meristems

Two classes of maize mutants are distinguished by the appearance of a larger SAM. The first class encodes members of the *clavata* class of genes described earlier. In maize, mutants in orthologs of *clv1* and *clv2* have been described. *clv3*-like genes are also expressed in the maize SAM, but to date no mutant phenotypes have been reported (Nagasawa et al., unpublished).

The maize *fasciated ear2* (*fea2*) and *thick tassel dwarf1* (*td1*) genes encode orthologs of *clv2* and *clv1*, respectively (Taguchi-Shiobara et al., 2001; Bommert et al., 2005). However unlike in *Arabidopsis*, where the *clv* mutations cause enlargement of

the SAM at all stages of development, mutations in *fea2* and *td1* result only in enlarged inflorescence meristems, leading to tassels with higher spikelet density and fasciated ears (see chapter on Inflorescences for images). Vegetative development is not affected in *fea2* mutants, and *td1* mutants have a shorter stature, with fewer leaves, and surprisingly have a smaller vegetative SAM (Lunde and Hake, personal communication). *td1* is not expressed in the maize seedling SAM, but in developing leaf primordia (Bommert et al., 2005; Nardmann and Werr, 2006). This pattern is distinct from *Arabidopsis clv1*, which is expressed in a specific domain in the central zone of the SAM (reviewed in Williams and Fletcher, 2005). Clearly more studies are needed to understand the spatial expression and functions of the *clv-wus* genes in maize, which so far do not appear to fit the canonical model from *Arabidopsis* (Bommert et al., 2005; Nardmann and Werr, 2006). Another maize mutant with fasciated inflorescences, *compact plant2*, also has a semi-dwarf phenotype, and in contrast to *td1* mutants these mutants do have a larger vegetative SAM (Peter Bommert and DJ, unpublished). In this case the larger SAM is correlated with wider leaves in the *ct2* mutants.

A second class of mutants with enlarged SAMs exhibit altered phyllotaxy. The first mutant described in this class, *abphyl1* (*abph1*) is recessive, and plants develop with opposite and decussate phylloyaxy (Fig. 2d, e; Jackson and Hake, 1999). The *abph1* SAM is about 1/3rd wider than normal in the seedling stage, and the meristems are also larger in the early embryo, before any leaves are initiated. *abph1* encodes a cytokinin inducible response regulator, and is thought to act as a negative regulator of cytokinin signaling (Giulini et al., 2004). Therefore *abph1* may regulate phyllotaxy by restricting SAM size through negative regulation of a cytokinin signal. This restriction of SAM size is proposed to regulate phyllotaxy by limiting the available space for leaf initiation. In the embryo, *abph1* is expressed throughout the SAM, but later its expression becomes restricted to a small region overlapping with the site of leaf initiation (Fig. 2j). This region is also characterized by up-regulation of the auxin polar efflux transporter *pinformed1* (*pin1*), and accumulation of auxin appears to be an instructive signal for leaf initiation (Reinhardt et al., 2000, 2003). Interactions between *abph1* and *pin1* at the P0 may be important in determining the site of leaf initiation (Lee et al, unpublished).

A second *abphyl* mutant, *Abph2,* also gives rise to plants with opposite and decussate phyllotaxy. *Abph2* mutants also have larger shoot meristems. *Abph2*, is dominant, and has been mapped to a region of chromosome 7, and map based cloning is in progress (Johnston et al., unpublished). Another phyllotaxy regulator is revealed by studies of *terminal ear1* (*te1*) mutants. These mutants have abnormal vegetative phyllotaxy and stem internodes, though specific effects on SAM size or organization have not been described in detail (Veit et al., 1998). *te1* encodes a predicted RNA binding protein that is expressed in arcs of cells opposite the leaf initiation sites, suggesting it may function to specify the size or position of leaf primordia.

3.3 Other Shoot Meristem Mutants

Several other shootless mutants have been described in maize, but their molecular characterization has not been reported (e.g., Pilu et al., 2002; Rivin et al., 1995).

Like *kn1*, the *early phase change/aborted shoot 3/narrow leaf 4* mutant is sensitive to genetic modifiers, producing shoots with only one or to leaves in certain genetic backgrounds, whereas in others they form adult plants but with early flowering and phase change defects (Vega et al., 2002). A mutant that is affected in epidermal development, *Extra cell layers1* (*Xcl1*) leading to the production of multiple epidermal layers, has a smaller SAM, indicating a possible link between L1 division patterns and SAM maintenance. Supporting this idea, double mutants between *Xcl1* and *crinkly4*, another mutant that affects epidermal development, are seedling lethal and fail to develop a functional SAM (Kessler et al., 2006). Interestingly, a number of *shootless* (*shl*) and *shoot organization* (*sho*) mutants have also been described in rice (Satoh et al., 2003). Recently, three of these genes were isolated, and found to encode different components of a plant specific small RNA regulatory pathway, the trans-acting siRNA (ta-siRNA) pathway. These findings suggest that ta-siRNAs regulate SAM initiation (Nagasaki et al., 2007).

In addition to these examples, a large number of embryo lethal mutants exist in maize (e.g., Clark and Sheridan, 1991), and some of these are likely to affect specific aspects of SAM development.

3.4 SAM Gene Expression

In addition to the insights from molecular genetic studies of specific mutants or of homologs of SAM genes identified in other species, recent efforts have focused on global transcript profiling of the SAM using laser capture microdissection (Ohtsu et al., 2007). The developmental complexity of the SAM is reflected in the fact that over 5,000 expressed sequenced tags were differentially expressed between the SAM and seedling transcriptomes. The differentially expressed genes included many interesting candidates for developmental regulators, including transcription factors, chromatin remodeling factors and components of the gene-silencing machinery. Many new SAM transcripts were also identified by high throughput "454"-sequencing, giving a near complete view of all SAM expressed genes. These new insights are extremely powerful and will enable future construction of regulatory networks, for example by comparing expression differences in specific mutant lines (e.g., Zhang et al., 2007).

4 Concluding Remarks

The last decade has seen a huge advance in our understanding of the molecular basis of shoot apical meristem development. Advances in maize genomics, in particular the ability to positionally clone mutants (Bortiri et al., 2006), is revolutionizing maize biology, with new insights emerging rapidly. The application of microarray analysis and high throughput transcript sequencing is revealing the landscape of gene expression that exists in the SAM, and the applications of such techniques to specific mutants should allow regulatory networks to be inferred. Clearly much

remains to be discovered, but the stage is set and tools are constantly emerging that one day will enable a complete understanding of the intricacies of the elusive shoot apical meristem.

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Development of the Inflorescences

Erik Vollbrecht and Robert J. Schmidt

Abstract The maize tassel and ear are complex structures that arise from a shared program of development involving a number of meristem identities and fates, yet emerge as distinct inflorescences that bear exclusively male and female flowers, respectively. Careful phenotypic and genetic studies of mutants that perturb meristem initiation, size, determinacy and identity or pathways of organogenesis such as sex determination, are elaborating the intricacies of these developmental programs by providing important insights into the underlying genes and gene interactions. Our understanding at the molecular level includes information from expression patterns of cloned genes coupled with knowledge of the gene products they encode and interact with, coalescing into molecular bases for the mechanisms underlying the formation of these remarkable structures. These studies show that some mechanisms are at least partially conserved with those elucidated in other model systems such as *Arabidopsis*, but many others are unique to the grasses. Permeating advances in our understanding of the maize inflorescence is a rich collection of mutants, some identified at the turn of the last century and new ones recorded in databases of productive EMS- and transposon-based screens, constituting a key genetic resource that will provide fertile ground for maize geneticists and developmental biologists for years to come.

1 Introduction

The transition from vegetative growth to flowering sets in motion one of the most dramatic developmental processes to occur during the life of a plant. This process in maize is additionally dramatic in that it produces two morphologically distinct inflorescences that bear separate male and female flowers. Many other grasses produce perfect flowers in a single inflorescence, yet the maize inflorescence remains an important and excellent model for the grasses, including other grain crops. The purpose of this review is to highlight some of the features that have made the study of maize inflorescence development productive. These attributes include meristem fates and identities that are unique to grass species, advancing molecular tools and a wealth of informative mutants that affect those meristem

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activities. Many of the underlying genes are now cloned, providing an emerging molecular framework for the developmental pathways regulating inflorescence architecture. In addition, maize manifests remarkable natural diversity in the tassel and ear among different inbreds. These features, and of course, the economic importance of inflorescence structures and the intense artificial selection that has influenced their morphology, all make the study of inflorescence development in maize an extremely exciting pursuit.

2 Features of the Mature Inflorescence

Modern maize produces two distinct mature inflorescences commonly referred to as the tassel and ear (Fig. 1a, b). The tassel bears staminate flowers and is borne at the apex of the mature plant, whereas the ear bears pistillate flowers. Ears are produced at the apex of a compressed branch originating in the axil of one or more leaves (Fig. 1b) and terminate all lateral branches, with the exception of branches in the tassel and a few vegetative basal branches called tillers (Kiesselbach, 1949). One obvious difference in morphology between the two inflorescences is the presence of a variable number of long branches originating at the base of the typical tassel. Ears lack these long branches. To produce flowers, or analogous floral shoots called florets in grasses, both inflorescences first make a pair of short branches called spikelets. The spikelet is the basic unit of grass inflorescence architecture and is characterized by an outer and inner glume (sterile bracts or modified leaves) that together enclose a variable number of florets. In maize, there are two florets (Fig. 1a). Each floret includes a floral shoot that originates from the axil of an additional bract called a lemma. The floret consists of a single lemma and palea, two lodicules, three stamens and a central pistil consisting of three fused carpels, surrounding a single ovule. During floral development, subsequent to initiating all floral organ types, the developing pistil aborts in tassel florets, and the developing stamens undergo a similar fate in ear florets. Additionally, the entire lower floret of each ear spikelet aborts, again soon after initiating floral organ primordia (reviewed in Dellaporta and Calderon-Urrea, 1994; Irish, 1996). The end result is a monoecious plant with distinct male and female inflorescences (Fig. 1a, b).

One of the intriguing aspects of maize inflorescence development is the wide range of natural variation that exists among different inbred lines, no doubt reflecting the great depth of allelic diversity that has been documented among these inbreds (Liu et al., 2003). For example, tassel branch number varies from an average of 3 to nearly 20, and the length and angle of tassel branches varies considerably (http://www.panzea.org). Similarly, the size and number of ears per plant varies considerably. This natural variation is being exploited to uncover the quantitative trait loci (QTL) underlying a variety of phenotypes (Upadyayula et al., 2006; Zhao et al., 2006) and through association mapping to identify statistical

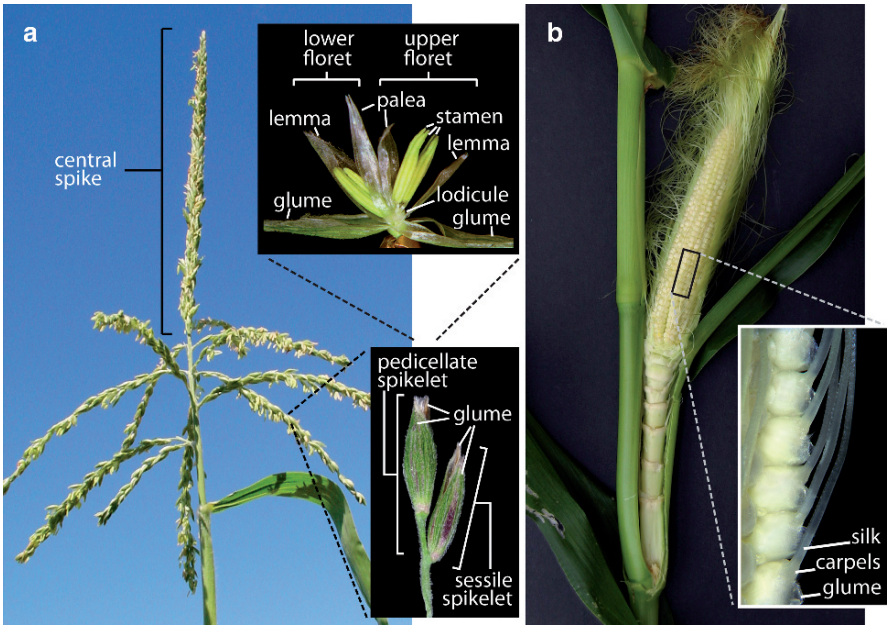

Fig. 1 General morphology of maize inflorescences. (**a**) a typical mature male inflorescence, or tassel, with several long branches at the base of the central spike. Long branches and the central spike produce rows of short branches or paired spikelets (lower inset); the pedicellate spikelet is atop a pedicel that is considerably longer than that which distinguishes the sessile spikelet. Each tassel spikelet produces two glumes, an outer and inner glume, that enclose two florets, an upper and lower floret (upper inset), each consisting of one lemma, one palea, two lodicules and three stamens. (**b**) a typical mature female inflorescence, or ear, with husk leaves removed to illustrate the ear's position atop several compressed internodes on an axillary branch. The ear is formed at the tip of the branch and produces rows (black rectangle) of paired spikelets; the distinction between sessile and pedicellate spikelet is subtle. Each ear spikelet consists of an upper and lower floret enclosed by a pair of glumes (inset). The lower floret and the stamens in the upper floret, degenerate. Only the upper floret matures to produce carpels, two of which fuse to form the long silk that emerges from within the surrounding glumes (inset). Thus each spikelet appears in (**b**) as glumes surrounding one pistillate floret. The lemma, palea and lodicules are present but obscured by the surrounding glumes.

associations between traits and genetic markers. Some 5,000 recombinant inbred lines have been generated between a core set of 25 diverse inbred lines and the common elite inbred B73. This resource is generating a wealth of genetic information that will be utilized to understand the genetic control underlying inflorescence diversity and many other traits. Identifying the genes controlling natural variation is of interest to developmental and evolutionary biologists alike. The importance of many of these inflorescence traits to seed yield underlies the intense interest by maize breeders as well.

3 Features of the Developing Inflorescence

The morphology of a mature structure in an organism is the end product of an array of contributing developmental processes. In plants, many of those core processes reflect the activity of meristems, small groups of undifferentiated, self-regenerating cells (Steeves and Sussex, 1989; Weigel and Jurgens, 2002). Two main meristems, the shoot apical meristem and the root apical meristem, establish the main axes of plant growth. Subsequently secondary meristems, formed in the axils of leaf primordia during both vegetative and reproductive development, may be either quiescent or active. When active, they are directly responsible for the formation of secondary axes of growth. In maize, these include lateral branches called tillers, and several specialized axes producing the tassel and ear. The ear and tassel have distinct morphologies at maturity, yet for much of the early stages of their development they appear remarkably similar. This similarity is due to sharing many common developmental processes, manifest as a shared set and arrangement of meristem types.

Normal maize inflorescence development has been described from a variety of perspectives (Collins, 1919; Bonnett, 1940, 1948; Mangelsdorf, 1945; Cutler and Cutler, 1948; Kiesselbach, 1949) including electron microscopy (Cheng et al., 1983). When the plant transitions from vegetative to reproductive development the terminal SAM becomes an inflorescence meristem (IM), committed to the formation of the tassel. The inflorescence meristem starts to produce lateral meristems called branch meristems (BMs), which develop into major branches at the base of the mature tassel (Fig. 2a, b). Coincident with their proximity to the vegetative shoot, BMs may be produced in the two-rowed phyllotaxy of the vegetative shoot (Giulini et al., 2004), or in the multiple rows that characterize the rest of the inflorescence. Regardless, only these first few lateral meristems are indeterminate and committed to form branches; the subsequent ones are initiated in multiple rows and acquire a different identity, that of the spikelet-pair meristem (SPM). Tassel branches form spikelet-pair meristems as well, but in a distichous pattern (Fig. 2b). Branch meristems are said to have less determinacy due to their capacity for continued growth of the axis, while spikelet-pair meristems have more determinacy and thus remain short (McSteen et al., 2000; Vollbrecht et al., 2005). Each spikelet-pair

Fig. 2 (continued) then converts to another SM. Each SM uniquely initiates two glume primordia (gp, panel **c**) followed by a lemma primordium in whose axil the lower floral meristem (Lower *FM*) forms (**e**). The upper SM then produces a second lemma and converts into a second FM (Upper FM, panel **e**) and the upper floret matures in advance of the lower floret. Each FM initiates one palea, two lodicule (lod), and three stamen primordia and then differentiates into the ovule and surrounding carpel wall or gynoecial ridge (**f**), which becomes the pistil (**h**). The schematics of the mature florets of the tassel (**g**) and ear (**h**) are oriented approximately in parallel to the microscope images in the panels above. Floral organs that degenerate as part of the sex determination program are shown in grey. The upper floret in each spikelet is depicted slightly larger than the lower floret. Lo, lodicules; st, stamens; ps, pistil. Black dots represent relative position of stem (rachilla). White scale bars are 200 μM.

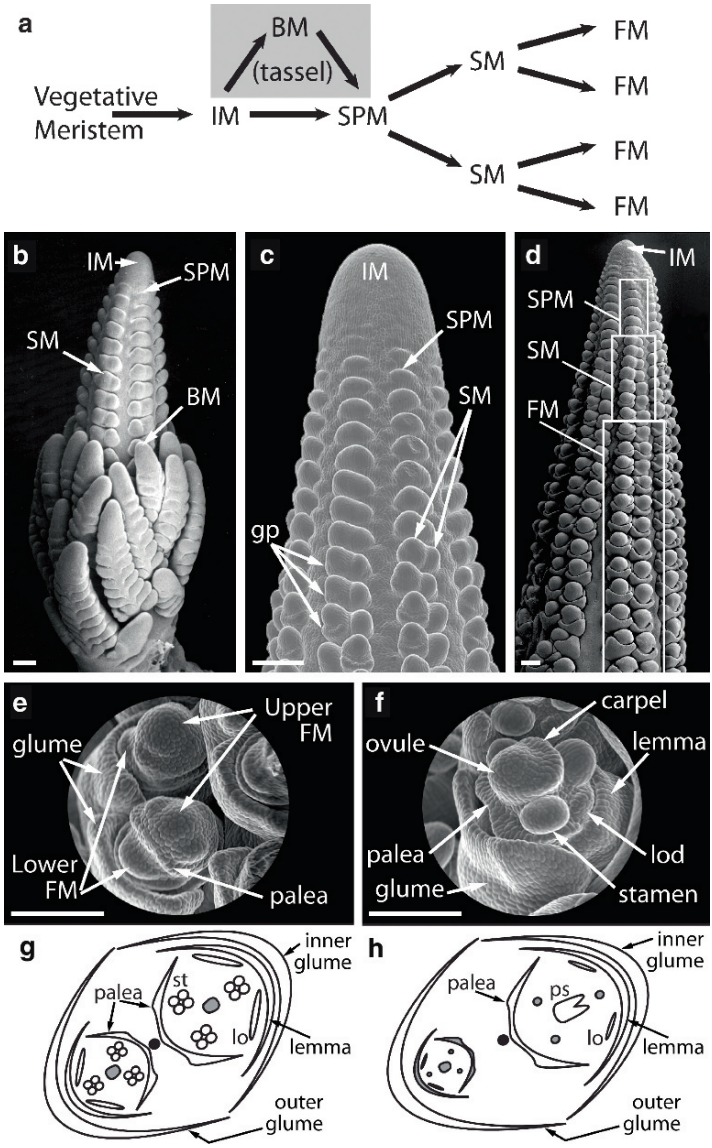

Fig. 2 Meristem identities during inflorescence development. (**a**) Schematic of the developmental progression of meristem identities during inflorescence development. (**b–f**) Scanning electron micrographs of developing tassel and ear through floral organ initiation. (**b**) developing tassel primordium, (**c**) close up of developing tassel primordium spike, (**d**) developing ear primordium, (**e**) floral meristems forming in spikelets, (**f**) initiation of floral organ primordia, (**g**) schematic of a mature staminate (tassel) spikelet, (**h**) schematic of a mature pistillate (ear) spikelet. The vegetative shoot meristem transitions to an inflorescence meristem (*IM*) that produces on its flanks the spikelet pair meristems (*SPM*) (**b–d**). In the tassel, the IM first produces a number of branch meristems (*BM*) that like the remainder of the tassel central spike, then recapitulate the developmental sequence indicated below. Each SPM produces a spikelet meristem (*SM*) on its flank and

meristem gives rise to a short branch that bears two spikelet meristems (SMs). The SM produces a glume primordium, a distinguishing feature of this particular meristem (Fig. 2c), and then forms two floral meristems (Fig. 2d, e), the upper and lower floral meristems (UFM and LFM). Each floral meristem subsequently forms the floral organs: a palea, two lodicules, three stamens and one pistil, all subtended by the lemma (Fig. 2e, f). Gynoecial development arrests and soon the gynoecium degenerates, resulting in an imperfect, staminate flower (Figs. 1a and 2g).

Each ear also originates from a meristem at the tip of a shoot, in this case a lateral shoot in the axil of a leaf (Fig. 1b). The lateral SAM becomes an ear IM within a few weeks after the floral transition at the tassel. One notable difference between the ear and tassel is that the ear's IM, while producing multiple rows of spikelet-pair meristems, does not form any basal branches. Spikelet initiation and development follows the same steps as in the tassel until an obvious divergence during floral development. As discussed earlier, in the ear the lower floret aborts as do the stamens of the upper floret, leaving one pistillate flower per spikelet with floral organs that appear less conspicuous then their tassel counterparts (Figs. 1b and 2h). In summary, four types of axillary meristems with different identity and fate, branch meristems (BMs), spikelet-pair meristems (SPMs), spikelet meristems (SMs) and floral meristems (FMs), give rise to structures in the maize inflorescence (Cheng et al., 1983; Irish, 1997; McSteen et al., 2000).

4 Significance as a Developmental System

Many classical mutants were described in the first half of the last century that specifically perturb aspects of maize inflorescence development (see Coe et al., 1988). Some of these altered the program of sex determination, whereas others affected various aspects of inflorescence morphology or some combination of features. The dramatic effect that many of these mutations have on inflorescence development (see examples, Figs. 3 and 4) is a consequence of changes in the activity of the various meristems that function during inflorescence development, in the differentiation of organs produced by meristems, or both. Broadly speaking then, these changes may affect basic properties of meristem initiation, size and/or maintenance, meristem identity or determinacy, or aspects of sex determination and floral organ specification (Table 1).

A central quest among maize developmental biologists is to understand mechanisms controlling these processes. How are the sites for new meristem initiation determined? Through what mechanisms are the identities of particular meristems established? What are the genes and gene interactions that dictate the fate of each meristem type? Answering those questions is key to understanding how the architecture of the maize inflorescence is orchestrated, and likely contains clues regarding those genes that were selected during grass evolution and the subsequent domestication of cereal crops. There are some 10,000 grass species, a few of which have been domesticated into our modern cereals. These domestications encompass a

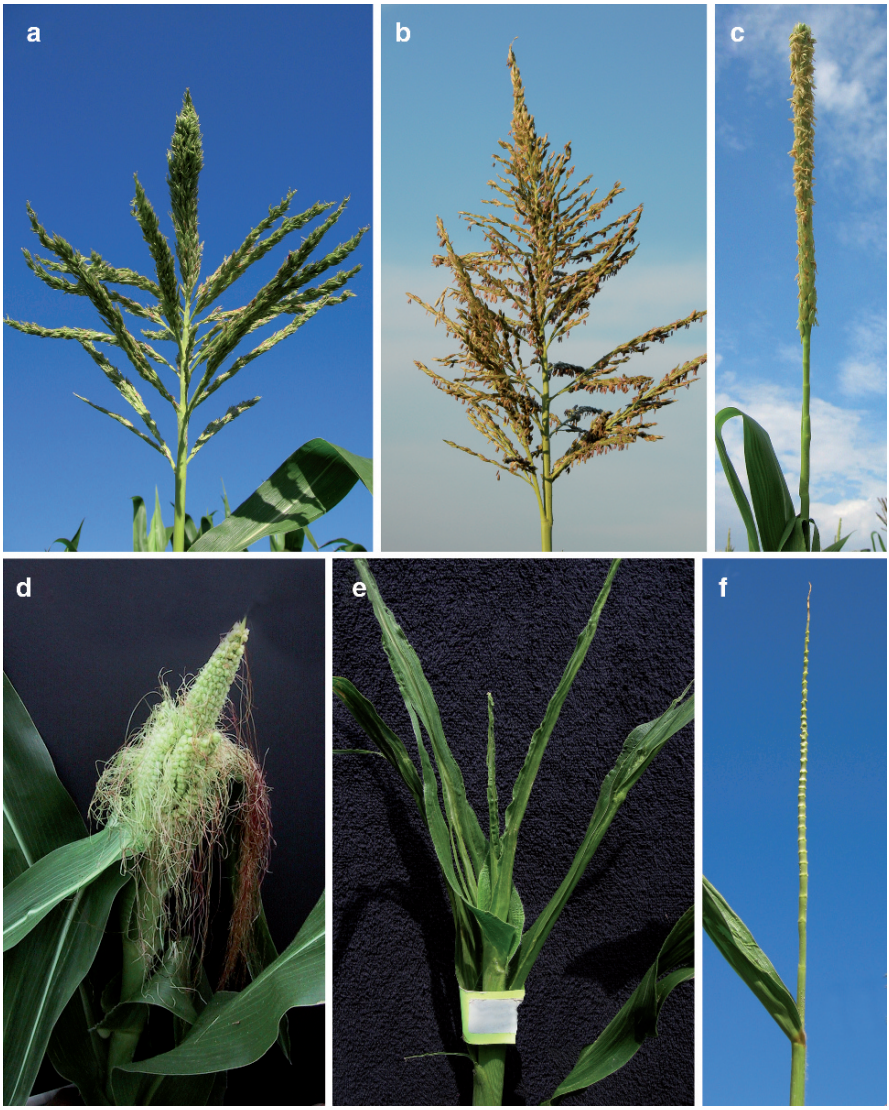

Fig. 3 Examples of mutant tassel phenotypes. (**a**) *branched silkless*, *bd1*, (**b**) *ramosa1*, *ra1*, (**c**) *unbranched*, *ub1*, (**d**) a tasselseed, (**e**) a tasselless, (**f**) *barren stalk1*, *ba1*

wide variety of inflorescence architectures, many of which resemble some of the abnormal phenotypes that accompany maize inflorescence mutants. One can speculate that subtle changes in the timing, levels or location of expression of key regulatory genes could underlie the architectural differences observed in the inflorescences of different grass species (Kellogg, 2000b). Understanding the genetic and molecular control of these developmental mechanisms in maize may therefore

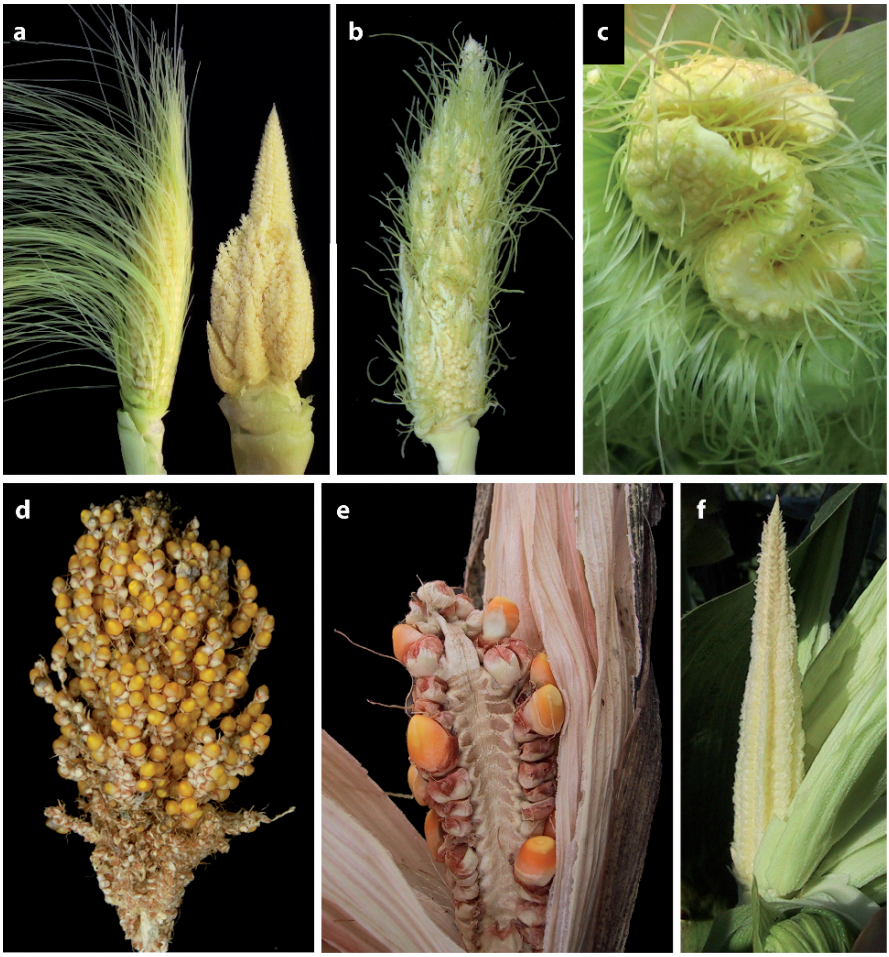

Fig. 4 Examples of mutant ear phenotypes. (**a**) normal ear (left) and *branched silkless1*, *bd1*, (**b**) *ramosa2*, *ra2*, (**c**) a fasciated ear, (**d**) *ramosa1*, *ra1*, (**e**) a barren inflorescence, (**f**) a silkless ear

provide valuable insights into the evolution of morphological variation. Tantalizing evidence to this effect has come from the molecular characterization of the *ramosa1* gene of maize and the examination of its expression in other grass species (Vollbrecht et al., 2005) where its timing of expression in different species is consistent with the variation in inflorescence branching among those species and the deduced role of this gene from genetic and molecular studies of its action in maize (see below).

Genetic and molecular analyses of maize flower development have already proven fertile ground for examining conservation in the developmental programs for angiosperm floral organ specification. Comparative studies of floral organ

Table 1 Selected mutants in maize that affect the inflorescence

Mutant symbol	Map location	Meristem function	Meristem identity	Organ dev	Sex determ	Gene product
an1	1.08			√	√	ent-kaurene synthase
bde	5.06		√	√	√	
Bif1	8.02	√				
bif2	1.05	√				S-T kinase
baf1	9.01	√				
ba1	3.06	√				bHLH (TF)
ba2	2.04	√				
ba3	Unplaced	√				
bd1	7.04		√			AP2-domain (TF)
Clt1	8.05		√			
ct2	1.02	√		√		
Cg1	3.02		√	√		Micro RNA
dlf1	7.06		√			bZIP (TF)
Dvd1	5.03	√				
d1	3.02			√	√	
d2	9.03			√	√	ent-kaurenoic acid hydroxylase
d5	2.02			√	√	
D8	1.10			√	√	DELLA (TF)
D9	5.02			√	√	DELLA (TF)
eg1	5.07			√		
fea2	4.05	√		√		LRR receptor-like kinase
fea3	3.04	√		√		
Fas1	9.05	√		√		
Fbr1	Unplaced	√				
Gn1	2.10			√		KNOX (TF)
ifa1	1.02		√	√		
ids	1.11		√			AP2-domain (TF)
id1	1.08		√	√		Zinc-finger (TF)
kn1	1.10	√		√		KNOX (TF)
Lfy1	3.09				√	
lg1	2.02	√	√			SBP-domain (TF)
lg2	3.06	√	√			bZIP (TF)
ns1; ns2	2.05; 4.08			√		WOX (TF)
Pn1	7.05			√		
Pt1	6.05		√	√		
rel1	1.07		√			
rel2	10.03		√			
ra1	7.02		√			C2H2 Zinc-finger (TF)
ra2	3.04		√			LOB domain (TF)
ra3	7.04		√	√		Trehalose phosphatase
rgo1	9.03		√			
Sdw1	8.04		√			
sk1	2.03			√	√	
si1	6.01			√		MADS box (TF)
spi1	3.08	√				YUCCA-like

(continued)

Table 1 (continued)

Mutant symbol	Map location	Meristem function	Meristem identity	Organ dev	Sex determ	Gene product
Sos1	4.02	√				
tls1	1.11	√				
tru1	3.06		√		√	
ts1	2.03				√	
ts2	1.03				√	Alcohol dehydrogenase
Ts3	1.09				√	
ts4	3.05		√		√	Micro RNA
Ts5	4.03				√	
Ts6	1.11		√		√	AP2-domain (TF)
tsh1	6.07	√		√		
tsh2	Unplaced	√		√		
tsh3	6L	√		√		
tsh4	7.03					
Tp1	7.03		√	√		
Tp2	10.04		√	√		
tb1	1.09			√	√	TCP (TF)
tga1	4.05			√		SBP-domain (TF)
te1	3.05		√	√	√	RNA binding
td1	5.03	√		√		LRR R-like kinase
Tu1	4.07			√		
ub1	Unplaced	√				
Vg1	1.07			√		
wcr	2L		√	√		
zag1	6.05		√	√		MADS box (TF)
zfl1; zfl2	10.06; 2.02		√	√	√	LEAFY class (TF)

Mutant symbol	**Gene name**	**Mutant symbol**	**Gene name**
an1	*anther ear1*	*rel1*	*ramosa enhancer locus1*
Bif1	*barren inflorescence1*	*rel2*	*ramosa enhancer locus2*
bif2	*barren inflorescence2*	*ra1*	*ramosa1*
baf1	*barren stalk fastigiate1*	*ra2*	*ramosa2*
ba1	*barren stalk1*	*ra3*	*ramosa3*
ba2	*barren stalk2*	*rgo1*	*reversed germ orientation1*
ba3	*barren stalk3*	*Sdw1*	*semi-dwarf plant1*
bde	*bearded ear*	*sk1*	*silkless ears1*
bd1	*branched silkless1*	*si1*	*silky1*
Clt1	*clumped tassel1*	*spi1*	*sparse inflorescence1*
ct2	*compact plant2*	*Sos1*	*supressor of sessile spikelets1*
Cg1	*corngrass1*	*tsh1*	*tassel sheath1*
dlf1	*delayed flowering1*	*tsh2*	*tassel sheath2*
Dvd1	*developmental disaster1*	*tsh3*	*tassel sheath3*
d1	*dwarf1*	*tsh4*	*tassel sheath4*
d2	*dwarf2*	*tls1*	*tasselless1*
d5	*dwarf5*	*tru1*	*tassels replace upper-ears1*
D8	*dwarf8*	*ts1*	*tasselseed1*

(continued)

Table 1 (continued)

Mutant symbol	Gene name	Mutant symbol	Gene name
D9	*dwarf9*	*ts2*	*tasselseed2*
eg1	*expanded glumes1*	*Ts3*	*tasselseed3*
fea2	*fasciated ear2*	*ts4*	*tasselseed4*
fea3	*fasciated ear3*	*Ts5*	*tasselseed5*
Fas1	*fascicled ear1*	*Ts6*	*tasselseed6 (ids1)*
Fbr1	*few-branched1*	*Tp1*	*teopod1*
Gn1	*gnarley1*	*Tp2*	*teopod2*
ifa1	*indeterminate floral apex1*	*tb1*	*teosinte branched1*
ids	*indeterminate spikelet1 (ts6)*	*tga1*	*teosinte glume architecture1*
id1	*indeterminate1*	*te1*	*terminal ear1*
kn1	*knotted1*	*td1*	*thick tassel dwarf1*
Lfy1	*leafy1*	*Tu1*	*Tunicate1*
lg1	*liguleless1*	*ub1*	*unbranched1*
lg2	*liguleless2*	*Vg1*	*vestigial glume1*
ns1; ns2	*narrow sheath1 and 2*	*wcr*	*wandering carpel*
Pn1	*papyrescent glumes1*	*zag1*	*zea AGAMOUS homolog1*
Pt1	*polytypic ear1*	*zfl1; zfl2*	*zea floricaula/leafy1 and 2*

Key to columns: Mutant symbol or Mut symbol, the abbreviated symbol for the most common mutant alleles (dominant alleles begin uppercase and recessive alleles begin lowercase); Gene name, the full name of the gene; Map location, chromosome and bin in which the gene has been mapped (for cloned genes, the bin is assigned by placement on the October 2007 sequence assembly of the maize genome). Each gene is classified according to our interpretation of its primary developmental implication(s): Meristem function, affects basic meristem properties such as initiation, maintenance and/or size; Meristem identity, affects processes of meristem determinacy and/or identity; Organ dev, affects organ development and/or identity; Sex determ, affects sex determination either specifically at the organ level (e.g., *tasselseed* genes) or generally by altering the sex of whole or parts of the inflorescence(s); Gene product, the functional protein or RNA encoded by the gene (*TF* putative Transcription Factor)

specification between grass species like maize and rice with those of core eudicots (*Arabidopsis* and *Antirrhinum*) have established conserved mechanisms of MADS-box gene expression and genetic control of floral organ specification (reviewed in Whipple and Schmidt, 2006). Finally, an understanding of the genetic and molecular mechanisms underlying maize inflorescence development will likely have important implications to the ongoing efforts to improve maize as one of the world's most important cereal crops. The links between inflorescence morphology and grain yield are readily apparent. Although rapid progress has been made during the past 5 years in cloning and characterizing the genes underlying a number of very informative inflorescence mutants, as is apparent from the discussion below and the list of mutants in Table 1, this is an area of study wide open with opportunities for research at the intersection of development, evolution, and applied agriculture.

5 Insights from Analyses and Gene Cloning of Mutants

Our intent here is to introduce mechanisms of inflorescence development by describing what we feel are a few of the more informative mutants, and by providing an update on the rapidly expanding pool of recently cloned genes and what is known regarding how their gene products function. For economy we will concentrate on work in maize, noting that recent, excellent review articles compare and contrast our current understanding of the genetic and molecular control of inflorescence development in maize to that in other grass species and the model Arabidopsis (Bommert et al., 2005a; Malcomber et al., 2006; Bortiri and Hake, 2007; Kellogg, 2007). Considering the striking similarities between many phases of tassel and ear development, it is perhaps not surprising that the majority of mutations affecting structural aspects of the inflorescence affect tassels and ears similarly. However, this should not be assumed to be the case for every mutant listed in Table 1.

5.1 Mutants Affecting the Transition to Flowering

In order to produce inflorescences a plant must undergo the transition to flowering, a process that integrates a combination of internal and external signals at the shoot apical meristem. This integration occurs after the SAM produces leaf primordia and stem tissue for a period of about 4–5 weeks post germination. At this point in time the SAM elongates and transitions into an inflorescence meristem. The IM still generates leaf primordia but in normal tassels the leaves remain greatly reduced, as miniscule bracts that are barely visible only near the inflorescence apex (Fig. 2c). In their axils are new meristems that quickly overtop the bracts and form the lateral structures that ultimately comprise the tassel and ear. In mutants like *barren stalk1*, where those axillary meristems are absent (Fig. 3f), or like *tassel sheath* or the dominant *Few-branched1* where bracts are derepressed, the bracts are clearly evident. From studies in *Arabidopsis*, the transition to flowering involves a complex network of interacting genes (Boss et al., 2004; Bernier and Perilleux, 2005; Kobayashi and Weigel, 2007), and a similar complexity can be expected in maize (see Colasanti and Muszynski). Indeed, more than 80 flowering time QTLs have been identified in maize (Chardon et al., 2004).

Among the several mutants that affect the transition to flowering, *indeterminate1* (*id1*), and *zea floricula/leafy1* (*zfl1*) and its duplicate *zfl2,* are cloned genes that in addition to showing a delay in the time to flowering show dramatic effects on the inflorescence (Colasanti et al., 1998; Bomblies et al., 2003). The *id1* gene encodes a Zn-finger putative transcription factor that is produced in young leaf primordia and is hypothesized to regulate production of a compound that acts elsewhere, at the meristem (Colasanti et al., 1998; Kozaki et al., 2004). *id1* mutants produce aberrant tassels with increased branch number accompanied by unusual reversion of tassel branch meristems to a vegetative state, including shoots and roots. *id1* mutants rarely

produce an ear at all. The *zfl1* and *zfl2* genes have a shared role in regulating the transition to flowering and aspects of inflorescence architecture, as well as in specifying floral meristem identity and organ development (Bomblies et al., 2003). Their floral patterning activities suggests that gene function is at least partially conserved with that of the eudicot *LEAFY* homologs, but the inflorescence role may be exclusive to maize or the grasses. On the other hand, *id1* appears to be a gene unique to the grasses with no apparent ortholog identified in eudicots (Colasanti et al., 2006).

Delayed flowering (*dlf1*), like the other mutants affecting this transition, shows a delay in the time to flowering and typically produces more leaves than in wild type. Tassel and ear development are also affected with extra branching and some feminization appearing in the tassel. The *dlf1* gene encodes a member of the basic leucine zipper family of DNA-binding proteins and appears to be a homolog of *FLOWERING LOCUS D* (*FD*) of *Arabidopsis* (Muszynski et al., 2006). *dlf1* is expressed in the shoot apex before, during and after the transition, with a peak of expression near the time of transition, consistent with the idea that a critical level of *dlf1* expression is required for the floral transition. Genetic analysis of *id1 dlf1* double mutants places *dlf1* downstream of *id1*, and suggests that *dlf1* may mediate the floral inductive signal transmitted between leaves and the shoot apex.

5.2 Mutants Affecting Meristem Size

Considerable progress has been made over the past decade in elucidating a number of the key genes regulating meristem size and maintenance. Prominent among these are members of the *CLAVATA* pathway (see Clark, 2001), originally described in *Arabidopsis* where it comprises three genes, *CLV1–3*. These work in concert in a signaling pathway in the central zone of the shoot apical meristem to restrict stem cell proliferation programmed through the transcription factor, *WUSCHEL* (reviewed in Williams and Fletcher, 2005). Because the BMs and SPMs are produced from the flanks of the inflorescence meristem, it is not surprising that more than a few mutants have been described in maize that affect inflorescence development by impacting the size or maintenance of the IM. Several of these encode homologs of the genes that regulate meristem size in *Arabidopsis*, suggesting conservation of this pathway across the angiosperms.

A *CLAVATA1*-like gene product is encoded by the maize *thick tassel dwarf1* (*td1*) gene, whose mutant phenotype results in a plant with shorter stature, a tassel with a distinctly thickened central spike, and variably fasciated ears. The sequence and developmental role of the TD1 protein appears similar to that of CLAVATA1 (Bommert et al., 2005b). Another mutant in maize called *fasciated ear 2* (*fea2*) encodes a *CLAVATA2* like gene product (Taguchi-Shiobara et al., 2001), a membrane associated leucine rich repeat (LRR) receptor-like protein. Mutations in *fea2* result in enlarged tassel and ear IMs and FMs and typically lead to ears with a markedly fasciated phenotype (Fig. 4c). The vegetative shoot meristem appears normal in *fea2* mutants, distinct from the effects observed in *clavata2* mutants in

Arabidopsis where all shoot meristems are affected. Thus, *fea2* appears to have evolved an inflorescence-specific role in regulating meristem size. Several other mutants in maize such as *fea3*, *compact plant2* and *Fascicled ear1* also affect aspects of meristem size (Table 1).

5.3 Mutants Affecting Meristem Initiation and Maintenance

Mutants that have an effect on a fundamental element of meristem function, may lead to a failure to initiate and/or maintain meristems. In a branch system like the inflorescence that involves multiple meristems, such failure may have various implications. The homeodomain transcription factor *knotted1* (*kn1*) plays a role in maintaining all SAMs, perhaps by positively regulating meristem size (Kerstetter et al., 1997; Vollbrecht et al., 2000). Thus, in recessive *kn1* mutants any of the different inflorescence meristems may fail, leading to inflorescences that are smaller, sparser due to missing structures or, in the case of the ear, sometimes absent entirely when the IM itself fails at an early developmental stage. Other mutants affect the initiation and/or maintenance of axillary meristems without significantly affecting the SAM or IM. These include *barren stalk1* (*ba1*), *barren inflorescence2* (*bif2*), *unbranched1* (*ub1*) and the dominant mutant *Suppressor of sessile spikelets1* (*Sos1*) among others (see Table 1).

Mutations in *ba1* prevent the formation of all axillary meristems, resulting in mutant plants lacking vegetative tillers, ears, tassel branches and spikelets (Fig. 3f), but otherwise have normal vegetative growth (Ritter et al., 2002). *bif2* mutants have a similar phenotype but typically produce rudimentary ears and tassels that occasionally produce spikelets (McSteen and Hake, 2001). Both genes have been cloned. *ba1* encodes a putative transcription factor belonging to the bHLH class of DNA binding proteins (Gallavotti et al., 2004) whereas *bif2* specifies a serine/threonine protein kinase (McSteen et al., 2007). Recent evidence indicates that application of inhibitors of polar auxin transport like NPA at or near the time of the floral transition phenocopies the *bif2* and *ba1* inflorescences phenotypes, suggesting that they act in a similar, auxin-related pathway. In addition, *ba1* expression is dependent on polar auxin transport whereas *bif2* transcript was still present in NPA-treated plants (Wu and McSteen, 2007). This suggests a model in which *bif2* is upstream of polar auxin transport and polar auxin transport is required for *ba1* expression. The BA1 protein may be a target of BIF1 activity (A. Skirpan and P. McSteen, personal communication), thus providing a link between their similar phenotypes. Consistent with this prediction, their patterns of expression overlap. *ba1* is expressed in a narrow arc of cells adaxial to the sites of new axillary meristem initiation (Gallavotti et al., 2004), while *bif2* is more globally expressed in all axillary meristems (McSteen et al., 2007). The restricted pattern of *ba1* expression adjacent to sites of new meristem initiation (see Fig. 5), and the failure in *ba1* mutants to form any axillary meristems, suggests an essential and general role for *ba1* in the formation of all axillary meristems, be they vegetative or inflorescence.

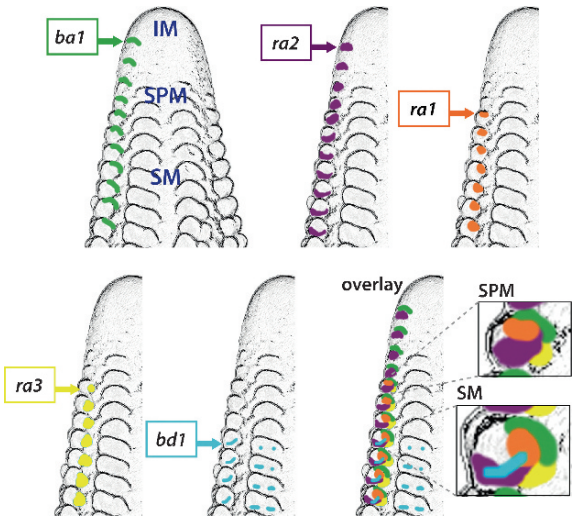

Fig. 5 An illustration of the expression patterns for several cloned genes affecting inflorescence meristem initiation and/or identity. The upper portion of a developing inflorescence is represented with the approximate expression patterns of *ba1*, *ra2*, *ra3,* and *bd1* portrayed in different colors, beginning with the onset of each gene's expression and continuing through formation of SMs. For clarity, expression is only depicted in some primordia, although the patterns are the same in every row. The overlay illustrates how the discrete patterns of each gene overlap in a pattern that surrounds the base of the SPM and/or SM.

ub1, lg2, and *sos1* are examples of genes that affect initiation of specific meristems. *ub1* mutants fail to initiate branch meristems, resulting in tassels that lack or produce only vestigial long branches but retain normal spikelet development along the central spike (Fig. 3c). *lg2* mutants also have altered BM function. They either lack long tassel branches altogether or initiate only one or two relatively normal ones, without the vestigial structures seen in mature *ub1* mutants. The *tassel sheath* mutants are similar, except that bract derepression accompanies the failed BMs. *lg2* encodes a bZIP transcription factor (Walsh et al., 1998), while *ub1* has not been cloned. A dominant, mutant allele of *Sos1*, another classic mutation awaiting molecular characterization, affects the formation of the sessile spikelet only, resulting in inflorescences with rows of single, unpaired and only pedicellate spikelets. Unpaired spikelets appeared in NPA-treated plants, again suggesting links between polar auxin transport and early events in inflorescence development (Wu and McSteen, 2007).

5.4 Mutants Affecting Meristem Identity and Determinacy

Apart from general properties such as meristem initiation and maintenance that are essential to all functioning meristems, the way a meristem behaves in a certain context is controlled by distinct properties commonly described as meristem identity.

Reflecting its array of meristem types, the maize inflorescence employs multiple genetic mechanisms to regulate meristem identity. Moreover, meristem identity, which classically defines qualities such as the number and types of primordia that a meristem will produce, is closely related in the inflorescence to the property of determinacy, which relates to the capacity for continued meristem activity. Branch meristems and the main IM possess indeterminacy as reflected in their continued cycles of initiation and generation of a stem axis, while each of the other meristem types in the inflorescence (SPM, SM and FM) show relative determinacy in that they produce only short branches with a small, defined number of organs. Genetic analysis suggests that indeterminacy is something of a ground state, because several mutants are known that clearly increase meristem indeterminacy but there are arguably few, if any, known mutants with increased determinacy. Put another way, the determinate meristem identities seem to be sequential and imposed by a number of genes (Irish, 1997) on a default, indeterminate fate (Vollbrecht et al., 2005).

For example, a normal function of the three *ramosa* genes (*ra1*, *ra2* and *ra3*) is to specify the determinate identity of the SPM, and thereby limit branch outgrowth. In each of the *ramosa* mutants SPMs have increased indeterminacy and assume fates more like a BM, leading to branched ears and to tassels with increased degrees of branching (Figs. 3b and 4b, d). Moreover, the mutants make novel branches whose length and identity is transitional between BM and SPM (Vollbrecht et al., 2005; Satoh-Nagasawa et al., 2006). This blurring of the normally sharp distinction between long branches and spikelet pairs demonstrates the interplay between identity and determinacy, where evolution has led to fates such as BM and SPM in maize, but to different permutations of determinacy, meristem order and other basic properties to produce related but distinct branching architectures across the grasses (Kellogg, 2000a; Vollbrecht et al., 2005; Malcomber et al., 2006). From work on the *ramosa* and other genes in maize, we now have clues about the underlying molecular mechanisms. *ra2* encodes a LOB domain transcription factor (Bortiri et al., 2006b) and is expressed in the incipient primordia of all axillary inflorescence meristems except the FMs, well before the developing meristems protrude as bumps on the lateral surface of the inflorescence (Fig. 5). This suggests that *ra2* has a fundamental and general role in axillary meristem specification, perhaps distinct from its effects on meristem determinacy. Consistent with this, *ra2* mutants have elongated branch and spikelet internodes, a feature not exhibited by other determinacy mutants. *ra1* also encodes a plant-specific transcription factor, one belonging to the C2H2 zinc finger protein class (Vollbrecht et al., 2005). Its expression initiates after but overlaps that of *ra2*, in a discrete domain associated with the base of initiating SPMs (Fig. 5). Consistent with the gene's initial expression, the *ra1* mutant phenotype is the most restricted among the *ramosa* mutants, being confined to effects on SPM determinacy. *ra3* encodes a trehalose 6-phosphate phosphatase whose expression also initiates in a basal domain concomitant with the emergence of SPMs, but like *ra2*, *ra3* is also expressed in later stages, and *ra3* affects determinacy of both SPMs and SMs (Satoh-Nagasawa et al., 2006). Each of the pairwise double mutants shows a synergistic effect on SPM determinacy and *ra1* expression is reduced in *ra2* single mutants and in *ra3* single mutants, but *ra2* and *ra3* expression is unaltered in the

other single mutants. Taken together, these data imply one genetic pathway in which *ra1* is downstream of *ra2* and *ra3* to impose determinacy on the SPM, while in parallel pathways, *ra2* and *ra3* regulate additional functions including determinacy of higher order meristem types. Whether or not *ra1* has other roles outside of *ra2* and *ra3*, awaits careful analysis of the triple mutant.

Identity and determinacy of one of the higher order meristems, the SM, is regulated in part by the *branched silkless1* (*bd1*) and *indeterminate spikelet1* (*ids1*) genes, which encode distinct transcription factors of the AP2-domain class (Chuck et al., 1998, 2002). In *bd1* mutants the SM is indeterminate in both the tassel and the ear, but with slightly different consequences. In the mutant tassel, the converted SM, now indeterminate, bears numerous spikelets as in the *ramosa* mutants, and eventually produces fertile flowers. The extra spikelets result in a mutant tassel (Fig. 3a) whose branches and central spike appear thicker in comparison to a normal tassel (Fig. 1a). But in the ear, the indeterminate SMs do not produce florets. Instead, the mutant SMs near the base of the ear often appear to take on an identity like that of the SPM, initiating new meristems in a distichous fashion, some of which in turn appear to again adopt a SPM fate, resulting in complex, compound branching (Fig. 4a). Interpretation of the phenotype is further complicated by the occurrence of additional indeterminate, SM-like meristems arising in a novel location, the axil of the normally sterile glume that subtends the SM on the main axis (Colombo et al., 1998; Chuck et al., 2002). On one hand, this mutant phenotype suggests a regression of SMs to BM identity, and therefore that *bd1* connotes SM identity (Chuck et al., 2002). However, it also suggests a more general role for *bd1* in SM determinacy and suppression of meristem outgrowth in the axil of the glume. This complex mutant ear phenotype further blurs the distinction between SM determinacy and identity. Intriguingly, like the *ramosa* genes, *bd1* is expressed in a specific domain, in an arc at the base of the meristem it regulates, the SM (Fig. 5).

ids1 is expressed more broadly in the SPM, SM and some floral primordia, perhaps reflecting its distinct role as a target of regulation by microRNAs (see below). But in *ids1* mutants only the spikelet axis shows increased indeterminacy, by producing multiple florets per spikelet rather than the requisite two (Chuck et al., 1998). A strikingly similar defect is shown by *reversed germ orientation1* (*rgo1*) mutants. Intriguingly, recessive alleles of *rgo1* and *ids1* exhibit nonallelic noncomplementation and double mutants show a synergistic, indeterminate phenotype in both SM and SPM, demonstrating a function for *ids1* in other meristems and fueling the hypothesis that meristem identity and determinacy may be regulated by factors that function in a dosage-sensitive manner (Kaplinsky and Freeling, 2003).

A novel mechanistic theme emerges from recent analyses of determinacy and other inflorescence gene expression patterns. For several genes, the domain of RNA expression is not within the meristem *per se* that the gene regulates, but in a boundary domain that surrounds the base and/or adaxial side of the meristem, as to visually separate the meristem from the main inflorescence axis. Such an expression pattern is observed for genes including *ba1*, a meristem initiation/maintenance gene, and the determinacy genes *ra1*, *ra2*, *ra3* and *bd1* (Fig. 5). In the cases of *ra2* and *ba1*, the genes are expressed even before the corresponding meristems are initiated. Further

characterization of the functions and locations of the encoded proteins as well as the transcriptional targets and corresponding gene networks, should help elucidate this mechanism. Nevertheless, the expression patterns suggest that in the inflorescence, disparate properties such as meristem initiation and determinacy are controlled by a mobile signal(s) whose activity may be gated or regulated spatially at the junction with the main inflorescence axis (Gallavotti et al., 2004; Vollbrecht et al., 2005). With the exception of *ra3*, all of these boundary domain genes encode putative transcription factors. It is tempting to speculate about the nature of the mobile signal. For example, the putative transcription factors are all small proteins that may themselves be mobile. Alternatively, sugar or hormone signaling or signal transduction may be involved, upstream and/or downstream of the transcription factors.

5.5 Mutants Affecting Sex Determination

The process by which strictly staminate (male) florets are produced in the tassel and pistillate (female) florets in the ear is termed sex determination, and a number of mutants affect this pathway (Table 1). The genes underlying the many andromonoecious dwarfs (*an1*, *d1*, *d2*, *d5*, *D8,* and *D9*) play a role in promoting stamen abortion in ear florets. Mutants typically produce anthers in the ear florets although these do not usually shed pollen. In addition, the mutants are generally characterized as dwarfs due to shortened internodes that produce broad and shortened leaves. The tassel branch number can also be reduced. The recessive andromonoecious dwarfs fail to synthesize normal levels of gibberellic acid (GA) and normal stature and floret development can be rescued by GA application (Phinney, 1956; Phinney and West, 1960). The specific enzymatic activity encoded by many of the dwarf genes has been predicted by careful feeding experiments (Spray et al., 1984); indeed, the cloning of *an1* (Bensen et al., 1995) confirmed its role in the biosynthetic pathway. The dominant *D8* and *D9* mutants, however, have defects in a DELLA repressor protein that interacts with the GA receptor and are hence unresponsive to GA applications (Harberd and Freeling, 1989; Peng et al., 1999). Clearly activity of the plant hormone, GA, is important to the formation of pistillate flowers in maize ears, but the mechanism remains unresolved. Insights into the connection between GA and sex determination should be facilitated by the molecular characterization of those genes with more specific effects on the sex determination pathway.

The group of tasselseed mutants (Table 1) shares in common the partial to nearly complete conversion of the normally male tassel spikelets into ones that bear female florets (Nickerson and Dale, 1955). This often results in seeds being formed on the tassel, sometimes as profusely as on the ear. Two groups of tasselseeds were established based on genetic analyses and morphological considerations (Irish et al., 1994), with *ts1*, *ts2*, *Ts3*, and *Ts5* falling in the group where sex reversal was not accompanied by extra branching, and *ts4* and *Ts6* affecting sex determination and causing proliferative inflorescence branching. *ts2* was the first tasselseed gene to be cloned (DeLong et al., 1993). It encodes a short chain alcohol dehydrogenase that is expressed in both ear and

tassel spikelets. This leads to an apoptotic-based degeneration of the gynoecium in the tassel florets as well as in the lower floret of the ear (Calderon-Urrea and Dellaporta, 1999). The *ts1* gene product is required for *ts2* mRNA accumulation placing *ts1* upstream of *ts2*. Interestingly, the *silkless ears1* (*sk1*) gene appears to be a key player in this pathway. *sk1* mutants fail to make a gynoecium and so produce ear florets and mature ears that lack silks (Fig. 4f). Surprisingly, the double mutant between *sk1* and *ts2* results in a silk-containing, essentially *ts2* phenotype. Thus *ts2* is epistatic to *sk1*, suggesting a model (Calderon-Urrea and Dellaporta, 1999) of sex determination in which TS1 promotes expression of *ts2* mRNA within cells of the developing pistils. The TS2 dehydrogenase then modifies a yet unidentified steroid that promotes pistil cell death. *sk1* protects the pistil in the upper ear floret by either interfering with TS2 activity directly, or by blocking a downstream step in the cell death response. The validation of this model will have to wait the cloning of *sk1*, but it predicts that the SK1 product will be specifically expressed in the upper floret only. Genetic analyses have shown *ts2* to be additive with *d1* although, as already indicated, epistatic to *sk1*. Thus, the formation of unisexual florets in maize involves an interplay of at least two apparently independent pathways, one involving GA and one involving TS1, TS2, and SK1 in an apoptotic cell death program.

The complexity of the sex determination process is highlighted by the recent cloning of two of the genes responsible for the proliferative tasselseed mutants. The recessive *ts4* and the dominant *Ts6* alleles affect tassel and ear development similarly (Irish et al., 1994; Irish, 1997), causing proliferative branching due to acquired indeterminacy of the SPM and SM, and the production of tassel florets bearing pistils. Molecular cloning of these genes (Chuck et al., 2007b) reveals *ts4* to encode a microRNA of the *miR172* family. One of the targets of this microRNA is the AP2 domain-encoding gene, *ids1*, discussed above with respect to SM determinacy. Remarkably, the dominant *Ts6* allele contains a single nucleotide change in the *ids1* gene, in the binding site for the *ts4*-encoded microRNA. Hence, *ids1* and *Ts6* are allelic. The nucleotide change renders the dominant *Ts6* allele free of *ts4*-mediated regulation and therefore leads to ectopic accumulation of *ids1* RNA and IDS1 protein. Double mutants of *ts4* with a recessive allele of *ids1* have restored, near normal tassel sex determination, but the SPMs and SMs still show some indeterminacy. This double mutant phenotype indicates that most of the *ts4* mutant phenotype is due to misregulation of *ids1*. However, the residual SPM and SM indeterminacy, indicates that there is at least one other target of *ts4*. The nature of the link between the branching pathway and sex determination is still unclear, but may be clarified by the cloning of the remaining tasselseed mutants and identification of downstream targets of *ids1*. However, it seems that additional links between inflorescence development and epigenetic regulation are likely. For example, mutants of the *required to maintain repression6* (*rmr6*) gene, initially identified on the basis of *rmr6*'s role in paramutation of anthocyanin regulatory genes, show a tasselseed phenotype. Genetic analysis showed that *rmr6* maintains maize's monoecious pattern of sex determination by restricting the activity of *sk1* (Parkinson et al., 2007). Although not demonstrated as for *ts4*, *rmr6* may also implement epigenetic repression by a mechanism that involves noncoding small RNA molecules.

5.6 Mutants Affecting Organ Specification and Floral Meristem Identity

There are several mutants of maize that act very late in the program of inflorescence development, affecting specifically the determinacy of the floral meristem or the specification of floral organs. One mutant with effects confined to a specific spikelet organ is *Vestigial glume1* (*Vg1*). In these mutants the tassel and ear appear normal with the exception that the glumes are dramatically reduced in size, leaving the stamens exposed on developing tassel florets, resulting in reduced fertility due to desiccation and premature sun exposure.

Other mutants are specific to development of floral organs. The *sk1* mutant, discussed above for its role in sex determination, has ears without silks, but otherwise produces a morphologically normal ear and tassel. The opposite phenotype is conditioned by the *silky1* (*si1*) mutant which produces numerous silks emerging from both tassel and ear spikelets. A mutant tassel producing silks is reminiscent of the tasselseed class of mutants. However, in the case of *si1* mutants the silks arise in place of stamens due to homeotic floral organ conversions. *si1* encodes a MADS-box gene related to the B-class floral homeotic MADS-box genes *APETALA3* of *Arabidopsis* and *DEFICIENS* of *Antirrhinum* (Ambrose et al., 2000). In the *si1* mutant tassel spikelets, the central gynoecium aborts as in wild type, but sterile carpels replace the stamens, and lemma/palea-like structures replace the lodicules. Unlike in the tasselseeds, the silks that form in place of stamens are sterile, and so never set seed. Similar organ conversions occur in *si1* ears such that the mutant ear spikelets each contain three additional silks in place of the stamens, and a functional primary silk.

zea agamous 1 (*zag1*) is another MADS-box gene that has been functionally characterized in maize. The *zag1-mum1* mutant was obtained through a reverse genetics approach that targeted one of the first MADS-box genes identified in maize, specifically a candidate ortholog of the *Arabidopsis* C-class floral homeotic gene, *AGAMOUS* (Schmidt et al., 1993). Mutants produce ears with greatly reduced fertility, resulting from production of sterile silks emerging from the indeterminate floral meristem of ear spikelets (Mena et al., 1996), indicating a role for *zag1* in floral meristem determinacy, a conserved function of C-class genes. No effects were observed on stamen identity, as might have been predicted for a loss of function mutation in an *AGAMOUS* homolog. This surprising result was explained based on the partitioning of the predicted C-function activities between *zag1* and its duplicate gene *zmm2*. The expression patterns of these two genes within developing stamens and carpels are distinct (Mena et al., 1996), suggesting that this floral organ identity function has been subfunctionalized during maize evolution. The double mutant between *zag1* and *si1* produced striking mutant tassels and ears with spikelets consisting of proliferating bracts having lemma and palea character (Ambrose et al., 2000), a phenotype reminiscent of the B- and C-function double mutants in *Arabidopsis* where a proliferation of sepals replaces normal floral organ development (Bowman et al., 1991). These and other data (reviewed in Whipple

and Schmidt, 2006) provide support for the conservation of the ABC program of flower development within maize.

Finally, a number of mutants discussed earlier (*bif2*, *kn1*, *zfl1, and zfl2*) and others like *indeterminate floral apex1* (Laudencia-Chingcuanco and Hake, 2002) have effects on floral organ development, but unlike the mutants discussed above, their effects are not restricted to this late stage of inflorescence development, but instead more globally impact all the meristems of the inflorescence. Worth noting here is the dramatic phenotype of double mutants between *ifa1* and *zag1* in which floral meristems within the developing ear revert to a BM identity producing ear spikelets from which a discrete inflorescence branch emerges (Laudencia-Chingcuanco and Hake, 2002). Since *ifa1* alone has only modest affects on floral organ development and floral meristem determinacy, the double mutant phenotype suggests a redundant role of both *ifa1* and *zag1* in promoting floral meristem identity, independent of their separate roles in floral meristem determinacy.

5.7 Mutants Lacking an Inflorescence

Lastly, there exists in maize several curious mutants where one or the other inflorescence fails to form altogether. *tasselless1* (*tl1*) mutants lack a tassel at maturity, but can produce ears and may otherwise appear normal (Fig. 3e). Anecdotal evidence suggests that this is a phototropic response that can be ameliorated by growth under short days, but detailed studies on this mutant are lacking. Similarly, the mutants *barren stalk2* and *3* (Pan and Peterson, 1992; Neuffer et al., 1997) lack ears and tillers but produce an otherwise normal plant including a normal tassel. In the case of *ba2*, at least, ears shoots are initiated but then arrest their development at such an early stage that the shoot never emerges from the axil of the leaf. Unlike *ba1*, which affects initiation of all axillary meristems, *ba2* and *ba3* are specific to the axillary shoot meristem, but affect the continued growth of the shoot rather than its initiation or formation.

6 Relationship of the Inflorescence to the Whole Plant

Thus far we have discussed the inflorescences more or less separately from the rest of the plant, but they develop in the context of and in response to signals generated by the whole plant. At one extreme are heterochronic mutants with pervasive effects on vegetative shoot architecture that have correlative alterations in inflorescences (*e.g. Teopod1* and *2*, *Corngrass1*). The recent cloning of the dominant *Corngrass1* (*Cg1*) mutation provides some insight into the basis of such a global effect on development. *Cg1* mutants (Fig. 6a) are a consequence of the ectopic expression of a noncoding regulatory RNA locus, *miR156*, whose apparent misexpression throughout development perturbs the transcript levels of at least 13 putative target genes belonging to the SPL family of transcription factors, with several

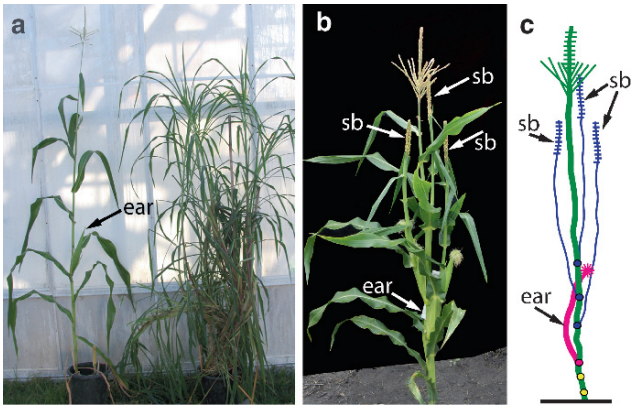

Fig. 6 Examples of mutants that affect whole plant and inflorescence architecture. (**a**) a normal plant (left) and a sibling *Corngrass1* (*Cg1*) mutant. (**b**) a *tassels replace upper-ears1* (*tru1)* mutant and (**c**) a schematic showing the fate of axillary meristems in the mutant shown in (**b**). In *Cg1* mutants axillary meristems are derepressed and proliferate throughout the plant, accompanied by heterochronic changes in shoot and organ identity. *tru1* mutants produce a relatively normal main shoot (represented in green in panel **c**) but axillaries are differentially derepressed. The normally pistillate ear shoot and a few axillaries below it develop instead as long, staminate branches (sb, blue axes) tipped by tassel-like inflorescences. An ear shoot (red axis) forms at a lower position, while the lowest axillary buds (yellow circles) remain repressed

of these normally expressed during both vegetative and inflorescence development (Chuck et al., 2007a). Considering the similar molecular bases of *Ts6* (discussed above) and *Cg1*, it seems likely that other dominant mutants of maize (see Chapter by Neuffer et al) with global effects on development may turn out to be alteration in pathways involving regulatory RNAs. As mentioned in Sect. 5.1, one integrative signal in plant ontogeny cues the transition to flowering and some mutants that affect this transition in maize, also show dramatic effects on inflorescence development (e.g., *id1*). When these mutants with pleiotropic phenotypes are considered as a group, it is evident that particular suites of inflorescence-related characteristics are affected, with different subsets changing in different mutants. Rather than list mutants with all of their phenotypes, in this section we will consider them as a group and discuss some of the themes that emerge from a synthetic perspective.

One unifying phenotype among mutants that demonstrate integration of whole plant and inflorescence development, is a derepression of axillary meristems. Just as inflorescence architecture arises from patterns of meristem determinacy, overall plant architecture reflects substantially the activity of vegetative axillary meristems (McSteen and Leyser, 2005). In maize, several axillary meristems are present on the main shoot at flowering but typically only the uppermost few grow out, to form the ear shoots (see Fig. 1b). Traversing down the plant (basipetally) from the leaf axil(s) that contain ears, sequential axillary meristems are repressed and remain quiescent, except that the lowermost may be released early in ontogeny to form tillers that recapitulate the main plant axis. The TCP transcription factor gene *teosinte*

branched1 (*tb1*) regulates the determinacy of these axillary meristems on the main shoot. In plants containing *tb1* alleles with reduced or no function, all axillary meristems up to and including those at the ear node are derepressed and grow out to produce long axes, each one much like a tiller (Hubbard et al., 2002). Thus a lateral branch that would normally be compressed and tipped by a pistillate ear, in *tb1* mutants is elongated with a staminate, tassel-like structure at its terminus. The *tassels replace upper-ears1* (*tru1*) mutant (Sheridan, 1988) similarly transforms the erstwhile ear branch to a long branch tipped by a staminate inflorescence (Fig. 6b, c), but does not strongly affect overall branching.

Indeed, the *tru1* mutation derepresses only a few axillary meristems, at and below the normal ear position. Below the uppermost elongated, tassel-tipped branch, the branches are progressively shorter and the lower axillaries do not grow out; *tru1* mutants do not show a marked tillering phenotype. The similarity of transformed lateral branches of *tru1* mutants to the staminate ear tips frequently seen in many normal maize lines, and often attributed to genotype by environment interaction (Coe et al., 1988) suggests an involvement of phytohormones in this phenotype. Flowering time mutants such as those corresponding to the transcription factor genes *dlf1* and *zfl1* and *2*, show a more subtle degree of derepression, of axillary meristems on the ear shoot itself. These mutants typically produce at the normal position, an shoot that is ear-tipped but whose derepressed axillaries lead to multiple, closely spaced ears attached to the same shank (Bomblies et al., 2003; Muszynski et al., 2006).

These and other mutants also suggest a general correlation between axis length and inflorescence sex, in which inflorescences that tip long axes tend to be staminate, while those on compressed axes tend to be pistillate, perhaps correlating with a physiology inherent to short branches (Iltis, 1983; Irish, 1996). *tb1* mutants provide the long branch example, typically with staminate inflorescences on all lateral branch termini. A series of transitional inflorescence forms may be evident in *tru1* mutants, where the uppermost and longest lateral branch shows the strongest and most consistent staminate transformation, sequentially lower branches are progressively shorter and more feminized and a normal ear forms at a somewhat lower-than-normal node (Fig. 6b, c). The same trend manifests in *terminal ear1* and *zfl* mutants but with respect to the length of the main shoot itself; both mutants have shortened vegetative internodes immediately preceding the tassel, so that the tassel is borne on a relatively shortened main axis, and both form strikingly similar feminized branches at the tip of this shortened axis, in the positions normally occupied by lower tassel branches (Veit et al., 1998; Bomblies et al., 2003). In analogous fashion, the long, staminate-tipped branches of *tb1* mutants bear small pistillate inflorescences laterally, especially in axillary positions of the shorter, uppermost branches off the main axis. Even *ts1* and *ts2* main stems, which are tipped by a pistillate inflorescence, tend to be shortened. Interestingly, the GA-related *dwarf* mutants provide a conspicuous counter-example; axis length is reduced in mutants, but inflorescences are always staminate or andromonoecious.

Finally, one group of mutants may be seen as altering the spacing or developmental coordination between the tassel and ear. This group includes mutants with intact axillary meristems but that otherwise do not elaborate a mature ear, discussed in Sect. 5.7. A similar ear phenotype occurs in some flowering time mutants. For example, dominant

Leafy1 mutants modestly increase flowering time and increase, by a few, the number of nodes below the ear and between the ear and the tassel, while strong mutants of the zinc-finger protein gene *id1* rarely form an ear at all, even when they do eventually produce tassels. These phenotypes demonstrate that coordinating whole plant architecture and placement of the ear relative to the tassel is regulated in part by an integrative component of the floral transition mechanism. Considered in this context, mutants like *ba2* and *ba3* that flower and make axillary meristems normally but do not elaborate an ear, may have ear development blocked in a related way.

7 Concluding Remarks

Considerable progress has been made in the last 5 years in the cloning and characterization of genes underlying many of the inflorescence mutants. This has provided valuable information on patterns of gene expression and the nature of the gene products, and enabled researchers to begin assembling testable models of gene networks. Interestingly, an overwhelming majority of these cloned genes encode transcriptional regulators, or regulatory molecules like small RNAs that have transcription factors as their targets. This lends support to the idea that subtle changes in the timing or pattern of expression of these genes may well underlie morphological variation in inflorescence structure that has evolved within the grasses (Doebley and Lukens, 1998; Vollbrecht et al., 2005). Establishing those associations will require the development of genomic resources in other grass species. The advent of positional cloning in maize (Bortiri et al., 2006a), made possible by the emerging genomic resources in maize and rice, has revolutionized the pace of cloning genes identified by mutant phenotypes. An additional challenge now will be to keep pace with the genetic analyses, so important to understanding the hierarchical relationships between genes. Linkages between plant hormones and inflorescence gene activities are becoming ever more apparent. Databases of cataloged mutants are providing new alleles of existing mutants and uncovering new genes for further study and integration into existing genetic frameworks. An expanding platform of reverse genetic resources (see Volume 2) is facilitating the identification of mutations in candidate genes that are identified through studies in other organisms and/or through expression and other analyses. Thus, we can anticipate that continued mutant analysis and genomics and other molecular approaches will flesh out the complex interplay of genes, ultimately leading to the identification of downstream targets and an understanding of relevant gene and protein interactions. These efforts will lead us closer to an understanding of the mechanisms underlying patterns of meristem activities that ultimately lead to the production of the tassel and ear.

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The Maize Floral Transition

Joseph Colasanti and Michael Muszynski

Abstract The floral transition is a critical developmental change in a plant's life cycle that is marked by the switch from vegetative to reproductive growth. The transition is induced by leaf-derived signals that translocate through the phloem to the shoot apex where the shoot apical meristem is reprogrammed to adopt a floral fate. In maize, this occurs when the vegetative shoot meristem ceases leaf initiation and becomes consumed in the production of the tassel inflorescence primordium. Upper axillary shoot meristems are converted into ear inflorescence primordia soon after this period. This review highlights current understanding of the genes and molecular mechanisms regulating the floral transition in maize. We relate flowering control in maize to its progenitor teosinte, provide an overview of the quantitative nature of flowering in maize germplasm and describe what is currently known about the molecular components of the maize floral transition genetic network.

1 Overview of Maize Flowering

Floral transition, the switch from vegetative to reproductive growth, marks a critical event in the life cycle of higher plants. During vegetative growth, the shoot apical meristem (SAM), a population of totipotent cells at the growing point of the plant, gives rise to leaves and other above-ground organs. To switch to reproductive growth, the SAM becomes committed to the production of reproductive structures, such as branched inflorescences bearing flowers (Fig. 1). The period when the SAM is reprogrammed is called the floral transition and the timing of the transition largely determines when a plant flowers.

Higher plants have developed sophisticated genetic mechanisms to ensure that flowering coincides with an optimal time for reproductive success. Early physiological studies revealed that environmental signals such as day length and temperature could alter the timing of the transition so that flowering occurs at the appropriate time. The underlying molecular components of floral inductive pathways are being elucidated at present, largely through the analysis of flowering time genes in the small model plant *Arabidopsis thaliana*. Although these studies show that diverse plant species share parts of the regulatory pathways defined in *Arabidopsis*, whether

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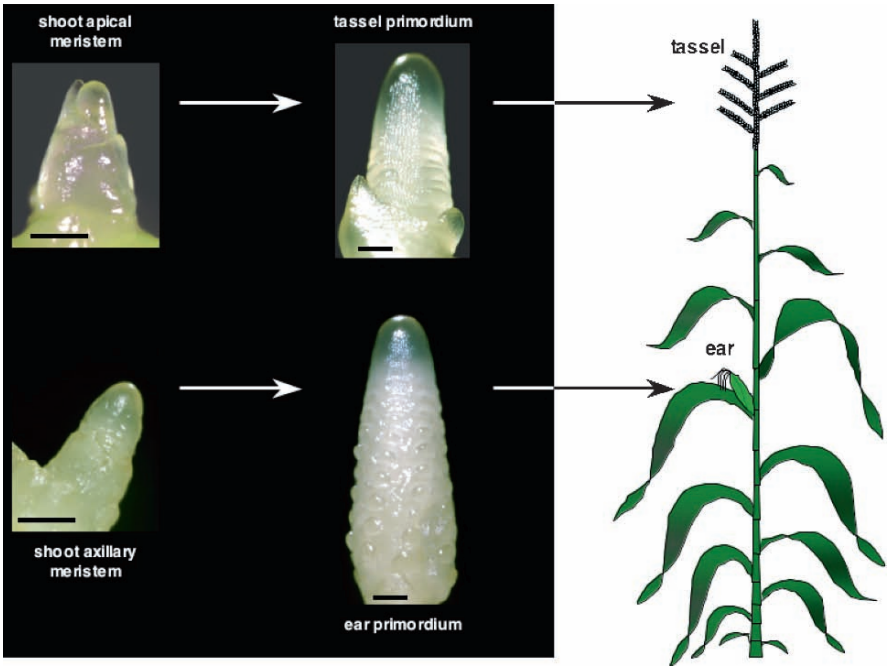

Fig. 1 Maize vegetative and reproductive meristems. The floral transition occurs when the vegetative shoot apical meristem switches to reproductive growth to become the tassel primordium and the upper axillary meristems transition to become ear primordia. Both apical and axillary primordia develop into the male inflorescence (tassel) shedding pollen at the same time the female inflorescence (ear) exserts silks. Scale bar = 200 μm

all components of these regulatory pathways exist in other plants is not apparent. Therefore further analysis of floral regulatory mechanisms in other species is required to define common pathways as well as uncover unique regulatory elements that may have evolved to accommodate particular environmental conditions and species-specific physiologies.

In maize, the floral transition is characterized by the cessation of leaf formation, the elongation of the SAM and the adoption of an inflorescence identity to create the tassel primordium (Fig. 1). A similar transition occurs later in time with the conversion of several axillary meristems into ear primordia. Schmidt and Vollbrecht describe the process of inflorescence formation in more detail in the Maize Development section, Chap. 2. Here, we focus on current understanding of the genetic and molecular events underlying floral induction in maize and some of the physiological changes associated with this key developmental process. Research on the maize floral transition has not received as much attention as other species; however, recent studies indicate that, while maize shares some common regulatory features with other model plants, it also employs unique components in a genetic network that controls flowering.

1.1 Teosinte: An Obligate Short-Day Plant

Domesticated maize is derived from a type of teosinte (*Zea mays* ssp. *parviglumis*), a sub-tropical, wild grass species that originates in southwest Mexico (Doebley, 2004). Although modern maize and teosinte appear quite dissimilar from each other, most of these morphological distinctions can be traced to a handful of genetic differences (Beadle, 1939). Relevant to this chapter, one major difference between teosinte and maize grown in more northerly latitudes is that modern maize flowers after making a particular number of leaves, regardless of photoperiod, whereas teosinte requires short day (SD) photoperiods to induce flowering. That is, unlike its tropical ancestor, temperate maize is primarily photoperiod insensitive and some varieties are day-neutral (DN). The discovery that some plants require a defined photoperiod to induce flowering follows the work of Garner and Allard (1920) with tobacco. Inspired by these studies, Emerson (1924) performed the first analysis of photoperiod effects on teosinte and tropical maize. He showed that teosinte would flower several months early if given short day treatments (10 h day/14 h night), and would only flower in mid- to late October (in Ithaca, NY) if untreated, presumably because the shorter days of September trigger floral inductive signals.

Therefore, as early Native American farmers migrated to higher latitudes, they selected for maize that is less dependent on short-day photoperiods to flower. This is a necessity given that tropical maize grown at 40° N latitude or higher will flower in October, and therefore almost certainly will be killed by frost before seed set. The question arises as to what genetic changes in maize were selected in the shift from photoperiod dependency to day neutrality. Specifically, were mechanisms common in other species modified to permit day-neutral flowering, or was a novel inductive mechanism co-opted for this purpose?

2 Breeding for Flowering Time

2.1 Quantitative Flowering Time Variation

Modern maize cultivars have been selected for a wide range in flowering time variation, allowing for adaptation to cultivation from short growing seasons at high latitudes to long growing seasons in tropical and subtropical climes. The earliest flowering maize variety, Gaspé Flint, reaches reproductive maturity, shedding pollen and exserting silks, in as few as 30–35 days after planting (Fig. 2). The floral transition in Gaspé Flint typically occurs within 7–10 days of germinating, with mature plants producing 7–9 leaves at maturity (Muszynski, unpublished observations). In contrast, late tropical varieties may require 4 months or more to flower, taking advantage of the longer growing season. Most U.S. Corn Belt lines flower between 75 and 120 days, with each line optimized to balance the extent of vegetative growth and duration of grain fill to its adapted geographic location.

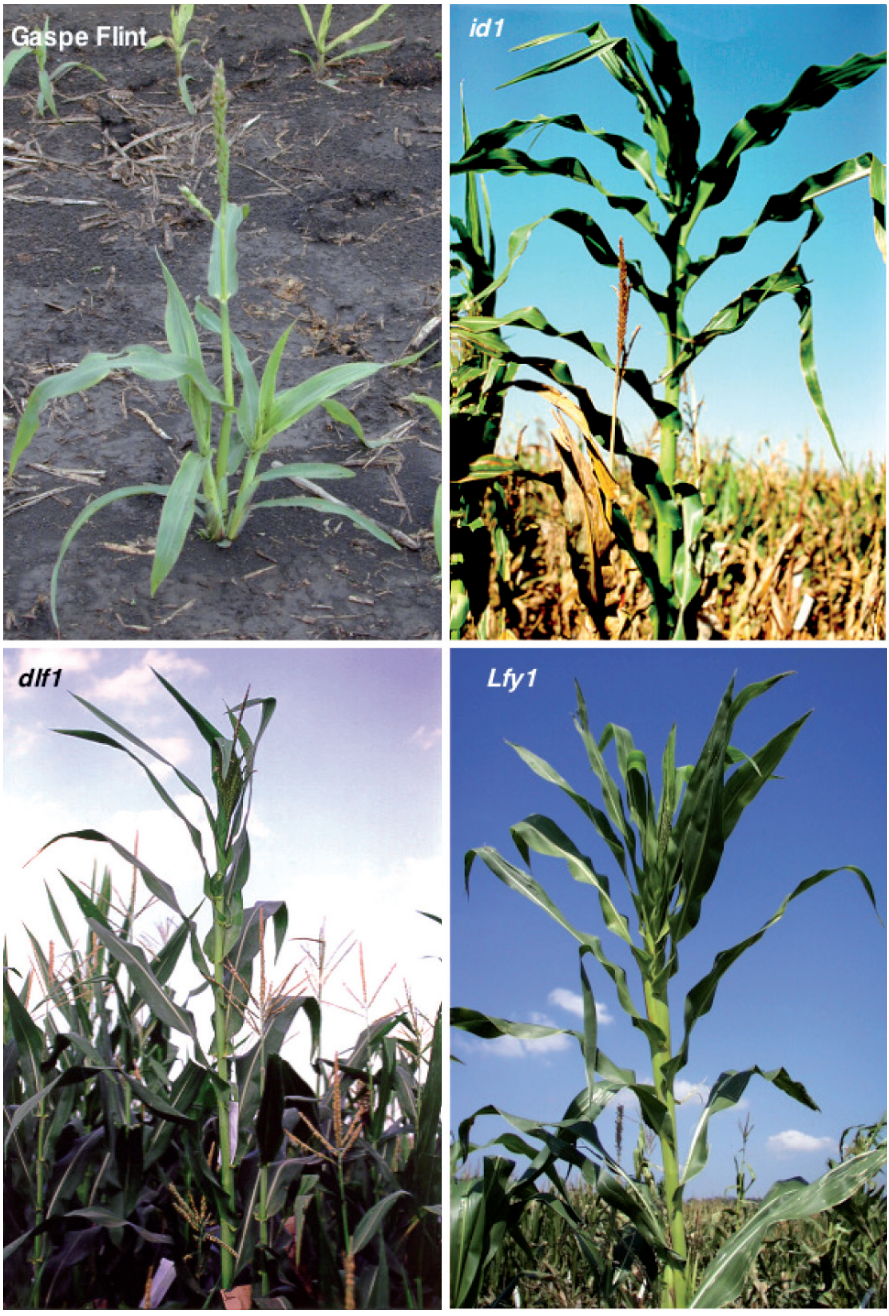

Fig. 2 Examples of maize flowering time variants. Gaspé Flint is an extremely early flowering variety, *indeterminate1* (*id1*) is an extremely late flowering recessive mutant, *delayed flowering1* (*dlf1*) is a moderately late flowering recessive mutant and *Leafy* (*Lfy*) is a moderately late flowering dominant mutant. All late flowering mutant plants produce more leaves and are taller than wild type siblings

Maize breeders have utilized the diversity in flowering time to study and manipulate this quantitatively inherited trait. Genetic mapping studies of quantitative trait loci (QTL) for flowering time indicate that this trait is controlled by the combined action of a limited number of loci with large effects and many loci with small effects (Veldboom et al., 1994; Austin et al., 2001; Chardon et al., 2004, 2005). Using near isogenic lines (NILs) developed from backcrossing Gaspé Flint into N28, a standard maturity inbred, and selection for early flowering, two QTL were identified on chromosome 8 termed *vegetative to generative transition1* (*Vgt1*) and *Vgt2* (Vladutu et al., 1999; Salvi et al., 2002). Both loci specifically affect the floral transition, with alleles from the Gaspé Flint parent mediating an earlier transition, leading to fewer leaves produced and days to shed pollen. Recent advances in positional cloning in maize allowed for the molecular isolation of the *Vgt1* locus and suggest a possible hypothesis for its mode of action (Salvi et al., 2007) (see below). A number of QTL mapping studies have detected flowering time loci on most of the maize chromosomes, with repeated identification of single large effect loci on chromosomes 1 and 9 and two large effect loci on chromosomes 8 and 10 (Chardon et al., 2004). Such results suggest that numerous genes participate in the floral transition and subsequent developmental processes regulating inflorescence development that affect the timing of pollen shed and silk exsertion. Future studies are required to define the molecular determinants underlying each QTL and dissect the role each plays in regulating flowering time.

2.2 QTL Corresponding to Specific Genes

Association genetic analysis offers a complementary approach to bi-parental mapping for the identification of loci controlling quantitatively inherited phenotypes. Utilizing the vast range of phenotypic variation found in diverse maize germplasm, association genetic analysis correlates molecular polymorphisms within candidate gene sequences to quantitative phenotypic variation. In a survey of 92 diverse inbred lines, sequence variation at the *dwarf8* (*d8*) locus, encoding a negative regulator of gibberellic acid (GA) signaling, was associated with quantitative variation for flowering time and plant height. Key to this result was the accurate estimation of population structure within this set of inbred lines (Thornsberry et al., 2001). Additionally, although linkage disequilibrium usually decays rapidly in maize, significant disequilibrium was detected for several polymorphisms within the *d8* gene, suggesting that one or more of these polymorphisms are responsible for the flowering time variation. Finally, the distribution of nonsynonomous polymorphisms within *d8* suggests that this locus has been under selection. Similar studies with a larger set of European inbreds and maize land races confirmed sequence polymorphisms in *d8* are associated with variation for flowering time in that germplasm and may have led to the adaptation of tropical maize to a more temperate climate (Camus-Kulandaivelu et al., 2006).

The variation in activity level of regulatory genes can have profound effects on development that may be a target of selection. Associations with flowering time and other quantitative traits linked with maize domestication were correlated with differences in gene copy number of the maize *FLORICAULA/LEAFY* homologous genes *zfl1* and *zfl2* (Bomblies and Doebley, 2006). Increasing functional copies of *zfl1* consistently showed earlier flowering as measured by total leaf number, a direct measure of the timing of the floral transition, as well as a modest but significant decrease in the number of days to pollen shed and silk exsertion. On the other hand, increasing the number of active copies of *zfl2* is associated more with changes in plant architecture and inflorescence traits, such as the number of ears or lateral branches per plant and the number of kernel rows per ear. As more flowering time genes are identified (discussed in more detail in Sect. 4), investigations using various statistical methodologies will identify the chromosomal regions and loci that underlie the quantitative inheritance of flowering time in diverse and elite germplasm. Such information will enable direct and precise selection for the determinants of flowering time variation using molecular breeding to modulate this trait in germplasm enhancement programs.

3 Long Distance Floral Inductive Signals

Early floral transition studies focused on the physiological changes associated with flowering and the signals that initiate the conversion of the shoot apex from vegetative to reproductive growth. Overall these studies established several key points regarding the transition (reviewed in Bernier and Perilleux, 2005). First, leaves are the source of the floral inductive signals; whether driven by environmental signals, such as photoperiod, or by endogenous signals, such as plant size or age. Second, the floral inductive signal, sometimes referred to as "florigen," is transmitted through phloem tissue to the shoot apex to cause flowering. Finally, the shoot apex, where the SAM resides, must be competent to receive the florigenic signal in order for the transition to occur. That is, maize must pass through a juvenile phase that is incapable of flowering, and into an adult phase that is competent to perceive the floral inductive signal (Poethig, 1990). For most temperate inbreds the juvenile phase consists of the first five to seven leaves. Evidence supporting the unresponsiveness of the juvenile apex to florigenic signals is based on *in vitro* culturing of shoot apices (Irish and Nelson, 1991). These experiments also gave the first clue that, like most physiological experiments with diverse plant species, maize leaves are the source of florigenic signals and the ability of maize to flower is not intrinsic to the shoot apex (Irish and Jegla, 1997).

A key tenet of the original florigen hypothesis is that floral inductive signals are universal and should act in the same way in diverse plants. However, until recently there was little evidence as to the biochemical nature of florigen. Now, recent discoveries, as described in Sect. 4, suggest that the movement of a small protein from leaf to apex through the phloem may act as a florigen. These studies of long

distance flowering signals involved plants whose flowering is greatly accelerated by inductive photoperiods such as *Arabidopsis* and rice. Whether a similar system exists in maize has yet to be shown. Since maize is a day-neutral plant that relies on internal (or autonomous) inductive signals such as leaf number or plant size to induce flowering, does it use the same florigenic signals used by photoperiod sensitive plants? Or does the autonomous pathway co-opt metabolic products of plant growth to signal the floral transition? This is particularly relevant to temperate maize grown at higher latitudes, which relies almost exclusively on endogenous signals to flower. Evidence in other plants suggests that the redirection of assimilates to the shoot apex can affect flowering time (Corbesier et al., 1998; Ohto et al., 2001). However, it is difficult to distinguish cause and effect; for example, do greater levels of sucrose at the apex activate flowering genes, or is the increased accumulation of assimilates required to sustain the higher metabolic activities of the florally-induced apex? Although there is evidence for both possibilities (Bernier and Perilleux, 2005), further evidence is required to define the mechanisms underlying the autonomous signaling pathway.

4 Molecular Mechanisms and Genetic Pathways

4.1 Photoperiod Effects on Flowering

Although most temperate maize varieties are considered to be day-neutral, they do retain some minor sensitivity to photoperiod. For most maize, sensitivity to SD photoperiod-induced acceleration of flowering is inversely correlated with distance from the equator. Under long-day (LD) photoperiods, different inbreds will flower after a genetically determined number of leaves are produced and growing degree units (GDUs) have accumulated. GDUs or heat units (HUs) are a measure of thermal time calculated from the average daily temperature and are a more accurate measure of flowering time than days alone (Zhang et al., 2005). Short-day (SD) photoperiods condition a minor reduction in leaf number and accumulated GDUs required to flower (Galinat and Naylor, 1951; Tollenaar and Hunter, 1983). The photoreceptor phytochrome is known to play a role in regulating flowering time in photoperiod sensitive species. In maize, a member of the *phytochromeB* (*phyB*) subfamily, *phyB2*, has been shown to play a predominant role in repressing flowering under LD and SD photoperiods (Sheehan et al., 2007). A naturally occurring deletion allele of *phyB2* was found in the early flowering Northern flint inbred F2. A similar deletion allele was found in many of the early flint lines, suggesting this mutation contributes to early flowering in this germplasm. In extreme cases, such as teosinte and tropical maize, which have an absolute SD requirement to induce flowering, the photoreceptors and the circadian clock mechanisms are intact. However, the molecular components of this photoperiod induction pathway have yet to be elucidated.

4.2 Maize Flowering Time Mutants

Unlike *Arabidopsis*, where more than 20 mutants with specific effects on flowering time have been described, relatively few mutant loci in maize are known that have a discrete effect in altering flowering time. However, as described above in Sect. 2, numerous QTL have been identified that are associated with altered flowering time (Chardon et al., 2004). The first mutation described to have a striking effect on maize flowering time, *indeterminate,* was discovered by Singleton (1946). Homozygous loss-of-function *indeterminate* mutants flower extremely late and often exhibit aberrant floral morphology, such as the absence of ears and reversion to vegetative growth (Galinat and Naylor, 1951) (Fig. 2). The *indeterminate* gene (later designated *indeterminate1* [*id1*]) was isolated by transposon tagging and found to encode a zinc finger protein (Colasanti et al., 1998). Subsequent analysis showed that ID1 protein is localized to nuclei and is able bind DNA *in vitro*, suggesting that ID1 has a role in regulating the transcription of other genes that control flowering time in maize (Kozaki et al., 2004; Wong and Colasanti, 2007), although the identity of ID1 target genes have yet to be established. Comparative genomic analysis suggests that the *id1* floral induction pathway may be unique to monocots, as no clear *id1* homolog is present in the *Arabidopsis* genome (Colasanti et al., 2006).

Two other mutations have been described which have discrete effects on maize flowering time; *delayed flowering1* (*dlf1*) and *Leafy* (*Lfy*) both postpone the floral transition, leading to late flowering (Shaver, 1983; Neuffer et al., 1997) (Fig. 2). *Leafy* (unrelated to the *Arabidopsis LEAFY* gene or similar homologous genes) is a dominant, late flowering mutation which increases the number of leaves on mutant plants, specifically between the uppermost ear and tassel. Most normal maize plants produce 5–7 leaves between the uppermost ear and the tassel, while mutant *Lfy* plants can have 9–15 or more leaves above the uppermost ear, depending on genetic background. The *Lfy* mutation can delay flowering by 10–20 days and switches inflorescence maturation such that mutant plants exsert silks prior to shedding pollen. This effect can become so pronounced that silks on *Lfy* mutant plants senesce before pollen is shed, thereby preventing self-pollination (Muszynski, personal observation). Little is known about the morphological and developmental aspects of this dominant mutation and it has yet to be molecularly isolated. Although relatively uncharacterized from a genetic perspective, it has been used to a modest degree in maize breeding programs in Canada to increase leaf biomass as a means to improve yield (Dijak et al., 1999; Andrews et al., 2000; Costa et al., 2002; Subedi and Ma, 2005a, b; Subedi et al., 2006).

Positional cloning and association mapping recently pinpointed the molecular position of *Vgt1,* a major flowering time QTL on chromosome 8, to an intergenic region ~70 kb upstream of an *APETALA2* (*AP2*)-like transcription factor, designated *ZmRap2.7* (Salvi et al., 2007). Variation in flowering time in various inbred lines was associated with sequence changes in this putative *cis* element that presumably controls the expression of *ZmRap2.7*. Transgenic analysis showed that

overexpression of *ZmRap2.7* cDNA caused late flowering, whereas down-regulation in antisense maize plants caused early flowering. Similarity to members of a family of *Arabidopsis AP2*-like genes that have a negative effect on flowering suggests a potential orthologous role for *ZmRap2.7* (Aukerman and Sakai, 2003). Further, this finding provides further evidence that some aspects of maize flowering may be controlled by conserved floral regulatory mechanisms.

4.3 Conserved Elements of Maize Floral Induction

Mutations in *dlf1* have a modest effect on flowering, with mutant plants flowering 1–2 weeks later than wild type sibs and also having minor inflorescence alterations. Molecular isolation and characterization of *dlf1* showed that it encodes a protein with homology to basic leucine zipper (bZIP) transcription factors and likely functions through binding DNA (Muszynski et al., 2006). The *dlf1* gene has high sequence similarity and a comparable expression pattern to the *Arabidopsis FLOWER LOCUS D* (*FD*) gene; *fd* mutants also exhibit a late-flowering phenotype. Thus, FD and DLF1 proteins are predicted to share co-orthologous functions. In maize, double mutant analysis indicates *dlf1* is downstream of *id1* activity and, consistent with this result, *dlf1* is misexpressed in *id1* mutants (Muszynski et al., 2006). These data, in concert with expression analysis, provide the preliminary components of a maize flowering time genetic network (Fig. 3). In the proposed model, ID1 protein, perhaps in response to intrinsic attributes such as leaf number or assimilate levels, regulates the production or transmission of floral inductive signals in leaves. Transmission of the signal to the shoot apex activates *dlf1* either transcriptionally or posttranscriptionally. The model network predicts several targets downstream of *dlf1*, including an early target (*x*) which feedback regulates *dlf1* expression and one or more *ZMM* MADS-box gene (Muszynski et al., 2006). Because both single *id1* and *dlf1* mutants and the *id1*/*dlf1* double mutant all flower eventually, an alternate floral induction pathway has been proposed that functions in parallel to the *id1*-*dlf1* pathway (Fig. 3). The components of the alternate pathway are not known, but data supports the idea that it converges with the *id1*-*dlf1* module downstream of *dlf1*. Likewise, the identity of the MADS-box gene(s) downstream of *dlf1* has not been unambiguously determined, but recent studies point to *ZMM4* and *ZMM15* as likely candidates for maize floral meristem identity genes (Danilevskaya et al., 2008). In fact, *ZMM4* and *ZMM15* cluster as the closest maize homologs within the *Arabidopsis FRUITFUL* (*FUL*) clade (Malcomber et al., 2006). In *Arabidopsis*, *FUL*, along with its paralogs *CALIFLOWER* (*CAL*) and *APETALA1* (*AP1*) have redundant roles in specifying floral meristem identity downstream of the floral activators *FD* and *FLOWER LOCUS T* (*FT*) (Abe et al., 2005; Wigge et al., 2005). The extent of overlap versus distinctiveness between the *Arabidopsis* and maize floral networks is a fruitful area for future research.

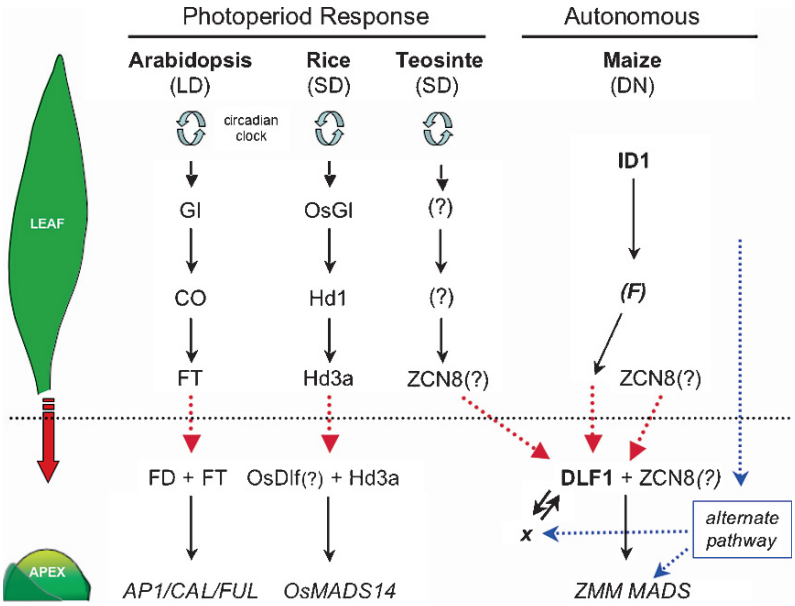

Fig. 3 Model comparing key floral induction and floral meristem identity genes in maize, rice and Arabidopsis. The circadian clock in Arabidopsis and rice drives signaling under inductive photoperiods to activate key leaf-expressed flowering time genes (GI and CO or OsGI and Hd1), which in turn activate expression of genes encoding the mobile floral stimulus (FT or Hd3a). Genes downstream of the circadian clock are unknown in Teosinte and designated by (?). The mobile floral stimulus protein transits from leaves through the phloem (red dotted line) to the shoot apex where it interacts with apex-expressed floral induction genes (FD or OsDlf1?) to activate expression of floral meristem identity MADS-box genes (AP1/CAL/FUL or OsMADS14). In day-neutral (DN) maize, a leaf-derived autonomous signal activates id1; the ID1 protein regulates the transmission or production of a mobile "florigenic factor" (F). The relationship between F and ZCN8, a possible maize FT ortholog, is not known. Signals downstream of F are transmitted to the shoot apex and regulate dlf1 expression or DLF1 activity. Interaction of DLF1 and ZCN8 presumably activates downstream targets x and the ZMM MADS-box floral identity genes. Redundant inductive signaling through an id1-independent alternate pathway converges downstream of dlf1 to activate x and the ZMM MADS genes too. The position of genes marked by (?) is speculative and the alternate pathway (blue dotted arrows) is hypothetical

4.4 Molecular Components of Maize Florigenic Signals

Recent studies suggest that the RAF kinase inhibitor-like *FT* gene of *Arabidopsis* and its co-orthologs in rice (*Heading date3a, Hd3a*) and cucurbits comprise a key mobile component of the leaf-derived floral stimulus in these species (Corbesier et al., 2007; Lin et al., 2007; Tamaki et al., 2007). Thus the long sought molecular identity of the phloem-mobile florigen may be partially solved. The *Arabidopsis FT* gene product, activated by the circadian-controlled CONSTANS (CO) transcription factor in the leaf, has been shown to migrate from the leaf via the phloem to the shoot apex where it interacts with the FD transcription factor. The FT-FD complex then directly

activates downstream floral meristem identity genes that mediate flowering (Fig. 3). Whether a similar CO-FT regulatory module exists in maize has not been shown. Similarly, a mobile maize FT-related protein has not been described. However, the maize genome does contain at least 25 *FT*-like and related paralogous *TERMINAL FLOWER* (*TFL*)-like genes, designated *ZCN* (for *Zea mays CENTRORADIALIS*), that could encode candidates for a conserved florigenic protein (Danilevskya et al., 2008, *Plant Physiology*, in press). Determining which family member(s) participate in florigenic signaling will require functional analysis of each gene but will be crucial for further elaboration of the maize genetic flowering time network.

One piece of the maize florigen puzzle that remains to be solved is the role of *id1* in controlling maize flowering. Like *CO*, *id1* is expressed and acts in leaves, specifically in immature, developing leaves (Colasanti et al., 1998). Therefore the ID1 transcription factor is believed to regulate the synthesis or facilitate the transmission of a leaf-derived mobile floral stimulus. In one scenario, ID1 may directly regulate expression of the maize equivalent of *Arabidopsis* FT; a possible candidate is the FT-related *ZCN8* gene (Fig. 3) (Danilevskya et al., 2007, *Plant Physiology*, in press). Alternatively ID1 may control the synthesis or movement of a yet-to-be-identified leaf-based florigenic factor ("F" in Fig. 3). Evidence suggests that the ID1 protein itself is localized and acts in the leaf and does not migrate from leaf to apex (Wong and Colasanti, 2007). Further, although *id1* defines a moderately sized zinc finger gene family found in all higher plants, the absence of a clear functional *id1* equivalent in distantly related plants suggests that *id1* may have a unique role in controlling flowering in maize and perhaps closely related species, such as sorghum and rice (Colasanti et al., 2006). Expression profiling of the molecular differences between normal maize and late-flowering *id1* mutants revealed a small number of downstream target genes that could be associated with long distance signaling (Coneva et al., 2007). One intriguing finding of this study is that a large proportion of the differentially expressed genes in *id1* mutants have roles associated with photosynthesis and C4 carbon assimilation. This preliminary finding suggests a possible link between assimilate partitioning and floral induction in maize. Further research into this connection, and the identification of direct targets of *id1* should shed more light on a flowering time regulatory network that may be unique to maize.

Extensive expression profiling, along with the release of a completed maize genome sequence will allow the identification of all putative flowering time genes from diverse plant species. This information, in combination with reverse genetics and refined QTL analysis, will eventually lead to a comprehensive understanding of the molecular components controlling maize flowering.

5 Future and Perspectives

Understanding the mechanisms that control the transition to flowering in maize may provide a glimpse into the evolution of a new regulatory function. The extremely rapid evolution of modern temperate maize from tropical teosinte

necessitated selection for flowering under non-inductive photoperiods that maize progenitors depend on to induce flowering. Current molecular evidence suggests that maize utilizes components of the floral transition regulatory pathway common to other plant species, but also may have developed a regulatory pathway that is unique to maize and perhaps other agronomically important grasses. The transition to flowering is a central event in the life of all higher plants; hence there are probably many inputs from both environmental and endogenous signals that can be co-opted to optimize flowering time. Most likely both endogenous (autonomous) and environmental mechanisms exist in all plants, and the balance can shift, depending more on one pathway or the other, conditioned by the geographic location and climate where a plant grows. Therefore, studies of maize flowering should reveal key pieces to the puzzle of what cause plants to flower that may not be apparent from studies of other model plants. Thus, elucidating the maize flowering gene network will be fundamental to translating knowledge from model systems to plants of economic importance.

In a more practical sense, understanding the molecular mechanisms underlying the control of flowering can be directly applied to improve crop productivity. Maize breeders select for maturities exquisitely adapted to different geographical locations to flower as late as possible, but early enough to assure that yield is maximized. Inbreds developed for one area of adaptation are rarely used in another due to limitations of flowering, grain fill or kernel maturation. Thus, breeding between inbreds with different maturities is uncommon, leading to a reduction in germplasm diversity and impeding the transfer of superior alleles to new lines. A comprehensive understanding of the genetic determinants regulating flowering would enable breeders to manipulate maturity through molecular breeding or transgenic methods and in this way increase the diversity of germplasm utilized in a selection program.

The intimate connection between flowering time and assimilate partitioning has a direct impact on maize yield. However, a plant that flowers too early produces fewer leaves and thus has less ability to capture sunlight and produce assimilates. It may be possible to characterize genes that control the rate of leaf production in order to develop maize that initiates leaves more quickly before flowering. A comprehensive understanding of the genetic and molecular mechanisms underlying flowering could lead to accentuated breeding where flowering time can be further fine-tuned to maximize yield. Further, a more sophisticated biotechnological approach could involve the creation of maize harboring floral transition genes under the control of promoters that allow the farmer to apply an external stimulus to promote or retard flowering in response to unforeseen but imminent abiotic stresses such as drought or prolonged cold periods that can have a severe impact on yield. Another future challenge will be to uncouple the floral transition from assimilate redirection to prolong the grain fill period. In any case, being able to adjust flowering to maximize yield would make maize an even better food, feed and fuel source.

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The Maize Male Gametophyte

Patricia A. Bedinger and John E. Fowler

Abstract The maize male gametophyte is a biologically complex component in seed production, and is of great interest for both practical and scientific reasons. In the anther of the stamen, a well-characterized series of developmental events produces the haploid pollen grain, which is released for pollination of the silk and subsequent fertilization of the embryo sac. Use of biochemical, cell biological, and genetic techniques has provided insight into the mechanisms underlying these developmental changes. Here, we provide a basic description of these events. We also highlight recent results that inform our understanding of male gametophytic development and function, as well as features of maize that make it an attractive alternative to other plant models for investigating the male gametophyte.

1 Introduction

Although the male gametophyte occupies a mere fraction of the angiosperm life cycle, it is of immense interest for both scientific and practical reasons. On the practical side, seed production (i.e., production of the next generation) depends on a functional male gametophyte achieving fertilization. This property explains why the male gametophyte has also been central in methods aimed at controlling fertility, e.g., through cytoplasmic male sterility (Schnable and Wise, 1998; Pelletier and Budar, 2007). More recently, propagation of transgenic plant cultivars has raised concerns about pollen spread and its role in introducing transgenes into nontransgenic populations (Watrud et al., 2004), or in creating unanticipated consequences by spreading transgene products in the environment (Rosi-Marshall et al., 2007). In addition, the male gametophyte provides ample opportunity to address questions of great biological interest. For example, in microgametogenesis, interaction between cell types results in deposition of a unique and complex cell wall, the pollen exine, which ultimately provides the first point of adhesion with the female (Blackmore et al., 2007). The pollen tube is the best-studied example of polarized cell growth in plants, exemplifying the use of cellular machinery and signaling for rapid and oriented growth (Cole and Fowler, 2006; Krichevsky et al., 2007). Notably, cell-cell

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communication between the male gametophyte and the female (both reproductive sporophytic and gametophytic cell types) is likely to be ongoing, as the male traverses from stigma to embryo sac. This communication provides the basis for such phenomena as self-incompatibility (Takayama and Isogai, 2005) and species specificity in fertilization (Swanson et al., 2004). Finally, the male gametophyte may provide a key point for selection of evolutionarily important traits (Mulcahy et al., 1996; Bernasconi et al., 2004), including those that lead to speciation (Swanson et al., 2004).

Maize is a valuable model for studying the male gametophyte, for biological and practical reasons. This chapter provides a basic description of male gametophyte development and function in maize, including steps occurring during formation of male meiocytes in the anther. (For a more detailed discussion of meiosis itself, see the chapter by Cande et al. in Vol. 2). We highlight some advantages of using maize as a model, and we provide an overview of results that advance our understanding and raise questions for future research.

2 Overview of Male Gametophyte Development

For simplicity, male gametophyte development can be divided into three phases, illustrated in Fig. 1: Premeiotic development of the microsporocytes and associated cells (a and b); microsporogenesis (c and d) and microgametogenesis (e and f); and progamic (postpollination) development (g and h). The terms microsporogenesis (development of the microspore) and microgametogenesis (development of the pollen grain) are used inconsistently in the literature, with differences in whether and when these phases overlap. Our usage in this chapter is that microsporogenesis ends upon the first pollen mitosis, at which point microgametogenesis begins. In maize, each staminate spikelet has two flowers, each containing three stamens, with the upper flower slightly more developmentally advanced than the lower one (Kiesselbach, 1949, and Chap. 2 of this volume). Male gametophytes, i.e., pollen, develop within the anthers (also called microsporangia), specifically within each of the four anther locules of each stamen. Pollen development in maize has been well-described at the cytological level (Beadle, 1932; Cheng et al., 1979; Albertsen and Phillips, 1981; Chang and Neuffer, 1989; Bedinger and Russell, 1994; Greyson, 1994; Sheridan et al., 1999) and is briefly summarized here (Fig. 1).

Premeiotic development within each anther locule follows a characteristic pattern of cell division and differentiation, first generating a central set of archesporial cells and a surrounding layer of primary parietal cells (Fig. 1a). The archesporial cells further differentiate to produce the pollen mother cells that will undergo meiosis; surrounding these is the tapetum, which is derived from the primary parietal layer (Fig. 1b). The pollen mother cells then undergo meiosis to produce the microspores, and the tapetal cells provide components for the pollen cell wall via secretion

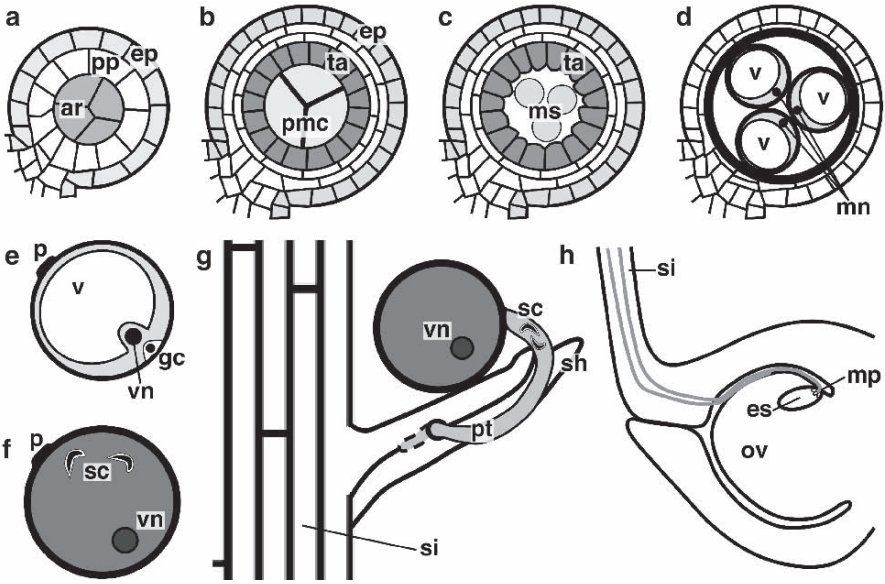

Fig. 1 Schematic of male gametophyte development, as detailed in the text. Cross-section of an anther locule: **a** Premeiosis, early stage; **b** Premeiosis, late stage, with all cell layers present; **c** Postmeiosis, early stage microspore maturation; **d** Late stage microspore maturation, with large vacuoles. In **a**–**d**, lighter shades correspond to more highly vacuolated cells. **e**, **f** Single gametophytes during microgametogenesis: **e** Bicellular gametophyte, after the first pollen mitosis; **f** Mature tricellular pollen, after the second pollen mitosis. **g**, **h** Progamic development: **g** Pollen tube germination on the silk; **h** Pollen tube growth (gray lines) near the ovule (integuments not shown). Labels: archesporial cells (*ar*); primary parietal layer (*pp*); epidermis (*ep*); pollen mother cells (*pmc*); tapetum (*ta*); microspores (*ms*); microspore nucleus (*mn*); vacuole (*v*); pore (*p*); vegetative nucleus (*vn*); generative cell (*gc*); sperm cells (*sc*); pollen tube (*pt*); silk hair (*sh*); silk (*si*); ovule (*ov*); micropyle (*mp*); embryo sac (*es*)

(Fig. 1c). The microspores enlarge, and each develops a central vacuole. Meanwhile, the surrounding sporophytic cell layers collapse (Fig. 1d). The asymmetric first pollen mitosis produces a small generative cell positioned adjacent to the pollen wall, and a large vegetative nucleus (Fig. 1e). Mature pollen contains starch granules and two sperm cells derived from the generative cell (Fig. 1f). Following anther dehiscence, pollen can land on a silk hair and germinate a pollen tube that will penetrate the underlying cell wall, and subsequently grow between cells toward one of the two transmitting tracts in the center of the silk (Fig. 1g). Pollen tube growth occurs along either tract toward the ovule at the base of the silk, and eventually across the ovary cavity to the micropylar region (Fig. 1h). Penetration through the micropyle brings the pollen tube to the embryo sac, where the tube ruptures and releases its two sperm cells for double fertilization. Each of these developmental stages is described in more detail below.

3 Premeiotic Development

The pattern of anther development in maize is typical of that found in most monocots (Davis, 1966). The anther primordium can be roughly described as a lobed rectangle (Fig. 2a). A large hypodermal cell in the corner of each lobe (arrow, Fig. 2a) undergoes a periclinal division to form a primary parietal cell adjacent to the epidermis and an archesporial cell to the interior (Fig. 2b). As the archesporial cells proliferate and differentiate into sporogenous cells, an additional periclinal division of the primary parietal cells results in the subepidermal endothecium and a secondary parietal layer of cells (Fig. 2c). The secondary parietal cells undergo a final periclinal division to form the two interior anther wall layers, the middle layer and the tapetum (Fig. 2d). Each of the four anther wall layers expands by anticlinal division and differentiates. The epidermis develops distinctive chemically resistant epicuticular ridges on the surface of each anther lobe (Fig 2e, box); the epidermal

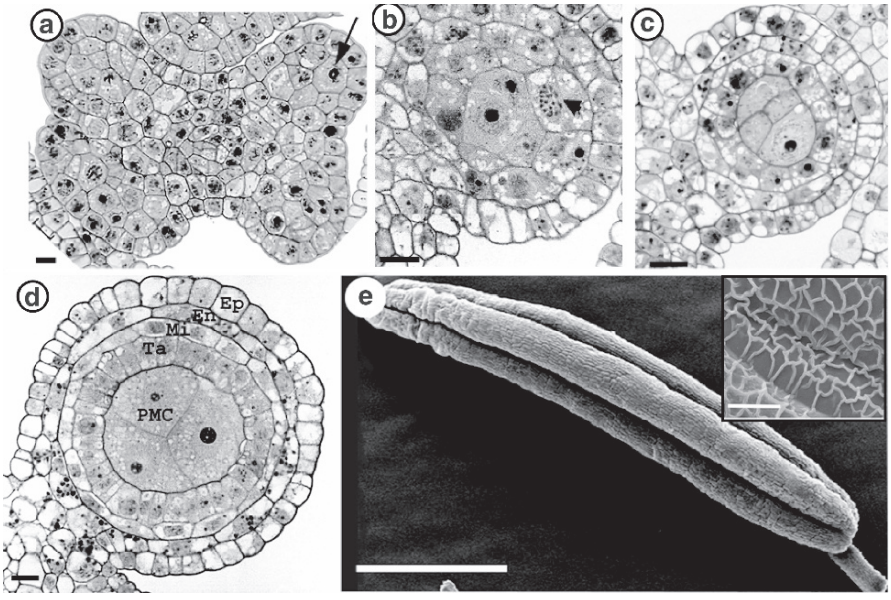

Fig. 2 Anther development in maize. **a** Cross-section, anther primordium. Arrow indicates large hypodermal cell that will undergo periclinal division. **b** A single developing anther locule, after the first periclinal division to form the primary parietal layer and the archesporial cells. Arrowhead indicates the beginning of the second periclinal division of the primary parietal layer. **c** Anther locule showing large interior sporogenous cells with dense cytoplasm and three anther walls. The secondary parietal cells will undergo another periclinal division to form the final two anther wall layers. **d** At this stage the four distinct anther wall layers are established, with pollen mother cells in the center of each anther locule. *Ep*; epidermis, *En*; endothecium, *Mi*; middle layer, *Ta*; tapetum, *PMC*; pollen mother cells. **e** Scanning electron micrograph of mature maize anther. Insert shows epicuticular ridges on the epidermis. Bars in **a**–**d**, and insert of E = 10 μm, bar in E = 1 mm. Modified from Chaubal et al. (2003)

cells between the anther lobes lack these ridges (Cheng et al., 1979). The subepidermal endothecium near the anther tip acquires secondary cell wall thickenings late in pollen development that are thought to be important in dehiscence. The middle layer remains fairly undifferentiated at the cytological level throughout pollen development. The tapetum, the innermost anther wall layer, undergoes a series of dramatic differentiation events. The tapetum of maize is the parietal secretory type (Pacini et al., 1985; Pacini, 1997). As meiosis progresses, tapetal cells develop a dense cytoplasm filled with secretory machinery. They become binucleate and polyploid (D'Amato, 1984), and lose their normal cellulosic cell walls on the locular and tangential sides. Channels between tapetal cells develop as the tangential cell walls are lost (Perdue et al., 1992). The tapetum is an ephemeral tissue that becomes crushed by the enlarging pollen after microspore mitosis, and undergoes programmed cell death (Wu and Cheung, 2000) prior to anther dehiscence. Many components of the pollen coat, including the *Zm*XYN1 xylanase (described in Sect. 7, Bih et al., 1999), are derived from the degenerating tapetum.

4 Microsporogenesis and Microgametogenesis

Microsporogenesis encompasses the developmental events that lead to the production and maturation of haploid microspores. Within the developing anther, archesporial cells divide by mitosis and differentiate into the male meiocytes (also known as microsporocytes, microspore mother cells or pollen mother cells). The male meiocytes are initially connected by cytomictic bridges, which are thought to play a role in synchronizing meiosis (Heslop-Harrison, 1966). These bridges become closed as the primary cellulosic cell wall (Fig. 3, Precallose) is replaced with a special cell wall composed of callose (β−1,3-glucan) (Owen and Makaroff, 1995). Callose is first deposited in the center of the locule and eventually surrounds each microsporocyte (Fig. 3, Central Callose and Callose). As described in detail by Cande et al. in Vol. 2, meiosis results in the formation of a tetrad of haploid microspores held together by an exterior callose wall (Fig 3, Tetrads). Soon thereafter a β-1,3-glucanase (callase) secreted from the tapetal cell layer (Steiglitz, 1977) releases each microspore from the callose-enclosed tetrad and free microspores with a central nucleus float within the fluid-filled anther locule (Fig. 3, Young Microspores). Microspore maturation proceeds, with the interplay of both haploid (microspore) and sporophytic (anther wall) tissues. Microspores with a central nucleus enlarge and begin to acquire an outer cell wall, or exine, with deposition of sporopollenin (Fig. 3, Microspores). Multiple small vacuoles are generated (Fig. 3, Multiple Small Vacuoles), eventually fusing into a single large vacuole that fills most of the cell (Fig. 3, Large Vacuole). At this point the microspore nucleus has migrated to a position near the cell wall opposite from the developing pore, which generally faces the tapetum (Chang and Neuffer, 1989).

The first pollen mitosis, a very asymmetric cell division that produces bicellular pollen, marks the beginning of microgametogenesis and coincides with a major

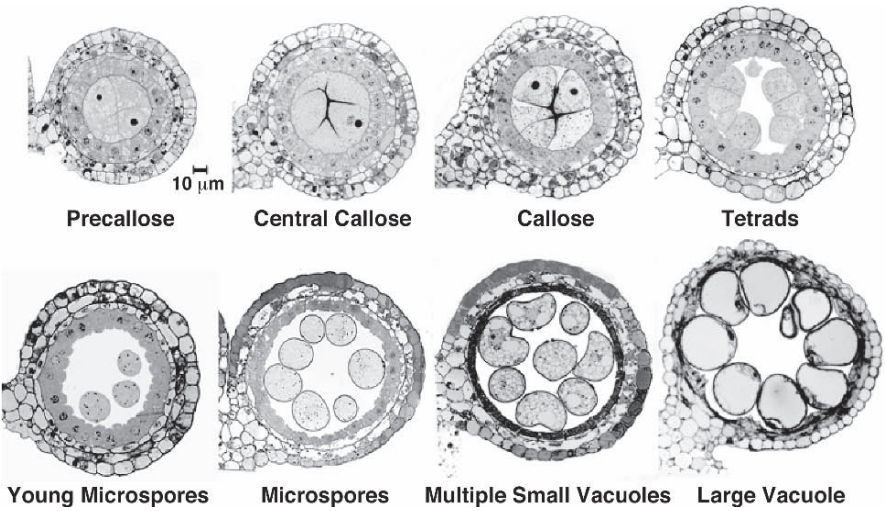

Fig. 3 Microspore development in maize. A single anther locule is pictured for each stage, each of which is described in the text

change in gene expression, as reflected by changes in protein populations (Bedinger and Edgerton, 1990). Bicellular pollen has a large vegetative (tube) nucleus with dispersed, transcriptionally active chromatin, and a smaller generative nucleus with more compacted chromatin (Chang and Neuffer, 1989). The generative nucleus, appressed against the pollen wall, becomes cellularized by an unusual hemispherically shaped phragmoplast and (later) callose-containing cell wall (Herrmann et al., 2006) (Fig. 1e). The small generative cell later migrates into the cytoplasm of the larger "vegetative" cell, with its callose cell wall still detectable by aniline blue staining (Herrmann et al., 2006).

Following the first pollen mitosis, the inner pollen wall, or intine, is formed between the plasma membrane and the exine. The intine is rich in cellulose, pectins and proteins. Starch granules begin to accumulate within the pollen, first in the region of the exine pore, eventually filling the entire pollen grain. The increase in starch content is accompanied by a developmentally specified increase in a number of carbohydrate metabolism transcripts (Datta et al., 2002). Cell volume increases, generating a pollen grain with a final diameter of about 100 μm. Further pollen maturation steps include extensive dehydration, and deposition of a lipid and protein-rich tryphine layer (pollen coat) derived from the tapetum onto the surface of the exine. During this time the callose associated with the generative cell becomes undetectable, and the cell undergoes mitosis, resulting in the formation of tricellular pollen with two sickle-shaped sperm cells (Fig. 6a). A ring-shaped callose-containing structure is detectable in mature pollen, surrounding or adjacent to the exine pore (Herrmann et al., 2006). Intriguingly, a green fluorescent protein (GFP) fusion with the pollen-specific and membrane-associated protein kinase ZmPTI1a shows dynamic co-localization with callose deposition throughout microgametogenesis.

RNAi-induced knockdown of ZmPTI1a-mRNA produces pollen with a strong, but not complete, gametophytically expressed transmission defect, indicating an important role for the protein; however, no specific defect in callose organization or any other pollen structure has been observed (Herrmann et al., 2006).

Dehiscence of the anther to release mature pollen coincides with anthesis (glume opening) and filament elongation. Dehiscence occurs at the anther apex, where the interlocular tissue (septum) separates (Cheng et al., 1979; Keijzer et al., 1996). An initial slit is formed by localized lysis of the inner anther wall and the epidermis in the tip septum. As the anther tip dehydrates, the opening becomes a spherical pore, allowing release of the spirally stacked mature pollen from the anther. The thickened cell walls of the endothecium in this region are likely to play a role in the formation of the anther pore.

5 Mutations That Affect Pollen Development

For decades, mutations have allowed the dissection of biological processes, from biochemical pathways to regulatory gene expression networks to developmental mechanisms. Male-sterile mutants of maize have likewise supplied essential tools for developmental (Loukides et al., 1995; Sheridan et al., 1996, 1999; Chaubal et al., 2000, 2003), molecular (Ma et al., 2007) and biochemical studies (Mo et al., 1992). In addition, the practical use of cytoplasmic male-sterility mutations to reversibly regulate pollen development has had an enormous impact on the production of hybrid seed (Schnable and Wise, 1998). Because cytoplasmic male-sterility has its basis in the mitochondrial genome, it is discussed in the chapter by Newton in Vol. 2. Genic male-sterility mutants can also form the basis of engineered reversible sterility systems (Unger et al., 2002). Thus, the male-sterile mutants of maize are a rich resource for both basic research and agronomic applications.

There are at least 40 different nuclear mutations that specifically cause male sterility in maize (Beadle, 1932; Cheng et al., 1979; Greyson et al., 1980; Albertsen and Phillips, 1981; West and Albertsen, 1985; Morton et al., 1989; Loukides et al., 1995; Chaubal et al., 2000, 2003). A few gametophytic male-sterile mutants that affect pollen development have been identified by screening for 1:1 normal:abnormal pollen (Sari-Gorla et al., 1996, 1997); gametophytic mutants that primarily affect the progamic phase are described in Sect. 7 (below). Most male-sterile mutations are recessive and sporophytic, such that heterozygotes are fully fertile.

An array of maize sporophytic male-sterile phenotypes is displayed in Fig. 4, in the order of their initial effect on anther/pollen development. The mutation with the earliest developmental consequences after anther primordia formation is *msca1* (*male sterile converted anther1*), wherein no archesporial and parietal cells are formed (Chaubal et al., 2003). Rather, the lobe-shaped structures produced in this mutant resemble a ground-state vegetative organ, with numerous epidermal stomata

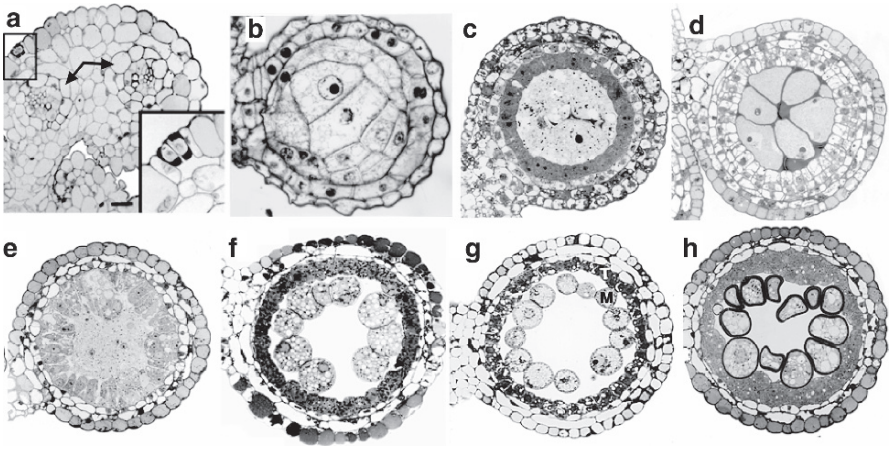

Fig. 4 Representative male-sterile mutants of maize. Single anther locules are shown for each mutant. **a** *msca1*, showing the formation of a vegetative-like organ rather than a normal anther, with none of the normal anther cell layers. Arrows, non-functional vascular strands; box = stoma on epidermal surface. Figure is modified from Chaubal et al. (2003). **b** *mac1*, which contains only primary parietal and archesporial cells. Figure is modified from Sheridan et al. (1999). **c** *ms9*, with densely staining bodies seen in pollen mother cells. **d** *ms23*, showing an extra anther wall layer and no normal tapetum. Figure is modified from Chaubal et al. (2000). **e** *ms8*, showing a syncytium of cells in the place of normal tetrads. **f** *ms25*, with an abnormal accumulation of lipid-rich bodies in tapetal cells **g** *ms26* showing premature vacuolation of tapetal cells. Figure is modified from Loukides et al. (1995). **h** *ms1*, with abnormal exine formation at the young microspore stage

and 1 or 2 strands of non-functional vascular tissues in each locule (Fig. 4a). Plants with the *mac1* mutation (Sheridan et al., 1999) form the primary anther parietal cells and the archesporial cells, but lack the further periclinal divisions that form the other specialized anther wall layers (Fig. 4b). The innermost cells develop into (abnormal) meiocytes and enter meiosis but do not proceed past early Prophase I. It should be noted that in addition to male-sterility, the *mac1* mutant is also partially female-sterile (although meiosis is normal in the female). In the *ms9* mutant, pollen mother cells at the precallose stage contain unusual densely staining bodies prior to meiosis (Fig. 4c), and it is not clear as to whether meiosis is initiated (Greyson et al., 1980). In both *ms23* and *ms32*, there is an extra periclinal division in the anther wall, producing five, rather than the normal four, anther wall cell layers, and there is no normal tapetum (Fig. 4d). Meiosis is initiated in these mutants, but does not proceed past Prophase I (Chaubal et al., 2000).

An unusual male-sterile mutation that affects cellular aspects of tetrad formation is *ms8* (Albertsen and Phillips, 1981). In this mutant normal cross-walls are not formed between meiotic products, leading to the production of a multinucleated syncytium (Fig. 4e). In *ms25*, tapetal cells accumulate abnormally large numbers of lipid bodies (Fig. 4f). A very common phenotype of male-sterile mutants is the premature vacuolation and death of tapetal cells, here represented by *ms26*

(Fig. 4g). Normal exines are not formed on microspore surfaces in either *ms25* or *ms26* (Loukides et al., 1995). In *ms1*, the exines are deposited in an aberrant fashion (Fig. 4h), as described below. The *ms45* mutant also affects cell wall deposition, and provides another clear link demonstrating the importance of tapetal function at this stage. Its corresponding gene encodes strictosidine synthase, a key enzyme in alkaloid biosynthesis, and is specifically induced in the tapetum during the vacuolated stages of microsporogenesis (Cigan et al., 2001).

Several male-sterile mutants affect the development of the exine, which is perhaps the most complex of plant cell walls (Blackmore et al., 2007). Pollen exines are particularly useful in paleobotany studies due to their remarkable chemical resistance and their distinctive ornamentation in each plant group. Maize pollen has an exine similar to that of other grasses, with a uniform outer layer (tectum) of spinules and a single pore (an aperture with a raised annulus and a central disk-like operculum) through which the pollen tube emerges upon germination. The development of the exine has been described in detail in maize (Skvarla and Larson, 1966) and also in wheat (El-Ghazaly and Jensen, 1985, 1986, 1987). The template for exine production (the primexine) is elaborated on the microspore surface while tetrads are still encased in their callose wall. The production of the primexine, which is fibrous and largely composed of polysaccharides (Rowley, 1973), coincides with the appearance of undulations in the microspore plasma membrane (Skvarla and Larson, 1966; El-Ghazaly and Jensen, 1986). As the callose wall of the tetrad is dissolved and microspores are freed into the anther locule, sporopollenin is rapidly deposited on the receptive primexine. Sporopollenin is a chemically resistant polymer of oxygenated aromatic monomers (Dominguez et al., 1999; Ahlers et al., 2000). It is thought that the tapetum produces sporopollenin precursors that can be polymerized either on the microspore primexine, or on small structures called Ubisch bodies (or orbicules) on the locular face of the tapetum.

Maize exine structure can be described as consisting of an outer tectum layer with evenly distributed spinules, a foot layer and columellae in between (Fig. 5a–c). Although several male-sterile mutants, such as *ms25* and *ms26* (Fig. 4f and g), affect pollen exine development due to obvious defects in tapetal function, there are at least two male-sterile mutants that appear to have more specific effects on exine structure (Fig. 5d–i). In *ms20* plants (Clokey and Anderson, 1938) no normal sporopollenin-based, osmiophilic exine is detected (Fig. 5d), and bundles of extracellular autofluorescent material are observed in microspores (Fig. 5e). The outer cell wall structure in *ms20* pollen is very abnormal compared to wild-type (Fig. 5f). In *ms1*, sporopollenin is produced, but it is not deposited on microspores in a regular pattern (Fig. 5g). Rather, there are regions on the surface that have more than twice as much sporopollenin deposited, and other regions that have essentially no exine (Fig. 5h and i). Thus, *ms1*, one of the first male-sterile mutants described (Beadle, 1932), appears to lack a normal primexine receptor complex on the microspore surface. Biochemical analysis of these abnormal microspore exines could be highly informative with regard to either primexine structure or sporopollenin polymerization.

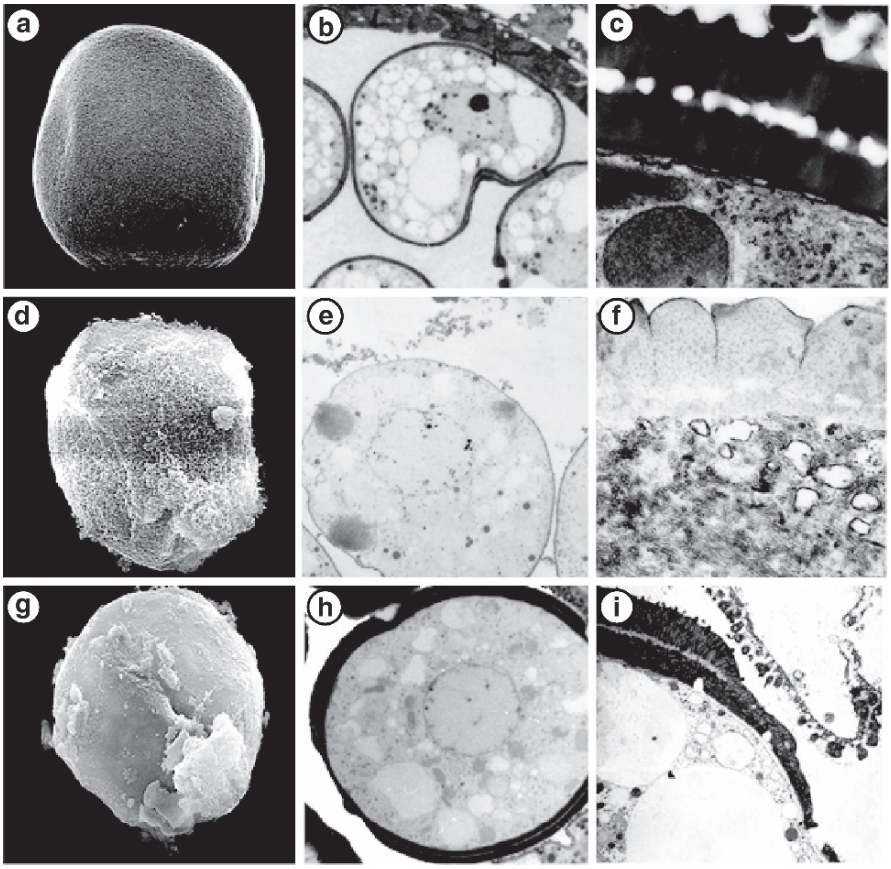

Fig. 5 Exine in normal and male-sterile mutant microspores. **a** SEM of normal maize microspore showing uniform distribution of exine spinules. **b** TEM of normal microspore at multiple small vacuole stage. **c** TEM of normal exine, showing outer tectum, columellae and inner foot layer. **d–f**. Same views of *ms20* microspores. **g–i**. Same views of *ms1* microspores

6 Transcriptomic Changes During Pollen Development

Due to the anther's specialized function and its uniquely differentiated cell types, numerous genes with roles in male gametophyte development and pollen maturation are likely to be expressed specifically in developing and mature anthers. A number of maize anther- and pollen-specific genes have been cloned using either molecular or transposon-tagging approaches (e.g., *ms45,* Cigan et al., 2001), and transgenic transcriptional silencing has been useful in testing some of them for effects on male fertility (Schreiber et al., 2004; Cigan et al., 2005). In addition, these same approaches have identified a few promoters with pollen- or anther-specific activity (e.g., *Zm13,* Hamilton et al., 1992; and *5126,* Cigan et al., 2005). The widely used maize *UBI* constitutive promoter is also active in pollen (Cigan et al., 2001).

In a more global analysis, gene expression was assessed in developing maize anthers using oligonucleotide microarrays with 13,000 sense and 5,000 antisense probes, representing ~25% of predicted maize genes (Ma et al., 2007). Three early developmental stages (mitotic proliferation, premeiosis, and meiotic prophase I) were interrogated with this array for wild-type, *msca1*, *mac1* and *ms23* anthers. About 6800 transcripts were detected in wild-type anthers at all developmental stages, and ~2400 additional temporally regulated transcripts were detected. Not surprisingly, the greatest differences between wild-type and mutant anther gene expression were detected with the *msca1* mutant (661 genes). 30% of the genes up-regulated in *msca1* (relative to wild-type) are expressed in juvenile leaves, including several involved in photosynthesis. All three mutants lack a normal tapetum (Fig 4a–d), and share 40 genes that are differentially regulated compared to wild type, including 10 that are down-regulated. Thus, this suite of genes, which includes *ms45* and a callase gene, could be required for tapetal development. Interestingly, all three mutants (including *msca1,* which lacks normal anther cells) express ABC floral organ determination MADS-box genes at all stages tested, as well as many meiosis-associated genes.

An earlier study using a similar microarray platform (Ma et al., 2006) assessed expression in mature pollen, comparing it to that of anthers and juvenile leaves in three maize lines (inbreds A619 and W23, and hybrid W23/ND101). Pollen transcripts showed significant hybridization with only about half as many probes as did transcripts from anthers and juvenile leaves. In addition, although the majority of pollen-positive probes also detected transcripts in the two other tissue types, the pollen transcriptome was judged most distinctive based on a clustering algorithm, reminiscent of results from studies of the pollen transcriptome in *Arabidopsis thaliana* (Pina et al., 2005). The distinctive and more limited nature of the pollen transcriptome could be due to the inherently restricted sample (i.e., developmentally synchronous, only three cells), and also to its dedicated biological role: fertilization via the pollen tube. In fact, the maize pollen transcriptome appears to be enriched for many of the processes associated with polarized cell growth: secretion and functioning of the secretory pathway, cell wall modification and cytoskeletal activities (Ma et al., 2006). Three hundred and fifty-six putative pollen-specific transcripts were identified, potentially useful as stage-specific markers (Ma et al., 2006). It should be noted that, due to the current incomplete nature of maize sequence databases, the arrays used in both transcriptome studies are also incomplete; comprehensive analyses will require next generation arrays or techniques.

Seventeen potential markers for maize sperm cells have also been identified out of a diverse set of sequences identified from FACS-purified sperm cells by EST sequencing (Engel et al., 2003; Engel et al., 2005). Follow-up studies of orthologous genes established that at least one ortholog is transcribed in sperm cells of mature *A. thaliana* pollen, arguing against the prevailing notion that those cells are transcriptionally quiescent (Engel et al., 2005). However, partitioning of sperm-specific transcripts into a largely transcriptionally quiescent sperm cell also remains a possibility, as several of the maize transcripts are detected at earlier stages of pollen development (Engel et al., 2003). Some of the sperm cell transcripts encode predicted secreted or plasma membrane proteins, and could play a role in events in the embryo sac (e.g., cell-cell recognition).

7 Progamic Development

Following anther dehiscence, tricellular pollen (Fig. 6a) is released in copious amounts to fall onto or be delivered to the silk (i.e., the maize stigma and style). The progamic phase of male gametophyte development then ensues, namely, generation of a pollen tube that carries the two sperm to the embryo sac for double fertilization. The ease of collecting large quantities of mature pollen is a primary advantage of using maize as a model: biochemical approaches to studying

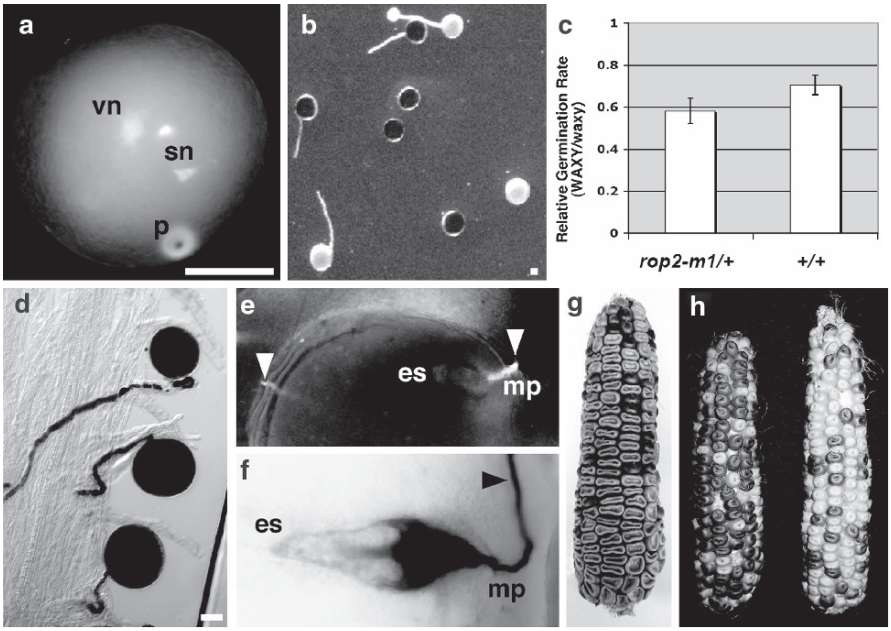

Fig. 6 Assessing progamic development. **a** DAPI staining reveals the compact sperm nuclei (*sn*) and more diffuse vegetative nucleus (*vn*) in a mature pollen grain; pore (*p*). **b** Pollen tubes germinate and grow in vitro, and can be stained with iodine to reveal the *waxy* (*wx*) versus wild-type starch phenotype (light vs dark). **c** A small but significant reduction in germination for *rop2* mutant pollen, linked to *Wx* using a marked reciprocal translocation. **d** Staining *Wx* pollen with iodine following germination in vivo allows visualization of pollen tube growth on the surface of a cleared silk, and penetration therein. Bars in **a**–**d** = 50 μm. **e**, **f** Pollen tubes (arrowheads) near the ovule, micropyle (*mp*) and embryo sac (*es*). **e** A pollen tube (revealed by aniline blue staining of callose in its cell wall) enters the ovary cavity, disappears behind the ovule, and reappears at the micropyle, where it enters the embryo sac. **f** Expression of a GUS reporter in a pollen tube allows visualization of its growth pattern and its successful penetration into the embryo sac. **g** The *ga*11* mutation, linked to *Bz1 Sh1* (purple, plump seed) in a heterozygous outcross, causes a male transmission defect, seen as non-Mendelian segregation on the ear. The mutant gametophytes are at an even greater disadvantage at the base of the ear, presumably due to defects in pollen tube growth. **h** Pollination using 1: 1 pollen mixes (test pollen: W22-*R* reference pollen, producing colorless and full color seed, respectively) demonstrates that pollen from inbred MS71 (left) is significantly less competitive than that from inbred P39 (right)

plant actin and profilin (Ren et al., 1997; Kovar et al., 2000) and pollen coat and cell wall proteins (Suen and Huang, 2007) have proved fruitful. In vitro pollen germination methods have also been productive (Fig. 6b), and were recently improved by a robust pollen germination medium that allows for transient transformation and expression of engineered constructs (Schreiber and Dresselhaus, 2003; Herrmann et al., 2006). Finally, an increasing number of mutants have been isolated that affect this stage, recognizable by their key phenotype: a gametophytically expressed defect in transmission through the male from a heterozygote, leading to non-Mendelian segregation, often detected using a linked seed marker (Fig. 6g).

The silk surface is covered with multicellular hairs that provide a surface onto which pollen grains adhere and germinate a pollen tube from the pore in the exine (Fig. 6d). Pollen hydration and germination can occur rapidly, within 5 min of landing on the silk. The polar-oriented, growing pollen tube expands on the surface of the silk, and then penetrates the cuticle and cell wall of either the silk hair or silk proper, eventually growing between silk cortex cells to reach one of the two transmitting tracts associated with the vascular strands in the silk. Pollen tubes that fail to penetrate the silk hair appear to lack a preferred direction of growth in the silk, implying that penetration of the hair assists in orienting tube growth toward the base of the silk (Heslop-Harrison et al., 1985; Booy et al., 1992). In many species the sperm cells and the vegetative nucleus travel together in the pollen tube as a linked "male germ unit" (e.g., Lalanne and Twell, 2002). However, it is unclear whether such a structure exists in maize (Heuer et al., 2000).

Several cellular components that facilitate germination, as well as some that function in pollen tube growth *per se*, have been identified. Internally, the actin cytoskeleton is clearly required, although its function in germination is less sensitive to pharmacological inhibition than its function in pollen tube growth, implying some distinction in its role in these two processes (Gibbon et al., 1999). This functional difference may be linked to the organization of the actin arrays in the two cell types: ungerminated pollen grains, but not pollen tubes, have a prominent patch of F-actin at the cortex adjacent to the pore (Gibbon et al., 1999). ROP GTPases are one class of actin cytoskeleton regulators active during pollen tube growth (Cole and Fowler, 2006). Reduction of ROP GTPase activity by mutation of the maize *rop2* gene impairs pollen function, as measured by the ability to successfully fertilize and produce seed when placed in competition with wild-type pollen (Arthur et al., 2003). The *rop2* competitive defect is due in part to a small, but measurable, effect on pollen germination (Fig. 6c, and KA Carroll and JE Fowler, unpublished results), demonstrating a role for ROP at the initiation of progamic development. The *waxy1* (*wx*) mutation (which affects pollen starch composition) can be used as a linked marker to compare mutant and wild-type pollen grains (e.g., *rop2* vs wild-type) from a heterozygous plant either in vitro or in vivo on a silk (Fig. 6d). This helps control for plant-to-plant variation and environmental effects, which can be quite profound. The availability of *wx*-marked reciprocal translocations for most of

the maize genome (Laughnan and Gabay-Laughnan, 1994) suggests this can be a general technique for following gametophytic mutants.

Extracellular components also play an important role in progamic pollen germination and growth. For example, the XYN1 pollen coat xylanase facilitates germination on solid media and in vivo, presumably by helping the grain to take up water (Suen and Huang, 2007). However, its primary function is to help the pollen tube penetrate the silk; penetration is significantly decreased in pollen in which xylanase levels are reduced via antisense-mediated knockdown. The xylan hydrolysis activity of the enzyme appears to help digest the cell wall of the silk, thus allowing penetration (Suen and Huang, 2007). Other extracellular proteins (e.g., β-expansin, Cosgrove et al., 1997; β-glucanase, Suen et al., 2003) may also assist in loosening or degrading the silk cell wall to facilitate pollen tube penetration and/or growth in the transmitting tracts. Consistent with this idea, a mutation in a single β-expansin gene (*expb1*) is associated with a ~30% reduction of β-expansin levels as well as a gametophytically expressed defect in pollen tube growth and competitive ability in vivo (Valdivia et al., 2007). The PEX (pollen extensin-like) glycoproteins, which are specifically localized to the cell wall of the growing pollen tube, are also potential participants in progamic development, acting to maintain cell wall integrity and/or aid in cell-cell recognition (Rubinstein et al., 1995).

A number of cytoplasmic components that promote maize pollen tube growth *per se* have also been identified. As mentioned previously, an uncompromised F-actin cytoskeleton is required to maintain growth (Gibbon et al., 1999). The organization of F-actin in maize pollen tubes mirrors that seen across angiosperms (Cole and Fowler, 2006; Krichevsky et al., 2007): the extreme tip of the pollen tube is relatively free of F-actin, with a dense collar of fine filaments just proximal to the apex, and thicker actin cables extending longitudinally along the tube cortex behind this collar (Gibbon et al., 1999). The actin cables are thought to act as tracks for the rapid tip-directed movement of organelles and vesicles along the pollen tube cortex (see http://bilbo.bio.purdue.edu/~cjslab/Pollentube.mov). A distinct class of the actin monomer-binding protein profilin (encoded by *ZmPRO1*, *ZmPRO2*, and *ZmPRO3*) is expressed specifically in pollen (Staiger et al., 1993), where it is hypothesized to help control F-actin organization in response to the tip-high calcium gradient in the pollen tube apex (Kovar et al., 2000). This model is based upon the quantified biochemical activities of actin and profilin (e.g., calcium-sensitive actin-binding) purified from maize pollen, and the concentrations of profilin and actin measured therein (Gibbon et al., 1999; Kovar et al., 2000). In a different approach, *Ac* transposon-induced mutagenesis revealed that the *aberrant pollen transmission1* (*apt1*) gene, which encodes a Golgi-localized protein, is crucial for pollen tube growth (Xu and Dooner, 2006). Using a mutable *Ds* insertion allele of *bz1* as a reporter for *Ac* activity in seeds, the *apt1-m1(Ac)* mutant phenotype was initially recognized based on decreased transmission of the inserted *Ac* through the male. The *apt1* mutant pollen grains show no obvious defects, but mutant pollen tubes grow slowly and are misshapen (Xu and Dooner, 2006). The APT1 protein is very large (2607 amino acids) and closely related to the SABRE and KIP proteins

from *A. thaliana*, which are involved in root and pollen tube cell growth, respectively (Aeschbacher et al., 1995; Procissi et al., 2003). APT1 is hypothesized to act in membrane trafficking, and may be crucial for generation of the Golgi-derived vesicles that are required for growth at the pollen tube tip (Xu and Dooner, 2006). The *maternal effect lethal1* (*mel1*) mutant also slows pollen tube growth, although the affected gene has yet to be identified; intriguingly, this mutation also causes a defect in seed development when transmitted through the female gametophyte, although it has no obvious effect on the female gametophyte itself (Evans and Kermicle, 2001a).

The isolation of *apt1* follows a precedent of using seed markers to analyze gametophytically expressed genetic functions. Several *gametophyte factor* (*ga*) loci that cause reduced male fertility (and thus, non-Mendelian segregation) have been described (e.g., *ga*11*, Fig. 6g; Fowler, 2003). In some, decreased transmission of the linked marker at the base of the ear, relative to the apex, suggests that the locus may cause a defect (e.g., in pollen tube growth) that is exacerbated by longer silks. Seed markers also enable quantitative assessment of pollen fecundity using a pollen mixing technique (Fig. 6h, Ottaviano et al., 1988). A series of gametophytic selection experiments supports the idea that pollen fecundity is affected both by sporophytically and gametophytically expressed genes, with sporophytic vigor providing a larger (positive) influence on pollen germination, and gametophytic expression affecting pollen tube growth (Ottaviano et al., 1988). Use of the pollen mixing assay with a set of recombinant inbred lines allowed detection of a number of quantitative trait loci with major effects on pollen function during the progamic phase (Sari-Gorla et al., 1992, 1994, 1995).

Not all *ga* mutants display the "generic" *ga* phenotype described above. Indeed, the first *ga* locus to be identified, *ga1* (linked to *sugary1*), is representative of several other *ga* loci in that the *ga*-conditioned male gametophytic defect is expressed only when crossed to a *Ga*/*Ga* or *Ga*/*ga* female (reviewed in detail in Nelson, 1994). This genetic system can be used to help isolate particular maize lines: for example, many popcorns harbor the *Ga1-s* ("strong") allele, and cannot set seed when pollinated by dent and flint lines, which are *ga1*/*ga1* (Nelson, 1952). Use of radioactively labelled pollen demonstrated that *ga1* pollen tubes grow more slowly, and eventually cease to grow at all, in *Ga1-s* silks (House and Nelson, 1958). A similar locus, *Teosinte crossing barrier1* (*Tcb1*), appears to play some role in reproductive isolation of teosinte and maize in Mexico: certain populations of teosinte harbor the *Tcb1-s* allele, restricting the ability of maize pollen (*tcb1*) to fertilize the teosinte (Evans and Kermicle, 2001b; Kermicle, 2006). However, in contrast to *ga1*, the effect is bidirectional: *Tcb1-s* pollen is at a competitive disadvantage on *tcb1* silks (Kermicle, 2006). The mechanistic bases for these effects are unknown, including whether these loci are single genes, or sets of tightly linked genes. Nonetheless, these observations highlight the complex interactions that occur between male gametophyte and carpel.

A different type of silk-pollen interaction may involve flavonols, a group of aromatic secondary metabolites produced in the tapetum (Mo et al., 1992; Pollak et al., 1995). Chalcone synthase (CHS) catalyzes the initial step in flavonoid

synthesis, and in maize is encoded by two loci, *white pollen1* (*whp1*) and *colorless2* (*c2*) (Franken et al., 1991; Pollak et al., 1995). Plants homozygous for both *c2* and *whp1* produce white pollen that lacks flavonols and is sterile in self-crosses and in outcrosses to most wild-type lines (Mo et al., 1992; Pollak et al., 1995). White pollen can germinate in vivo, but its pollen tubes contain irregular, dense deposits of callose, and arrest growth after 12–24 h (Pollak et al., 1995). However, the fertility defect can be rescued either by shortening the silks (Pollak et al., 1995), or by mixing white pollen with flavonols prior to pollination (Mo et al., 1992). In contrast to petunia, in which the stigma produces flavonols that can rescue flavonol-deficient pollen (Pollak et al., 1993), flavonols have not been found in maize silks. However, it is possible that certain maize lines do produce flavonols in the silks, and these correspond to the few lines that do support white pollen fertility. The specific role of flavonols in the pollen tube is not clear, although several hypotheses have been proposed (e.g., signaling in pollen-pistil recognition, pollen wall synthesis) (Pollak et al., 1995).

The final phase of male gametophyte development, arrival of the pollen tube at the embryo sac and fertilization (Fig. 6e), is the least understood. However, the generation of transgenic tools such as β-glucuronidase (GUS) -expressing pollen (Brettschneider et al., 1997) allows not only visualization of pollen tube growth at the ovule and micropyle, but also robust assessment of pollen tube reception at the embryo sac (Fig. 6f, and Márton et al., 2005). Indeed, one of the most exciting recent discoveries in male gametophyte development is more directly associated with the female gametophyte: expression of the *Zea mays EGG APPARATUS1* (*ZmEA1*) gene is required in the embryo sac to guide the pollen tube through the micropyle to the embryo sac (Márton et al., 2005). *ZmEA1* encodes a short, secreted protein that is localized to the micropylar region, based on GFP fusion expression, and thus could be a signal that directly influences pollen tube growth at this late stage (Márton et al., 2005).

Maize sperm cell behavior in this final phase, and in particular, the question of whether the two sperm cells randomly fuse with their female targets (egg and central cell), has been investigated using both genetic and cell biological approaches. Marked translocations between a supernumery B chromosome and an autosome (A), which often undergo non-disjunction in the first pollen mitosis to produce two genetically non-identical sperm (one carrying two copies of the B-A translocation, the other none), have been used to address this question. It has long been known that a sperm cell carrying two copies of the B-A translocation will preferentially fertilize the egg, compared to a sperm with no B-A translocation (Roman, 1948); however, the basis for this discrimination was unknown. More recently, development of techniques to isolate sperm and egg cells for in vitro fertilization (Faure et al., 1994) indicates that discrimination between the two types of sperm cell is not due to differences in their ability to fuse with the egg (at least in vitro) (Faure et al., 2003). Thus, although the mechanism remains a mystery, the use of chromosomal deletion derivatives has identified a region of the B chromosome that is responsible for preferential fertilization (Carlson, 2007), raising the possibility that further investigations can further characterize this region, and thus, gain mechanistic insights.

8 Conclusion

Our discussion in this chapter has made clear the breadth of techniques – biochemical, cellular, molecular, genetic, and now genomic – that have expanded our knowledge of the maize male gametophyte. As a counterpart to work in the dicot model *A. thaliana*, this research has been relevant not only to maize and other monocot crops, but also to our understanding of male gametophytes throughout the plant kingdom. In particular, recent advances in our knowledge of sperm cell function, pollen wall biochemistry, actin cytoskeleton regulation and interactions between the male and female gametophytes are based on results from maize, and have broad implications. We foresee these contributions continuing, not just because of the compelling practical rationale for studying maize, but also because some of its features make it a useful alternative model for approaches that are more difficult in *A. thaliana*. For example, the ease of isolating large quantities of developmentally synchronous cells throughout most of the progression described here (Fig. 1) not only facilitates biochemistry, but should also make transcriptomic and proteomic techniques very feasible. Maize pollen tubes are significantly more amenable to microscopic analysis and in vitro culture than those of *A. thaliana*. Development of a robust transient transformation method for maize pollen to enable functional analyses similar to those in *Nicotiana tabacum* (e.g., Wu et al., 2001) would further enhance this advantage. Finally, the availability of the maize genome sequence and the increasing number of public genomic resources (e.g., sequence-tagged mutant collections) should make it easier to identify the molecular basis for known male sterile phenotypes, as well as for gametophytically active genes isolated in screens for transmission defects. Thus, the prospects for a better mechanistic understanding of the maize male gametophyte, particularly in those areas that allow a synergistic application of all of these approaches, appear to be very promising.

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The Maize Megagametophyte

Matthew M.S. Evans and Ueli Grossniklaus

Abstract The life cycle of plants alternates between a diploid and a haploid generation. In flowering plants the haploid gametophytes are sexually dimorphic and produce the gametes, which fuse to produce the diploid sporophyte of the next generation. The megagametophyte of maize follows the *Polygonum*-type pattern of development: one of the four meiotic products, the functional megaspore, undergoes three free nuclear divisions to produce a polarized, eight-nucleate syncytium. Cellularization produces seven cells that differentiate into four cell types: two synergids, three antipodals, and the two female gametes, the egg cell and the central cell. The position of the nuclei in the syncytial phase and the position and differentiation of cell types after cellularization follow stereotypical patterns, suggesting a tight genetic regulation of the cellular processes involved. Recent genetic evidence demonstrates that many of these cellular processes are regulated by the activity of the haploid genome of the megagametophyte itself, rather than the parental diploid genome from which it originates. The functions performed by the megagametophyte includes both basic cellular functions and functions that unique to the megagametophyte, such as pollen tube guidance and reception, as well as processes associated with double fertilization and the maternal control over seed development. In this chapter we describe the development and functions of the megagametophyte, and what is known the regulation of the underlying processes.

1 Megasporogenesis

The life cycle of plants alternates between a diploid sporophytic and a haploid gametophytic generation. The sexually dimorphic, multicellular gametophytes produce the gametes and are, thus, at the center of plant reproductive biology. Male and female gametophytes develop in the sexual organs of the flower, the anthers and ovules, respectively. The development and function of the male gametophyte (microgametophyte, pollen) is described by Bedinger and Fowler. It delivers the two sperm cells to the female gametophyte where they participate in double fertilization. The female gametophyte (megagametophyte, embryo sac) also produces two gametes, the egg and central cell, which after fertilization give rise to the

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embryo and endosperm, respectively. Together with maternal tissues of the sporophyte, the pericarp, the two fertilization products develop into the mature seed (chapter by Scanlon). Although the formation of spores, which subsequently develop into the gametophytes, is under the control of the sporophyte, it is included in this chapter because it immediately precedes the formation of the megagametophyte and is central to the understanding of gametophyte development.

Within the nucellus of the maize ovule one hypodermal cell, the archesporium, undergoes meiosis to produce one functional megaspore per ovule. The functional megaspore develops into the embryo sac, located at the midline of the ovule toward the tip of the ear. The ovule is polarized along the micropylar–chalazal (m–c) axis, a polarity that is shared by the megagametophyte, individual cells of the megagametophyte, and the embryo. The archesporial cell is easily distinguished from its neighbors because of its larger size and more prominent nucleus. The archesporial cell directly differentiates into the megasporocyte, or megaspore mother cell (MMC), and elongates perpendicular to the ovule surface along the m–c axis before meiosis (Fig. 1a). In the maize *multiple archesporial cells1* (*mac1*) mutant several adjacent cells develop as archesporial cells, suggesting that archesporial cell fate is actively suppressed in cells adjacent to the archesporium (Sheridan et al., 1996). Polar distribution of cytoplasmic components along the long m–c axis of the megasporocyte is present in the MMC before meiosis. While the rough ER is concentrated at the micropylar pole, mitochondria and plastids are more prevalent at the chalazal pole (Russell, 1979). The MMC is also reported to lack plasmodesmata connections with the nucellus (Diboll and Larson, 1966), or to only retain a few plasmodesmata at its chalazal end (Russell, 1979). The walls of the MMC are higher in callose than the surrounding nucellar cell walls.

The MMC divides asymmetrically along the m–c axis in meiosis I, producing a small micropylar dyad cell and a larger chalazal dyad cell (Fig. 1b). The micropylar dyad remains high in callose compared to the chalazal dyad. Meiosis II is more variable than meiosis I. The micropylar dyad may degenerate without finishing meiosis. When the micropylar dyad does divide, it typically divides periclinally, but occasionally does so anticlinally or obliquely, producing a tetrad ranging from linear to T-shaped. The chalazal dyad completes meiosis II with an asymmetric, periclinal division to produce two megaspores (Fig. 1c). The only functional megaspore (FM) is the largest, chalazal-most megaspore, which is lower in callose than the non-functional megaspores, particularly at its chalazal pole (Russell, 1979). The micropylar megaspores (and/or dyad) quickly degenerate and collapse (Fig. 1d).

Meiosis leads to the production of the functional megaspore and the subsequent development of the megagametophyte is under the control of the haploid genome. Meiosis is a very complex process described in detail in the chapter by Cande et al. and its disruption usually results in sterility. This may be because certain developmental checkpoints ensure proper progression through reproductive development. In the *elongate1* (*el1*) mutant, however, a dyad cell initiates gametogenesis and produces a functional, diploid megagametophyte (Rhoades and Dempsey, 1966; Barrell and Grossniklaus, 2005). This finding illustrates that megagametophyte

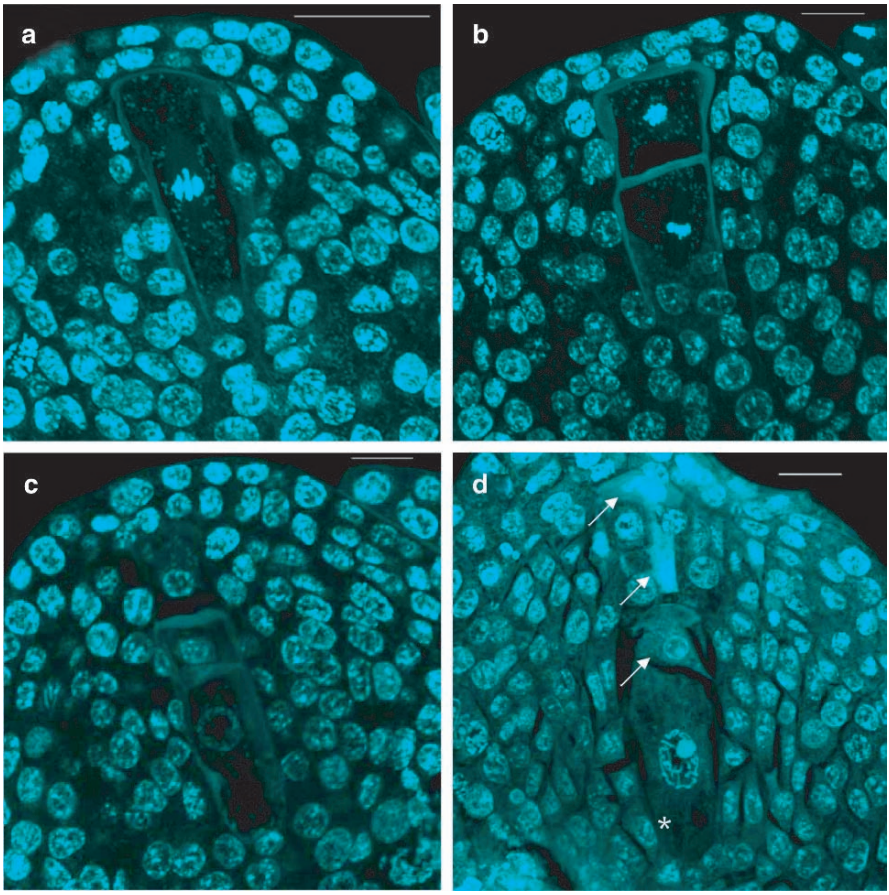

Fig. 1 Maize megasporogenesis. The micropyle is up; the chalaza is down. (**a**) megaspore mother cell in metaphase of the first meiotic division; (**b**) the two dyad cells in metaphase of the second meiotic division; (**c**) tetrad of megaspores; (**d**) the three micropylar megaspores degenerate (arrows), while the chalazal-most functional megaspore initiates megagametogenesis, characterized by the formation of a vacuole (star). Pictures courtesy of P. Barrell

development does not depend on the ploidy level or reduction, but rather is controlled by a specific set of genes characteristic for the megagametophyte.

2 Megagametophyte Development and Function

Megagametogenesis is initiated after the second meiotic division is complete. Only the chalazal-most megaspore survives and the FM initiates a series of mitotic divisions to form the mature megagametophyte. The fact that most deficiencies arrest at the FG1 stage

(Fig. 2) immediately after meiosis, indicates that a large number of genes is required for megagametogenesis and that this process is largely under the control of the haploid, gametophytic genome (Patterson, 1978; Coe et al., 1988; Buckner and Reeves, 1994).

2.1 Megagametophyte Growth and Development

After meiosis, a central vacuole forms in the FM, and the megagametophyte enlarges throughout megagametogenesis primarily through enlargement of this vacuole. The surrounding nucellar cells degenerate and collapse, leaving a halo of appressed nucellar cell walls around the embryo sac (Russell, 1979). This vacuole forms chalazal to the single nucleus (Barrell and Grossniklaus, 2005; Russell, 1979; Vollbrecht and Hake, 1995). The FM then undergoes three rounds of synchronous, free nuclear divisions to produce an eight-nucleate coenocyte. The last round of division is rapidly followed by cytokinesis to produce an eight-nucleate, seven-celled megagametophyte. As in the MMC, the nuclei of the free nuclear stages are larger and possess more distinct nucleoli than surrounding nucellar cell nuclei.

The first nuclear division is parallel to the m–c axis. The two nuclei are separated by the central vacuole producing a two-nucleate embryo sac with micropylar and chalazal domains. A second vacuole often forms chalazal to the chalazal nucleus at this stage (Barrell and Grossniklaus, 2005; Vollbrecht and Hake, 1995). Polarity of the developing megagametophyte along the m–c axis can be seen by the asymmetric distribution of plastids concentrated around the micropylar nucleus (Huang and Sheridan, 1994; Vollbrecht and Hake, 1995). In the second division, the chalazal nucleus divides along the m–c axis, but the micropylar division is perpendicular to this axis producing a T-arrangement of nuclei (Barrell and Grossniklaus, 2005; Huang and Sheridan, 1994; Lin, 1981; Vollbrecht and Hake, 1995). In the third division both poles reiterate the pattern of the second division. At each pole, one division is parallel to the m–c axis (each producing one internal and one peripheral nucleus) and the other is perpendicular, producing a T arrangement of nuclei at each pole (Huang and

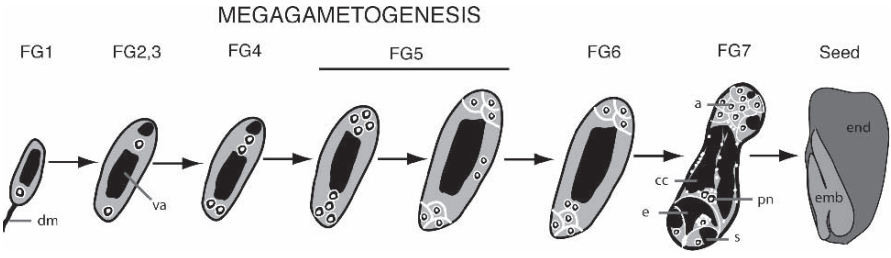

Fig. 2 Maize megagametophyte development. The micropyle is down; the chalaza is up; and the tip of the ear is left in stages FG5 and later. *dm* degenerated megaspores, *a* antipodal cells, *cc* central cell, *pn* polar nuclei, *e* egg cell, *s* synergid, *end* endosperm, *emb* embryo, *va* vacuole, *FG1-FG7* female gametophyte stage 1–7 (stages adapted from Christensen et al., 1997).

Sheridan, 1994). The two central nuclei from the m–c axis divisions are the two polar nuclei of the future central cell, and the peripheral sister of the micropylar polar nucleus becomes the egg nucleus. During the free nuclear phase microtubules are perinuclear with connections between the sister nuclei at the poles (Huang and Sheridan, 1994). This microtubule arrangement may help maintain nucleo-cytoplasmic domains within the syncytial megagametophyte. Phragmoplasts form between the nuclei at each pole immediately after the third division, even between non-sisters, followed by cytokinesis to form seven cells (Huang and Sheridan, 1994).

2.2 Megagametophyte Maturation and Cell Differentiation

The seven cells formed are, from the micropyle to the chalaza, the two synergids, the egg cell, the binucleate central cell, and the three antipodals (Figure 3). The egg cell and synergids together make up the egg apparatus at the micropylar pole. Each embryo sac contains two gametes: the egg cell, which is fertilized to produce the embryo, and the central cell, which is fertilized to produce the triploid endosperm. After cellularization the embryo sac cells mature to their fully differentiated state in preparation for fertilization. While plasmodesmata connect the cells of the megagametophyte to one another, there are reportedly no plasmodesmata connecting megagametophytic to nucellar cells (Diboll and Larson, 1966). The lack of cytoplasmic connections between the megagametophyte and the surrounding nucellar cells has been interpreted to be important for establishing a peculiar environment for gametophyte development and to be related to the gametophyte belonging to a distinct generation from the surrounding sporophyte (Diboll and Larson, 1966).

The factors controlling pattern formation of the embryo sac and the establishment of polarity across the whole embryo sac and within the individual embryo sac cells is largely unknown. Additionally, our understanding of how the individual embryo sac cell identities are determined is also in its early stages. A few genes have been identified that shed light on these processes. The maize *indeterminate gametophyte1* (*ig1*) gene encodes a transcription factor that is known to control the polarity of leaf primordia in maize and Arabidopsis, suggesting it may play a similar role in the maize embryo sac (Evans, 2007). In Arabidopsis the *LACHESIS, CLOTHO*, and *ATROPUS* genes restrict gametic cell fate to the egg and central cells (Gross-Hardt et al., 2007; Moll et al., 2008), and synergid cell differentiation depends on the *MYB98* gene (Kasahara et al., 2005).

2.2.1 Antipodal Cells

At the chalazal pole of the embryo sac lie the antipodal cells, which are believed to function as transfer cells for the embryo sac. The antipodal cells are densely cytoplasmic compared to the neighboring nucellus and central cell (Figure 3). The antipodal cells can be multi- or uninucleate and the cell walls separating them are

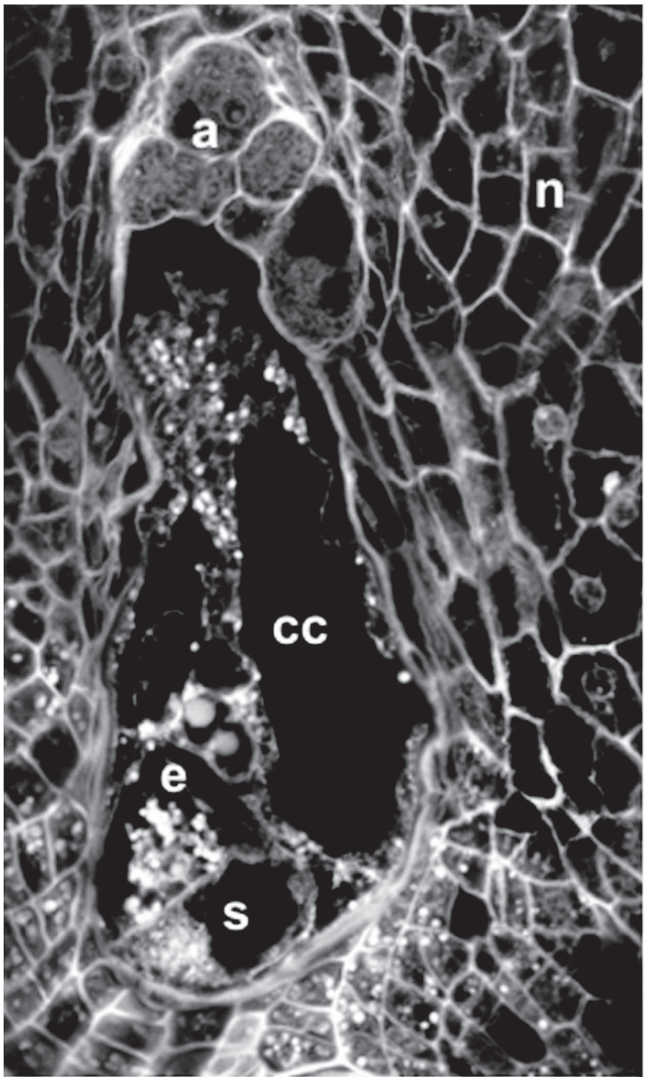

Fig. 3 Nearly mature cellularized embryo sac. The micropyle is down; the chalaza is up; and the tip of the ear is to the left. *a* antipodals, *cc* central cell, *e* egg, *s* synergid, *n* nucellus . Picture by M. Evans

sometimes incomplete (Diboll and Larson, 1966). The antipodal cells continue to divide during embryo sac maturation reaching a final number of 20 to 100 cells with one to four nuclei each. The antipodal cells can even persist and continue dividing after fertilization during kernel development (Randolph, 1936). The size of the antipodal cell vacuoles is also variable (Diboll, 1968). The nuclei, of the antipodal cells are smaller with less prominent nucleoli than the polar nuclei, and

more closely resemble the nuclei of the surrounding nucellus. The microtubules of the antipodal cells are randomly oriented (Huang and Sheridan, 1994). The cell walls of the antipodal cells adjacent to the nucellus are papillate, supporting a role for the antipodals as transfer cells for the embryo sac. However, the function of the antipodal cells has not been experimentally determined and in other plants, like Arabidopsis, they degenerate without proliferation prior to fertilization during embryo sac maturation (Murgia et al., 1993; Christensen et al., 1997). This could mean that antipodal cell function is taken over by other cells of the embryo sac or that they complete their role before degenerating, or a combination of both.

2.2.2 Central Cell

In the center of the embryo sac lies the large homo-diploid central cell, characterized by a very large vacuole (Fig. 3). After cellularization the two polar nuclei typically migrate to the center of the embryo sac along the wall away from the ovule surface (the future abgerminal side of the endosperm), partially fuse, and then migrate to the micropylar end of the central cell adjacent to the egg apparatus near the midline of the central cell. The central cell is traversed by cytoplasmic strands, one of which typically connects the two polar nuclei to the chalazal end of the central cell along its midline. The two polar nuclei typically remain unfused until fertilization, although fusion of the two to form a secondary endosperm nucleus has been reported in some maize lines (Gutierrez-Marcos et al., 2006; Huang and Sheridan, 1996; Mol et al., 1994). The polar nuclei are exceptionally large with larger nucleoli than other cells in the ovule. Plastids brightly stained with Schiff reagent are distributed throughout the central cell cytoplasm. Microtubules of the central cells are primarily cortical and transverse in orientation possibly in connection with central cell expansion (Huang and Sheridan, 1994). The walls of the central cell next to the antipodals are papillate like those of the antipodal cells adjacent to the nucellus, suggesting a metabolite flow from the nucellus to the antipodals to the central cell (Diboll and Larson, 1966). The boundary between the central cell, egg, and synergids is typically membranous, likely to ease fertilization by one of the two sperm cells delivered by the pollen tube, initiating endosperm development. Mutant analysis in Arabidopsis shows that endosperm development is actively repressed in the central cell until being initiated by fertilization (Chaudhury et al., 1997; Grossniklaus et al., 1998; Köhler et al., 2003; Luo et al., 1999; Ohad et al., 1999; Ohad et al., 1996). Pattern information along the future apical–basal axis of the endosperm also appears to be predetermined in the central cell prior to fertilization (Gutierrez-Marcos et al., 2006).

2.2.3 Egg Cell

At cellularization the egg cell is densely cytoplasmic, and the egg nucleus is in a mid-lateral position (Fig. 3). The egg cell is polarized with the bulk of the cytoplasm and the nucleus displaced from the micropylar pole by a vacuole(s) (Diboll and

Larson, 1966). As the egg cell matures, many vacuoles form in the periphery of the cell, first at the micropylar end followed by formation of a large chalazal vacuole, and these changes in the egg cell are accelerated by pollination (Mol et al., 2000). The egg cell nucleus is typically larger with a more distinct nucleolus than all of the other ovule nuclei except those of the central cell. The egg cell cytoplasm also has a distinct organization with perinuclear, starch-containing plastids and a distinct cytoskeletal arrangement of a few randomly oriented cortical microtubules and an absence of interior microtubules (Hoshina et al., 2004). The egg cell wall is thickest at the micropylar end and membranous adjacent to the central cell and synergids (Diboll and Larson, 1966). In Arabidopsis, an enhancer detector line (Sundaresan et al., 1995) with expression of the *GUS* reporter gene in the egg cell has proven useful for isolating the *LACHESIS, CLOTHO,* and *ATROPUS* genes required to restrict egg cell fate (Gross-Hardt et al., 2007; Moll et al., 2008). The molecular mechanisms of cell specification are largely unknown. Currently, only four *Arabidopsis* mutants are known to affect cell specification. Three of these lead to ectopic expression of an egg cell-specific marker and were shown to affect the core splicing machinery, indicating that their effects are rather indirect (Gross-Hardt et al., 2007; Moll et al., 2008). The forth mutant, *eostre*, was found to lead to overexpression of a homeodomain transcription factor, which is, however, not usually expressed in the embryo sac (Pagnussat et al., 2007). Nevertheless, the *eostre* mutant has revealed that the mis-expression of homeodomain target genes can convert a synergid cell into an egg cell. The signaling events underlying cell specification in the female gametophyte are, however, completely unknown.

Along with the synergids, the maize egg cell expresses the *Zea mays Egg Apparatus1 (ZmEA1)* gene suggesting that, in maize, the egg could contribute to pollen tube attraction (see below) (Marton et al., 2005). Egg apparatus specific enhancers also exist in Arabidopsis indicating that the existence of a transcriptional domain encompassing the egg and synergids is conserved in monocots and dicots (Yang et al., 2005). In Arabidopsis, at least, fertilization of the egg cell not only initiates embryo development but also is sufficient to stimulate endosperm development in the central cell (Nowack et al., 2006).

2.2.4 Synergids

The synergid cells occupy the most micropylar position in the embryo sac (Fig. 3). The function of the synergids in pollen tube guidance and reception has been demonstrated by a combination of mutant and laser ablation studies (Higashiyama et al., 2001; Huck et al., 2003; Kasahara et al., 2005; Marton et al., 2005; Rotman et al., 2003). In particular, the filiform apparatus at the micropylar end of the synergids is critical for pollen tube attraction (Kasahara et al., 2005). The filiform apparatus is a specialized polysaccharide-rich structure at the micropylar end of the synergids. The synergid cell wall is thin at the chalazal end and thickest at the micropylar end, and the plasma membrane is highly invaginated adjacent to the filiform apparatus. The maize synergids are polarized with the bulk of the cytoplasm at the micropylar pole

of the cell and a large vacuole at the chalazal end. The cytoplasm itself is also polarized with small vesicles, plastids, and mitochondria concentrated at the micropylar end near the filiform apparatus (Diboll, 1968) and with microtubules longitudinally oriented and also concentrated near the filiform apparatus (Huang and Sheridan, 1994). The organization of the synergid cytoplasm has been attributed to the need to export materials to form the filiform apparatus at the micropylar pole. The synergid cell nuclei are less distinct and more difficult to visualize than other embryo sac nuclei. Lectin binding oligosaccharides are more concentrated on the plasma membrane of the synergids than other embryo sac cells and are especially concentrated in the filiform apparatus (Sun et al., 2002). In many maize lines one or both of the synergids degenerate during embryo sac maturation (Lin, 1978); in other cases however, the synergids persist until pollination, with one of them degenerating as the pollen tube arrives (Diboll, 1968; Guo et al., 2004; Mol et al., 1994).

3 Double Fertilization

Double fertilization, first described more than 100 years ago, is the distinguishing feature of flowering plants (Nawaschin, 1898; Guignard, 1899). One sperm fuses with the egg cell and the second with the central cell to form the zygote and the primary endosperm, respectively. To achieve double fertilization the two sperm cells have to be delivered to the mature megagametophyte, deeply embedded in the female reproductive tissues. The early steps of this process, pollination and pollen tube growth during the progamic phase, are described in the chapter by Bedinger and Fowler. Here we focus on the last steps in which the megagametophyte plays an active role.

3.1 Pollen Tube Guidance and Reception

The synergids play a crucial role in double fertilization as they are involved in attracting the pollen tube to the megagametophyte as well as in its reception. After the pollen tube penetrates the degenerating, receptive synergid, the pollen tube ceases to grow and bursts to release the two sperm cells; processes that are collectively referred to as pollen tube reception. Insights into the signaling processes that guide the pollen tube to the megagametophyte have mainly come from studies in Arabidopsis and *Torenia fourneri*, which has an egg apparatus that is not covered by integuments (Higashiyama et al., 1997). Arabidospis mutants with ovules lacking a mature megagametophyte do not attract pollen tubes, suggesting that the megagametophyte is the source of the attractive signal (Hülskamp et al., 1995; Ray et al., 1997; Shimizu and Okada, 2000). Detailed observations of the growth behavior of the pollen tube suggest the existence of a long-range signal, guiding the pollen tube along the funiculus into the neighborhood of the micropyle, and a short-range signal

that guides it into the micropyle itself (Shimizu and Okada, 2000). In *T. fourneri*, pollen tube guidance can be studied in an *in vitro* system and laser ablations studies have shown that the synergid cell is essential for the production of the pollen tube attractant (Higashiyama et al., 1998; 2001). Recent studies in Arabidopsis, however, also suggest an involvement of the central cell in this process, as *central cell guidance* (*ccg*) mutants, disrupting a gene expressed exclusively in the central cell, are defective in this process (Chen et al., 2007). *CCG* encodes a nuclearly localized protein with features of a transcription factor. Its exact mode of action is not known, but it is likely that pollen tube guidance involves communication processes between the central cell and the synergids.

Not much is known about the molecular nature of the signals involved in pollen tube guidance. To date, only one gene encoding such an activity has been identified, the *ZmEA1* gene expressed in the cells of the egg apparatus (Marton et al., 2005). *ZmEA1* encodes a member of a conserved family of small, secreted proteins that is localized to the filiform apparatus and secreted into the micropylar nucellar cell walls (Marton et al., 2005; Gray-Mitsumune and Matton, 2006). Down-regulation of *ZmEA1* in transgenic maize plants results in female sterility due to a loss of short-range pollen tube guidance into the micropyle, demonstrating that *ZmEA1* is required for pollen tube attraction (Marton et al., 2005). In Arabidopsis it has been proposed that gradients of the small organic molecule γ-aminobutyric acid may serve as an attracting signal (Palanivelu et al., 2003), but the fact that the guidance signals are species-specific suggests that they are of a more complex biochemical nature (Palanivelu and Preuss, 2006; Higashiyama et al., 2006).

Once the pollen tube penetrates the receptive synergid, tube growth is arrested and the tube bursts in an explosive process to release the two sperm cells (Higashiyama et al., 2000; Rotman et al., 2003). Until recently, pollen tube rupture was thought to be a mechanical process possibly mediated by a lytic intracellular milieu in the degenerating synergid cell. The discovery of two mutants in Arabidopsis, *feronia* (*fer*) and *sirene* (*srn*), however, has shown that pollen tube reception is an active process under the control of the female gametophyte (Huck et al., 2003; Rotman et al., 2003). In these mutants the pollen tube is guided normally and enters the micropyle, but instead of discharging the sperm cells, it fails to arrest and continues to grow inside the megagametophyte. The two mutants were recently shown to be allelic and *FER* encodes a receptor-like kinase consistent with its role in a signaling process involving the pollen tube and the synergid (Escobar-Restrepo et al., 2007). The FER protein is weakly expressed in sporophytic cells and at a high level in the synergids, where it localizes to the filiform apparatus, through which the pollen tube enters the receptive synergid. It has been proposed that pollen tube reception relies on an interaction between a putative pollen-produced ligand and the FER receptor-like kinase. This specific interaction may play a role in maintaining crossing barriers between related species, a hypothesis that is consistent with the finding that some interspecific crosses phenocopy the *fer* phenotype (Escobar-Restrepo et al., 2007).

The sperm cells of flowering plants are immotile, such that once they have been discharged into the receptive synergid, they need to be transported to the female

gametes. The molecular basis of this transport is not clear but may involve an actomyosin-based process (Russell, 1996). Immunocytological and ultrastructural analyses have shown that two actin coronas form in the megagametophyte, outlining the future trajectory of the male gametes. In tobacco and maize, these coronas are formed prior to the arrival of the sperm cells (Huang et al., 1993; Huang and Russell, 1994; Huang and Sheridan, 1994). One of these actin tracks forms at the chalazal pole of the receptive synergid connecting the middle lateral region with the area near the egg nucleus, the second forms between the egg cell and the central cell. In barley, unfused sperm cells were found to be associated with electron-dense bundles that may reflect these actin tracks (Mogensen, 1982). The timing of their appearance and their arrangement suggests that actin coronas may interact with the sperm cells to transport them to site of syngamy (gamete fusion). It is not known how the sperm cells acquire myosin on their external surface to allow movement along these actin tracks. Since sperm cells are negatively charged on their surface, it is possible that myosin from the disrupted pollen tube or the receptive synergid attaches to the sperm cells though electrostatic interactions (Zhang and Russell, 1995).

3.2 Fertilization

In maize, the fertilization process *sensu strictu*, i.e. syngamy, occurs between 16 and 24 h after pollination, depending on the genetic background and environmental conditions. The two fusion events appear to occur simultaneously or nearly so, but the timing of the first division of the two fertilization products differ greatly (Randolph, 1936). While the zygote does not divide until 10–12 h after syngamy, the primary endosperm nucleus divides within 3–5 h after fertilization and proliferates rapidly (Kiesselbach, 1949). We know very little about the molecular basis of gamete fusion in plants and currently only one mutant is known that disrupt this process. The Arabidopsis mutant *generative cell specific1/hapless2* (*gcs1/hap2*) affects the male gametophyte and sperms that are delivered into the receptive synergid fail to fuse with their female targets (Mori et al., 2006; von Besser et al., 2006). Since *GCS1* encodes a putative transmembrane protein it has been proposed to play a role in sperm-egg adhesion or recognition (Mori et al., 2006).

Usually, only one pollen tube enters the micropyle and the two sperm cells that participate in double fertilization are genetically identical. However, it is possible that the sperm cells originate from different pollen tubes, such that the resulting embryo and endosperm can be non-identical if the pollen donor was heterozygous. This phenomenon is termed heterofertilization and occurs at frequencies of 1–10% depending on the genetic background (Sprague, 1932; Coe et al., 1988). That heterofertilization can generate embryo and endosperm of distinct genotypes can be used to determine whether a mutant affecting kernel development affects primarily embryo or endosperm development. In Arabidopsis, such experiments are currently not possible but recent studies using the *cdka;1* mutant, which produces only a single sperm cell, have shown that the embryo can develop to the globular stage in

the presence of only a few diploid endosperm nuclei (Nowack et al., 2006). In this mutant, the single sperm cell always fertilizes the egg cell and the primary endosperm nucleus starts dividing in the absence of fertilization, indicating a signal from the zygote that activates endosperm formation. This independence of early embryo from endosperm development is also observed in the *glauce* mutant, where a globular embryo develops in the complete absence of endosperm (Ngo et al., 2007).

While the single sperm of the *cdka;1* mutant in Arabidopsis always fertilizes the egg cell (Nowack et al., 2006), it is not clear whether the two sperm cells during normal double fertilization fuse randomly with the female gametes or whether there is a fertilization preference. Such a preference could be based on the capacity of only one of the sperm cells to fuse with the egg cell. Alternatively, both sperms may be able to participate in syngamy with the egg cell, but one would be better poised for competition. In fact, sperm cells do show dimorphisms with respect to shape, size, and organelle number in several species and in *Plumbago zeylandica* there is clear evidence for non-random fertilization. The plastid-rich sperm fertilizes the egg cell in about 95% of the cases, whereas the larger, plastid-poor sperm fertilizes the central cell (Russell, 1984, 1985). However, *P. zeylandica* is a special case as its megagametophyte does not have any synergid cells and the mechanism of fertilization is quite different from the one in maize. In maize, non-random fertilization has been inferred from segregation analyses using B-chromosomes. Non-disjunction in the mitotic division producing the two sperm cells leads to one sperm carrying two B-chromosomes while the other has none (Roman, 1947). An excess of kernels where the embryo carries the B-chromosomes indicates non-random fertilization (Roman, 1948). However, when such distinct sperm cells were used in *in vitro* fertilization experiments, no difference in fusion behavior with the egg cell could be detected. Thus, both sperms appear capable of fertilizing the egg cell at least under these specific conditions (Faure et al., 2003).

Essentially everything we know about the subsequent steps of the fertilization process stems from *in vitro* fertilization experiments, which have been conducted mainly in maize and some other grasses. Such experiments became possible through the isolation of viable gametes and their fusion through electrofusion or chemical induction. These procedures, however, do also lead to fusion with nongametic cells (Kranz et al., 1991; Kranz and Lörz, 1994). In contrast, if the gametes are brought into close contact by micromanipulation in the presence of low concentrations of calcium, gamete specificity is maintained (Faure et al., 1994). Egg and centrals cells fuse with the sperm cell within seconds after bringing the gametes into close contact with each other (Faure et al., 1994; Kranz et al., 1998). After fusion a calcium influx initiates at the fusion site and propagates across the egg cell as is typical of animals (Antoine et al., 2000). While the manipulation of calcium influx though pharmacological agents affects certain aspects of egg activation, an increase in intracellular calcium is not sufficient to trigger the first division of the zygote (Antoine et al., 2001). This is in contrast to animal systems, where such a calcium wave is also observed and an increase in the calcium concentration is sufficient to trigger parthenogenetic development of the egg (Miyazaki and Ito, 2006). In animals, the fusion of multiple sperms with an egg cell is prevented by a block to polyspermy, consisting of a fast electrophysiological block and a slow mechanical block (Tsaadon et al., 2006).

Although the cell biological events occurring at fertilization seem very similar in plants and animals, it is not clear whether a block to polyspermy exists at the gamete level in plants. It was reported that *in vitro* fertilization products were unable to fuse with further sperm cells, indicating a possible slow block to polyspermy, since the manipulations for a second fusion take a few minutes (Faure et al., 1994). Within this time cell wall material is deposited around the zygote, providing a strong physical barrier to further fusions.

We know relatively little about the events occurring after gamete fusion. The male and female pro-nuclei must come into contact to undergo karyogamy. Karyogamy is completed about 2 h after *in vitro* fusion in both the egg and the central cell (Faure et al., 1993; Kranz et al., 1998). Using sperm cells from transgenic plants carrying GFP under the control of the 35S promoter, it was shown that the activation of this viral promoter coincided with the decondensation of the paternal chromatin and mRNAs could be detected as early as 4 h after fusion, indicating an early activation of this paternal locus in *in vitro* fertilization products.

4 Parent-of-Origin Effects

Kernel development can be affected by parent-of-origin effects that stem from events occurring during megagametogenesis. Other chapters will discuss such effects in detail but given their gametophytic nature, they will be briefly mentioned here. Parent-of-origin effects are manifested through distinct phenotypes in reciprocal crosses. Maternal effect mutants only show a phenotype when the mutant allele is inherited from the mother. Because either the sporophytic tissues or the megagametophyte can exert such effects, we distinguish between sporophytic and gametophytic maternal effects (Grossniklaus and Schneitz, 1998). While it was known for long that endosperm development is sensitive to dosage effects (reviewed in Birchler, 1993), the large number of gametophytic maternal effect mutants uncovered over the last years in Arabidopsis and also maize has been unexpected. Because plant cells can easily form somatic embryos in culture, it was generally thought that embryogenesis relies neither on a specialized cytoplasm nor on a distinct epigenetic chromosomal constitution of the gametes. Because seeds usually contain genetically identical embryo and endosperm, it is difficult to distinguish between direct effects on embryogenesis and indirect effects mediated by the endosperm. However, many of the gametophytic maternal effect mutants in Arabidopsis show very early effects on embryogenesis (Moore, 2002; Pagnussat et al., 2005). Given that the Arabidopsis embryo can develop to the globular stage in the absence of an endosperm, it is likely that some of these effects are direct. Thus, zygotic embryogenesis depends on factors controlled by the megagametophyte, but these requirements can be overcome in somatic embryogenesis or androgenesis.

The mechanisms underlying gametophytic maternal effects are diverse. They could be related to dosage effects of various kinds, including haplo-insufficiency, altered parental genome balance in the endosperm, or an imbalance of the

megagametophytic to the zygotic genome (Birchler, 1993; von Wangenheim and Peterson, 2004; Grossniklaus, 2005; Dilkes and Comai, 2004). Alternatively, embryo and/or endosperm development may depend on maternally produced products that are stored in the gametes and required after fertilization. Finally, such effects may be due to genomic imprinting, which leads to a functional non-equivalence of maternal and paternal genomes. In imprinted loci, the activity of an allele depends on its parental origin, with maternally and paternally inherited alleles showing different activities. The first locus for which regulation by genomic imprinting was unambiguously shown was the *R* locus in maize (Kermicle, 1970), which leads to a fully colored aleurone if inherited maternally, but a mottled phenotype when inherited paternally (Fig. 4). Several imprinted loci have been described in maize and Arabidopsis, including some that are essential for seed development. Their regulation involves complex epigenetic mechanisms based on both DNA methylation and chromatin modifications. A detailed description of imprinted loci and their regulation can be found in the chapter by Springer.

A few years ago, studies in Arabidopsis have revealed that early seed development may be largely under maternal control (Vielle-Calzada et al., 2000). For a large number of reporter genes and some endogenous loci, no paternal activity could be detected during the first few divisions of the zygote, after *in vitro* fertilization (Vielle-Calzada et al., 2000; Luo et al., 2000; Springer et al., 2000; Sorensen et al., 2001; Baroux et al., 2001; Golden et al., 2002; Yadegari et al., 2000). Although there is a low basal activity of paternal alleles at these early stages (Baroux et al., 2001) and

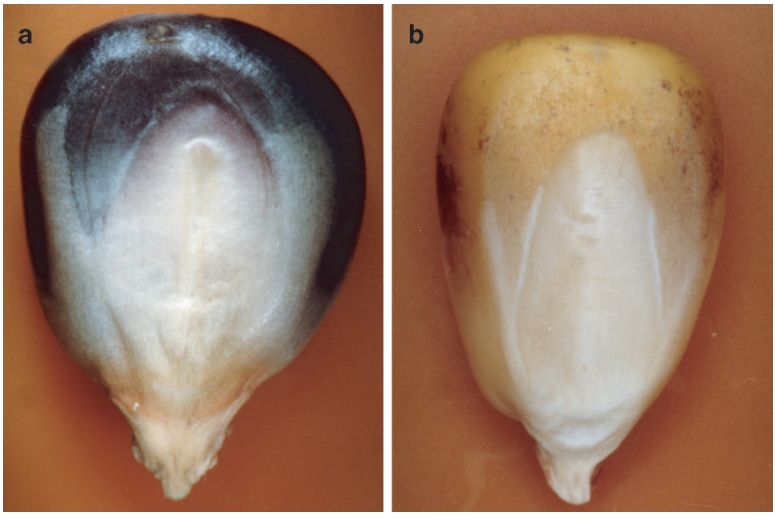

Fig. 4 Parent-of-origin effects. Kernels derived from reciprocal crosses with a colorless *r-g* tester line show different phenotypes dependent on the parent-of-origin of the *R-r* allele. (**a**) If inherited maternally, the kernel is fully colored; (**b**) if inherited paternally, it shows a mottled phenotype. *R-r* was shown to be regulated by genomic imprinting (Kermicle, 1970). Here, *R-r* was exposed to *R-st* in the previous generation, enhancing differential expression. Picture by U. Grossniklaus.

some paternally inherited alleles become activated already after the first or second division (Weijers et al., 2001), there is a clear non-equivalence in maternal and paternal expression even in these cases (Baroux et al., 2002; Grossniklaus, 2005; Walbot and Evans, 2003). At present, it is not known whether this predominantly maternal expression is due to maternally stored products, is related to a general but transient imprinting phenomenon, or is a combination of both (Vielle-Calzada et al., 2000). In maize, microarray analyses have revealed a similar dominance of maternal expression during early seed development (Grimanelli et al., 2005). There are, however, also many loci that show bi-parental expression in the zygote after *in vitro* fertilization (Meyer and Scholten, 2007). Thus, there appear to be two distinct classes of early acting genes in maize, but it is not known whether their distinct regulation is related to a specific function. This difference with respect to the maternal control of early seed development between maize and Arabidopsis may be linked to the fact that heterosis effects are seen very early in maize embryogenesis but are not manifest in Arabidopsis until after germination (Meyer and Scholten, 2007; Wang, 1947; Meyer et al., 2004).

5 Molecular and Genetic Analysis of Megagametophytes

Several studies show that large portions of the genome are required during the gametophytic phase of the life cycle in maize. Patterson and others exploited more than 850 reciprocal translocation stocks in maize, producing ~1700 deficiencies, to establish that about 90% resulted in embryo sac lethality (Patterson, 1978). Two separate strategies are being used to identify the genes involved in megagametophyte development and function: (1) identification of genes expressed in embryo sac cells and (2) isolation of mutants impaired in megagametophyte development or function.

Several technological advances have assisted the identification of genes expressed in the embryo sac. One of these is the ability to isolate viable gametophyte cells and make cDNA libraries from single embryo sac cell types. Several labs have taken advantage of this technique to isolate embryo sac RNA; sequencing and expression analyses of these cDNAs have identified several thousand embryo sac transcripts, including ones specific to the egg and central cell (Cordts et al., 2001; Le et al., 2005; Sprunck et al., 2005; Yang et al., 2006) – unknown, hypothetical, and novel proteins are encoded by a large portion (30%) of these sequences (Yang et al., 2006). Microarray technology allows the analysis of many thousands of genes simultaneously. Comparisons of gene expression between Arabidopsis ovules with and without embryo sacs using microarrays were used to identify over 1,200 embryo sac-expressed genes (Johnston et al., 2007; Jones-Rhoades et al., 2007; Kasahara et al., 2005; Yu et al., 2005). However, these microarray experiments do not give a complete description of the megagametophyte transcriptome, because genes expressed in both the embryo sac and diploid ovule tissue are not distinguished from genes expressed only in the diploid tissue. Additionally, genes with low expression levels may be missed in a complex tissue sample. The maize embryo sac cell purification techniques allow for a description of all of a cell's RNAs

but are limited to more mature embryo sac cells. The use of laser capture microdissection (Kerk et al., 2003; Day et al., 2005) will allow the collection of highly purified megagametophyte tissue of any stage for a complete description of the megagametophyte transcriptome, particularly in combination with the high throughput sequencing technologies now available.

Mutations in genes required in the megagametophyte result in characteristic fertility phenotypes and modes of transmission in mutant heterozygotes. Female gametophyte mutants have reduced fertility and seed set. In a heterozygote, half of the embryo sacs inherit the mutant allele, and the seeds are not in well-ordered rows, because the wild-type embryo sacs producing the seed are randomly distributed. Additionally, when mutant heterozygotes are crossed as females, both the mutant allele and the alleles of loci linked to it in *cis* are found at a lower frequency in the progeny than the wild-type allele, because embryo sacs carrying the mutant chromosomal region fail to make seeds. In the special case of gametophytic maternal effect mutants, heterozygotes produce up to 50% defective kernels regardless of pollen genotype, while mutant pollen has no effect on seed development. These features allow for three different means to identify megagametophyte mutants: (1) reduced seed set of ears with disrupted rows of kernels; (2) segregation ratio distortion of the visible genetic markers linked to the gene; and (3) seed phenotypes that only occur when the mutation is maternally transmitted.

The ability to study mutations in genes required in the gametophyte depends on the ability to produce and maintain heterozygous, diploid sporophytes (i.e., the ability to transmit the mutation). For this to occur the gametophyte mutant phenotypes must be either sex-specific or otherwise partially transmittable, thus allowing the mutation to pass through the haploid stage to the next generation at some frequency. If the new mutant only produces defective gametophytes and particularly defective gametes, a heterozygous seed would rarely if ever be produced. Thus, many mutant alleles cannot be generated and studied under common genetic practices. Because of the requirement to transmit mutations through a haploid generation, all screens for gametophyte mutants in standard diploids are biased against mutants with no or very low transmission through both gametophytes, and thus mutations in gametophyte-essential genes will be missed, because they are rapidly culled from standard stocks. This problem can be circumvented by using duplications of chromosomal regions so that some gametophytes are heterozygous for wild-type and mutant alleles and, therefore, viable. Maize is an excellent system for this purpose because translocations between any of the standard A chromosomes and the supernumerary B chromosome can be used to create a tertiary trisomic plant carrying duplications of whole chromosome arms (Auger and Birchler, 2002).

Some gametophyte genes have been identified in maize, but because of the difficulties involved, they are relatively few in number (Table 1). The reduced fertility phenotype is useful for screening the whole genome, but is not distinct from chromosomal rearrangements and some sporophytic effects, requiring tests to determine the cause of the reduced fertility (Moore et al., 1997). Segregation ratio distortion has the advantage of identifying the map position of the affected gene, verifying that the reduced fertility is caused by reduced transmission of a

Table 1 Published maize megagametophyte mutants

Mutant	Embryo sac morphology	Male gametophyte defect	Maternal effect	Sporophyte defect	References
indeterminate gametophyte1	Excess proliferation and abnormal fertilization	N	Y	Y	(Evans, 2007; Huang and Sheridan, 1996; Kermicle, 1971; Lin, 1978, 1981)
lethal ovule1 (lost)	nd	Y	N	nd	(Singleton and Mangelsdorf, 1940)
lethal ovule2	Early arrest	Y	N	nd	(Nelson and Clary, 1952; Sheridan and Huang, 1997)
small pollen1 (possible new allele)	Early arrest	Y	N	Y	(Singleton and Mangelsdorf, 1940; Evans, unpublished)
maternal effect lethal1	Normal	Y	Y	Y	(Evans and Kermicle, 2001)
baseless1	Abnormal central cell morphology	Y	Y	Y	(Gutierrez-Marcos et al., 2006)
Zmea1 (by RNAi)	Pollen tube attraction defect	nd	nd	nd	(Marton et al., 2005)
dappled1	nd	Y	Y	nd	(Stinard and Robertson, 1987)
dap2-dap7	nd	Y	Y	nd	(Gavazzi et al., 1997)

mutant allele on one chromosome, and easing identification of mutant heterozygotes in the next generation.

Many gametophyte mutants have been identified in Arabidopsis. Research for gametophyte mutants in Arabidopsis has taken advantage of the transmission defect phenotype, using segregation distortion of the insertional mutagen itself. Mutagenesis with T-DNA or *Ds* elements carrying an easily scored herbicide resistance gene facilitates scoring the ratio of homozygous wild-type to heterozygous mutant progeny, regardless of whether the insertion occurs near a visible marker. This strategy takes advantage of the least ambiguous phenotype of the mutants and eases their subsequent cloning. However, it does not discriminate between male and female gametophytic mutants and, therefore, two-step screens relying on reduced seed set and segregation ratio distortion have been used to enrich for mutants affecting megagametogenesis (Moore et al., 1997; Page and Grossniklaus, 2002). The use of insertional mutagens has not yet been exploited in maize because of the difficulty of transformation. But, with the easily scored endosperm markers available in maize for transposition and inheritance of a single copy transposable element, it could be

done very efficiently. The ease of transforming Arabidopsis has also facilitated the establishment of lines carrying GUS or GFP reporters specifically expressed in the embryo sac or individual cell types of the embryo sac (Gross-Hardt et al., 2007; Huanca-Mamani et al., 2005; Punwani et al., 2007; Yang et al., 2005, Ngo et al., 2007; Vielle-Calzada et al., 1998). With these markers, gene expression in the embryo sac can be analyzed more rapidly than by *in situ* hybridization, permitting their use in mutant screens (Gross-Hardt et al., 2007). Development of a similar suite of maize reporters will be very useful for maize gametophyte research.

The array of described mutants combined from maize and Arabidopsis contains at least one mutation associated with each of the developmental stages depicted in Fig. 1. Five broad classes of mutants are those that: (1) are affected during the nuclear division phase; (2) are affected during maturation of the gametophyte; (3) appear normal but fail to achieve fertilization; (4) have maternal effects on embryo and/or endosperm development; and (5) affect pollen tube growth (for those affecting the male gametophyte). Mutations have been identified from screens for gametophyte mutants in EMS-treated, irradiated, *Ds* mobilized, or T-DNA insertion lines in forward genetic screens for gametophyte phenotypes, as well as in T-DNA populations using reverse genetic analyses of genes of interest (see also Brukhin et al., 2005). Table 2 summarizes the effects of published gametophyte mutants in maize and Arabidopsis. Certain trends emerge in looking at the collection of mutants from multiple projects: many mutants affect both male and female gametophytes even if the original screen was for female semi-sterility, and the most common megagametophyte phenotype is disruption during the nuclear division phase (primarily arrest). Moreover, a surprisingly high number of mutants show a maternal effect phenotype confirming the strong maternal influence on early seed development.

Table 2 Developmental stage and sex affected by published gametophyte mutants[a]

Gender affected			Stage affected				
Female	Male	Both	Division phase	Maturation phase	Fertilization	Maternal effect	Pollen tube growth
25	36	82	90	31	28	88	50

[a]The number of mutants in each class is shown. Phenotype totals do not match sex-affected totals because not all mutants were examined for all aspects of transmission or phenotype, and some mutants belong to more than one phenotypic class
(Acosta-Garcia and Vielle-Calzada, 2004; Arthur et al., 2003; Bonhomme et al., 1998; Brukhin et al., 2005; Capron et al., 2003; Chaudhury et al., 1997; Chen and McCormick, 1996; Choi et al., 2002; Christensen et al., 1997, 1998; Ebel et al., 2004; Evans and Kermicle, 2001; Feldmann et al., 1997; Goubet et al., 2003; Grini et al., 2002; Grossniklaus et al., 1998; Guitton et al., 2004; Gupta et al., 2002; Gutierrez-Marcos et al., 2006; Hejatko et al., 2003; Holt et al., 2002; Howden et al., 1998; Huanca-Mamani et al., 2005; Huang and Sheridan, 1996; Huck et al., 2003; Jiang et al., 2005; Kasahara et al., 2005; Kim et al., 2005; Kwee and Sundaresan, 2003; Lin, 1981; Luo et al., 1990; Marton et al., 2005; Moore, 2002; Moore et al., 1997; Mouline et al., 2002; Nelson and Clary, 1952; Niewiadomski et al., 2005; Ohad et al., 1996; Pagnussat et al., 2005; Park et al., 1998, 2004; Pischke et al., 2002; Procissi et al., 2001, 2003; Redei, 1965; Sari-Gorla et al., 1996, 1997; Sheridan and Huang, 1997; Shi et al., 2005; Shimizu and Okada, 2000; Singleton and Mangelsdorf, 1940; Springer et al., 1995; Xu and Dooner, 2006)

6 Future Directions

Although our understanding of megagametophyte development has increased dramatically over the last few years, particularly with regard to the identity of some of the genes required, a great many aspects of megagametophyte biology are still incompletely understood. Our understanding of this phase of the maize life cycle is going to increase dramatically in the next few years simply by applying the knowledge gained from other systems and through continued identification and analysis of maize gametophyte mutants, but more is needed for a complete description of the megagametophyte. Combining recent technologies like laser capture microdissection and genome-wide expression analysis will provide a complete description of the transcriptome of all stages of megagametophyte development, allowing the dynamic modeling of these processes through network inference analyses. But additional tools need to be developed in order to describe other details, such as the proteome, interactome, methylome and metabolome, and the cellular compartments in which they occur. Such studies are greatly impaired by the limited and inaccessible material of the embryo sac and require the development of novel approaches. In particular, the ability to examine the function of proteins in living embryo sacs in a genetic system like maize will be critical for answering many of the remaining questions.

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Patterning of the Maize Embryo and the Perspective of Evolutionary Developmental Biology

Judith Nardmann and Wolfgang Werr

Abstract Genetic and molecular analyses in the dicot model plant *Arabidopsis thaliana* have identified numerous genes and different regulatory networks involved in embryonic patterning. Many genes encode members of transcription factor families. Orthologous genes can be identified by phylogenetic reconstructions based on conserved protein domains and functionally substantiated by gene expression patterns and mutant analyses. However, interspecies comparisons are necessary to validate networks identified in model species on the evolutionary scale. Comparative analyses between maize and *Arabidopsis* reveal both a significant conservation of gene expression patterns and thus presumably function as well as the evolutionary freedom for adaptation in the course of plant speciation.

1 Introduction

The life cycle in maize starts with a double fertilization event, which is characteristic for angiosperms. One sperm cell fuses with the egg cell whereas the second sperm cell fuses with the binucleate central cell of the embryo sac to give rise to the endosperm, which is discussed in Chap. 6. Grasses such as maize exemplify the most complex embryogenesis in plants resulting in the development of grass-specific organs like the scutellum, the coleoptile, and the coleorhiza. The homologies of these organs have been the subject of longstanding investigations and controversial discussions (Weatherwax, 1920) starting with Malpighi (1687). We will not outline these different views here but discuss some selected aspects later in association with molecular markers, which shed some new light on these evolutionary interesting questions.

2 Histology

2.1 Early Embryo Development

The diploid zygote, the fertilised egg cell, follows a strictly cellular division pattern with a first division about 40 h after pollination. This first division is asymmetric and oriented perpendicular to the micropylar/chalazal axis of the embryo sack. It

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results in a small apical or semi-lateral cell and a large basal cell (Randolph, 1936), which have different fates: the apical cell develops into the *embryo proper*; the basal cell will form the suspensor. Only the plane of the first division is predictable in the maize zygote, all subsequent cell divisions in the maize embryo appear random although characteristic morphological stages are established. In contrast, a stereotypic cell division pattern in the early *Arabidopsis* embryo results in the establishment of apical/basal polarity and radial organization in the *embryo proper* (Jurgens, 1992; Berleth and Chatfield, 2002). The typical sequence of maize embryonic stages is depicted in Fig. 1; the terminology is according to Abbe and Stein, 1954, recently compared to *Arabidopsis* in Vernoud et al., 2005.

Cells descending from the small apical cell remain small and cytoplasm-rich, whereas basal suspensor cells vacuolize and enlarge. This cell size difference discriminates the apical *embryo proper* from the subtending suspensor sharing a multicellular boundary in the ovoid proembryo. Both, the *embryo proper* and the suspensor enlarge through ongoing cell division to form the club-shaped transition stage maize embryo. Histologically, little evidence exists for further differentiation up to midtransition stage.

2.2 *Establishment of the Embryonic Axis: Shoot and Root Meristems*

About 7–9 days after pollination (dap) at the face opposite to the developing endosperm and thus oriented toward the main axis of the ear (adaxial) a small group of cells remains densely packed in the *embryo proper* whereas surrounding

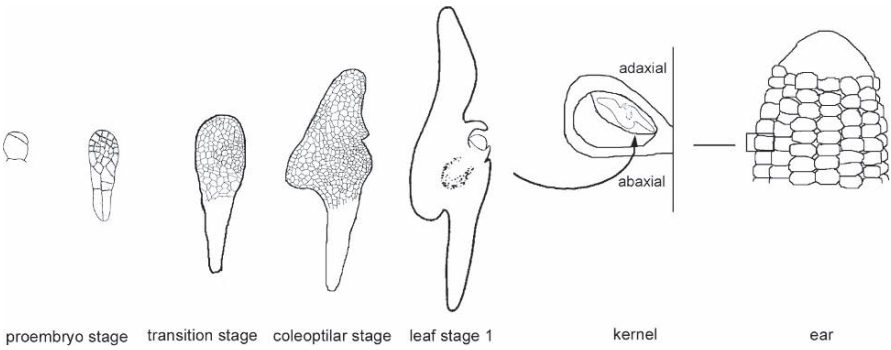

Fig. 1 The histology of maize embryogenesis. Schematic drawings of successive stages in maize embryogenesis (from left to right; adjusted from Abbe and Stein, 1954) and the position of the embryo in an adaxial compartment of the kernel relative to the ear. After fertilization, embryogenesis starts with an asymmetric division of the zygote resulting in a small apical and a large basal cell (left). During the proembryo stage, the apical cell gives rise to the embryo proper whereas the basal cell develops into the suspensor. Histological differences between the scutellum and the prospective SAM become apparent during the transition stage when cells in the scutellum enlarge whereas cells at the opposing adaxial face of the embryo remain small and cytoplasm-rich. At the coleoptilar stage, a notch above the SAM indicates the development of the coleoptile. Later embryonic stages are named according to the number of initiated leaf primordia (leaf stage 1–6)

cells start to enlarge. This is an important morphogenetic decision as the enlarging cells will form the scutellum and thus the future morphogenic shoot/root axis is placed to the opposing, adaxial face of the *embryo proper*. In this domain of greater cytoplasmic density, the shoot apical meristem (SAM) is initiated at a lateral position. It becomes apparent in longitudinal sections of coleoptilar stage embryos as a small protuberance with a notch above it, the apical tip of the emerging coleoptile.

To the end of the coleoptilar stage, which depends on the genotype and the environmental conditions (10–15 dap), the scutellum, which is considered as the single grass cotyledon, has enlarged apically, basally, and laterally. In a frontal view the SAM is positioned central of the scutellum and encircled by the now-recognizable coleoptilar ring, which still remains to be closed basally (Fig. 1). In median longitudinal sections, the shoot/root axis is elaborated oblique to the apical basal polarity of the embryo with the SAM at a superficial lateral position. The root meristem (RM) has now acquired its characteristic histology at a central, basal position of the *embryo proper* on top of the suspensor but still forms a continuous cell array with the surrounding embryonic tissue.

2.3 Development of Embryonic Leaves and Maturation

In contrast to dicots such as *Arabidopsis* where the SAM achieves activity only after germination, the embryonic SAM in maize starts with the initiation of 5–6 leaves. Later embryonic stages are named according to leaf number (leaf stage 1–6). The first leaf is always initiated at the basal face of the SAM where the coleoptilar ring closes. This position is opposite to that of the coleoptile and reflects the alternate leaf phyllotaxy (Fig. 1). Subsequent embryonic leaves are initiated either at the apical (2, 4, 6) or at the basal (3, 5) position of the embryonic SAM with a new leaflet becoming apparent approximately every 2 days. At maturity the embryonic plumule consists of the SAM enclosed by 5 or 6 leaf primordia and protected within the coleoptile for emergence through the soil.

The primary root elongates and the characteristic root histology with central pith, vascular elements, endodermis, and cortex is fully established. The primary root delaminates laterally from the surrounding embryonic tissue but cells subtending the root quiescent centre (QC) remain in contact with the QC and connect the miniature plantlet with the basal suspensor until it finally degenerates.

The last phase of embryo development is maturation. Storage products such as lipids accumulate in the scutellum, which increases greatly in size and becomes a major portion of the embryo. Apoptosis, or programmed cell death, contributes to degeneration of the suspensor and accounts for shape changes that set apart the primary shoot and root from enshrouding embryonic, mainly scutellum tissue (Giuliani et al., 2002). During this phase, the capacity to withstand desiccation is established before ultimately dormancy occurs after massive dehydration of the embryo during maturation of the maize kernel.

3 Cellular Decisions and the Perspective of Evolutionary Developmental Biology

Morphogenesis is the read out of developmental decisions implemented earlier on the level of a single cell or groups of cells. Such decisions rely on the genetic program and altered gene expression. RNA transcripts or proteins – so-called molecular markers – can visualize differences or similarities between cells in time and space. Although the suitability of the individual gene is unpredictable, molecular markers may originate via various approaches. *KNOTTED1 (KN1)* for example was isolated as a dominant leaf developmental mutant (Vollbrecht et al., 1991) but marks the origin of the SAM in the maize embryo (Smith et al., 1995). As genome sequence information is nowadays rapidly accumulating, the modern field of evolutionary developmental biology (EvoDevo) provides an intriguing perspective. Based on sequence phylogeny and functional analyses, it compares homologous gene functions between species and addresses the question of how regulatory networks have been maintained or changed during evolution. For example, *KN1* in maize is considered to encode a homologous gene function to *SHOOT MERISTEMLESS (STM)* in *Arabidopsis*, which is essential to establish and maintain the SAM (Barton and Poethig, 1993).

Embryogeny is an attractive candidate for comparative approaches as the single cell zygote is a unique starting point of the new life cycle and the establishment of SAM and RM is the common aim in higher plant embryonic programs. There is also evidence that fundamental principles in meristem function have been evolutionary conserved (Kerstetter et al., 1997). The genetic model organisms *Arabidopsis* and maize are especially suited for such a comparative analysis because our knowledge about the embryonic program is detailed and embryonic stages are readily accessible in silique or ear. These studies are supported by full or near complete genome sequences information, which will identify the shared gene repertoire. Not only similarities but also differences in the patterning programs will be of interest as these may elucidate evolutionary modulations between monocots and dicots, the latter considered comprising the more ancient situation.

At the first glance the oblique angle of the shoot/root axis in the maize embryo seems a minor change relative to the embryonic program of dicots, however, it relates to a fundamental difference. The initially stereotypic cell division pattern in *Arabidopsis* establishes radial symmetry and the apical/basal polarity of the zygote prepatterns the shoot/root axis. As the SAM is established at the apical tip of the globular embryo the symmetry-breaking event is the initiation of two cotyledons, which converts radial symmetry into two planes of bilateral symmetry. In contrast, the SAM in maize is established at a lateral position and a single plane of bilateral symmetry through the midrib of embryonic leaf primordia splits the scutellum into two mirror-image halves. The specification of the lateral SAM position requires some adaxial/abaxial information to be implemented in the zygote or in the proembryo.

3.1 Early Proembryonic Cell Types

Irradiation experiments revealed that the cell lineages of the morphogenic axis and the scutellum do not become separate until some time after the second longitudinal division of the apical precursor cell of the *embryo proper* (Poethig et al., 1986). The high frequency of early-induced whole-plant sectors suggests that the shoot meristem arises from the progeny of only one of the cells produced by the first longitudinal division, however it does not provide information about the timing of cellular decisions, which is only deducible from chromosomal breaks during later developmental stages. The resulting clonal sectors are consistent with the assumption that SAM founder cells (1–3) are specified during proembryo stage involving the protodermal and subprotodermal layers. The number of cells internal of the SAM increases during the transition stage when concentric circles of cells in the epidermal layer apparently prepattern positional information along the apical basal axis of the mature plant. Sectors confined to the tassel relate to 2–3 cells central of the embryonic SAM, which are already specified at late transition stage (Poethig et al., 1986). These data imply that positional information for the shoot is laid down in early embryogeny although cells are not ultimately determined and can be recruited for different fates.

Excellent molecular markers for early cell fates in the *Arabidopsis* embryo are members of the *WOX* (*WUSCHEL* related homeobox) gene family (Haecker et al., 2004). After the first asymmetric division of the zygote, *AtWOX2* transcripts are specific for the apical embryo proper cell and mark the apical cell fate toward the 16-cell stage when *WUSCHEL* (*WUS*) is activated in subepidermal cells. In contrast, *AtWOX8* and *AtWOX9* are expressed in the basal suspensor precursor cell and remain coexpressed in the hypophyseal cell, which gives rise to the root QC and columnella root cap (Haecker et al., 2004). Slightly later, *AtWOX5* performs a similar function in the QC as *WUS* contributes in the SAM (Sarkar et al., 2007). Most *Arabidopsis WOX* gene family members find orthologues in maize (Nardmann and Werr, 2006) and several mark early embryonic cell fates (Nardmann et al., 2007).

ZmWOX2A, one of two paralogues, is already transcribed in the zygote and marks the apical domain of the early embryo proper (Fig. 2). The expression domain then starts shifting laterally and at 5 dap the expression domain faces adaxial to the main rachis of the ear, where the SAM will be initiated. At the early transition stage transcription is restricted to the L1 layer at the adaxial face of the embryo proper, where the SAM marker *KN1* will be activated slightly thereafter in subtending cell layer (Smith et al., 1995). According to the *ZmWOX2A* expression pattern, lateral adaxial positional information is implemented between 2 and 4 days after pollination in the maize proembryo.

Early asymmetry is also evident with protodermal cell fate markers. Randolph (1936) characterized the protoderm as a single layer of homogeneously sized peripheral cells surrounding the embryo proper in the late proembryo stage. A number of molecular markers are available for the outer cell layer of the maize proembryo. A prominent example is the *lipid transfer protein2* (*LTP2*) gene (Sossountzov et al., 1991).

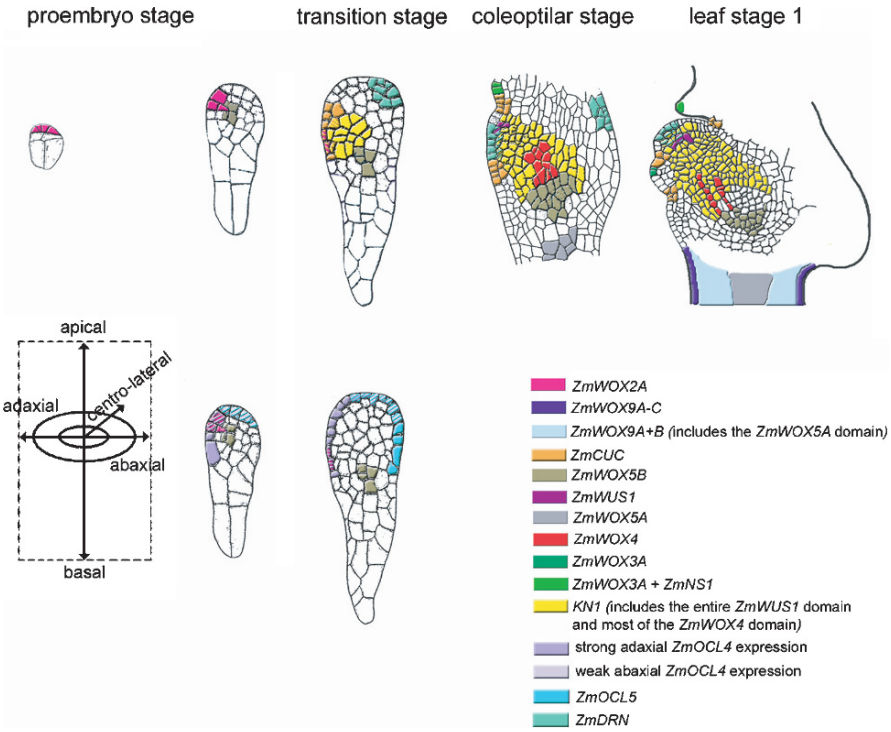

Fig. 2 Cell type specification in the maize embryo. Three different axes of the maize embryo (bottom left) are established early in embryogenesis, which is implied by gene expression patterns represented by individual or mixed colors. Depicted in the schematic drawings is the medial longitudinal plane of bilateral symmetry. Adaxial/abaxial polarity is visualized by the gene expression patterns of *ZmWOX2A*, *ZmOCL4, ZmOCL5,* and *ZmDRN* from the proembryo stage onwards until it becomes evident histologically during transition stage. Radial symmetry may persist at the base of the *embryo proper* (black arrow) and the subtending suspensor as indicated by the central *ZmWOX5B* expression domain in the late proembryo. At later embryonic stages, *ZmWOX5B* marks the QC and provascular strands of the root whereas *ZmWOX4* expression starts slightly later in the developing vasculature of the hypocotyl. Discrete outer or inner regions of the basal suspensor are marked by the expression of *ZmWOX5A* and *ZmWOX9A-C* during later embryonic stages. Maize *CUC* orthologues are coactivated with *KN1* at late proembryo/early transition stage and delineate a ring-shaped boundary between SAM and coleoptile and later between the SAM and leaf primordia. The coleoptile emerges outside this ring of *ZmNAM1/2* or *ZmCUC3* expressing cells and is marked at the margin by *NS1* and *ZmWOX3A* transcription. However, the *NS1* and *ZmWOX3A* patterns differ significantly in anlagen of true leaf primordia (schematic drawing in part modified from Nardmann et al., 2007)

Plant epidermal cells are covered with cuticle and cutin monomers as other lipids are directionally transported to the outer cell wall (Thoma et al., 1993). *LTP2* transcripts are confined to peripheral cells of the *embryo proper*, but are absent in outer suspensor cells. Series of longitudinal sections through maize proembryos showed that distribution of the *LTP2* transcript is not symmetrical in the outer cell layer of the embryo proper. At the position of the prospective shoot apical meristem (SAM), where

ZmWOX2A is transcribed, a small protodermal area adaxial in the embryo proper is free of *LTP2* transcripts (Bommert and Werr, 2001).

Different protodermal cell fates are also indicated by overlapping expression domains of two members of the *Zea mays outer cell laye*r (*ZmOCL*) transcription factor family (Ingram et al., 1999). The *ZmOCL* genes as its close relative *AtML1* (*Arabidopsis thaliana meristem layer 1*) are expressed exclusively in protodermal layers of the embryo and characterized by a DNA-binding homeodomain, a leucine zipper dimerization motif, and the lipid-binding START domain. Sagittal sections through the late maize proembryo showed *ZmOCL4* transcripts preferentially in the protoderm at the SAM face whereas *ZmOCL5* RNAs accumulated at the opposite scutellum face of the *embryo proper*; apically the protodermal *ZmOCL4* or *ZmOCL5* expression domains overlap (Ingram et al., 2000; Fig. 2). The asymmetries detected by *ZmWOX2A*, *LTP2,* and *ZmOCL4* or *ZmOCL5* strongly argue against radial symmetry to persist after the proembryo stage. Based on *ZmWOX2A* expression lateral positional information is realized 4 days after pollination.

Prepatterning the position of the SAM via *ZmWOX2A* raises the question about specification of the root. Here *ZmWOX5B*, which later marks the root QC, provides the earliest available marker. Expression starts at 5 dap in a central domain of the embryo proper above the uppermost tier of vacuolized suspensor cells in the proembryo (Nardmann et al., 2007). Positional information for the root is therefore realized in a central and basal domain of the *embryo proper*. The cellular histology and different proembryonic molecular markers therefore demonstrate existence of at least three major axes in the maize proembryo: apical-basal, radial and adaxial/abaxial relative to the main axis of the ear (Fig. 2).

3.2 Establishment of the SAM and the Scutellum Fate

Bilateral symmetry in *Arabidopsis* is prepatterned by the expression of the three *CUP-SHAPED COTYLEDON* (*CUC1, 2, 3*) genes, which redundantly control *STM* activation and functional establishment of the SAM (Aida et al., 1997, reviewed in Berleth and Chatfield, 2002). *NO APICAL MERISTEM (NAM)*, the *CUC2* orthologue in *Petunia hybrida*, contributes a similar function and takes a central phylogenetic position in the *CUC1/2* branch between the *Arabidopsis* members and grass orthologues, 2 paralogues in maize *(ZmNAM1 + 2)* and a single orthologue, *OsNAM*, in rice. In addition, both grass genomes contain single *CUC3* orthologues, the *CUC3* genes comprise a paraphyletic branch and thus existed prior to the dicot/monocot split (Zimmermann and Werr, 2005).

Analyses of the expression patterns in maize revealed no *ZmNAM1/2* or *ZmCUC3* gene transcriptional activity prior to the activation of *KN1* in the embryo when *ZmNAM1* and *2* are coactivated with *KN1* in a small centro-lateral domain of the midtransition stage embryo (Fig. 2). Later, at the end of transition stage, the expression patterns of *KN1* and *ZmNAM1/2* are mutually exclusive. *KN1* transcripts mark the prospective SAM but are excluded from the L1 layer cells which express

ZmNAM1/2 and now also *ZmCUC3* in a ring-shaped expression domain enclosing the future SAM and delineating a boundary to the outer embryonic tissue. Histologically, the coleoptile later emerges outside of this ring of *ZmNAM1/2* or *ZmCUC3* positive cells.

The *ZmNAM1/2* and *ZmCUC3* patterns in the maize embryo are reminiscent of the late *CUC1, 2* or *3* patterns in the *Arabidopsis* embryo from torpedo stage on. In *Arabidopsis* as in maize, *CUC* gene expression demarks a boundary between the SAM and the cotyledons or the coleoptile, respectively. In *Arabidopsis* the failure of *stm* mutants to initiate a SAM and the fact that these mutants are blocked just prior to the point at which the SAM becomes histologically distinct is consistent with the hypothesis that the initiation of the SAM coincides with its histological differentiation (Barton and Poethig, 1993). Cotyledons developing in the absence of the SAM in *stm* or *cuc* mutant consequently could not be derived from the SAM. An alternative model assumes the entire apical half of the globular stage *Arabidopsis* embryo to comprise the SAM; the first structures produced by the SAM thus would be cotyledons. However, *CUC* gene activity is necessary for *STM* activation and their expression pattern divide the apical region of the embryo in three distinct subregions: the SAM, the cotyledon boundaries and the cotyledons (Aida et al., 1999). The two cotyledons gain cellular identity in the globular *Arabidopsis* embryo when *CUC* gene activity focuses to a stripe between the prospective cotyledons and prior to *STM* transcriptional activation. This prepatterning aspect of *CUC* gene activity in *Arabidopsis* is lacking in the maize embryo, where *KN1* and *ZmNAM1/2* are coactivated simultaneously during early to midtransition stage. The regulatory network toward SAM anlagen thus is altered significantly in grasses compared to *Arabidopsis*, which is consistent with the need to implement lateral positional information for specifying the anlagen of SAM and scutellum to different faces of the maize proembryo.

An early marker for the scutellum fate is the maize orthologue of the *DORNRÖSCHEN/ENHANCER OF SHOOT REGENERATION 1* (*DRN/ESR1*) gene acting upstream of auxin during embryonic patterning (Kirch et al. 2003; Chandler et al., 2007). *ZmDRN* gene expression is detected in the late proembryo at the scutellum face of the *embryo proper* about 5–6 dap (Zimmermann and Werr, 2007). *ZmDRN* transcripts remain associated with cells at the apical tip of the scutellum throughout the transition stage resembling expression of *DRN/ESR1* in the tips of the cotyledons in the heart stage *Arabidopsis* embryo. When the scutellum emerges during the coleoptilar stage, *ZmDRN* activity ceases in the scutellum, similar to *DRN/ESR1* expression which ceases in the cotyledons but transcriptional activity is regained in the functional maize or *Arabidopsis* SAM associated with the anlagen of new leaf primordia.

3.3 Formation of Root Meristem and Coleorhiza

As outlined above *ZmWOX5B* expression provides the first evidence for the anlagen of the root in the 5–6 dap proembryo. *ZmWOX5B* is activated only slightly earlier

than *ZmSRC*, the maize orthologue of *SCARECROW* in *Arabidopsis* (Lim et al., 2000). Initially expressed in the same central domain as *ZmWOX5B*, transcripts of *ZmSCR* soon become confined to a single U-shaped cell file, the endodermis (Zimmermann and Werr, unpublished results). In contrast, the *ZmWOX5B* expression domain remains globular until late coleoptilar stage. At transition stage, the U-shaped *ZmSCR* pattern and the globular *ZmWOX5B* domain overlap in a few basal embryonic cells, which will give rise to the root QC and where both markers remain coexpressed later. In contrast to *AtWOX5*, which is exclusive to QC cells in the *Arabidopsis* root, its maize orthologue *ZmWOX5B* remains also expressed in basal provascular bundles of the embryonic root system.

The coleorhiza is an embryonic achievement without obvious counterpart in the *Arabidopsis* embryo. However, expression of the *ZmNAC5/6* paralogues, which are close relatives to *ZmNAM/ZmCUC3*, shed light on the development of the coleorhiza. Both paralogues are activated during early transition stage in an expression domain subtending the *ZmSCR* and *ZmWOX5B* domains (Zimmermann and Werr, 2005). During leaf stage 1, *ZmNAC5/6* transcripts are detected in a large domain below the primary root. At its base the embryonic root remains in contact with the subtending embryonic tissue whereas laterally the primary root delaminates from surrounding embryonic cells. Basal cells in contact with the root QC comprise the coleorhiza and express *ZmNAC5/6* during late embryonic stages. Noteworthy, one of the two paralogues, *ZmNAC5*, is postembryonically expressed in the calyptrogen of the seedling root cap. The embryonic coleorhiza and the regenerative cell layer of the root cap thus share cellular identity, suggesting the coleorhiza to comprise an embryonic root cap. However, expression of the *ZmNAC5/6* markers relative to *ZmSCR/ZmWOX5B* show that the specification of prospective coleorhiza cells starts in the early transition stage embryo, before the prospective root QC is obvious histologically.

3.4 Elaboration of the Root Shoot Axis

So far the *KN1* and *ZmWOX5B* patterns were discussed independently and relative to the anlagen of the SAM and RM, respectively. However, the primary SAM and RM comprise the opposite poles of the plant body and the patterns of *ZmWOX5B* and *KN1* exhibit striking complementarity and spatial changes in transition and coleoptilar stage embryos during the elaboration of the plant body axis. In the midtransition stage embryo, *KN1* expressing cells comprise a club-shaped, centrolateral domain, which is centrally subtended by a globular *ZmWOX5B* expression domain. When the embryo enlarges the *ZmWOX5B* domain remains globular and shifts basally whereas the *KN1* domain expands until the *ZmWOX5B* domain is contained in an inverted cup-shaped *KN1* domain at the end of the coleoptilar stage. This *KN1/ZmWOX5B* expression domain comprises the entire shoot/root axis of the maize embryo from the L2 layer of the SAM toward the root QC and indicates a common cellular identity, which separates the prospective morphogenic axis from the surrounding embryonic tissue.

Radial organization of this morphogenic axis is detected by the *ZmSCR* pattern (Zimmermann and Werr, 2005; Lim et al., 2005), which overlaps with the *KN1* expression domain at the periphery and with the *ZmWOX5B* pattern in the basal cell tier. These most basal cells coexpress *ZmWOX5B* and *ZmSRC* and specify the root QC after leaf stage 1, when the root is histologically elaborated. Specific for maize is *ZmWOX5B* transcription in basal provascular bundles which is different to *AtWOX5* in *Arabidopsis* and *QHB* in rice (Haecker et al., 2004; Kamiya et al., 2003). Further elongation of the shoot/root axis is reflected in the activation of *ZmWOX4* during leaf stage 1 initially in a broad domain between SAM and RM. *ZmWOX4* transcripts become restricted to the vascular system at the height and above the scutellar node. *ZmWOX4,* therefore, provides a marker specific for the vascular system of the hypocotyl, whereas *ZmWOX5B* is expressed in the vasculature of the subtending primary root. The *ZmWOX4* and *ZmWOX5B* patterns in the embryo are non-overlapping and the delayed activation of *ZmWOX4* relative to *ZmWOX5B* indicates that cells within the morphogenic axis acquire novel fates during elongation.

In the SAM region further evidence for differentiation is provided by the *WOX3* orthologues; this branch of the WOX phylogenetic tree in maize contains the two *NARROWSHEATH1/2* paralogues and two additional grass-specific paralogues, *ZmWOX3A/B* (Nardmann et al., 2004, 2007). Together with *ZmNS1* the grass specific *ZmWOX3A/B* paralogues are expressed in marginal cells of the coleoptile starting apical of the prospective SAM and outside of the ring-shaped *ZmNAM/ZmCUC3* boundary (Fig. 2). The redundancy of *ZmNS1* and of *ZmWOX3A/B* may explain why *ns1/ns2* double mutants are phenotypic in leaves but aphenotypic in the coleoptile. In respect to evolutionary considerations, however, more important is the orthology between *PRESSED FLOWER* in *Arabidopsis* or the *NS1/2* paralogues and their common expression domain in marginal cells of the emerging cotyledons or the coleoptile. No evidence exists for *NS1/2* or *ZmWOX3A/B* to be expressed in the scutellum. Therefore the coleoptile (*NS1 + ZmWOX3A/B)* and the cotyledons (*PRS*) share cellular identity at their margins, which may relate to the morphogenetic programs both relying on apical growth.

After the coleoptilar stage, *NS1/2* and *ZmWOX3A/B* are transcriptionally active at the periphery of the SAM. Their expression patterns are very dynamic and relate to leaf development. Although presumably acting redundantly in the coleoptile, the *NS1/2* or *ZmWOX3A/B* patterns differ in the SAM. *ZmNS1/2* activity in the shoot apex is restricted to small foci where lateral leaf domains are initiated and will detach from the apex (Nardmann et al., 2004). In contrast, *ZmWOX3A/B* expression marks an earlier cellular decision in the SAM, where a ring of peripheral cells is recruited for the P_0 primordium (Nardmann et al., 2007). Expression starts and ceases first at the midrib position of a new leaf primordium and then extends to the opposite face of the SAM. Members of the WOX3 branch are good markers for SAM function in leaf stage embryos but due to the dynamics and the alternate phyllotaxy of maize, the patterns may differ tremendously.

In contrast to *WUS* in *Arabidopsis*, which permanently marks the stem cell organizing centre in the SAM, the maize *ZmWUS1* and *ZmWUS2* paralogues provide no reliable embryonic markers; on a cellular resolution only *ZmWUS1* is

expressed transiently during embryogeny, in a short time window when the SAM achieves functional activity at leaf stage 1 (Nardmann and Werr, 2006).

3.5 Cell Types Outside the Morphogenic Axis

At later embryonic stages, the scutellum, which is often considered the single cotyledon of grasses, comprises a major fraction of the maize embryo. According to clonal analyses the morphogenic axis and the scutellum do not become separate until some time after the second longitudinal division in the *embryo proper* (Poethig et al., 1986). Clonal sectors either comprised more than half or less than half of the scutellum and some extended into the embryonic shoot/root axis, which in most cases was of the same phenotype as the majority of the tissue in the scutellum. Based on differently shaped sectors, *embryo proper* cells appear uncommitted during earliest stages of zygotic development. This is consistent with the early *ZmWOX2A* transcriptional activity throughout the *embryo proper*, which is only later confined to the face of the prospective SAM (Nardmann et al., 2007). With the exception of *ZmDRN* being transiently expressed from the late proembryo to midtransition stage there is no other marker for the early scutellum fate (Zimmermann and Werr, 2007; Fig. 2). In contrast, the discrete shoot/root axis is visualized continuously by *ZmWOX2A* or *ZmWOX5B* expression from the early proembryo stage onwards and then by *KN1* activation during transition stage thus implying that the maize embryo is divided very early into two independent domains.

This discrete shoot/root axis relates to the controversial and longstanding discussion whether the cotyledons and the scutellum represent orthologous organs (Weatherwax, 1920). The alternative hypothesis, that the scutellum may be an invention of grasses and consequently may find no counterpart in dicot embryos, has also been raised (Reeder, 1953). The shared expression of *ZmNS1* or *ZmWOX3A/B* and *AtWOX3/PRS* in the margins of coleoptile or cotyledons and the boundary between SAM and coleoptile or cotyledons provided by orthologous *CUC* gene activity in the maize and *Arabidopsis* embryo are in support of the latter view. Strikingly, different types of molecular markers highlight similarities between the development of coleoptile and cotyledon but with the exception of *DRN/ESR1* and *ZmDRN* provide little support for a homology between scutellum and cotyledons. A joining hypothesis considers the scutellum as the apical domain and the leaf-like coleoptile as the basal domain of the single grass cotyledon (Weatherwax, 1920; Kaplan, 1973).

Another embryonic organ is the suspensor, which traces back to the large basal cell originating from the first zygotic division. It is thought to position the embryo within the developing caryopsis and to provide nutrients to the developing embryo, although conclusive experimental evidence is still missing. A hint may be provided by the expression pattern of *ZmWOX5A*, the paralogue of the QC-specific *ZmWOX5B*. Expression is detectable in the leaf stage 1 suspensor region in 4–6 cell layers below the root QC (Fig. 2). The intervening cells express the coleorhiza/calyptrogen marker *ZmNAC5/6* (Zimmermann and Werr, 2005). Although the

coleorhiza laterally delaminates from the primary embryonic root, it remains connected to the subtending embryonic suspensor at its basal end. The *ZmWOX5A* gene is transcribed in cells subtending the central coleorhiza domain, which forms a continuous cell array with the root. Concerning the function of these cells in the suspensor, the transcriptional activity of *ZmWOX5A* in the early endosperm may be of some interest as it is restricted to the basal transfer layer shortly after cellularization (Nardmann et al., 2007). This specialized endosperm domain provides nutrient supply from maternal into the filial tissue i.e. endosperm and embryo (Hueros et al., 1995). By analogy, the *ZmWOX5A* transcriptional activity in the suspensor might indicate a cell type specialized for acropetal transport of nutrients through the root into the growing miniature maize plantlet. Consistent with this assumption is the *ZmWOX5A* expression in the root rhizodermis allowing ions and water uptake from the rhizosphere into the root. Early markers for the suspensor cell fate such as *AtWOX8/9*, which mark the basal cell after the first zygotic division in the *Arabidopsis* embryo, have so far not been described in maize.

4 Perspectives

Starting from a single cell, the zygote, plant embryonic patterning programs elaborate the SAM, the RM and some of the cellular complexity of the primary morphogenic axis. Although differing largely between species (Johansen, 1950; Wardlaw, 1955) the embryonic program in each plant species is highly reliable and provides a unique entry point for addressing questions about cell type specification or cellular differentiation in the course of development. Pioneering histological analyses in the early 20th century involved numerous species from different branches of the green tree of life. Nowadays two main models have remained: firstly *Arabidopsis* the dicot model where our knowledge is far advanced and often is approaching a cell biological resolution, and secondly maize the monocot or grass model. A large repertoire of developmental including embryonic mutants exists and various embryonic stages are easily accessible on the ear. Moreover, being an important crop plant research on maize frequently relates to applied aspects e.g. lipid storage in the scutellum of the embryo. Foreseeable molecular mechanisms contributing to the functional establishment of SAM and RM in the maize embryo will be of relevance during vegetative and reproductive phase and to elaborate plant architecture. As our technique and method repertoire has tremendously improved within the last century we should revisit plant embryogeny and take the challenge to firstly identify the principles of fundamental importance during plant embryonic patterning and secondly those, which were free to evolve and to give rise to the phenotypic pleiotropy we observe in the plant kingdom. Numerous embryonic mutants exist in *Arabidopsis* and maize or can be identified in other species. Although such embryonic phenotypes are frequently lethal (Clark and Sheridan, 1991) and mutations need to be propagated heterozygously, this lethality identifies a bottleneck relating to a single gene function. Amongst those, the so-called house keeping genes may

not appear interesting from a developmental biology perspective (Magnard et al., 2004). However, at a molecular marker-improved resolution any retardation in growth can easily be discriminated from the mis specification of individual cell types in a timely or spatial fashion.

The bilateral comparison between maize and *Arabidopsis* as outlined above shows significant conservation on the level of the gene repertoire but also adaptations in gene expression patterns in the course of speciation. An emerging question relative to monocots and dicots is how lateral positional information is established in the maize proembryo, which is interpreted by establishment of the SAM at one face and the formation of the single scutellum at the opposing face of the embryo proper. In the early *Arabidopsis* embryo directed auxin transport contributes to apical basal polarity (Friml et al., 2003) thus raising the pressing questions whether the auxin distribution or perception is changed in the early maize embryo. With the completion of the maize genome sequence the auxin related gene repertoire, e.g. *PINFORMED* or *AUXIN RESPONSE FACTOR* orthologues, is phylogenetically accessible and this question will be open to a functional analysis.

Given, however, the common aim in all species to establish essential cell types and regulatory networks for the elaboration of the plant body, the perspective inherent of the plant embryonic patterning programs extends much further. Starting from the single-celled zygote the level of complexity increases sequentially, which is a unique position for comparative studies on cellular decisions and the underlying molecular mechanisms. With the accumulating sequence information of additional plant genomes and a careful selection of key species representing nodes in the green tree of life reliable gene phylogenies will be achievable and the plant embryonic program must be considered one of the best areas for comparative analyses on the evolutionary scale. Both, conservation and adaptation are of interest as they may have been subject of selection and might identify general and fundamental principles or new inventions in the course of plant evolution.

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Kernel Biology

Michael J. Scanlon and Elizabeth M. Takacs

Abstract Maize kernels contain a persistent endosperm and a developmentally precocious embryo, each of which is comprised of a relatively small number of distinct tissue types. Clonally distinct yet genetically identical structures, the embryo and endosperm are an exquisite experimental system for analyses of developmental interactions. Several decades of classical and molecular genetic research utilizing an especially abundant array of informative mutants have provided unique insight into the developmental mechanisms of maize kernel morphogenesis.

1 Introduction

1.1 Maize Kernel Structure

The kernel of *Zea mays* L. is a single-seeded fruit comprised of a persistent endosperm and a large embryo enclosed within an adherent pericarp, the mature ovary wall (Fig. 1). The endosperm accounts for 85% of the kernel mass at maturity, and is of prime agronomic importance. Histologically simple yet developmentally complex, the maize endosperm contains just four tissue types; the starchy endosperm; the aleurone outer layer; the basal endosperm transfer layer (BETL); and the embryo-surrounding region (ESR). Typical of grasses, the maize embryo is developmentally precocious. At seed maturity, maize embryos contain a root meristem, a shoot meristem, and several (5–6, depending upon genetic background) small leaf primordia enclosed within a protective covering called the coleoptile. The embryonic root is likewise surrounded in a protective sheath, termed the coleorhiza. A highly modified structure of controversial homology, the spade-shaped scutellum functions as a digestive organ upon seed germination. As the first lateral organ to differentiate from the embryonic shoot, the scutellum may comprise the upper component of the single grass cotyledon whereas the leaf-like coleoptile forms the sheathing cotyledonary base (Weatherwax, 1920; Kaplan, 1973).

Mature maize kernels are a composite of maternally derived tissues (pericarp, placenta, pedicel), endosperm tissues and embryonic structures that provides an

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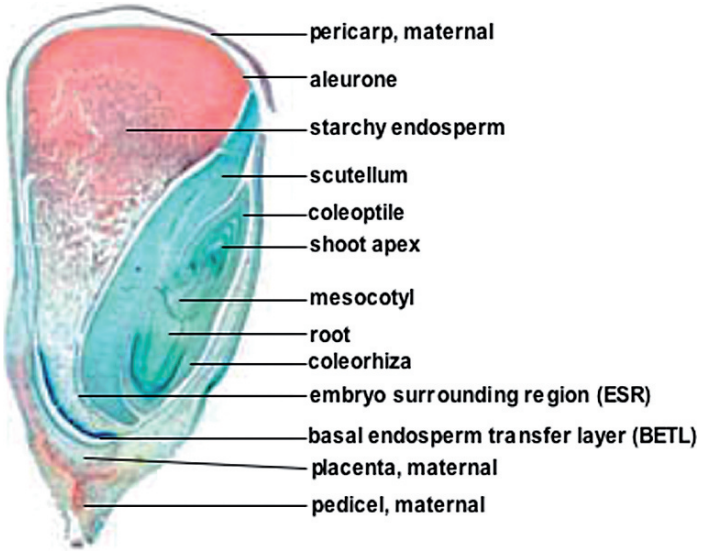

Fig. 1 Maize kernel structure. The mature maize kernel is comprised of multiple tissues and organs within the embryo and endosperm, in addition to maternally derived structures

exquisite experimental system for developmental analyses. Differentiation of the endosperm and embryo occurs side by side, within distinct developmental compartments. Although they are clonally and functionally separate from their inception, genetic analyses reveal that the embryo and endosperm interact extensively throughout their development. Thus the maize kernel offers a unique opportunity to study developmental signaling between the embryo, endosperm, and maternal tissues.

1.2 Double Fertilization Generates the Embryo and the Endosperm

As described in a preceding chapter (see Chapter 5: The maize megagametophyte), all of the eight haploid nuclei within the maize embryo sac are derived from three successive, mitotic divisions of a single megaspore. Double fertilization of both the egg cell nucleus and the two polar nuclei of the central cell generates a diploid embryo and the triploid endosperm, respectively. The vast majority of double fertilization events in maize are enacted by genetically identical sperm nuclei contained within a single pollen grain (see Chapter 4: The maize male gametophyte), although hetero-fertilization (i.e., pollination of the egg cell and the central cell by different pollen grains) does occur at a measurable frequency (1–10%; Sprague 1932). Two competing hypotheses exist concerning the evolution of double fertilization and the endosperm in angiosperms (reviewed in Friedman, 2001). One theory considers the endosperm to be a supernumery embryo, whereas a rival theory suggests that the endosperm is derived from an extended development of the megagametophyte that is augmented by a second

fertilization event. The discovery of both diploid and triploid endosperm species among basal angiosperms (Williams and Friedman, 2002) has opened the possibility that endosperm has evolved more than once during angiosperm evolution. A thorough understanding of endosperm morphogenesis in maize will enhance comparative analyses across multiple taxa and provide insight into the origin of endosperm tissue.

1.3 Genetic Analyses of Kernels: An Abundance of Informative Mutants

Investigations of maize kernel structure, function, and development are enabled by a large inventory of genetic mutants affecting all aspects of kernel biology. This chapter will focus on developmental genetic analyses of the maize kernel, with an emphasis on signaling between kernel compartments during the differentiation of endosperm tissue types. Discussions of embryo development, endosperm starch biosynthesis, and seed storage proteins are presented in separate chapters of this book.

Kernel mutations were the first maize genes to be molecularly cloned, including seed storage genes (Geraghty et al., 1981; Burr et al., 1982; Pedersen et al., 1982), starch biosynthetic loci (Shure et al., 1983), and genes required for kernel pigmentation (Fedoroff et al., 1984; O'Reilly et al., 1985). Analyses of defective seed mutants (Jones, 1920; Mangelsdorf, 1923) and germless mutations (Demerec, 1923) were among the earliest genetic studies conducted in maize. Both of these mutant classes are grouped within a larger collection of defective kernel (dek) mutants, which harbor defects in endosperm development, embryogenesis, or both (Neuffer and Sheridan, 1980; Scanlon et al., 1994). Several hundred *dek* mutations are identified although relatively few have been cloned, probably due to the inherent difficulties of working with embryo-lethal alleles. Cloned *dek* genes include *empty pericarp2* (*emp2*), which encodes a heat shock factor binding protein required for negative regulation of the heat shock response (Fu et al., 2002, 2006). Mutant kernels are developmentally arrested at about 14 days after pollination (DAP; Scanlon et al., 1997), concomitant with the developmental stage at which maize first becomes competent to elicit a heat shock response. Analyses of clonal sectors of emp2 mutant tissue confirm a separate function for EMP2 during maize shoot development, outside of its role in heat shock regulation (Fu and Scanlon, 2004). Another embryo lethal mutation, *discolored1,* also aborts kernel development before 20 DAP; mature dsc1 seed are necrotic and germless (Scanlon and Myers, 1998). *Dsc1* encodes an ARF-GAP protein putatively involved in vesicle-mediated intercellular trafficking, although nothing is known concerning the cargo of DSC1-mediated transport (Takacs et al., unpublished data). Two additional cloned *dek* mutations (the CALPAIN-encoding gene *dek1* and *supernumerary aleurone layer1*, which encodes a sorting protein required for membrane trafficking) function during aleurone development and are described in Sect. 2.4 of this chapter (Lid et al., 2002; Shen et al., 2003).

The majority of *deks* as yet described are recessive zygotic mutations, in that the mutant phenotype is dependent upon alleles contributed by both parents. However an ever-increasing number of maize kernel mutations display parent-of-origin effects, in

which the mutant phenotype is dependent solely upon the genotype of the maternal allele. Adding to this complexity are mutations such as *maternal effect lethal1* (*mel1*), wherein the defective kernel phenotype is dependent upon an interaction between the maternal parent-of-origin effect gene *mel1* and the genetically redundant zygotic effect genes *sporophytic enhancer of mel1* (*snm1*) and *sporophytic enhancer of mel2* (*snm2*). No obvious phenotype is observed in the haploid embryo sacs harboring the *mel1* mutation, and the inviable kernel phenotype is only observed when the *mel1* mutation is inherited through the female and the seeds are also homozygous for mutations in either *snm1* or *snm2* (Evans and Kermicle, 2001). Although the mechanisms of these genetic interactions are unknown, the mel1 mutant phenotype suggests that kernel development is under biparental (i.e., maternal and zygotic) control.

2 Endosperm Development

2.1 Cellularization and Growth of the Endosperm

During the first 72 h following fertilization, the triploid nucleus undergoes several rounds of synchronized mitotic divisions without cytokinesis to form a single-celled syncytium comprised of hundreds of endosperm nuclei surrounding a large central vacuole. Cellularization of the maize syncytial endosperm occurs via a different process than that observed during *Drosophila* embryogenesis. In *Drosophila*, more than 5,000 nuclei accumulate in the embryonic syncytium, whereupon they migrate to the periphery of the blastoderm surrounding the yolk sac (reviewed in St Johnston and Nüsslein-Volhard 1992). Subsequently plasma membranes invaginate the syncytium and enclose each nucleus to form individual cells. During maize endosperm development, mitosis ceases within the syncytium after 72 h and a radial microtubule system (RMS) extends out from each nuclear membrane of the coenocyte (Fig. 2). Free-growing cell walls are then initiated in the absence of mitosis via the fusion of vesicles that accumulate between daughter and non-daughter nuclei alike. Cytoplasmic phragmoplasts are deposited between each RMS to form tube-like structures known as alveoli that open toward a central vacuole. Mitotic divisions resume and are accompanied by periclinal cell wall deposition (i.e., new walls parallel to the surface of the existing cell wall), forming an outer cell layer completely encompassed by cell walls and an inner layer of alveoli opened toward the central vacuole. Mitotic divisions and cell wall deposition proceed in centripetal fashion from the outer endosperm toward the kernel center, until the endosperm is completely cellularized (Fig. 2; reviewed in Olsen 2001).

Following cellularization, differentiation of the four-endosperm tissue types becomes apparent (Fig. 2). The starchy endosperm undergoes an expansive period of cell division and cell elongation that terminates first in the central endosperm at around 12 DAP, and finally in the kernel periphery at 20–25 DAP (Duvik, 1961). Synthesis of endosperm starch and storage proteins peaks during the onset of kernel maturation at 12–15 DAP, and continues throughout later stages of kernel development as discussed in separate chapters of this book (see Chapter 22: Seed starch

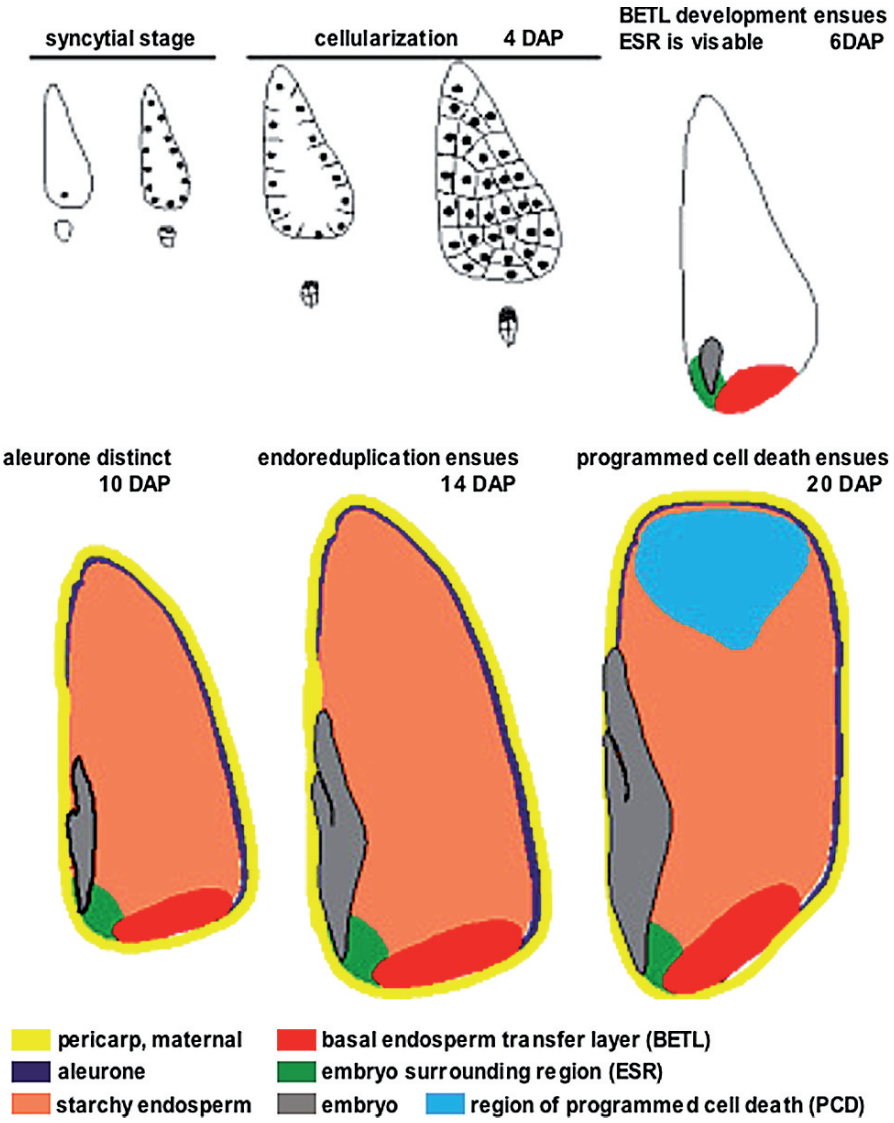

Fig. 2 Time-points in maize kernel development. Details are provided in text. The size of the developing embryo (drawn below the endosperm) relative to the endosperm during the syncytial and cellularization stages is shown. Images created by M. Evans, Carnegie Institution, Stanford University

synthesis). An unusual pattern of nuclear DNA replication termed endoreduplication initiates in the starchy endosperm after the cessation of cell division (Fig. 2), during which nuclear DNA content increases greatly in the absence of mitosis (Kowles and Phillips, 1985). Finally, endoreduplication is followed by programmed cell death (PCD) of starchy endosperm tissues, as described below.

2.2 Specification of Cell/Tissue Fate in the Maize Endosperm

Cell fate specification in the maize kernel depends upon the transduction and correct interpretation of internal and/or external signals. In contrast to the cellular migration that typifies animal development, the plant protoplasm is enclosed within rigid cell walls that are permanently cemented into place following cell division. Accordingly, the abundance of evidence derived from genetic mosaic and laser ablation analyses demonstrate that cell fate acquisition in plants is determined by positional information, rather than cell lineage. All endosperm tissue types are ultimately derived from fertilization of the two polar nuclei within the central cell of the megagametophyte, generating a triploid nucleus. Clonal analysis of maize endosperm development reveals that the first mitotic division of the fertilized central cell establishes the right and left halves of the endosperm, suggesting that positional cues are received during the syncytial stage of development and may indeed be initiated in the embryo sac (McClintock, 1978).

An ever-increasing cache of genes involved in pattern formation in the maize endosperm exhibit maternal parent-of-origin effects, which correlates with extensive hypomethylation of maternally transmitted alleles in the maize kernel (Lauria et al., 2004). As detailed below, endosperm tissues undergo cell fate specification and differentiation at different developmental stages. Whereas the ESR and BETL are established early, differentiation of starchy endosperm and aleurone cell fate occur relatively late during kernel development (Fig. 2). Once established, BETL cell specification appears to be fixed, whereas aleurone and starchy endosperm cell fates are readily reversible. Figure 2 depicts a general model outlining the developmental timing of cell fate specification in the maize endosperm; more detailed descriptions are presented below.

2.3 BETL Cell Fate is Patterned Early in Endosperm Development

The BETL forms in the basal endosperm directly over the maternal vasculature, and facilitates the unidirectional transport of nutrient solutes from the symplasm of maternal placenta to the apoplasm of the developing endosperm (Figs. 1 and 2). Secondary cell wall ingrowths extend into the first two to three cell layers closest to the kernel pedicel, thereby increasing the surface area of the BETL plasma membrane up to 20-fold (Fig. 3b; reviewed in Olsen 2004). Two cell wall INVERTASE-encoding genes [*cell wall invertase1* (*invcw1*) and *miniature1* (*mn1*)] that function to hydrolyze sucrose into hexose sugars are expressed in the BETL, in keeping with its role during uptake of solutes from the pedicel (Cheng et al., 1996; Taliercio et al., 1999). Mutations in *mn1* cause disintegration of the maternal placenta, presumably due to disruptions in cellular osmotic pressure induced by deficient INVERTASE activity (Miller and Chourey, 1992).

Differentiation of BETL-specific morphology becomes obvious beginning ~6DAP. Variation in the normal 2:1 maternal to paternal genomic ratio that typifies endosperm tissue impairs BETL development and leads to the formation of ectopic BETL cell patches in apical endosperm domains (Charlton et al., 1995; Hueros et al., 1999; Gutierrez-Marcos et al., 2003). Multiple gene functions contribute to BETL development, many of which display maternal parent-of-origin expression. Recent evidence suggests that although expression of many BETL-associated genes begins during the syncytial stage of endosperm development, maternal signals may actually initiate BETL patterning within the polar nuclei of the central cell.

Analyses of BETL development in barley led to the identification of *endosperm1* (*end1*), which is expressed in the basal region of the syncytial endosperm in the presumptive BETL domain (Doan et al., 1996). Although the function of END1 is unknown, it was proposed that a transient maternal signal emanating from the underlying placenta induces *end1* expression and BETL differentiation. The apparent *end1* ortholog in maize exhibits a homologous expression pattern in the endosperm syncytium, and serves as a useful marker for early BETL development (Gutierrez-Marcos et al., 2006). Recent studies employed in vitro endosperm culture techniques to demonstrate conclusively that both *end1*expression and BETL development require interaction with maternal tissues (Gruis et al., 2006).

Another molecular marker for BETL differentiation is the multi-locus *maternally expressed gene* (*meg*) cluster, which encode small, glycosylated, cysteine-rich polypeptides that accumulate within the cell wall ingrowths of the BETL (Gutierrez-Marcos et al.,2004). Although the function of MEG1 is unknown in the absence of genetic data, MEG1 may play a role in BETL structure, pathogen defense, or molecular signaling. Transcription of *meg1* is first detected at 4 DAP. Only the maternal allele of *meg1* is expressed at this early stage, and this parent-of-origin expression is not dependent upon the dosage of paternal-maternal genomes in the developing endosperm. At later stages (12–20 DAP) *meg1* expression becomes biallelic. A similar stage-specific pattern of parent-of-origin gene expression is seen in a maize homolog of *fertilization independent expression1* (*fie1*). First described in *Arabidopsis* as a repressor of endosperm differentiation prior to fertilization (Ohad et al., 1996; 1999), maize contains two paralogs of *fie* (*fie1* and *fie2*). Whereas only the maternal allele of *fie2* is transcribed in the embryo sac and during early kernel development, after 5 DAP both parental alleles of *fie2* are transcribed in the maize endosperm (Danilevskaya et al., 2003). Gutierrez-Marcos et al. (2004) speculate that the transient parent-of-origin expression of genes such as *meg1* and *fie2* may be related to widespread changes in chromatin structure or DNA methylation that coincide with the onset of endoreduplication (Dilkes et al., 2002; see section on endoreduplication below).

The novel *myb-related DNA binding protein1* (*mrp1*) gene is transcribed exclusively in the developing BETL, beginning in the basal syncytial endosperm ~10 h after fertilization and continuing until at least 29 DAP. MRP1 trans-activates the BETL-specific expression of at least four genes that are implicated

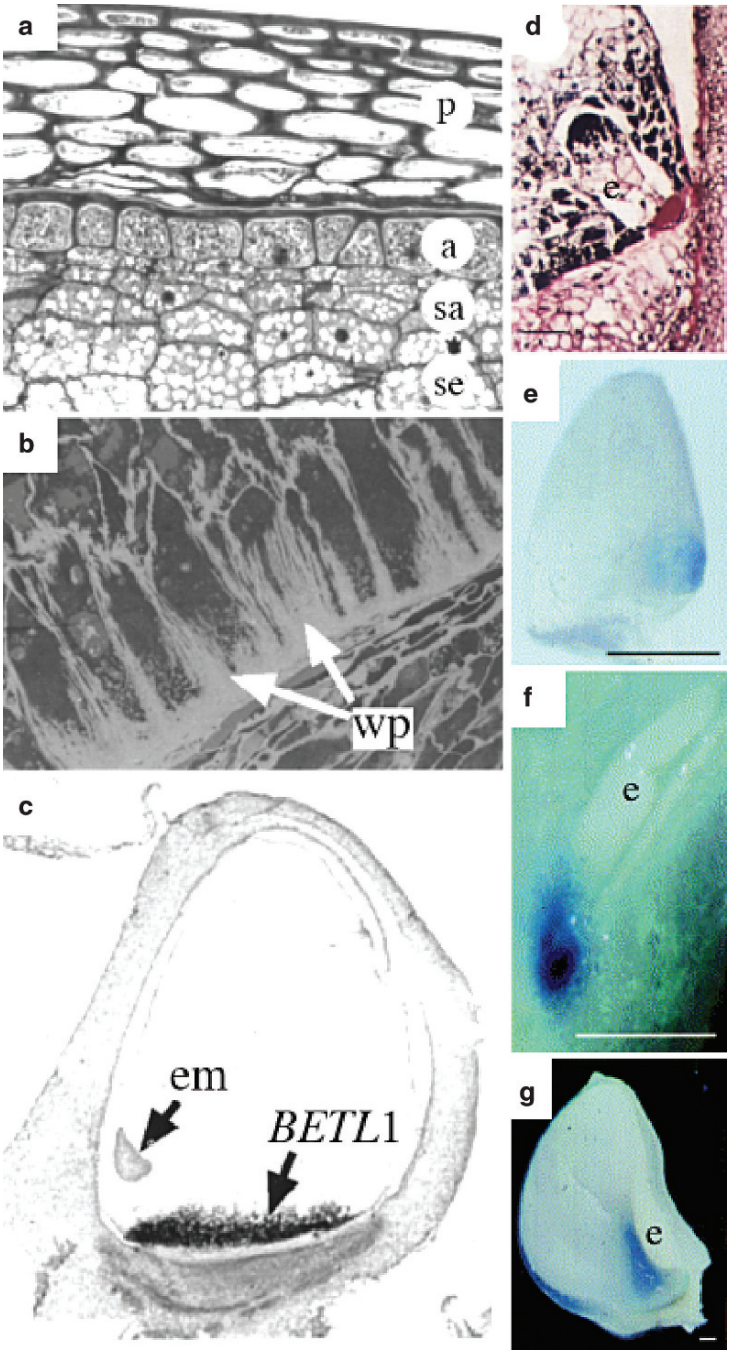

Fig. 3 Maize endosperm tissues. **a** The aleurone (*a*) forms the single-celled outer layer of the endosperm, and functions in the catalytic degradation of starch reserves during embryo germination. The sub-aleurone layer (*sa*) separates the aleurone from the starchy endosperm (*se*), which

during transfer cell differentiation or function, including *meg1*, *basal endosperm transfer layer1* (*betl1)*, *basal layer-type anti-fungal protein2* (*bap2*), and *transfer cell response regulator1* (*tcrr1*) (Gomez et al. 2002; Gutierrez-Marcos et al., 2004; Muñiz et al., 2006; Hueros et al., 1995, 1999; Serna et al., 2001). Two MRP1-regulated genes, *betl1* (Fig. 3c) and *bap2*, may encode proteins involved in pathogen defense although molecular signaling functions are also possible. Transcription of *bap2* is BETL-specific, however the BAP2 protein is secreted and localizes to the placento-chalazal cells of the pedicel (Serna et al., 2001). In contrast, the defensin-like protein BETL1 localizes to the transfer cell walls (Hueros et al., 1999; Cai et al., 2002). TRRC1 is an atypical type-A response regulator, which is predicted to function noncell autonomously (Muñiz et al., 2006). Expression of *trrc1* is localized exclusively to the differentiating BETL region between 8–14 DAP. Although transcribed in the BETL, TRRC1 protein also localizes to conductive tissues located several cell layers above the BETL, and implicates an intercellular trafficking system during BETL development (Muñiz et al., 2006).

Expression of the paralogous *embryo sac/basal endosperm transfer layer/ embryo surrounding region* genes *ebe1* and *ebe2* initiates within the central cell prior to fertilization and continues throughout the syncytial and cellularization stages of endosperm development (Magnard et al., 2003). Transcribed in both the BETL and ESR, allele specific RT-PCR reveals that *ebe1* shows maternal parent-of-origin expression in the endosperm whereas *ebe2* does not. Although *ebe1* and *ebe2* encode proteins of unknown function, their expression patterns implicate a role during BETL development and suggest that this function may begin in the megagametophyte (Magnard et al., 2003).

Mutations in *globby1* (*glo1*) disrupt both embryo and endosperm development, suggesting a model wherein irreversible patterning of BETL cell fate begins during the syncytial stage and is inherited thereafter in a cell-lineage dependent manner (Costa et al., 2003). An abnormal accumulation of nuclei observed in the basal domains of the glo1 mutant syncytium is followed by aberrant endosperm cellularization, reflecting a role for GLO1 during mitosis, cytokinesis, and cell-cycle regulation. The expression of multiple BETL-specific markers is reduced or patchy in glo1 mutant kernels, and differentiation of the BETL is variably disrupted. Discontinuous patches of BETL tissue form in apical regions of glo1

Fig. 3 (continued) accumulates the starch and endosperm proteins comprising the bulk of the endosperm mass. The kernel is encased in a multilayered pericarp (p), maternal tissue derived from the mature ovary wall. **b** Extensive cell wall projections (*wp*) characterize the BETL cells, which are specialized to transport solutes from the maternal placental tissues. **c** in situ expression of the transfer layer-specific marker *BETL1* in the basal endosperm of an immature kernel. The embryo (em) is seen in the germinal face of the developing kernel. **d** The ESR is identified by the densely stained cytoplasm of the cells surrounding the embryo (e) at 5 DAP. **e-g**. GUS-reporter gene expression is driven by the *esr2* gene promoter. Expression is seen in the basal endosperm surrounding the embryo at 5 DAP (**e**), 12 DAP (**f**) and 15 DAP (**g**). (Images in **a**–**c** are taken from Becraft, 2001; **d** is from Opsahl-Ferstad et al., 1997; **e–f** are from Bonello et al., 2000)

mutant endosperm, which is reminiscent of the clusters of BETL tissue that develop in seed containing an excess contribution of the paternal genome following interploidy crosses. Gutierrez-Marcos et al. (2003) interpret these data to mean that BETL cell fate differentiation is irreversibly assigned by positional cues sent from the maternal placental tissue to the basal syncytial endosperm during a narrow developmental window. Once assigned, BETL cell identity is proposed to be fixed and inherited in a lineage-dependent manner, owing to the fact that ectopic BETL cells formed in apical endosperm domains fail to respond to apical-positional cues and don't revert to apical cell types. Evidence for lineage-dependent cell fate acquisition during maize development is rare (Kessler et al. 2002). Although this model for BETL cell fate acquisition is intriguing, it remains virtually untestable until the identity of the predicted maternally derived BETL differentiation signal(s) is discovered.

Mutations in the maternal effect gene *baseless1* (*bsl1*) disrupt patterning within the female gametophyte and during BETL development in the endosperm (Gutierrez-Marcos et al., 2006). The central cell of bsl1 mutant embryo sacs is markedly displaced toward the chalazal pole, although the efficiency of fertilization is not affected. Expression of BETL-specific transcripts marking syncytial (*end1* and *mrp1*) and cellular (*meg1*) stages of endosperm development are abnormally patchy in bsl1 mutants, and mutant endosperms exhibit delayed and incomplete differentiation of the BETL. Tetraploid endosperms in which the maternal contribution of *bsl1* mutant alleles is increased correlate with enhanced abnormalities in BETL development. BSL1 also affects embryo morphogenesis, although the retarded development of bsl1 mutant embryos is a likely consequence of poor nutrient transfer to BETL-defective kernels (Gutierrez-Marcos et al., 2006). Taken together with the glo1 mutant phenotypes and the expression of *ebe1* and *ebe2* in both the megagametophyte and the developing BETL, the bsl1 mutant phenotypes further suggest that correct patterning of the BETL actually begins in the embryo sac and is dependent upon maternal gametophytic factors.

2.4 Differentiation of the Aleurone and the Starchy Endosperm: Reversible Cell Fates are Specified According to Positional Information

The aleurone layer of a mature maize kernel consists of a single file of cuboidal, densely cytoplasmic cells that occupies the outer layer of the endosperm surrounding the starchy endosperm (Figs. 1, 2, and 3a). Unlike the starchy endosperm that comprises the bulk of the kernel, the aleurone tissue remains viable throughout kernel development. Upon germination, the embryo generates gibberellic acid that stimulates the aleurone layer to synthesize a variety of catabolic enzymes, including hydralases, glucanases and proteinases. In turn, the enzymes produced in the aleurone mobilize the protein and starch reserves of the starchy endosperm cells and

thereby nourish the emerging maize seedling (reviewed in Olsen 2001, 2004). Thus, the aleurone layer performs an essential function in enabling the embryo-nutritive role of the maize endosperm during seed germination. Development of aleurone cell morphology is distinct by 10 DAP. Recent studies reveal that aleurone cells are clonally related to the underlying starchy endosperm and are reversibly differentiated in response to surface-positional cues and cell-to-cell signaling from the internal endosperm tissue.

Becraft and Asuncion-Crabb (2000) analyzed mosaic kernels expressing genetic markers for aleurone (*C1*) and starchy endosperm (*Wx*) identity to demonstrate convincingly that these distinct endosperm tissues share a common cell lineage. A similar strategy exploited mutant alleles of *defective kernel1* (*dek1*), a gene encoding a CALPAIN-type proteinase required for embryogenesis and aleurone development (Lid et al., 2002), to demonstrate that differentiation into aleurone and starchy endosperm cell fates is reversible. Loss of DEK1 function within X ray-induced clonal sectors in the peripheral endosperm resulted in the conversion of previously differentiated aleurone cells into starchy endosperm. Likewise, reversion of the mutable *dek1-PIA* allele to functional *Dek1* resulted in late-stage differentiation of starchy endosperm into aleurone cells, even after mitosis had ceased in the outer endosperm layer. Therefore endosperm cell fate is flexible, and dependent upon positional cues to the outer endosperm cell layer that must be maintained throughout kernel development (Becraft and Asuncion-Crabb, 2000). Analyses of connated kernels that are fused at the abgerminal face (i.e., back to back) showed that aleurone identity is absent along the fusion plane, wherein would be peripheral cells are instead buried between layers of starchy endosperm (Geisler-Lee and Gallie, 2005). These kernels also demonstrate that positional cues ascribe aleurone identity to cells occupying the endosperm periphery.

Several lines of evidence suggest that the positional cues signaling aleurone cell fate emanate from within the endosperm and not from maternal tissues such as the pericarp. In addition to the previously described defects in BETL development, *glo1* mutations also disrupt the aleurone (Costa et al., 2003). Defects include sporadic formation of multiple aleurone layers, kernel patches devoid of aleurone, and small pockets of aleurone cells that develop deep within the starchy endosperm. The formation of internal aleurone cells surrounded by starchy endosperm suggests that maternal signals alone cannot be responsible for establishing aleurone cell fate. More recently, Gruis et al. (2006) developed an in vitro endosperm organ culture system in which developing embryo sacs were excised at 6 DAP and grown in the absence of maternal tissues. Fluorescent markers of aleurone and starchy endosperm identity revealed that both these tissue types differentiate normally in culture, illustrating that maternal tissues are not the source of the aleurone specification signal(s).

Although descriptions of the precise mechanism of aleurone cell fate acquisition remain elusive, a growing number of aleurone developmental mutations correspond to genes predicted to be involved in cell-cell signaling. Identified by mutations affecting aleurone differentiation and seedling epidermal development,

crinkly4 (*cr4*) encodes a receptor kinase-like molecule with similarity to human tumor necrosis factors (Becraft et al., 1996; Jin et al., 2000). These data suggest that an unknown ligand predicted to bind the CR4 receptor might comprise the elusive positional cue specifying aleurone differentiation. The *defective kernel* gene *dek1* encodes a membrane spanning protein containing a cytosolic domain with high similarity to CALPAINs, calcium-regulated cysteine proteases previously described in animals (Lid et al., 2002). Null mutations in *dek1* are lethal, and condition shootless globular embryos within a chalky endosperm devoid of aleurone. The embryo-lethal mutant phenotype and ubiquitous expression of *dek1* suggest that, like CR4, DEK1 functions during intercellular signaling at multiple stages of maize development. A third gene required for aleurone and epidermal patterning is *supernumery aleurone layer1* (*sal1*), which encodes a ubiquitously expressed class E vacuolar protein putatively involved in membrane vesicle trafficking (Shen et al., 2003). Kernels harboring *sal1* mutations develop multiple aleurone layers, suggesting that SAL1 performs a cell-cell signaling function that restricts aleurone identity to the endosperm periphery. An exciting area of current research is focused on elucidating the mechanism(s) whereby CR4, DEK1 and SAL1 may interact and contribute to specify aleurone cell fate in maize endosperm. Detailed investigations of the potential role of additional genes controlling aleurone cell shape (including *dappled*, Stinard and Robertson, 1987; Gavazzi et al., 1997; *collapsed2* and *opaque12*, Becraft and Asuncion-Crabb, 2000) and aleurone layer patterning (including *extra cell layer1*, Kessler et al., 2002; *disorganized aleurone layer1* and *2,* Lid et al., 2004; and *paleface,* Becraft and Asuncion-Crabb, 2000) await the molecular cloning of the corresponding genes. The multiplicity of mutations affecting both aleurone development in kernels and epidermal development in seedlings implies that common developmental pathways control the differentiation of outer cell layers in multiple plant organs (Neuffer and Sheridan, 1980; Scanlon et al., 1994; Becraft et al., 1996, Kessler et al., 2002; Lid et al., 2002; Shen et al., 2003).

2.5 Endoreduplication: Dosage Effects and Cell Cycle Regulators Control DNA Content of Endosperm Nuclei

Mitotic activity declines markedly in the maize endosperm at around 10 DAP; at 14 DAP nuclear divisions effectively cease (Fig. 2; Kowles and Phillips, 1985; reviewed in Olsen, 2001). Coincident with the decline in mitosis the process of endoreduplication begins, and continues throughout the terminal differentiation stage of kernel development until 21–27 DAP. Endoreduplication is characterized by repeated, alternating cycles of DNA replication and non-replication in the absence of chromatid separation, nuclear division, or cytokinesis. Endosperm cell size and mass increase markedly during endoreduplication, which coincides with the onset of rapid accumulation of starch and storage

proteins (Schweizer et al., 1995). Endoreduplication begins in the central endosperm and spreads gradually to cells in kernel periphery (Dilkes et al., 2002; Kowles and Phillips, 1985). DNA content in endosperm nuclei of mature maize kernels averages about 12.8 C and may reach ploidy levels as high as 200 C in some inbred lines (Kowles and Phillips, 1985; Kowles et al., 1990). Initiation of endoreduplication corresponds with a spike in endosperm auxin levels, and exogenous application of auxin confers the early onset of endoreduplication in maize seed (Lur and Setter, 1993).

Flow cytometric data suggested that the entire genome is duplicated during a given endoreduplication cycle (Kowles et al., 1990). Detailed cytological inspections confirmed these predictions (Bauer and Birchler, 2006). Euchromatin and heterochromatic regions such as knobs and centromeres are all endoreduplicated, and chromatin strands remain associated throughout the length of the chromosomes after replication. Overall, the endoreduplicated chromosomes are less condensed than normal, especially at knobs and centromeres (Kowles and Phillips, 1985; Bauer and Birchler, 2006). Initial speculation concerning the function of endoreduplication focused on a possible mechanism to increase the metabolic capacity of endosperm during the peak of kernel maturation. In this view, a large-scale increase in the amount of nuclear DNA with an open chromatin conformation might enhance transcription of multiple genes required for starch and protein accumulation (Sugimoto-Shirasu and Roberts, 2003). Others speculated that endoreduplication provides a means to store phosphates and nucleotide reserves in the seed, to be utilized by the embryo upon germination (Kowles et al., 1992).

Endoreduplication is disrupted by interploidy crosses (Kowles et al, 1997; Leblanc et al., 2002). Crossing tetraploid males x diploid females induces a delay in the onset of endoreduplication, whereas crosses between tetraploid females and diploid males accelerate the process. Given these observations, researchers concluded that maternal genomic excess leads to mitotic arrest in endosperm cells and forces an early transition to endoreduplication. In contrast, paternal genomic excess allows for mitotic divisions to continue, thereby delaying or preventing endoreduplication (Leblanc et al., 2002). In keeping with these conclusions, Dilkes et al. (2002) analyzed the inheritance of endosperm DNA content in lines of maize that differed greatly in the extent of endoreduplication. Measuring endosperm DNA content as a quantitative trait, these authors concluded that endoreduplication displays maternal parent-of-origin effects in addition to maternal sporophytic effects.

Mechanistically, the process of endoreduplication requires an alteration in the cell cycle progression from G1-S-M phases. Specifically, the loss of M-phase cyclin-dependent kinase activity is predicted to induce endoreduplication, together with oscillations in the activity of S-phase cyclin-dependent kinases (reviewed in Edgar and Orr-Weaver, 2001; Larkins et al., 2001). Progression through the eukaryotic cell cycle is regulated by various CYCLIN-DEPENDENT KINASES (CDKs), whose functions are relatively well conserved in yeast, animals, and plants (reviewed in Dewitte and Murray, 2003). As predicted, Grafi and

Larkins (1995) demonstrated that S-phase-related protein kinase activity is increased in endoreduplicating maize endosperm cells, which also accumulate an inhibitor of the M phase promoting factor (MPF). As the maize endosperm transitions from mitosis to endoreduplication, expression of the maize cyclin gene *cycZme1* declines abruptly whereas expression of many S-phase genes is maintained (Sun et al., 1999a). In addition, the onset of endoreduplication is correlated with the S-phase kinase-induced phosphorylation of the maize tumor-suppressor-like protein ZmRB (Grafi et al., 1996). Unphosphorylated (i.e., active) retinoblastoma (RB) proteins in mammals function as negative regulators of the G_1-S transition during the cell cycle, suggesting that inactivation of ZmRB in the maize endosperm coincides with continuation of the endoreduplication cycle. Likewise, expression of the maize of ortholog of *wee1*, a yeast CDK-inhibitor that represses cell division and promotes an increase in cell size, is also upregulated during endoreduplication (Sun et al., 1999b). Taken together, these data reveal that endoreduplication in maize endosperm is dependent upon the differential expression cell cycle regulators whose functions are markedly conserved during eukaryotic evolution.

Leiva-Neto et al. (2004) created a dominant-negative mutation in the maize *cdka* gene that reduced the endosperm nuclear DNA content by 50% when expressed between 12 and 15 DAP. Subsequent analyses further implicated a role for CDK-inhibitors during maize endoreduplication. Over-expression of KRP1, an endosperm protein that inhibits numerous cyclin-associated CDK activities in maize, caused an extra round of DNA replication without mitosis in embryogenic callus tissue (Coelho et al., 2005). Surprisingly, although endoreduplication was effectively halved in the cdka mutant seed, no significant decrease was observed in the accumulation of starch, storage proteins, or endosperm transcripts. These data suggest that endoreduplication may not function to enhance endosperm gene expression during kernel expansion, but rather to increase the reserves of phosphates and nucleotides during kernel germination (Leiva-Neto et al., 2004).

2.6 Programmed Cell Death of Endosperm Tissues

Following endoreduplication and prior to desiccation and quiescence, the starchy endosperm cells of the maize kernel undergo programmed cell death (PCD). PCD is characterized by specific nuclease and protease activities culminating with the internucleosomal degradation of nuclear DNA characteristic of apoptosis (Young and Gallie, 2000a). The aleurone is exempt from PCD, and survives to degrade the protein and starch reserves within the starchy endosperm cells upon kernel germination. It is hypothesized that PCD of the starchy endosperm cells has evolved to allow the aleurone-derived hydrolases to gain access to the carbohydrate stores within these kernel tissues (Young and Gallie, 2000a,b, Gallie and Young, 2004).

PCD initiates within the centrally localized starchy endosperm cells at ~16 DAP, and progresses to the crown before finishing toward the kernel base at ~24–40 DAP (Fig. 2). Young et al. (1997) showed that elevated levels of ethylene lead to the onset of endosperm PCD. Interestingly, the ethylene precursor is produced in both the endosperm and the embryo of nonmutant kernels, but this precursor is only converted to ethylene in the endosperm (Gallie and Young, 2004). ABA is implicated as a negative regulator of ethylene-induced PCD, as demonstrated by the early PCD-onset phenotypes of the ABA insensitive mutant vp1 and the ABA deficient mutant vp9 (Young and Gallie, 2000; McCarty et al., 1989; Hattori et al., 1992). Likewise, the starch biosynthetic mutant shrunken2 exhibits elevated levels of kernel ethylene resulting in premature PCD in the endosperm and ectopic cell death in the mutant embryo (Young et al., 1997).

3 Embryo–Endosperm Interactions

3.1 Interactions Revealed by Analyses of Discordant Kernels

The persistent endosperm of maize kernels develops alongside the embryo, which forms 5–6 leaves prior to kernel quiescence. A normal embryo cavity forms in the base of the endosperm even in embryo-less mutant kernels, revealing that the endosperm is genetically programmed to receive the embryo and may not require signaling from the embryo itself (Clark and Sheridan, 1991; Heckel et al., 1999). Although their cell lineages are clonally separated following megasporogenesis, the endosperm and embryo do interact extensively during kernel morphogenesis and represent an excellent system to study signaling networks between these developmentally distinct compartments. A large number of *dek* mutations disrupting both embryo and endosperm development are available in maize, and serve as useful genetic tools to study the kernel interactome. Such analyses are enhanced by the use of maize B-A translocation stocks, which enable the easy manipulation of chromosome arm dosages in both the endosperm and the embryo. Discordant kernels resulting from the use of B-A translocation stocks contain either a hyperploid endosperm (i.e., two doses of a specific chromosome arm segment contributed by the male) and a hypoploid embryo (i.e., no dose of a specific chromosome arm contributed by the male), or vice-versa. For analyses of endosperm/embryo interactions, discordant kernels allow one to study the effect of a dek mutant embryo on the development of a nonmutant endosperm, as well as the effect of a nonmutant embryo on a dek mutant endosperm.

In their large-scale study of EMS-induced kernel mutations, Neuffer and Sheridan (1980) utilized the B-A translocations to analyze endosperm/embryo interactions in discordant kernels of nineteen dek mutants. In four of nineteen cases examined, development of the mutant hypoploid embryo was improved by

interaction with a nonmutant hyperploid endosperm, as compared to concordant dek mutant kernels. For the opposite situation, in three of nineteen cases the nonmutant embryo enhanced development of the mutant endosperm, whereas in seven of nineteen cases the normal embryo rendered a more extreme mutant endosperm phenotype. These studies revealed that in the majority of cases the development of the embryo and endosperm is dependent upon their compartment-specific genotypes, although in some cases an embryo-nurturing role for the endosperm or an endosperm-nurturing role for the embryo is observed.

The phytohormone abscisic acid (ABA) inhibits the gibberellic acid-induced (GA) expression of *alpha-amylase* in the aleurone, thereby preventing precocious germination of cereal kernels (Jacobson and Chandler, 1987). A large number of maize viviparous genes are described and cloned, which function to regulate seed germination (Robertson, 1955; McCarty et al., 1991; Hable et al., 1998; Porch et al., 2006; Suzuki et al., 2006). VP1 is a transcriptional activator of ABA-regulated genes in the maize kernel; vp1 mutant seed germinate prematurely. Hoecker et al. (1999) used discordant vp1 mutant kernels to demonstrate that the embryo is the source of a non-VP1 signal that contributes to repression of *alpha-amylase* expression in the aleurone during kernel quiescence. These conclusions are supported by the phenotype of emb-2008 mutant kernels, in which the embryo is genetically ablated but the endosperm is normal. Enhanced levels of *alpha-amylase* gene expression observed in emb-2008 kernels indicate that full repression of *alpha-amylase* in the aleurone requires signaling from a nonmutant embryo, even in the presence of nonmutant VP1.

3.2 The ESR May Mediate Embryo–Endosperm Interaction

The ESR was first identified as a region of small, densely cytoplasmic cells that surrounds the basal endosperm cavity holding the young embryo (Fig. 3d; Schel et al., 1984; Opsahl-Ferstad et al., 1997). A defined function for the ESR is not described, although this domain may play a role in embryonic defense, nutrition, or apoplastic signaling between the embryo and endosperm. A growing number of maize genes are shown to exhibit ESR-localized expression [including *esr1, esr2, esr3, androgenic embryo1* (*ae1*)*, androgenic embryo3* (*ae3*)*, invertase inhibitor homolog1* (*invinh1*) *and esr6*], however in the absence of genetic data their functions remain somewhat speculative. The mechanisms underlying ESR cell fate specification are also unknown, however ESR development appears to be dependent upon signaling from the embryo. Although an embryo cavity still forms within the endosperm of embryo-less kernel mutants, no *esr1, 2, 3* gene expression is detected and the cavity lacks specialized ESR cellular morphology (Opsahl-Ferstad et al., 1997).

Identified in a differential display for endosperm-enriched transcripts, *esr1, esr2,* and *esr3* are expressed in the ESR between 4 DAP and 28 DAP (Opsahl-Ferstad et al., 1997; Bonello et al., 2000). During early stages of embryogenesis

the domain of *esr1, 2, 3* expression surrounds the entire proembryo; at later stages expression is limited to endosperm cells near the basal and suspensor regions of the enlarged embryo (Fig. 3e–g). The *esr1, 2, 3* genes encode small, highly homologous proteins that bear little homology to proteins of known function. However ESR3 does share a conserved motif of 15 amino acids with CLAVATA3, which may function as a ligand for the CLAVATA1/CLAVATA2 receptor kinase complex (Fletcher et al., 1999). Thus ESR3 belongs to a large family of more than 40 rapidly evolving CLE proteins (CLAVATA3/ESR-related) that may be involved in intercellular signaling (Cock and McCormick, 2001; Sharma et al., 2003). In support of a proposed signaling function, the ESR1, 2, 3 proteins are secreted and localize to cell walls of the ESR (Bonello et al., 2002). Likewise, *ae1* and *ae3* are also predicted to encode excreted ESR proteins of unknown function (Magnard et al., 2000). Yet another exported ESR protein is INVERTASE INHIBITOR HOMOLOG1, which is purported to regulate the developmental rates of the endosperm and embryo via the selective, apoplastic compartmentalization of invertase activity (Bate et al., 2004). Finally, an embryo protective function is predicted for *esr6*, which encodes an ESR-excreted defensin protein during early embryogenesis (Balandin et al., 2005). During later stages of kernel development ESR6 accumulates in the placental cells, although the ESR remains the source of *esr6* transcription.

4 Future Prospects

Despite the difficulties encountered in molecular genetic analyses of embryo-lethal alleles, *dek* mutations comprise a bounty of essential gene functions during plant development. Mutations disrupting both embryo and endosperm development enable analyses of molecular interactions during the coordinated development of the various tissues and organs in the maize kernel. Exhaustive yet elegant molecular genetic analyses of embryo-lethal mutations in *Drosophila* served as the foundation for fundamental discoveries in fly developmental biology (Nüsslein-Volhard and Wieschaus, 1980; reviewed in St Johnston and Nusslein-Volhard, 1992). Logically, analogous studies of the largely untapped maize *dek* mutations promise to yield essential breakthroughs in our understanding of the development in this important crop plant. Although this review has focused on the developmental biology of the endosperm and embryo, the maize kernel forms within a much larger developmental field comprised of a variety of floral organs that likewise contribute to the developing seed (Fig. 4 Future studies of this larger kernel interactome will be enhanced by sequencing of the maize genome, and improved resources for reverse genetics and transformation in maize. Cell-specific and tissue-specific sampling of developing seed via laser microdissection and cell-sorting techniques will enable genomic and proteomic analyses of specific kernel domains, which promises to advance our understanding of the genetic and epigenetic networks involved in kernel morphogenesis and evolution.

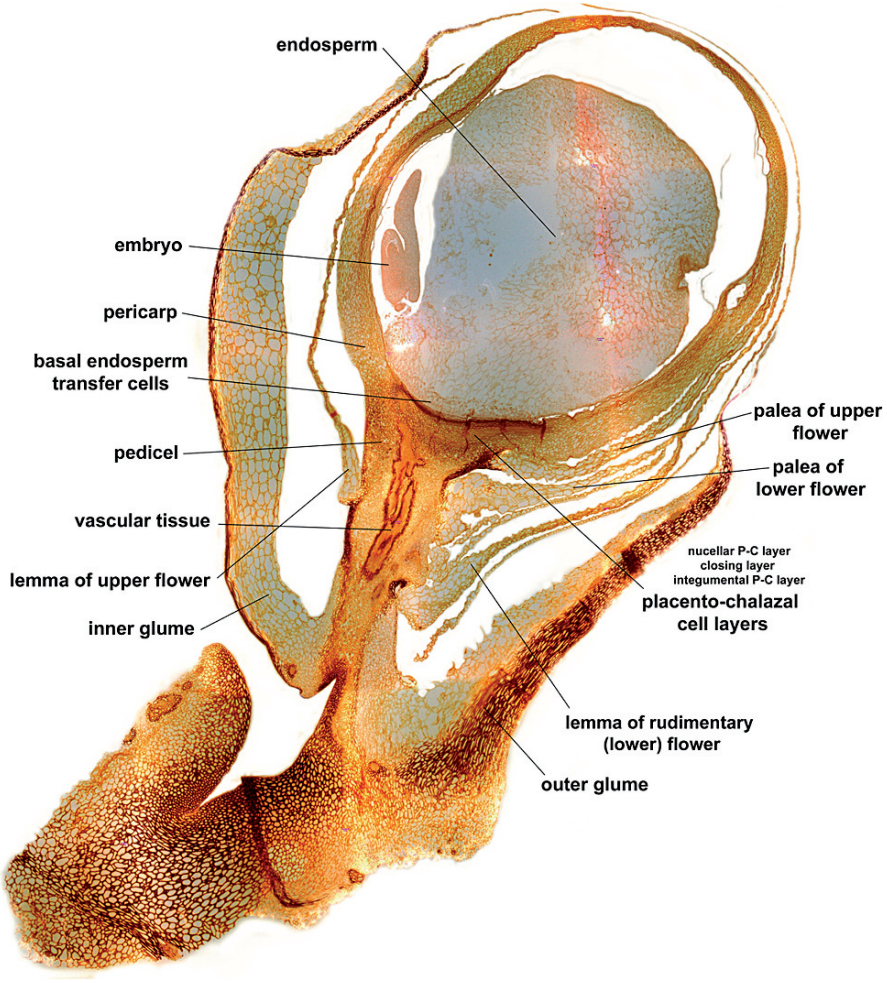

Fig. 4 Maize kernel development: the interactome. The maize kernel at 10 DAP is part of a larger, interacting, developmental field comprised of a variety of maternal tissues in addition to the embryo and endosperm. Original drawing by Evgueni Ananiev

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The Maize Root System: Morphology, Anatomy, and Genetics

Frank Hochholdinger

Abstract Maize root system architecture is determined by distinct embryonic and postembryonic root types that are formed during different phases of development. While embryonically preformed roots dominate the early seedling root system, the adult root stock is determined by an extensive shoot borne root stock. Although the cellular organization of all root types is similar specific mutants imply complex genetic programs that regulate maize root system formation. Recently, the first genes that are involved in shoot borne root formation and root hair formation have been cloned. Moreover, proteome and cell type specific transcriptome analyses gave initial cues on the complex molecular networks involved in maize root system development.

1 Morphology of the Maize Root System

The maize root stock has a unique architecture which secures the efficient uptake of water and nutrients and provides anchorage (Lynch, 1995; Aiken and Smucker, 1996). The formation of the maize root system is regulated by an endogenous genetic program (Feix et al., 2002; Hochholdinger et al., 2004a, 2004b, 2005a) but also by interactions with the rhizosphere (Bais et al., 2006; Watt et al., 2006) and the flexible adaptation to changing environmental cues (Drew and Saker, 1975, 1978; Hawes et al., 1998; McCully, 1999).

The root system of maize consists of roots that are formed during embryogenesis and roots that are formed during postembryonic development (Abbe and Stein, 1954; Feldman, 1994). The embryonic root system consists of a primary root that is formed at the basal pole of the embryo and a variable number of seminal roots that are laid down at the scutellar node (Fig. 1a). The postembryonic root system is composed of shoot borne roots that are formed at consecutive shoot nodes and lateral roots that are initiated in the pericycle of all roots (Fig. 1a and b).

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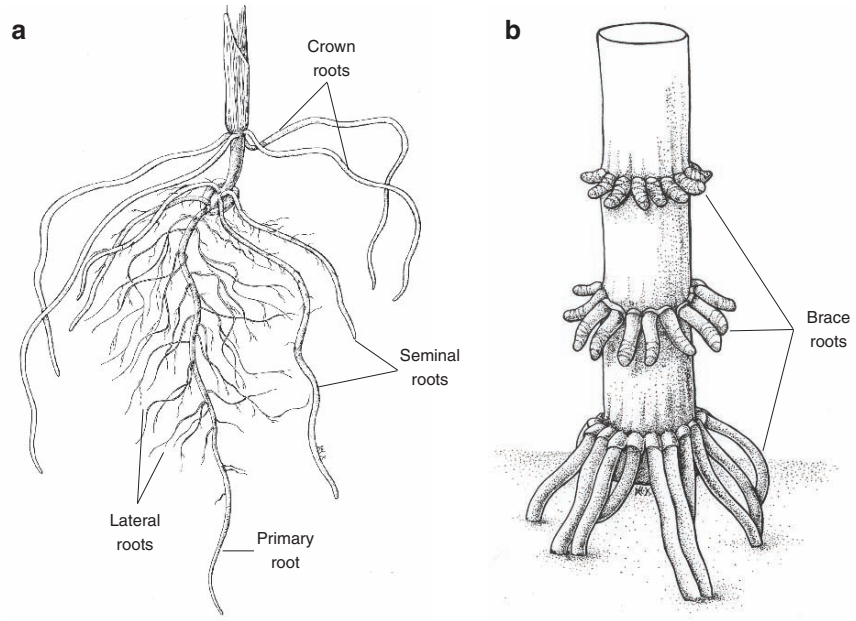

Fig. 1 **a** Embryonic primary and seminal roots and postembryonic lateral and crown roots are already visible in 14-day-old wild type maize seedlings. **b** Aboveground shoot borne brace roots of a 6-week-old plant

1.1 The Embryonic Primary and Seminal Roots

The *Poaceae* is the only family in the plant kingdom that forms an endogenous primary root (Fig. 1a) deep inside the embryo (Tillich, 1977; Yamashita, 1991). During germination the primary root tip of maize has to penetrate other tissues which form the coleorhiza at the proximal end of the newly developed primary root (Tillich, 1977; Hochholdinger et al., 2004b). This is in contrast to most other angiosperms which form their primary root exogenously from the outermost cell layer of the embryo. A unique feature of maize is the formation of seminal roots at the scutellar node (Fig. 1a). These roots are laid down endogenously in the embryo between 22 and 40 days after pollination (Sass, 1977; Erdelska and Vidovencova, 1993; Feldman, 1994). Since the scutellar node tissue is already differentiated and can easily be penetrated seminal roots do not form a coleorhiza. The number of seminal roots per seedling depends on the genetic background of the seedling and can vary between 0 and 13 (Kiesselbach, 1949; Sass, 1977; Feldman, 1994). The fate of the embryonic root system depends on the genetic background. While in some inbred lines the primary and seminal roots remain functional during the whole life cycle (e.g., Kiesselbach, 1949; Kausch, 1967; Kozinka, 1977; McCully and Canny, 1985) in other inbred lines these roots become obsolete with the emergence of the shoot-borne root system (e.g., Lawson and Hanway, 1977; Feldman, 1994).

1.2 The Postembryonic Shoot Borne Crown and Brace Roots

While the embryonic primary and seminal roots make up the major portion of the seedling root stock in the first weeks after germination, later in development the postembryonic shoot-borne roots form the major backbone of the maize root system (Fig. 1a and b). The maize root stock develops about 70 shoot-borne roots during its life cycle which are organized on average in six whorls of underground crown roots and two to three whorls of aboveground brace roots (Hoppe et al., 1986). Shoot borne roots are endogenous and their primordia are formed opposite to collateral vascular bundles (Martin and Harris, 1976). Shoot borne roots that are formed below the soil level are named crown roots (Fig. 1a) while aboveground shoot borne roots are designated brace roots (Fig. 1b). The mean diameter and number of shoot-borne roots per whorl increases at higher nodes (Hoppe et al., 1986). The first four underground internodes are very short (Hoppe et al., 1986). Therefore, the shoot nodes are located closely together which leads to a dense root stock. Crown roots at lower nodes first grow in horizontal orientation before they bend and follow the gravitropic vector (Kiesselbach, 1949; Feldman, 1994). In contrast, roots from higher nodes grow directly downwards (Feldman, 1994). The dense crown root stock is the basis for the lodging resistance of the adult maize plant (McCully and Canny, 1988). Typically, only the first two whorls of the aboveground brace roots penetrate into the soil and only those brace roots form lateral roots (Feldman, 1994). Brace roots formed at higher nodes only prevent plants that have fallen sideways from complete lodging in most instances (Feldman, 1994).

1.3 The Postembryonic Lateral Roots

Lateral roots (Fig. 1a) are per definition all roots that are initiated from the pericycle of other roots (Esau, 1965). Lateral roots have a strong influence on root architecture (Lynch, 1995) and are responsible for the major part of water and nutrient uptake of the maize plant (McCully and Canny, 1988; Wang et al., 1991, 1994; Varney and Canny, 1993) due to their branching capacity. In contrast to the main roots of maize lateral roots are typically very short (Varney et al., 1991). Moreover, lateral roots are more responsive to drying by transpiration (Wang et al., 1991) and they are characterized by their early determination due to the loss of their meristem (Varney and McCully, 1991). Finally, they have an open late metaxylem for most of their length (Wang et al., 1994) which is responsible for their dominant role in water uptake (Wang et al., 1995). In maize, lateral roots are initiated from pericycle and endodermal cells opposite of phloem poles in the differentiation zone of other roots. While, endodermal cells give rise to the newly formed epidermis and columella (Bell and McCully, 1970), all other root cell-types are formed by the pericycle (Fahn, 1990). An exact prediction which pericycle cells will divide and give rise to new lateral root meristems in maize is difficult (Charlton, 1991).

1.4 Exogenously Induced Adventitious Roots

The root types described in 1.1–1.3 are all determined by the endogenous developmental program of the maize plant. However, maize displays a considerable plasticity by forming roots as an adaptive mechanism at uncharacteristic sites of the maize plant as for instance at the mesocotyl as a response to environmental factors like wounding or hormone application. These roots induced by environmental factors are referred to as adventitious roots. Previously, all roots that were formed postembryonically were described as adventitious roots (e.g., Kiesselbach, 1949). However, this nomenclature is misleading because it does not distinguish between root types that are genetically determined and roots that are formed as a reaction to environmental stimuli. We therefore suggest to designate genetically determined roots with the terms introduced in 1.1 (primary and seminal roots), 1.2 (crown and brace roots), and 1.3 (lateral roots) and call only roots that are induced by exogenous factors as adventitious.

2 Cellular Organization of Maize Roots

2.1 Radial Organization of Maize Roots

In general, the cellular organization of all maize root types is similar. Mature maize roots exhibit a polyarch organization of the central vascular cylinder with six to ten differentiated late metaxylem vessels (Fig. 2a: LMX) which are the largest vascular elements and are responsible for the transport of water and nutrients. Shoot borne roots display an increasing number of late metaxylem arms at successively higher nodes (Hoppe et al., 1986) with up to 48 late metaxylem elements in the largest roots (Tillich, 1992). The differentiated early metaxylem elements (Fig. 2a: EMX) which are initiated from undifferentiated protoxylem elements are peripheral to and encircling the late metaxylem elements (Feldman, 1994). Their number is variable, but in general two to three early metaxylem strands, which are alternating with the primary phloem that functions in the transport of photosynthates (Fig. 2a: PP), are arranged per late metaxylem element (Feldman, 1994). The central cylinder is enclosed by the pericycle (Fig. 2a: P). The ground tissue consists of a single layer of endodermis (Fig. 2a: En) with the casparian strip that represents a barrier to the radial flow of water and nutrients (Hose et al., 2001) and eight to fifteen layers of cortex tissue (Fig. 2a: C). The epidermis, sometimes referred to as rhizodermis (Fig. 2a: Ep), which consists of root hair forming trichoblasts and non-root hair forming atrichoblasts is the outermost cell layer of the maize root and connects the root with the rhizosphere. Arrangement of tricho- and atrichoblasts in maize is irregular so that predictions which cells will form root hairs are difficult (Row and Reeder, 1957). The short-living epidermis is in older roots replaced by a lignified, suberinized exodermis (Fig. 2a: Ex), which develops from the outermost cortical cells and forms an additional casparian band

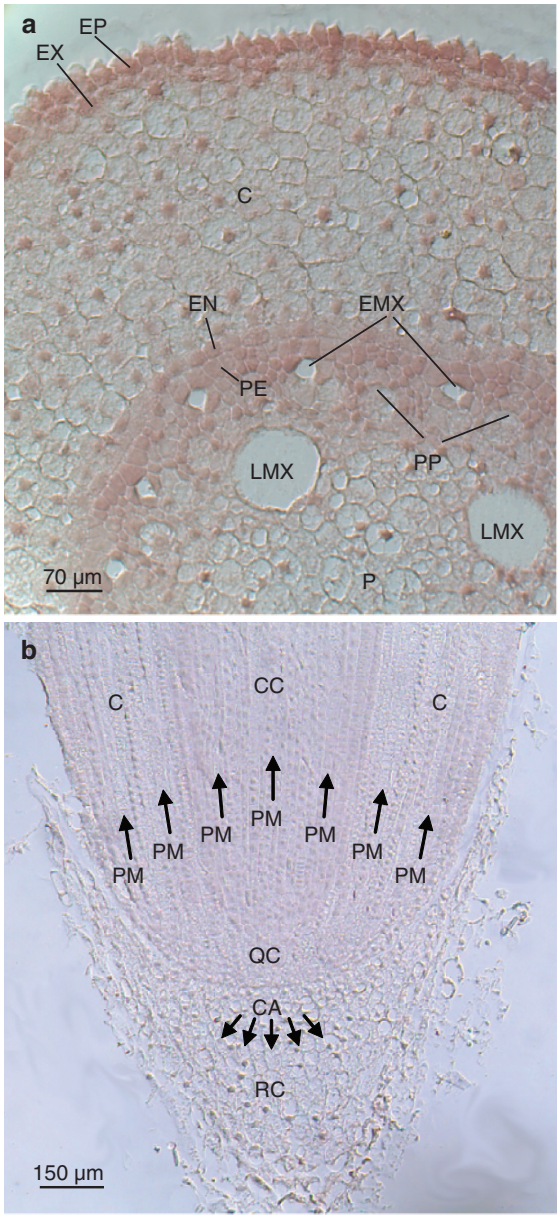

Fig. 2 **a** Transverse section through a maize primary root. The central cylinder is composed of the pith (*P*), early (*EMX*) and late (*LMX*) metaxylem elements, primary phloem (*PP*), and the pericycle (*PE*) as its outermost cell layer. The ground tissue is made up of a single endodermis layer (*EN*), multiple layers of cortex (*C*) tissue and single exodermis (*EX*) and epidermis (*EP*) cell layers. **b** Longitudinal section of the primary root tip. Arrows indicate proliferation of root tissue by the arc shaped proximal root meristem (*PM*) and proliferation of root cap (*RC*) tissue by the calyptrogen (*CA*). *QC*: meristematically inactive quiescent center; *CC*: central cylinder; *C*: cortex

(Feldman, 1994). In aboveground formed brace roots the epidermis persists and forms a protective cuticula. Like the roots of most monocotyledonous plants, maize does not display secondary root growth.

2.2 Longitudinal Organization of Maize Roots

The longitudinal structure of the maize root is characterized by various in part overlapping zones of development including the root cap at the terminal end, the meristematic zone comprising the root apical (proximal) meristem (Fig. 2b: PM), the elongation zone and the maturation or root hair zone (Ishikawa and Evans, 1995). The root cap (Fig. 2b: RC) is laid down early during embryogenesis and may consist of up to 10,000 cells (Feldman, 1994). The root cap has multiple functions. First, it covers and thus protects the root tip. Moreover, it secrets mucilage which facilitates the movement of the roots in the soil. Finally, the root cap is a sensor for gravity, light, temperature and moisture gradients (Feldman, 1994). New root cap cells are proliferated from the meristematically active cell layer of the calyptrogen (Fig. 2b: CA). Calyptrogen cells have the highest cell division rate in the maize root with an average cell cycle time of 12 h (Feldman, 1994). Newly formed root cap cells are displaced through the root cap. After reaching the surface of the root cap these cells disintegrate and are finally shed into the soil as a mucilage ("slime") that covers the root tip. This displacement process takes on average 5–8 days (Feldman, 1994). The subterminal root apex consists of 800–1200 cells (Jiang et al., 2003) of the mitotically inactive quiescent center (Fig. 2b: QC) which is neighbored by the proximal meristem that has an "arc-like" structure (Fig. 2b: PM). Cells that have left the proximal meristem move into the elongation zone in which the newly generated cells do not divide any more but start to elongate. This zone plays also an important role in the response of maize roots to a variety of exogenous environmental signals (Ishikawa and Evans, 1995). The maturation zone (not displayed in Fig. 2b) is positioned proximately to the elongation zone. This zone is marked by differentiated cells that form root hairs (Ishikawa and Evans, 1995).

3 Genetic Dissection of Maize Root Formation

Forward genetic screens allow for the identification of mutants from large mutagenized F_2-populations. This approach has been successfully applied to a considerable number of aboveground traits of maize (Neuffer et al., 1997). However, this strategy imposes difficulties when adapted to the analysis of maize root development because soil grown roots and are not directly accessible for phenotypic analyses, their growth is very variable, and their extraction from soil without damaging them is tedious. Therefore, screens for maize root mutants were thus far performed with seedlings germinated in paper rolls (e.g., Wen and Schnable, 1994; Hetz et al., 1996; Hochholdinger and Feix, 1998a; Hochholdinger et al., 2001; Woll et al., 2005).

An advantage of this approach is that roots can be inspected without damaging them and that temperature, humidity and light conditions can be strictly controlled thus minimizing environmental variation. Moreover, despite the complexity of the maize root system (Kiesselbach, 1949), analysis of 10–14-day-old seedlings grown in distilled water already allow for the inspection of primary, seminal, crown and lateral roots (Fig. 1a). Analyses at this early stage of development also allow for large scale screens because of the small size of the seedlings. Different maize root types are formed at different phases of development at different positions of the plant. In the following section we will classify the mutants spatially according to the affected root types (Table 1). However, one needs to keep in mind that this classification is somehow artificial since several mutants are affected in different root types and during different stages of root development.

3.1 Shoot Borne Root Formation

Shoot-borne roots dominate the adult root stock and are responsible for lodging resistance and the major part of water and nutrient uptake via their lateral roots (McCully and Canny, 1988). In maize, the non-allelic mutations *rootless 1* (*rt1*;

Table 1 Characteristics of maize root mutants

		Affected root features					
Mutant	Primary root	Seminal root	Shoot borne roots	Lateral roots	Root hairs	Affected gene	References
rt1			•			nd	Jenkins, 1930
rtcs		•	•			LOB domain protein	Hetz et al., 1996; Taramino et al., 2007
rum1		•		•		nd	Woll et al., 2005
lrt1				•		nd	Hochholdinger and Feix, 1998
slr1				•		nd	Hochholdinger et al., 2001
slr2				•		nd	Hochholdinger et al., 2001
rth1					•	SEC3 homolog	Wen and Schnable, 1994; Wen et al., 2005
rth2					•	nd	Wen and Schnable, 1994
rth3					•	COBRA-like protein	Wen and Schnable, 1994 Hochholdinger et al., submitted

nd not determined

Jenkins, 1930) that maps to chromosome 3 (Emerson et al., 1935) and *rootless concerning crown and seminal roots* (*rtcs*; Hetz et al., 1996) that maps to chromosome 1S lead to defective shoot borne root formation (Fig. 3a). The monogenic recessive mutation *rootless1* (*rt1*) is exclusively affected in shoot borne root formation.

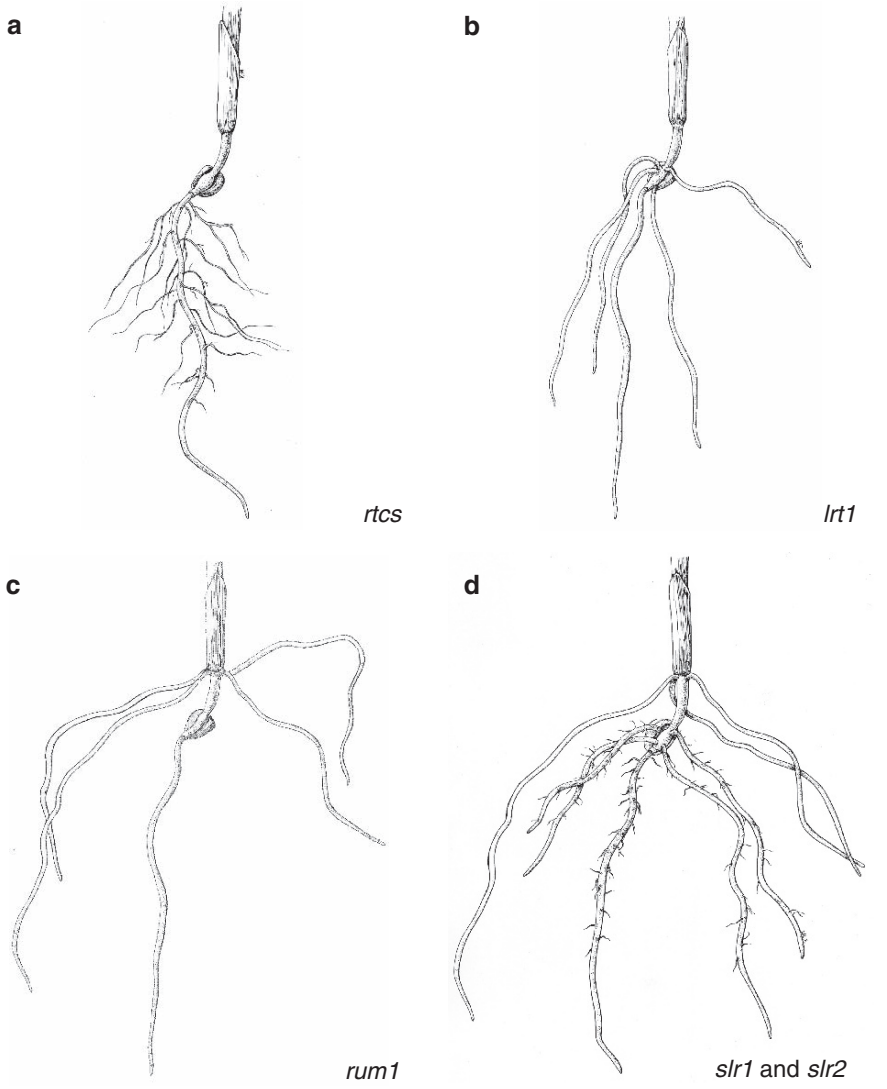

Fig. 3 **a** The shoot borne root initiation mutant *rtcs* **b** The lateral root initiation mutant *lrt1* **c** The lateral and seminal root initiation mutant *rum1* **d** The lateral root elongation mutants *slr1* and *slr2*. For details on the mutant phenotypes please refer to the text. Modified according to Hochholdinger et al. (2004b)

While the formation of the aboveground brace roots is the most prominent phenotype of *rt1*, the number of crown roots is only slightly reduced (Jenkins, 1930). In contrast, the mutant *rtcs* (Hetz et al., 1996) is affected in the initiation of all not be rescued by the exogenous application of auxin (Hetz et al., 1996). Hence, the only root that remains in *rtcs* is the primary root and its lateral roots. Remarkably, despite this drastic reduction of the root system *rtcs* can be propagated in the greenhouse (Hetz et al., 1996). The finding that the four maize cyclins *cyc1a*, *cyc1b*, *cyc2*, *cyc3* (Renaudin et al., 1994) are not expressed in the coleoptilar node of the mutant (Hochholdinger and Feix, 1998b) is in line with the observation that the *rtcs* mutation acts before the initiation of crown roots. In contrast, *cdc2a* (Colasanti et al., 1991), which is a marker for cell division competence (Colasanti et al., 1993), displays similar expression levels in *rtcs* and wild-type coleoptilar nodes (Hochholdinger and Feix, 1998b). Moreover, outcrossing of the *rtcs* mutant locus to *gaspe flint*, an excessively tillering inbred line, resulted in *rtcs* mutants that showed shoot tillering at the lower nodes (Hochholdinger and Feix, 1998c). These results demonstrated that the *rtcs* gene does not generally control cell division in shoot nodes, but only cell divisions associated with crown root formation.

Cloning of the *rtcs* gene via a map based approach revealed that it encodes a LOB-domain protein (Taramino et al., 2007). The *rtcs* gene has been duplicated during evolution. The paralogous *rtcl* (*rtcs-like*) gene maps on chromosome 9, and displays 72% sequence identity with the *rtcs* gene on the protein level (Taramino et al., 2007). Both genes are preferentially expressed in roots. In the coleoptilar node, expression of *rtcs* is confined to emerging shoot-borne root primordia. The *rtcs* and *rtcl* promoters share auxin response elements (AuxRE) and are inducible by exogenously applied auxin (Taramino et al., 2007). This implies that the *rtcs* gene might be an early auxin response gene which plays an important role in auxin mediated shoot borne and seminal root initiation. This notion was also supported by the results of comparative proteome analyses of the most abundant soluble wild-type and *rtcs* proteins (Sauer et al., 2006). Identification of differentially accumulated proteins extracted from 5- and 10-day-old coleoptilar nodes via ESI MS/MS mass spectrometry identified several proteins involved in auxin signal transduction (Sauer et al., 2006). ABP1 (Auxin-Binding Protein 1), a putative high-affinity auxin receptor (Chen, 2001) that might act in the earliest stages of auxin signal transduction, displayed a high expression in 5-day-old and a much lower expression in 10-day-old wild type coleoptilar nodes. In the mutant *rtcs,* the gene and protein maintained a constantly high expression level between 5 and 10 days after germination (Sauer et al., 2006) which might imply a defect in a putative feedback inhibition of ABP1 in the mutant *rtcs*. Furthermore, calmodulin, which binds to the SAUR class of early auxin responsive genes (Yang and Poovaiah, 2000), and a subunit of a heterotrimeric G-protein, which is assumed to stimulate cell division in response to high auxin concentrations when coupled to a low affinity auxin receptor (Chen, 2001), were down regulated in 10-day-old wild-type coleoptilar nodes (Sauer et al., 2006).

3.2 Lateral Root Formation

Lateral roots are initiated via dedifferentiation of pericycle cells in the differentiation zone of other roots (Esau, 1965). In maize, the identification of mutants specifically affecting lateral root-initiation and elongation in the embryonic but not the postembryonic roots suggests that there are at least two pathways of lateral root formation or two different sensitivities to signals involved in lateral root formation (Hochholdinger et al., 2004b). The *lateral rootless 1* (*lrt1*; Hochholdinger and Feix, 1998a) mutant is affected in early postembryonic root development comprising the initiation of lateral roots on primary and seminal roots and the formation of crown roots at the coleoptilar node (Fig. 3b). Crown root formation at higher shoot nodes and lateral root formation at crown roots emerging from these shoot nodes is not affected in *lrt1*. Remarkably, lateral roots cannot be induced by auxin in the mutant *lrt1* (Hochholdinger and Feix, 1998a). However, inoculation with the arbuscular mycorrhizal fungus, *Glomus mosseae* or growth in high phosphate environment induces lateral roots in this mutant (Paszkowski and Boller, 2002). Similarly, the mutant *rootless with undetectable meristems 1* (*rum1*; Woll et al., 2005) is affected in the initiation of seminal and lateral roots at the primary root while lateral root formation is normal in the shoot-borne root system (Fig. 3c). The mutant *rum1* displays severely reduced auxin transport in the primary root and a delayed gravitropic response (Woll et al., 2005). Exogenously applied auxin does not induce lateral roots in the primary root of *rum1* (Woll et al., 2005). In the mutants *short lateral roots 1* and *2* (*slr1* and *slr2*; Hochholdinger et al., 2001) reduced lateral root elongation is also confined to the embryonic roots while normal lateral roots are formed at the shoot-borne root system (Fig. 3d). In *slr1* and *slr2* the lateral root primordia and meristems cannot be distinguished from their wild-type counterparts. However, lateral roots show reduced longitudinal elongation and cortex cells display only about one fourth of the corresponding size of wild-type cells (Hochholdinger et al., 2001).

Different aspects of lateral root formation in maize have been studied on the proteomic level (Hochholdinger et al., 2006). First, in a comparative proteomic study of 9-day-old wild-type versus *lrt1* mutant primary roots 10% of the detected proteins were preferentially expressed in the mutant (Hochholdinger et al., 2004c), hence demonstrating the influence of lateral roots on the proteome composition of the primary root. In a second analysis, the abundant soluble proteins of 2.5-day-old primary roots of seedlings of wild-type and the lateral root initiation mutant *rum1* were compared before the initiation of lateral roots (Liu et al., 2006). Differentially accumulated proteins were involved in lignin biosynthesis, defense and the citrate cycle. Remarkably, for the corresponding genes of the differentially accumulated proteins no expression differences were detected on the RNA level (Liu et al., 2006). This suggests that these differences occurred posttranscriptionally for example via protein modification, different rates of protein synthesis or different protein stability. Third, in a reference dataset of 5-day-old primary roots of maize, the 81 most abundant soluble proteins were identified via MALDI-ToF (Hochholdinger et al., 2005b). This study highlighted considerable changes in the accumulation of abundant soluble proteins during the early stages of lateral root formation in the maize primary root.

Plant organs are characterized by their composite structure made up of different cell types. Each of these cell types expresses a unique transcriptome (Schnable et al., 2004). Microarray analyses of whole roots therefore provide only gene expression levels integrated over all cell types which bears the potential to mask genes of interest that are expressed in a particular cell type. This problem can be overcome by the isolation of particular cell types via cell type specific analyses for example with laser capture microdissection (Schnable et al., 2004).

Pericycle cells of wild type and mutant *rum1* seedlings that do not initiate lateral roots were isolated via laser capture microdissection (LCM) before wild type seedlings initiate lateral root initiation. Subsequently, their transcriptomes were subjected to microarray experiments (Woll et al., 2005). This study revealed 90 genes preferentially expressed in the wild-type pericycle and 73 genes preferentially expressed in the *rum1* pericycle. Among the annotated genes predominately expressed in wild-type pericycle cells, 37% were involved in signal transduction, transcription and cell cycle. This study defined a framework of genes that is active in the pericycle and might thus play a role in lateral root initiation (Woll et al., 2005).

Pericycle cells are the only root cells that maintain the competence to divide after they leave the meristematic zone. Comparison of the transcriptomes of LCM captured wild type (B73) pericycle cells versus nonpericycle maize primary root cells identified genes preferentially expressed in pericycle versus all other root cells that have left the apical meristem (Dembinsky et al., 2007). Transcription and protein synthesis represented the most abundant functional categories among these pericycle specific genes. EST sequencing of pericycle transcripts revealed that there are no dominant genes expressed in the pericycle and that sequencing cell type specific transcripts can contribute the discovery of transcripts not yet deposited in databases (Dembinsky et al., 2007). Among the most abundant soluble pericycle proteins separated via 2-dimensional electrophoresis, 20 proteins were identified via ESI MS/MS mass spectrometry, thus defining a first reference dataset of the maize pericycle proteome (Dembinsky et al., 2007). Remarkably, among those, two proteins were preferentially expressed in the pericycle. In summary, these pericycle specific gene expression experiments define the distinct molecular events during the specification of cell-cycle competent pericycle cells prior to their first division and demonstrate that pericycle specification (Dembinsky et al., 2007) and lateral root initiation (Woll et al., 2005) might be controlled by a different set of genes.

3.3 Root Hair Elongation

Root hairs are unicellular structures of the epidermis that play an important role in water and nutrient uptake (Schiefelbein, 2003). The maize mutants *roothairless 1* to *3* (*rth1, rth2* and *rth3*; Wen and Schnable, 1994) are defective in root hair elongation. The mutant *rth1* initiates normal looking root hair primordia that fail to elongate (Fig. 4a). The mutant *rth2* also initiates normal root hair primordia that elongate to about 20–25% of the normal wild-type length. The mutant *rth3* also fails to

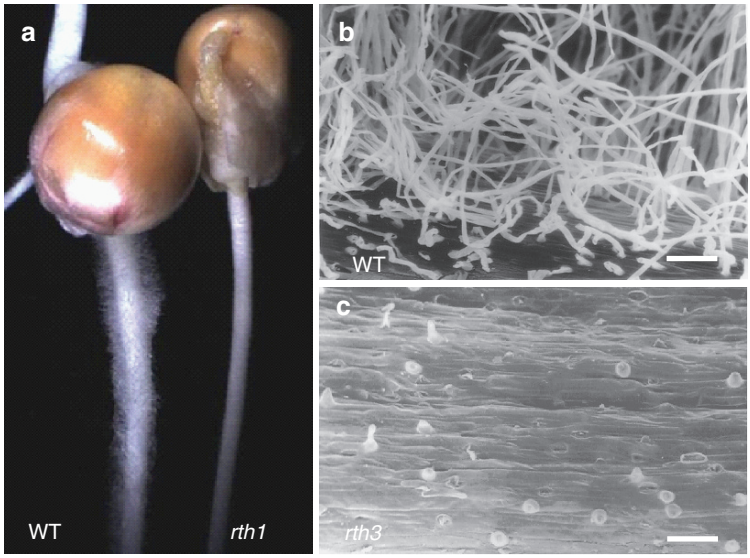

Fig. 4 **a** The mutants *rth1*, *rth2*, and *rth3* display a similar phenotype which is characterized by the inability to form phenotypically visible root hairs. **b** and **c** Scanning electron microscopy reveals that *rth3* initiates root hairs but fails to elongate them. Size bars: 20 μm

elongate the root hairs like *rth1* but already seems to be disturbed during the establishment of root hair primordia (Fig. 4b and 4c). While the mutants *rth2* and *rth3* display a normal aboveground phenotype the mutant *rth1* displays symptoms of nutrient deficiency.

Root hairs elongate via localized tip growth, a process which is mediated by polar exocytosis of secretory vesicles. Cloning of the *rth1* gene via a transposon-based DNA gel blot approach revealed that the gene encodes a SEC3 homolog (Wen et al., 2005). In yeast and mammals SEC3 is a subunit of the exocyst complex, which tethers exocytotic vesicles prior to their fusion. The cloning of the *rth1* gene links a component of the postulated exocyst complex to root hair elongation and supports the notion that the exocyst complex might be involved in plant exocytosis (Elias et al., 2003). The preferential accumulation of a prohibitin, which is a negative regulator of the cell-cycle in the *rth1* mutant proteome, may at least partially explain the delayed development and flowering of the *rth1* mutant (Wen et al., 2005).

Cloning of the *rth3* gene via a transposon based PCR approach revealed that it belongs to a monocot specific clade of the COBRA-like gene family (Hochholdinger et al., 2008). Members of this plant-specific glycosylphosphatidylinositol (GPI)-anchored gene family appear to be involved in various types of cell expansion and cell wall biosynthesis (Brady et al., 2007). Despite the specific root hair phenotype the *rth3* gene is expressed in almost all tissues at very low levels with the highest expression in young primary roots (Hochholdinger et al., 2008). *In situ* hybridization experiments confine *rth3* expression to root hair-forming epidermal cells and lateral

root primordia. Remarkably, in replicated field trials the *rth3* mutant conferred, on average, a 35% reduction in grain yield.

4 Conclusion

The identification of mutants specifically affected in different aspects of maize root development and the progressive cloning of the affected genes contributes to a better understanding of the developmental checkpoints of root stock architecture. In the future, the identification of the molecular context of these genes will reveal the complex molecular networks involved in maize root formation. High throughput reverse genetic dissection of root-specific genes and the analysis of root traits that are inherited in a quantitative way will further enhance the knowledge of functional networks involved in maize root formation.

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Axial Patterning of the Maize Leaf

Toshi M. Foster and Marja C. P. Timmermans

Abstract The characteristic morphology and anatomy of the maize leaf reflects the outcome of developmental patterning along three axes, proximodistal, mediolateral, and adaxial-abaxial, which are specified relative to the main axis of the plant. The past decade has seen a dramatic increase in our understanding of the genetic control of leaf development. Gene regulatory networks involved in the specification of organ polarity are beginning to emerge. These are distinguished by contributions of highly conserved, often redundant transcription factor families whose expression or activity are modulated to give rise to the distinctive maize leaf. Small regulatory RNAs, hormones, as well as proteins that selectively trafficking between cells have emerged as candidate signals conveying positional information within the shoot to the newly initiated leaf. This chapter outlines findings of both classical genetic and recent molecular studies that have led to a framework for axial patterning of the maize leaf.

1 Introduction

The leaf has three major axes along which morphological differentiation occurs; proximodistal, mediolateral, and adaxial–abaxial (dorsoventral). The proximodistal axis lies along the length of the leaf and is the primary axis of growth. The mediolateral axis, defined as midrib to margin, can also permit extensive growth. By contrast, the adaxial–abaxial axis, which extends across the leaf from the upper to the lower surface, comprises only a handful of distinct cell layers. Leaves are derived from cells on the flank of the shoot apical meristem (SAM) and consequently a fundamental positional relationship exists between the leaf and the SAM. The adaxial surface of the leaf, which generally faces up, develops in closer proximity to the tip of the SAM than the abaxial surface. Similarly, the proximal end of the leaf is attached to the shoot whereas the distal end extends away (Fig. 1).

In most plant species, characteristic anatomical and morphological asymmetries develop along each axis that optimize different regions of the leaf for specialized functions. The maize leaf is typical of the grasses, consisting of distal blade and proximal sheath. The sheath wraps around the stem, providing mechanical support for the blade, which projects outwards to catch the light and is optimized for photosynthesis.

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DOI: 10.1007/978-0-387-79418-1_9,

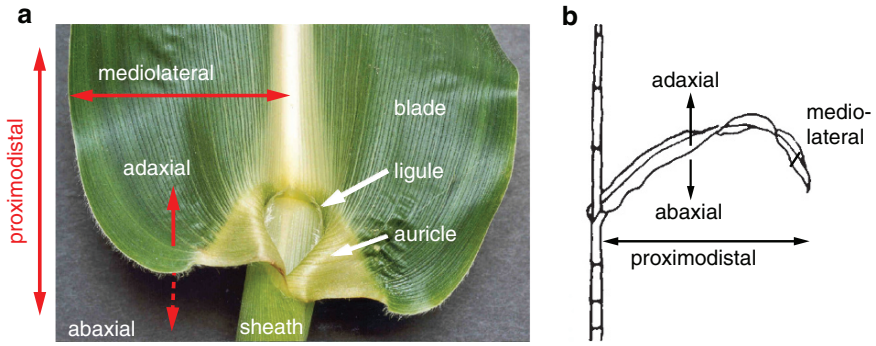

Fig. 1 The tissues and major growth axes of the maize leaf. (**a**) Image of the adaxial (upper) surface of the maize leaf showing distal blade and proximal sheath, separated by the ligule/auricle region. (**b**) Cartoon illustrating the axes of the maize leaf relative to the main axis of the plant

Blade and sheath are separated by the ligule and auricle. The latter serves as a hinge that allows the blade to bend outwards, whereas the ligule is an adaxial fringe that protects the axillary bud and apex from debris and predators. Each of these tissue types has characteristic morphological and histological features that are easily distinguished and serve as markers to study the genetics of leaf development. A more comprehensive analysis of maize leaf cell types is presented in Chap. 10.

Early studies of leaf development were largely descriptive. These led to the formulation of the "leaf base" model, which describes how the contribution of different regions of the leaf primordium to the mature leaf varies among species (Troll, 1955; Kaplan, 1973). The model proposes that the monocot leaf blade is homologous to the dicot petiole and sheathing base, in that both are derived from the "lower leaf zone" of the primordium. Later, surgical, irradiation and tissue culture experiments helped to define some of the central concepts in leaf development. For instance, classical surgical experiments suggest that positional signals from the SAM are important for axial patterning in developing leaves (Sussex, 1954) and that cell fates in lateral organs are determined gradually (Sachs, 1969). Recently, genetic and phenotypic analysis of mutants that alter leaf morphology and anatomy have greatly advanced our understanding of the major processes of leaf development; leaf initiation, the establishment of polarity, and growth and differentiation. In this chapter, we describe advances in understanding the genetic pathways leading to axial patterning of the maize leaf.

2 Leaf Initiation – Recruitment of Leaf Founder Cells from the SAM

Maize has a distichous phyllotaxy, in which successive leaves are initiated at 180° from one another. The term plastochron describes the temporal pattern of leaf initiation and can be used to designate the age of a leaf primordium. For example,

the first visible leaf is a plastochron 1 (P1) leaf, the next older is plastochron 2 (P2), and the incipient primoridium within the SAM is termed P0.

The first histological signs of leaf initiation are periclinal cell divisions (parallel to the surface of the SAM) in the epidermal (L1) and subepidermal (L2) layers. These divisions are initiated in a position that corresponds to the future midrib, and are propagated laterally in both directions until they encircle the entire SAM. As a result, the maize leaf primordium appears first as a crescent shaped protrusion and then as a ridge of cells that encircles the apex (Sharman, 1942; Esau, 1965).

Cell lineage analysis has revealed patterns of cell division both within the meristem and in developing organs, and provided estimates of the number of initials (founder cells) that are recruited to become a leaf. Lineage analysis requires that a cell be marked with a heritable, visible, cell-autonomous marker, which allows clonal derivatives of the marked cell to be identified (Fig. 2). Genetic mosaics of albino mutations are commonly used for lineage mapping in leaves (Steffensen, 1968; Langdale et al., 1989; Poethig and Szymkowiak, 1995). A number of clonal analysis studies in maize have demonstrated that approximately 200 founder cells recruited from at least two cell layers of the SAM contribute to the development of the leaf and associated subtending node, internode, and axillary bud. (Steffensen, 1968; Poethig, 1984, 1987; McDaniel and Poethig, 1988; Poethig and Szymkowiak, 1995).

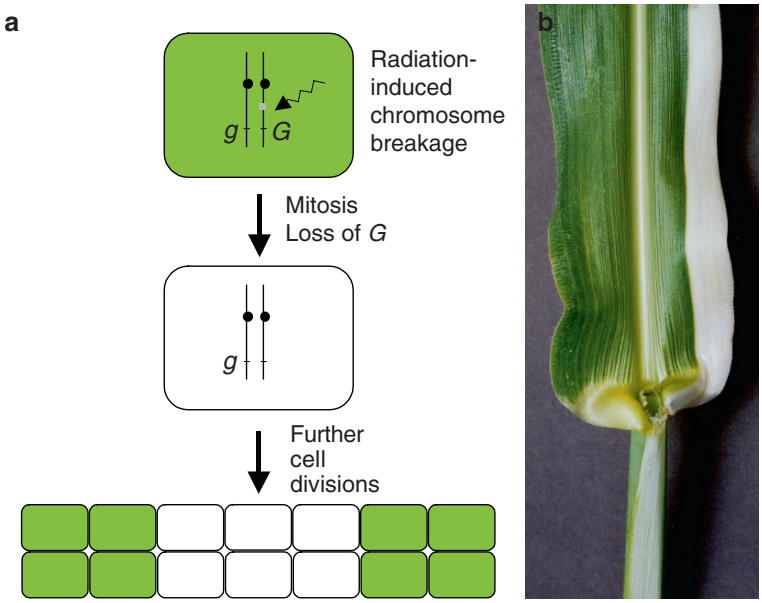

Fig. 2 Generation of albino sectors for clonal analysis. (**a**) Seeds or seedlings that are heterozygous for a recessive albino mutation (*g*) are irradiated causing chromosome breakages. Loss of the chromosome arm carrying the wild-type allele (*G*) uncovers the albino allele. Derivatives of this cell are white, while the rest of the plant is green. (**b**) Image of an albino sector on the leaf margin. Margin sectors often include one margin of the blade, but both margins of the sheath, indicating that the founder cells that form the sheath margins overlap the flank of the SAM

Mutational analysis and expression patterns of class I *knox* genes suggest that KNOX proteins promote developmental indeterminacy and the acquisition and/or maintenance of meristematic fate (Sinha et al., 1993; Jackson et al., 1994; Chuck et al., 1996; Long et al., 1996; Kerstetter et al., 1997). *knotted1* (*kn1*) and other members of the class I *knox* family are expressed in the SAM but are excluded from developing leaf primordia (Jackson et al., 1994; Schneeberger et al., 1995; Foster et al., 1999b; Muehlbauer et al., 1999). Detailed expression analysis indicates that *kn1* is downregulated in a ring of cells that corresponds to size and position of the incipient leaf primordium (Smith et al., 1992; Jackson et al., 1994). KN1 protein downregulation occurs first in the cells that give rise to the future midrib, and this pattern spreads toward both margins (Smith et al., 1992, 1995). Loss of KN1 expression thus provides a molecular marker for founder cell recruitment, and is in fact one of the earliest changes that mark this process, occurring before changes in cell division are apparent.

3 Proximodistal Patterning

Based on clonal analyses and direct observations of cell division patterns by scanning electron microscopy (SEM), three stages of primordium development are apparent (Poethig, 1984; Sylvester et al., 1990). Each stage is characterized by distinctive progressions in epidermal, ground tissue, and vascular development. With regard to proximodistal patterning, cell division initially occurs evenly throughout the leaf primordium, and the sheath and blade regions are morphologically indistinguishable. During the second stage of primordium development (P2-P4), a localized increase in cell division rate, accompanied by a decrease in cell expansion, generates a band of small cells, termed the preligular band, near the base of the leaf. Formation of the preligular band visually separates the future blade and sheath. Periclinal divisions by epidermal cells at the base of the preligular band triggers formation of the ligule, whereas the remaining cells form the auricle. The third development stage is marked by a basipetal arrest of cell divisions and terminal differentiation of epidermal and subepidermal cell types (Sharman, 1942).

3.1 Recessive Liguleless Mutations

The *liguleless1* (*lg1*) and *lg2* genes act in a pathway that specifies ligule and auricle tissues. Loss of either gene function results in deletion of both tissues (Emerson, 1912; Brink, 1933). Using SEM, Sylvester et al. (1990) have shown that LG1 acts early in primordium development, as the localized cell divisions associated with formation of the preligular band are absent in *lg1* mutant leaves. *lg1* and *lg2* mutant leaves, however, retain distinct regions of blade and sheath, although the boundary between them is displaced and diffuse (Becraft et al., 1990; Harper and Freeling, 1996). This suggests that both genes may respond to proximodistal patterning cues to sharpen or fix the sheath-blade boundary and induce ligule-auricle formation. *lg1*

encodes a nuclear localized protein with homology to SQUAMOSA BINDING PROTEINs from *Antirrhinum* (Moreno et al., 1997), whereas *lg2* encodes a predicted transcription factor of the basic leucine zipper class (Walsh et al., 1997).

Mosaic analysis provides information about where and when a gene product acts, and whether it acts noncell autonomously to effect change in adjacent cells. Mosaic analysis involves generating organisms that are genetic mosaics with normal and mutant sectors of a gene of interest as well as a linked, cell-autonomous visible marker. Ligule formation is initiated on either side of the midvein and proceeds both toward the margins and the midvein (Becraft et al., 1990). Mosaic analysis indicates that *lg2* acts noncell autonomously, suggesting that LG2 is involved in intercellular signaling (Harper and Freeling, 1996). *lg1* acts cell-autonomously to specify ligule and auricle tissues, perhaps by responding to the LG2-dependent inductive signal, but may be required to laterally propagate the signal to initiate ligule and auricle (Becraft et al., 1990; Becraft and Freeling, 1991).

3.2 Dominant Knox Mutations

Overexpression or ectopic expression of class I *knox* genes outside the SAM results in a range of phenotypes that involve delayed cell fate acquisition and indeterminate patterns of growth. In maize, dominant gain-of-function mutations in *knox* genes that cause ectopic expression in leaves, disrupt proximodistal patterning (Smith et al., 1992; Schneeberger et al., 1995; Foster et al., 1999b; Muehlbauer et al., 1999). These mutants are characterized by the differentiation of proximal tissue types, such as sheath, ligule and auricle, in the distal blade (Sinha and Hake, 1990; Becraft and Freeling, 1994; Schneeberger et al., 1995; Fowler John and Freeling, 1996; Foster et al., 1999b). Some cells in the leaf also overproliferate, producing outgrowths and abnormal boundaries between leaf domains (Fig. 3).

Fig. 3 Dominant *knox* mutants exhibit delayed cell fate acquisition and disrupted proximodistal patterning. (**a**) Abaxial view of a nonmutant leaf. (**b**) *Knotted1* (*Kn1*) blades develop localized tissue overgrowths or knots that are a mosaic of sheath, ligule and auricle tissue. Similar tissue overgrowths occur in the auricle and proximal blade of (**c**) *Rough Sheath1* (*Rs1*) mutants and in the sheath of (**d**). *Gnarley* (*Gn1*) mutants

Mosaic analysis of the dominant *knox* mutants, *Kn1, Gnarley1* (*Gn1*) and *Lg3* indicate that ectopic expression in one mesophyll layer is sufficient to condition a mutant phenotype involving all layers of the leaf (Hake and Freeling, 1986; Muehlbauer et al., 1997; Foster et al., 1999a). These *knox* genes act noncell autonomously between cell layers (adaxial–abaxial), but generally act cell-autonomously in the mediolateral dimension. Only *Rough sheath1* (*Rs1*) acts completely noncell autonomously, which is surprizing considering that *rs1* and *gn1* are duplicate loci (Becraft and Freeling, 1994). Interestingly, plasmodesmatal trafficking of *kn1* RNA and protein contributes to the noncell autonomous activity of *Kn1*, identifying selective mobility of transcription factors as a novel mechanism of cell–cell communication in plant development (Lucas et al., 1995; Kim et al., 2002).

The phenotypes of the dominant *knox* mutants and observations from mosaic analyses have been reconciled into the "maturation schedule hypothesis" for proximodistal patterning (Freeling, 1992; Muehlbauer et al., 1997). In this model, regions of the primordium progress through a series of competency states, which enable cells to respond to inductive signals and differentiate first as sheath, then as ligule/auricle, and lastly as blade. Proximal leaf regions progress through these competency states more slowly than distal regions, such that at the time of differentiation, the primordium is made up of sheath, ligule/auricle, and blade competencies along the proximodistal axis. If so, ectopic *knox* expression in the leaf would inhibit the normal progression of cells through subsequent competency stages. A key aspect of this model is that *knox* activity does not inhibit differentiation itself.

3.3 Negative Regulators of Knox Expression in Leaves

rough sheath2 (*rs2*) negatively regulates *knox* expression in maize. *rs2* loss-of-function mutants ectopically express *knox* genes and have leaf phenotypes similar to those resulting from gain-of-function *knox* mutations (Schneeberger et al., 1998; Timmermans et al., 1999; Tsiantis et al., 1999b). *rs2* encodes a MYB-domain transcription factor orthologous to PHANTASTICA and ASYMMETRIC LEAVES1 from *Antirrhinum* and *Arabidopsis*, respectively (Timmermans et al., 1999; Tsiantis et al., 1999b). *rs2* expression is first detected in the incipient primordium. However, *knox* downregulation precedes the onset of *rs2* expression and the arrangement of leaves in *rs2* mutants is normal. Thus, RS2 acts after leaf initiation to maintain *knox* gene silencing in developing leaves.

In *rs2*, *knox* genes become reactivated in a variegated clonal pattern, such that *rs2* null leaves are mosaics of $knox^{+}$ and $knox^{-}$ sectors. This pattern of *knox* reactivation is reminiscent of several classic epigenetic phenomena (e.g. transposon silencing or position effect variegation), and suggest that RS2 represses *knox* expression during organogenesis by maintaining a somatically stable silenced chromatin state at the *knox* targets (Timmermans et al., 1999). Such "cellular memory" of *knox* repression would propagate early patterning events throughout the many rounds of cell division associated with leaf development. Consistent with

an epigenetic mode of *knox* gene repression, RS2 was found to interact with the chromatin-remodeling factor HIRA (Phelps-Durr et al., 2005).

RS2 also interacts with the LOB-domain protein INDETERMINATE GAMETOPHYTE1 (IG1), which is a close homolog of ASYMMETRIC LEAVES2 (AS2) from *Arabidopsis* (Phelps-Durr et al., 2005; Evans, 2007). AS1 and AS2 are known to act together in the repression of *knox* genes during *Arabidopsis* leaf development (Lin et al., 2003; Xu et al., 2003). Recessive *ig1* mutants exhibit a range of phenotypes, most obvious of which are defects in female gametophyte development and male sterility (Evans, 2007). Flag leaves on *ig1* mutant plants, however, frequently resemble those of *Knox* and *rs2* mutants or show adaxial-abaxial polarity phenotype. Like the *rs2* mutant leaves, phenotypic *ig1* flag leaves misexpress multiple *knox* genes. *ig1* is expressed in the adaxial domains of lateral organs, which may explain why *ig1* mutants, unlike other mutants that condition ectopic *knox* expression, can show defects in adaxial-abaxial polarity.

SEMAPHORE1 (SEM1) and CORKSCREW1 (CKS1) are both negative regulators of *knox* gene expression that seem to act in a developmental pathway separate from RS2 and IG1. Curiously, recessive *cks1* mutants accumulate *knox* transcripts in the leaf, but ectopic KNOX proteins cannot be detected (Alexander et al., 2005). Loss of *sem1* function causes pleiotropic defects, disrupting leaf, embryo, endosperm, lateral roots, and pollen development (Scanlon et al., 2002). These phenotypes are associated with ectopic expression of a subset of *knox* genes, specifically the duplicated *knox* loci *rs1* and *gn1,* in P4 and older leaf primordia. Polar auxin transport (PAT) is strongly reduced in *sem1* mutant shoots. Moreover, seedlings grown on chemicals that disrupt PAT develop *rs2*-like phenotypes in the absence of *knox* misexpression (Tsiantis et al., 1999a). Together, the data suggest that *knox* misexpression perturbs a PAT-dependent mechanism contributing to proximodistal patterning.

3.4 Other Proximodistal Mutants

Several mutations that affect proximodistal patterning independent of *knox* regulation have been described, e.g. the *extended auricle1* (*eta1*), *Lax midrib1-O* (*Lxm1-O*), and *Wavy auricle in blade1* (*Wab1*) mutants. The proximodistal patterning defects of *eta1* enhance the *Knox* and *lg1* phenotypes, and cause a dose-dependent interaction with *lg2* (Osmont et al., 2003). Expression of *lg1* and *lg2* seems unaffected in *eta1*. Based on these data, as well as observations from mosaic analysis, it is thought that *eta1* acts downstream in the same pathway as *lg1* and *lg2*.

The dominant *Wab1* mutation not only displaces proximal sheath and auricle tissues into the blade, it also reduces the width of the leaf as early as P3-P5 (Hay and Hake, 2004). The *lg1* gene is misexpressed in *Wab1* leaves, which may condition the ectopic auricle tissue seen in mutant leaves, and suggests *wab1* as an upstream regulator of *lg1*. However, the *Wab1* phenotype is enhanced by the loss of *lg1* or *lg2* function, as double mutant leaves are reduced to narrow strips of sheath-like tissue. This suggests that *lg1* activity also suppresses some effects of the *Wab1* mutation (Foster et al. 2004).

Detailed phenotypic and genetic analyses suggest that *Lxm1-O* is a heterochronic mutation that accelerates leaf developmental programs, including the proposed "maturation schedule" for proximodistal patterning (Schichnes and Freeling, 1998). The cloning and molecular characterization of these genes should clarify their role in leaf development, and may identify novel genetic pathways involved in proximodistal patterning.

4 Adaxial–Abaxial Patterning

A central theme that has emerged from leaf development research is the interdependence of growth along the three axes. Waites and Hudson first suggested a model in which the juxtaposition of adaxial and abaxial domains is required for subsequent lamina outgrowth along the mediolateral axis (Waites and Hudson, 1995). In a variety of plant species, mutations that cause the complete loss of adaxial or abaxial identity result in radialized lateral organs, whereas partial loss of polarity leads to ectopic outgrowths along the new adaxial–abaxial boundaries. Adaxial–abaxial patterning also leads to differentiation of distinct cell types on the upper and lower sides of the leaf. The adaxial surface of the maize leaf expresses several distinctive characters, including the ligule, macrohairs, and bulliform cells. Polarity is also visible in the internal leaf tissues. For instance, in vascular bundles, xylem is adaxially positioned relative to phloem, and schlerenchyma cells develop on just the abaxial side of intermediate veins.

The adaxial–abaxial axis is established by several redundant genetic pathways involving antagonistic factors that define and elaborate the boundary between adaxial and abaxial domains. These genetic pathways are mostly conserved between dicots and maize. However, a variable dependence on individual polarity pathways between plant species has been noted (Kidner and Timmermans, 2007), which may relate to fundamental differences in the way monocot and dicot leaves develop.

4.1 HD-ZIPIII Genes Specify Adaxial Cell Fate

The Class III *homeodomain-leucine zipper* (*HD-ZIPIII*) genes specify adaxial identity in lateral organs in dicot species as well as maize. HD-ZIPIII proteins contain a homeodomain-leucine zipper domain and a predicted sterol/lipid-binding domain similar to the mammalian START domain (Sessa et al., 1998; Pontig and Aravind, 1999; McConnell et al., 2001; Juarez et al., 2004a). As in *Arabidopsis,* maize *HD-ZIPIII* genes are expressed at the tip of the SAM and on the adaxial side of incipient and leaf primordia (Juarez et al., 2004b). In older primordia, polarized expression persists in the vasculature and at the growing margins.

Semi-dominant mutations in the maize *HD-ZIPIII* gene, *rolled leaf1* (*rld1*) cause an upward curling of the leaf blade due to adaxialization or partial reversal of

adaxial–abaxial polarity in sectors of the leaf (Nelson et al., 2002). *Rld1* leaves develop ectopic ligules and small blade outgrowths on the lower leaf surface, and epidermal cell types are displaced from the adaxial to the abaxial side. In *Arabidopsis*, dominant gain-of-function *HD-ZIPIII* alleles all result from mutations in a small, highly conserved motif in the START domain. Initially, it was proposed that these mutations caused HD-ZIPIII proteins to be constitutively active in the absence of a ligand; perhaps the hypothetical adaxializing signal from the SAM first proposed by Sussex (McConnell et al., 2001). However, subsequent investigations indicate that mutations in this conserved motif overlaps with a microRNA (miRNA) complementary region, which disrupts miRNA-dependent degradation of the *hd-zipIII* transcripts (Juarez et al., 2004b).

4.2 Regulation of HD-ZIPIII Genes by Mirnas

miRNAs are endogenous ~21 nucleotide RNAs that act in *trans* to regulate the expression of target genes. miRNAs are processed by DICER-LIKE1 (DCL1) from a stem-loop structure in long, noncoding, RNA-polymerase II-dependent primary transcripts. The mature miRNA becomes incorporated into a complex with ARGONAUTE1 (AGO1), which mediates the homology-dependent cleavage or translational repression of target transcripts (for review, see Jones-Rhoades et al., 2006).

The *HD-ZIPIII* genes are targets for the miR166 class of miRNAs, which direct the cleavage of *HD-ZIPIII* transcripts (Rhoades et al., 2002; Emery et al., 2003). This mechanism of miR166-mediated *HD-ZIPIII* regulation is conserved throughout all land plants, and as in *Arabidopsis*, dominant *Rld1* alleles have nucleotide changes in the miR166-complementary site (Juarez et al., 2004b). Consequently, transcripts derived from the dominant *Rld1-O* allele are misexpressed on the abaxial side of leaf primordia. In maize, miR166 accumulates abaxially in a pattern complementary to that of the *HD-ZIPIII* genes. At the side of leaf initiation, miR166 accumulates most abundantly immediately below the incipient leaf, but a gradient of weaker expression extends into the abaxial domain of the incipient primordium. The genetic and expression analyses suggest that miR166 acts as a polarizing signal that establishes organ polarity by spatially restricting *HD-ZIPIII* expression to the adaxial side of developing primordia (Juarez et al., 2004b).

4.3 Ta-Sirnas Specify Leaf Polarity Through Regulation of Mir166

Recessive *leafbladeless1* (*lbl1*) mutants show a variable loss of polarity, ranging from the development of thread-like, abaxialized leaves to the formation of leaves with ectopic lamina at the boundary of abaxialized sectors on their adaxial surface (Timmermans et al., 1998) (Fig. 4a). The *Rld1* and *lbl1* phenotypes are mutually

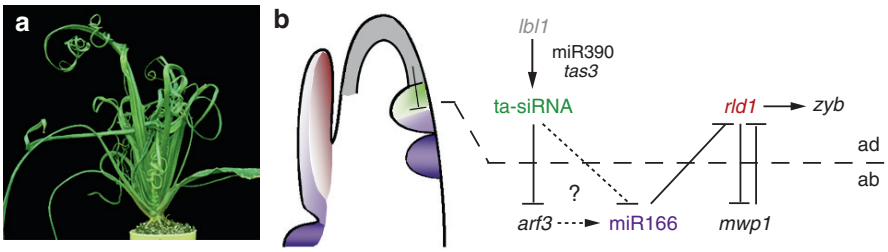

Fig. 4 *leafbladeless1* (*lbl1*) establishes leaf polarity by demarcating the domains of *hd-zipIII* and miR166 accumulation. (**a**) *lbl1* plant with thread-like abaxialized leaves. (**b**) Cartoon illustrating genetic interactions and expression domains of factors controlling adaxial–abaxial polarity in maize. Please see text for details

suppressive, and *HD-ZIPIII* expression is reduced in *lbl1* mutants (Juarez et al., 2004a). Thus, *lbl1* acts upstream of the *HD-ZIPIII* genes in the specification of adaxial cell fate (Juarez et al., 2004a). *lbl1* mutants affect *HD-ZIPIII* expression through changes in the pattern of miR166 accumulation, which in the mutant accumulate uniformly throughout incipient and developing leaf primordia (Nogueira et al., 2007).

lbl1 encodes a homolog of SUPPRESSOR-OF-GENE SILENCING3 (SGS3), which is required for the biogenesis of a class of plant-specific small RNAs termed *trans*-acting small interfering RNAs (ta-siRNAs) (Nogueira et al., 2007). Briefly, miRNA-guided cleavage of ta-siRNA precursor (*TAS*) transcripts marks them for entry into an *RNA-DEPENDENT RNA POLYMERASE6* (*RDR6*) and *SGS3*-dependent pathway that leads to formation of double stranded RNAs, which are processed by *DCL4* into phased, 21 nucleotide ta-siRNAs (Allen et al., 2005; Xie et al., 2005; Yoshikawa et al., 2005). Ta-siRNAs guide the cleavage of target transcripts, similar to the action of miRNAs. Interestingly, the *TAS3*-derived ta-siRNA, tasiR-ARF, regulates the expression of *AUXIN RESPONSE FACTOR3* (*ARF3*) and *ARF4*, which, in *Arabidopsis*, act redundantly to promote abaxial cell fate (Allen et al., 2005; Pekker et al., 2005; Williams et al., 2005).

LBL1 is required for the biogenesis of ta-siRNAs, such as tasiR-ARF, demonstrating an essential role for the maize ta-siRNA pathway in specification of adaxial-abaxial polarity. This is in contrast to *Arabidopsis*, where mutations affecting ta-siRNA biogenesis cause no obvious polarity defects (Peragine et al., 2004; Adenot et al., 2006; Hunter et al., 2006). In maize, tasiR-ARF accumulates on the adaxial side of the incipient leaf and restricts the expression domains of abaxial determinants, including miR166 (Fig. 4b). As the biogenesis of tasiR-ARF is triggered by miR390 (Allen et al., 2005), adaxial-abaxial polarity in maize is established by a cascade of opposing small RNAs, which presents a novel mechanism of pattern formation.

The prevalent contribution of small RNAs to adaxial-abaxial patterning does not preclude a role for a ligand that activates HD-ZIPIII proteins in adaxial regions of lateral organs. Regulation by miRNAs and a ligand produced in the SAM could act together to specify and refine the domain of active HD-ZIPIII protein (Emery et al., 2003;

Juarez et al., 2004b). Alternatively, small RNAs themselves may act as mobile signals that convey positional information inherent within the SAM to the developing leaf primordium.

4.4 KANADI Genes Specify Abaxial Cell Fate

KANADI (*KAN*) genes belong to the GARP family of transcription factors and promote abaxial identity in both dicots and maize (Kerstetter et al., 2001; Candela et al., 2008). *KAN* genes are expressed abaxially in lateral organs, and repress expression of the *HD-ZIPIII* genes in the abaxial side of developing primordia. The maize genome includes at least 11 *KAN* family members (pers. comm., Hector Candela and Sarah Hake). *ZmKAN2*, the maize homologue of *Arabidopsis KAN2*, is expressed on the abaxial side of young leaf primordia in a pattern complementary to *rld1* (Henderson et al., 2006). However, unlike miR166, *ZmKAN2* expression is first detected in the P1 rather than the incipient leaf primordium.

milkweed pod1-R (*mwp1-R*) is a recessive mutation in a maize *KANADI* (*KAN*) gene that specifically disrupts adaxial-abaxial patterning of sheath tissue. *mwp1-R* husk leaves and the sheaths of vegetative leaves develop pairs of outgrowths on the abaxial surface associated with regions of adaxialized tissue. Given the number of *KAN* genes in maize, a high level of redundancy and subfunctionalization in the gene family is to be expected (Candela et al, 2008). Thus, *mwp1* may act primarily in sheath tissue, with other *KAN* family members fulfilling a similar role in blade tissue.

4.5 Interactions Between HD-ZIPIII and KANADI Genes

Genetic interactions suggest that the *Arabidopsis HD-ZIPIII* and *KAN* genes act mutually antagonistically to maintain defined adaxial and abaxial domains. *rld1* is misexpressed in *mwp1-R* leaf and husk primordia, and *Rld1*; *mwp1* double mutants develop more severely adaxialized organs than either single mutant. In the double mutant, ectopic leaf flaps on the abaxial surface extend into the blade and the polarity defects continue into the stem, with invaginated regions that show irregular vasculature patterning. These finding suggests that the mutually antagonistic relationship between *KAN* and *HD-ZIPIII* family members is conserved in maize (Fig. 4b).

5 Mediolateral Pattering and Lamina Outgrowth

Several aspects of axial patterning differ significantly between maize and dicots. Maize leaf founder cells occupy a complete ring around the SAM, thus the maize leaf has a flattened lamina from inception. In contrast, the incipient primordium

of a typical dicot only occupies a small portion of the SAM, and emerges from the meristem as a radial structure that subsequently elaborates the flattened leaf blade. These differences in founder cell recruitment and leaf morphology likely reflect divergence in the regulation or function of patterning genes during plant evolution.

5.1 *NARROW SHEATH Mediates Lateral Founder Cell Recruitment*

Characterization of the *narrow sheath* (*ns*) mutant phenotype demonstrates that mediolateral patterning of the maize leaf occurs in the SAM during founder cell recruitment (Scanlon et al., 1996). Mutations in the *ns* duplicate gene pair delete a lateral domain of the leaf, including the margin (Fig. 5a). *ns* leaves are very narrow and lack normal marginal characters, such as sawtooth hairs and tapered leaf edges. Clonal analysis in *ns* plants demonstrates that cells, which would normally give rise to the lateral and marginal domains of the leaf, are not recruited into *ns* leaves (Scanlon and Freeling, 1997). Consistent with a defect in founder cell recruitment, in the *ns* mutant, KNOX downregulation does not occur on the marginal side of the SAM.

The homeologous *ns1* and *ns2* genes encode WUSCHEL1-like homeodomain transcription factors (Nardmann et al., 2004). *ns* transcripts are detected in two foci in the L1 layer of the initiating primordium and later in the margins of developing leaves. This expression pattern supports the conclusions from mosaic analysis of *ns1*, which demonstrated that NS1 acts in two lateral foci in the SAM to non-cell autonomously initialize the cells for the lateral and marginal domains of the leaf

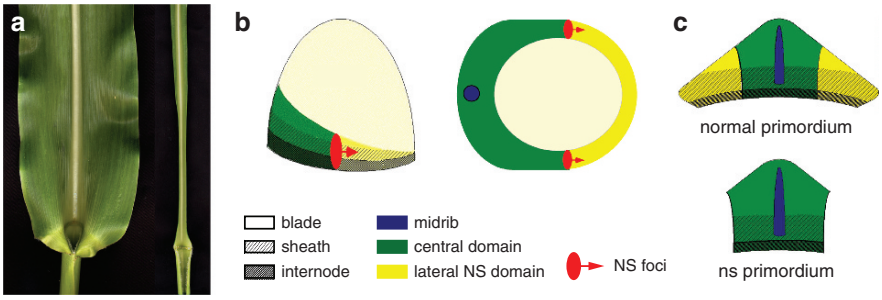

Fig. 5 *narrow sheath 1* (*ns1*) and *ns2* recruit leaf founder cells from a lateral compartment of the SAM. (**a**) Mature leaf from a nonmutant (left) and an *ns* mutant (right) showing deletion of the leaf margins in *ns*. (**b, c**) A model of NS function. (**b**) Recruitment of leaf founder cells from the SAM begins in the midrib domain and progresses toward the opposite flank. NS acts at two foci (red ovals) to initialize founder cells from the lateral domain of the SAM, which gives rise to both leaf margins. (**c**) The *ns* leaf primordium contains the central domain of the phytomer, but lacks the margin domains shown in yellow on the nonmutant leaf. Thanks to Mike Scanlon for the images shown in this figure

(Scanlon, 2000) (Fig. 5b, c). Microarray analysis of laser microdissected meristems suggests that NS-mediated induction of lateral leaf initials involves multiple signaling pathways (Zhang et al., 2007). Several studies in *Arabidopsis* and maize implicate PINFORMED-mediated auxin transport in control of phyllotaxy, founder cell recruitment and associated KNOX repression (Reinhardt et al., 2000, 2003; Scanlon, 2003). Microarray analysis and expression studies support a role for TIR1-auxin receptor mediated signaling in the NS-dependent lateral propagation of leaf initiation, and further implicates a role for cytokinin and jasmonate signaling in this process.

lbl1 and the recessive *ragged seedling2* (*rgd2*) mutants have leaf phenotypes that range from very narrow to radially symmetric, and both show aberrant patterns of KNOX downregulation in the SAM, indicating defective founder cell recruitment (Timmermans et al., 1998; Henderson et al., 2005). Surprizingly, even the strap-like *lbl1* leaves develop normal margins, suggesting that perhaps the *ns* expression foci are displaced in *lbl1*. Depending on the severity of the phenotype, one or both foci of *ns* expression are missing in *rgd2*. Loss of mediolateral growth in *lbl1* seems to be a consequence of defects in adaxial-abaxial patterning. However, in contrast to *lbl1*, the *rgd2* leaves retain correct adaxial-abaxial polarity (Henderson et al., 2005). *rgd2* mutant leaves show polarized expression of both *HD-ZIPIII* and *KAN* genes, although at much lower levels than normal. One interpretation of this observation is that *rgd2* leaves fail to grow laterally because they are not competent to respond to signals induced by the juxtaposition of adaxial and abaxial domains.

5.2 Maize YABBY Genes Promote Outgrowth of the Lamina

YABBY proteins are candidate components in the pathway that directs lateral outgrowth in response to adaxial-abaxial patterning. The maize *YABBY* orthologues, *zyb9* and *zyb14*, are expressed on the adaxial side of incipient and young leaf primordia, opposite to the abaxial expression pattern of *YABBY* genes in *Arabidopsis, Antirrhinum* and tomato (Juarez et al., 2004a). In older leaf primordia, *zyb* expression persists at the growing margins and in the central layer of provascular tissue at the presumptive adaxial-abaxial boundary. Expression of *zyb9*, but not *zyb14*, also persists in the differentiating vasculature.

Genetic interactions and expression analyses indicates that the *YABBY* genes act downstream of the initial polarity pathways. *zyb9* and *zyb14* expression is increased in *Rld1-O* and reduced in *lbl1*, suggesting that these genes are positively regulated by *rld1* and the maize ta-siRNA pathway (Juarez et al., 2004a). More interestingly, z*yb* expression is associated with abaxial outgrowths on *Rld1-O* leaves as well as adaxial outgrowths in *lbl1*. The *Rld1-O* outgrowths occur at the boundaries of *zyb*-expressing and nonexpressing cells of the central layer. A role in promoting abaxial fate is clearly not conserved between the *Arabidopsis* and maize *YABBY* genes. However, in both species, polarized *YABBY* expression is associated with regions of lateral blade outgrowth (Juarez et al., 2004a). Consistent with a role in mediolateral

outgrowth, *rgd2* leaves show aberrant and reduced expression of *zyb14*, and a distinct *YABBY* family member is downregulated in the *ns* mutant SAM (Henderson et al., 2005; Zhang et al., 2007). The regulation or function of *YABBY* genes thus seems to have diverged between the grasses and dicots, which may reflect or perhaps contribute to their distinct leaf development programs.

6 Conclusion

Our understanding of leaf development has progressed enormously in the past decade. Key regulators of axial patterning of the maize leaf have been cloned and the genetic pathways that specify organ polarity are beginning to emerge. The prevalence of redundant or partially redundant gene families reflects the evolution of plants by gene duplication and divergence but may also signify the importance of axial patterning for survival.

Cell fate in plants is largely determined by positional cues rather than cell lineage. Cell–cell communication is a central theme in axial patterning. Mobile signals are thought to convert positional information from the SAM into differential gene expression patterns in the incipient primordium. Hormone signalling, as well as the selective trafficking of proteins and perhaps small RNAs, have emerged as candidate signals in axial patterning.

Lastly, epigenetic regulators are being recognized as important for development. Chromatin modification can impart cellular memory of gene expression patterns through many rounds of cell division, ensuring that regional identity is maintained long after the primoridium grows away from the SAM. The repression of *knox* genes by the RS2 complex provides an example. Recessive *required to maintain repression6* (*rmr6)* mutants provide further evidence that epigenetic modification maintains axial identity. *rmr6* mutants show ectopic leaf outgrowths and adaxialized leaf fates and enhances *Rld1-O* phenotypes (Parkinson et al., 2007).

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Cell Biology of Maize Leaf Development

Anne W. Sylvester and Laurie G. Smith

Abstract The maize leaf has a simple cellular architecture that is amenable to cell biological study. Combined with the spatially defined growth gradients in the leaf, maize cells are useful for investigating how cell division and expansion are controlled spatially and temporally. Here we present recent advances in our understanding of molecular controls of cell division and expansion, particularly as mediated through the dynamic functions of the plant cytoskeleton and via analysis of mutants. We contend that the maize leaf epidermis is a particularly useful platform for study due its ordered growth pattern and the linear array of cells. Current advances and emerging tools are discussed that will allow the power of maize genetics to be fully realized at a cell biological level.

1 Overview

Maize leaves are uniquely simple and orderly structures. The leaf is divided into three major regions, an upper blade separated from the lower sheath by a joint-like ligule and auricle. Maize cells within each region are functionally distinct and are typified by characteristic shapes and sizes. Contributing to the simple structure, leaf cells are highly oriented and aligned in files that in turn run parallel with the elongate leaf shape. Furthermore, leaf cells grow from inception at the shoot apical meristem in a predictable pattern of cell division coupled to directional cell expansion, thus generating the spatially defined developmental gradient unique to the grasses and relatively easy to study in maize due to its large size. These features of development, cell patterns and shape relationships recommend maize leaves as models for cell biology study. The leaf epidermis is particularly useful to address questions concerning mechanisms of cell division and expansion because a minimal number of epidermal cell types grow in stereotyped patterns amenable to genetic dissection and direct observation at the leaf surface. In this chapter, we discuss cellular features of the maize leaf and their developmental origins, highlighting the epidermis as a particularly useful platform for clarifying molecular mechanisms that govern cell division and morphogenesis.

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2 Cellular Organization

Maize leaves are constructed to accomplish C4 photosynthesis and to support the plant prior to reproductive development. The bulk of the plant biomass during this vegetative phase is sustained by leaf, rather than stem tissue, as is characteristic of dicotyledonous plants. Consequently, virtually all visible portions of a developing maize plant consist of leaf cells and tissues that primarily serve a photosynthetic function in the blade and structural support in the lower sheath. The ligule/auricle region projects the blade from the sheath at an angle optimal for light reception. This basic architecture is repeated in each newly emergent primordium at the shoot apex. The distinct linear array of cells arises from oriented patterns of division followed by directional expansion from their earliest inception at the meristem (Sylvester et al., 1990; Smith et al., 1996; Mitkovski and Sylvester, 2003).

The leaf blade consists of adaxial (top) and abaxial (bottom) epidermal tissue enclosing mesophyll and vascular tissues (Sharman, 1942; Mauseth, 1988; Steeves and Sussex, 1989; Freeling and Lane, 1994). Anatomy of the mature (nongrowing) leaf is consistent with function of each tissue: epidermal cells are externally encased in varying degrees of cuticular and epicuticular waxes for protection and punctuated by stomatal complexes to permit gas and water exchange. There are essentially two major groups of epidermal cell types: specialized cells and nonspecialized intercostals cells (Fig. 1). Specialized cells include stomatal complexes and three types of hairs: large macrohairs, microhairs and bicellular hairs, all contributing to water regulation and likely serving a protective function (Dickison, 2000). Under environmental conditions, cork and silica cells form between the end walls of intercostals cells. Also parallel to the underlying veins are long rows of bulliform cells, which are flanked by the single-celled microhairs. Long macrohairs emerge from basal cells within the bulliform rows. The thin-walled bulliform cells roll the leaf inwards when osmotically stressed, thus likely preserving a water vapor boundary layer on the adaxial surface during drought conditions (Dickison, 2000). Adaxial and abaxial surfaces share cellular linearity but different cell types: stomata are dispersed on both adaxial and abaxial surfaces, but other specialized cells are restricted to the adaxial epidermis only.

Leaf sheath anatomy reflects its primary support function. In cross-sectional view, sheaths are thicker than blades and inwardly curved due to reinforcement of lignin in sclerenchyma cells along the entire adaxial inner surface (Fig. 1b). Emergent sheaths have photosynthetic cells lining the outer surface and nonphotosynthetic cells associate with the inner surface. Vascular tissue is distributed as in the blade but generally with increased interveinal spacing. Sheath curvature is reinforced by the secondary walls in vessels of the xylem as well as extensive ribbing due to epidermally directed sclerenchyma fibers. Adaxial and abaxial epidermis of the sheath lack specialized cells such as bulliform cells, cork and silica cells, bicellular hairs and microhairs. Macrohairs accumulate on the outer surface in some lines and under some growth conditions. Abaxial epidermal cells are simple rectangles, lacking deep crenulations and stomatal guard cells are more rotund than elongate.

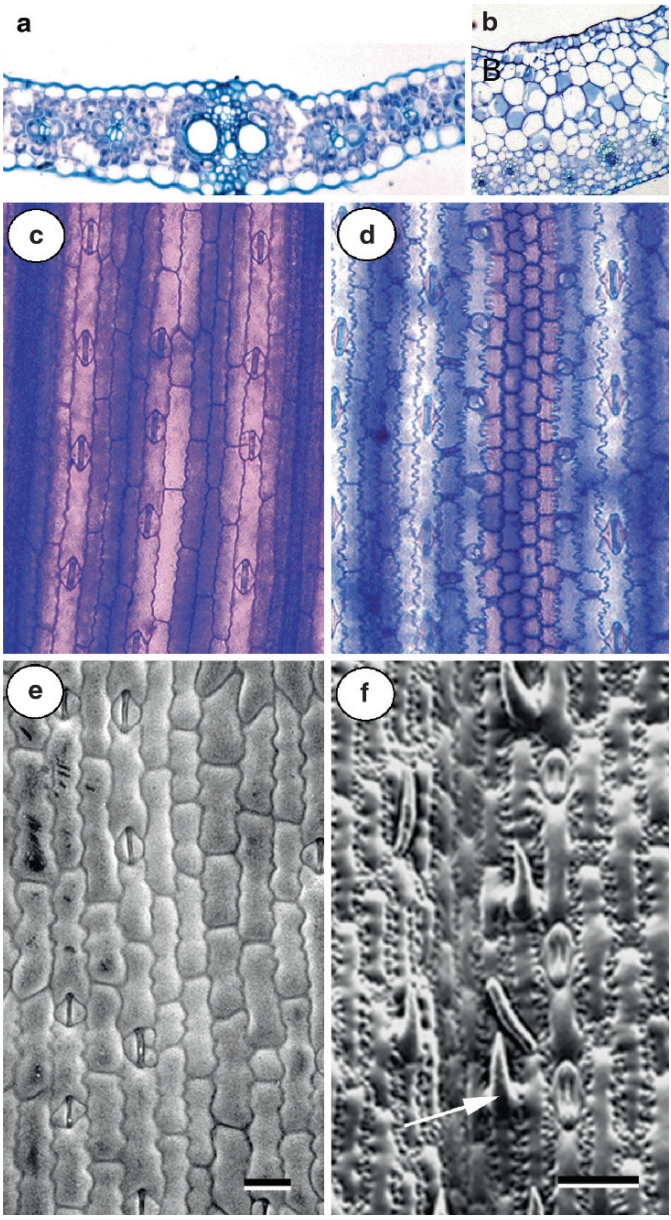

Fig. 1 Cells in the leaf. (**a**) Cross-section of an adult leaf blade shows the arrangement of vascular bundles surrounded by bundle sheath and mesophyll cells compared with (**b**) sheath. Comparison of juvenile leaf anatomy (**c**, **e**) with adult leaf anatomy (**d**, **f**) in leaves cleared and stained with TBO (**c**, **d**) and scanning electron micrographs (**e**, **f**). In (**d**), purple cells are bulliform rows. In (**f**), arrow points to microhair adjacent to the bulliform row; arrowhead points to bicellular hair. Macrohairs not visible. Scale bars = 50 μm. Modified from Reynolds et al. (1998) (**a**), Julio Ramirez (thesis, UC Berkeley) (**b**), and Sylvester et al. (2001) (**c–f**)

Adaxial epidermal cells have mild crenulations when fully mature. Stomata are present on both the abaxial (outer) and adaxial (inner) sheath of the leaf.

Epidermal cell types and characteristics vary with vegetative phase of the plant (Fig. 1). Juvenile leaves emerge first after seed germination and show a distinct glossy character due to extensive accumulation of epicuticular waxes on the adaxial blade surface. The juvenile adaxial epidermis also lacks specialized hairs, bulliform and cork or silica cells; Juvenile intercostal cells are monochromatic when stained with Toluidene Blue (TBO), and wall abutments are wavy rather than interlocking. Adult blades, on the other hand, lack epicuticular waxes and are pubescent with all three hair types present on the adaxial surface. In addition, intercostal cells in the adult blade have deeply crenulated, interlocking walls and exhibit differential staining with TBO, reflecting biochemical differences in cell wall composition between cell and leaf types (Sylvester et al., 1990, 2001; Abedon et al., 2006).

Juvenile and adult leaf anatomy is coupled to reproduction in maize and other grasses (Sylvester et al., 2001; Moose and Sisco, 1996), as evidenced by the fact that maize mutants with altered reproductive timing also show changes in numbers of juvenile leaves. For example, the *glossy 15* (*gl15*) mutant truncates juvenile growth and speeds up flowering (Moose and Sisco, 1996), similar to the *early phase change* (*epc*) mutation (Vega et al., 2002). *gl15* is downregulated at the juvenile to adult phase transition, with a concomitant induction of adult cellular traits, by a maize version of miR172, a microRNA originally shown to regulate phase change in *Arabidopsis* (Lauter et al., 2005). How *gl15* inhibits adult cell differentiation or conversely, promotes the juvenile cellular state, is unclear. Connection has been made between GL15 function and macrohair initiation through study of the *macrohairless1* (*mh1*) mutant, which reduces or completely eliminates the initiation of macrohairs in the adult leaf blade. *gl15* is epistatic to *mh1* and both mutant phenotypes are influenced by the growth regulating hormone, gibberellin (Moose et al., 2004). Gibberellin also influences trichome production in *Arabidopsis* (Gan et al., 2006) and could be acting through its potential role in aligning microtubules (Foster et al., 2003), which contribute to trichome initiation and development in *Arabidopsis* along with actin (Qiu et al., 2002; Lu et al., 2005; Szymanski, 2005). However, there is as yet no clear evidence that *Arabidopsis* trichomes and maize macrohairs share similar morphogenetic strategies. The unique maize macrohair is an ideal subject for comparative cell biology and should be exploited for descriptive, genetic and experimental study.

Internal leaf tissues consist of two compartments: vascular and mesophyll (Fig. 1a, b). Conducting vascular tissues are made up of xylem, phloem and associated cells, and are surrounded by closely appressed, photosynthetic bundle sheath cells. Leaf vascular bundles are connected to the epidermis by bundle sheath extensions, consisting of highly lignified sclerenchyma fibers that provide structural support between vascular bundles and each epidermal layer. Spongy mesophyll and bundle sheath cells produce different photosynthetic enzymes and cooperate to achieve C4 photosynthesis, producing the distinct chloroplast ultrastructure characteristic of Kranz anatomy. In the mesophyll cells, carbon is first fixed by PEP carboxylase into oxaloacetate and ultimately malate, which is transported to the bundle sheath cells, where it is be decarboxylated releasing $C0_2$ for fixation into a C3 sugar by

RUBISCO. Cell-specific ultrastructure and enzyme localization distinguishes bundle sheath from mesophyll cells. Study of mutants such as *bundle sheath defective2* (*bsd2*; Roth et al., 1996), *argentia* (*ar*; Langdale et al., 1988) and *golden2* (*g2;* Cribb et al., 2001; Rossini et al., 2001) has revealed that chloroplast ultrastructure is established in response to sequestration of enzymes. In the absence of the *bsd2* or *g2* product, C4 enzymes fail to accumulate in bundle sheath cells leading to atypical chloroplast ultrastructure. Interestingly, mutant bundle sheath cells still differentiate as a unique cellular layer surrounding the vascular tissue, even though C4 enzyme expression in maize precedes development of Kranz anatomy (Langdale et al., 1988). This suggests that the pathways establishing overall cell position and shape are separate from pathways regulating physiological and biochemical function for C4 photosynthesis. Bundle sheath cells are required to maintain hydraulic conductance within the vascular bundle (Fricke, 2002), and thus represent an ancestral anatomical arrangement in plants. The ultrastructural dimorphism in maize is a good example of anatomical adaptation, where a particular cellular arrangement is co-opted for a new biochemical function.

3 Growth Patterns

3.1 Proliferative Cells

Cellular order in maize is maintained from leaf inception to maturity through a developmental gradient that is typical of all grass leaves. The gradient can be identified in the epidermis due to characteristic changes in cell shapes from base to tip of a developing leaf. The growth gradient is established early, if not coincident with the organ polarity associated with leaf initiation (Sylvester et al., 1990, and elsewhere herein). When the primordium first emerges, the entire structure is meristematic, with cells dividing and expanding throughout. A meristematic region, defined here as a proliferative zone, becomes gradually restricted to the base while a "formative zone" is established at the primordium tip, establishing the initial apical to basal gradient. Divisions in the formative zone give rise to specialized cell types such as stomatal complexes, hairs etc. (Fig. 2; Reynolds et al., 1998; Mitkovski and Sylvester, 2003). With continued growth, the proliferative zone splits to occupy a region at the base of the blade, and independently, at the base of the sheath (termed the leaf meristem by Fiorani and Beemster, 2006). The basal sheath proliferative zone is associated with the intercalary meristem within the shoot axis, which is eventually activated to elongate the stem during reproductive growth. The division zone at the base of the expanding leaf is thus partitioned into blade and sheath basal proliferative zones separated by the ligule and expanding sheath (as depicted in Fig. 2).

Once established, the blade proliferation zone remains as a distinct band immediately above the ligule during much of leaf elongation. Within this zone, cells

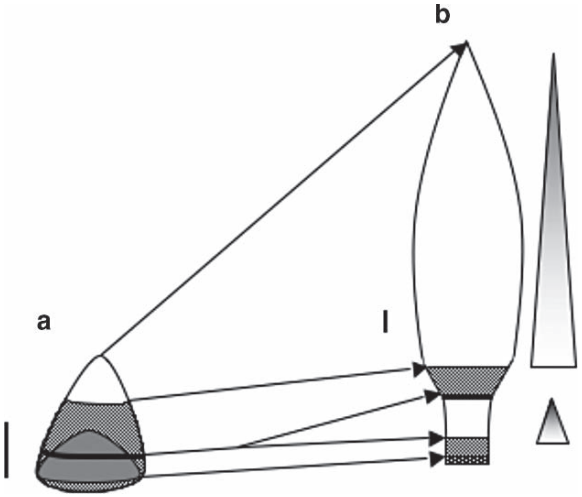

Fig. 2 Schematic illustration of the growth gradient in the developing leaf. (**a**) A leaf primordium in which the tip (white) has begun to differentiate. The proliferative zone (hatched) is above the forming ligule (black line) and extends into the incipient sheath. The meristem is shaded. (**b**) During early leaf elongation, the proliferative zone is located at both the blade and sheath base, with intervening differentiation (white). Shaded pyramids represent the growth gradient with shaded point site of differentiation and clear base site of increased division and expansion. Scale bars = 0.5 cm

divide symmetrically with new cross walls oriented primarily transverse or longitudinal to the cell or leaf long axis (Fig. 3). Cells then expand anisotropically generating a gradient of increasingly longer rectangular cells from the base to upper edge of the proliferative zone (Fig. 3, Reynolds et al., 1998; Mitkovski and Sylvester, 2003). Changes in cell shape within the proliferative zone may reflect changes in cell cycle duration, with a longer time spent in G1 allowing more cell expansion to occur before mitosis intervenes. Quantitative analyses support this hypothesis by showing more cross walls per unit area when normalized against cell size in the basal vs. distal reaches of the proliferative zone (Reynolds et al., 1998; Mitkovski and Sylvester, 2003). Further analyses will clarify aspects of cell cycle regulation relevant to understanding leaf development.

Distal to the blade proliferation zone (~1 cm above the ligule), formative divisions begin (Fig. 3). At this position on the leaf, procambial cells (precursors to vascular tissue) have already begun to differentiate within internal tissue layers. Within the formative zone of the epidermis, walls are not yet crenulated but asymmetric divisions establish the future positions of specialized cells such as hairs and stomatal complexes in a characteristic pattern. As development proceeds, the blade proliferation zone is eventually replaced by a formative zone, and ultimately by a zone of cellular differentiation (Mitkovksi and Sylvester, 2003).

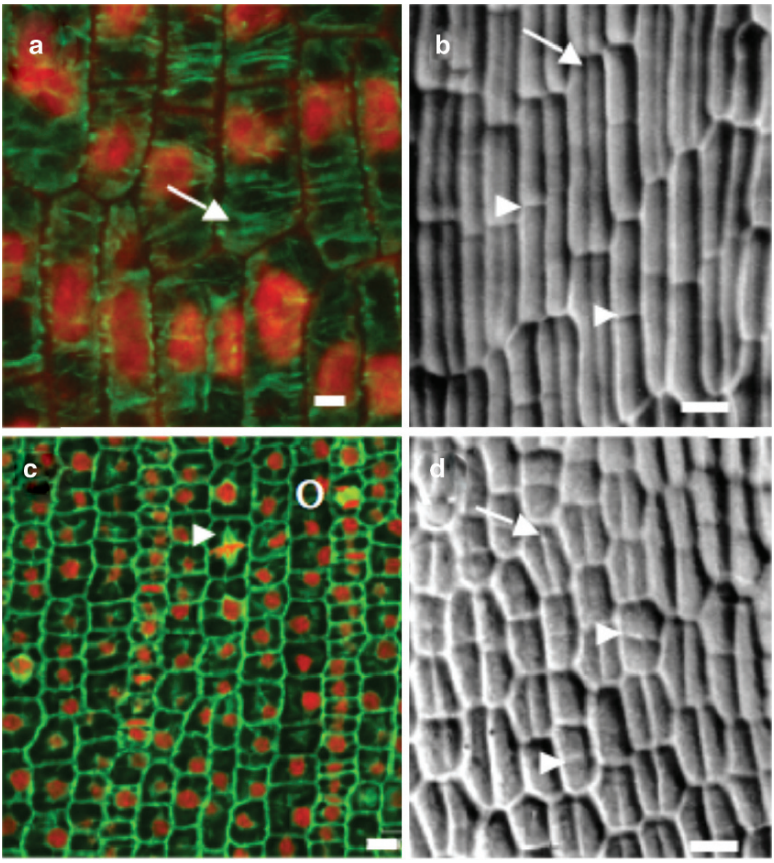

Fig. 3 Proliferative zone cells. (**a**, **c**) Whole mount staining of microtubules (green. labeled with antitubulin) and nuclei/chromosomes (red, labeled with propidium iodide) in the epidermal layer of a maize leaf primordium. Arrowheads point to spindles; O, oblique spindle in (**c**). Arrow points to aligned cortical microtubules in (**a**). (**b, d**) Comparable stages to (**a, c**), showing epidermis of proliferative zone in scanning electron micrographs. Arrowheads point to transverse new cross-walls. Arrows point to longitudinal new cross walls. Scale bars = 20 μm. Modified from Reynolds et al. (1998) (**b, d**); Zhang and Sylvester (unpublished) (**a, c**)

3.2 Leaf Elongation

Maize leaf elongation is eventually restricted to the leaf base near the site of leaf insertion (e.g., Fricke, 2002). Measurements of relative elongation and widening rates have been used to characterize normal leaf growth patterns for the purpose of contrast with mutants and for comparative purposes among the grasses, which share similar anatomy and morphology with maize (Schnyder et al., 1987; Fiorani et al., 2000; Sylvester et al., 2001). These studies measure and locate the site of leaf elongation near the leaf base (Muller et al., 2007). Other grasses differ from maize

with respect to blade:sheath ratios, suggesting that finer scale developmental gradients work independently in blade and sheath (Sylvester et al., 2001). Studies by Beemster and colleagues suggest that large scale elongation in grasses (and by extrapolation, maize) is driven by the size of the division zone at the base of the sheath, as well as the size of the mid expanding zone (Fiorani et al., 2000).

The gradient of leaf elongation has been exploited to identify molecular markers of growth and candidate growth regulators. For example, leaf-specific expansins, proteins required to loosen the cell wall during expansion, are differentially expressed near the expanding middle and tip of a growing leaf as expected (Muller et al., 2007). Transcriptional profiling of growing maize leaves also identified 43-cell cycle regulatory genes and clusters of chloroplast specific genes differentially expressed along the large scale gradient (Rymen et al., 2007; Cahoon et al., 2007). These genes provide a useful set of markers for cellular studies of leaf growth and its response to biotic and abiotic stress (Perchorowicz and Gibbs, 1980; Ben-Haj-Salah and Tardieu, 1995; Tardieu et al., 2000; Rodríquez et al., 2002; Rymen et al., 2007; Muller et al., 2007).

Large scale growth gradients have been measured in maize and other grasses by physical marking (Schnyder et al., 1987) and kinematic methods (Tardieu and Granier, 2000; Muller et al., 2001), by identifying and measuring cell production in the sheath proliferative zone only (Fiorani and Beemster, 2006), and by localization of molecular markers of growth zones (Granier et al., 2000; Muller et al., 2007; Rymen et al., 2007; Cahoon et al., 2007). In contrast, direct observation of cell sizes, shapes and cross wall orientations uncovers specific gradients of cell size within the proliferative zone immediately above the ligule (Fig. 2; Sylvester et al., 1990; Mitkovski and Sylvester, 2003). The precise function or contribution of these local gradients to overall leaf elongation is not known. It is possible that this subzone of the leaf serves to reset a new developmental gradient in the blade, permitting separate developmental programs to function in the blade versus sheath. Detailed study of the blade proliferative zone using established methods (Tardieu and Granier, 2000; Mitkouski and Sylvester, 2003; Fiorani and Beemster, 2006; Horiguchi et al., 2006) will further clarify the relative role of proliferative cell divisions and cell expansion in blade growth. Ultimately, this information will be essential to uncover molecular controls and mechanisms of cell growth.

3.3 Epidermis As a Cell Biology Model

The maize leaf epidermis is particularly useful for addressing cell biological questions due to its orderly cell arrangement. A given position on the developing leaf provides a population of cycling cells for sampling. The fact that orthogonal cell shapes are propagated from proliferation to maturity (Fig. 3) means that mutants can be identified that alter cell pattern in the mature nongrowing (and fully accessible) blade (Fig. 4). The flat structure of the epidermis is also useful for cytological study

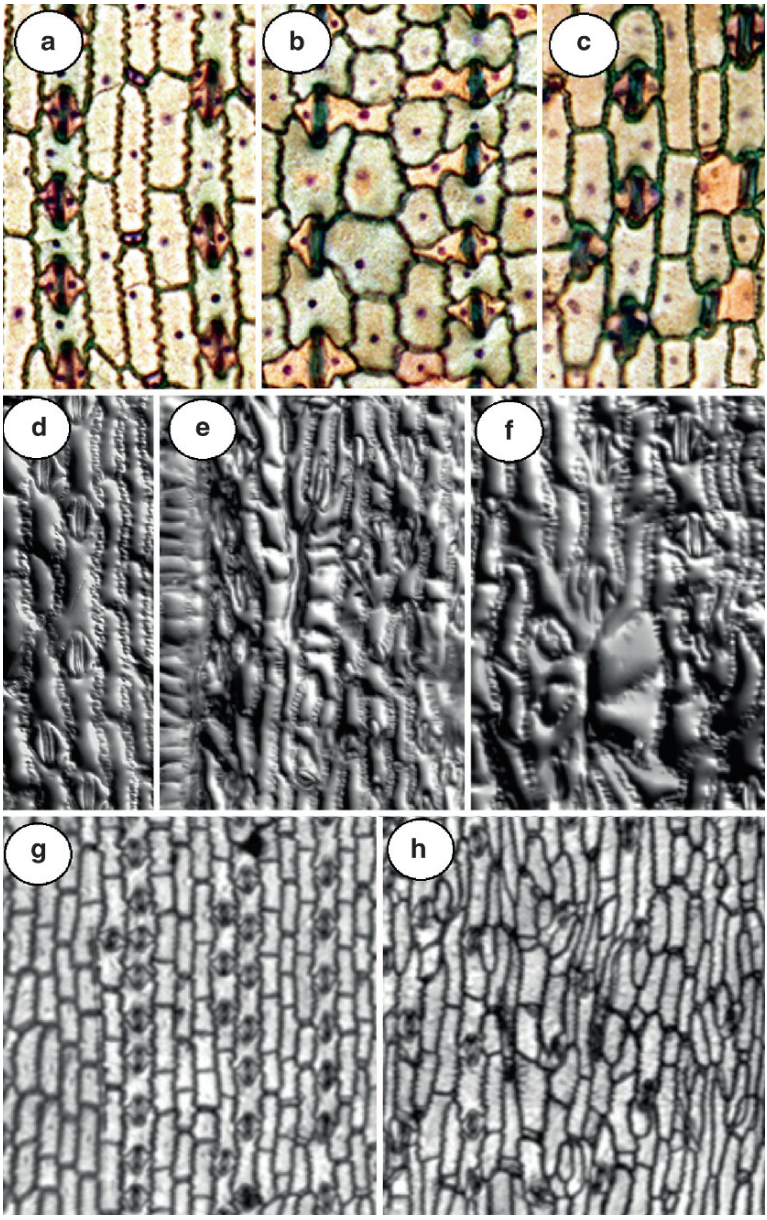

Fig. 4 Phenotypes of cell pattern mutants showing altered division of stomatal complexes (**b, c**), expansion defects (**e, f**) and misplaced cell walls (**h**), as discussed in text. Top panel compares adaxial epidermis, cleared and stained with TBO of (**a**) wild type compared with *dcd* (**b**) and *pan* (**c**) mutants. Middle panel compares SEMs of dental wax impressions molded with epoxy of wild type (**d**) compared with the *chao*s (*chs*) mutant (**e**) and *wty* (**f**). Bottom panel compares cleared DIC images of wild type (**g**) compared with *tan* mutant (**h**)

as shown in Fig. 3. Mutant screens have been thus designed by clipping blade segments from mutagenized plants, preparing epidermal impressions using superglue, nail polish or dental wax, then scanning for altered cell shape and size under the microscope (Fig. 4; Williams and Sylvester, 1994; Smith et al., 1996; Reynolds et al., 1998). Mutant progeny can then be dissected and phenotypes characterized in proliferative and formative zones. There, the cellular gradient serves as a useful platform to investigate cell biology of the normal compared with the mutant process (Smith et al., 1996; Reynolds et al., 1998). These screens are also useful to select for mutations in genes that may have pleiotropic effects such as transcription factors or hormonal regulators. The approach has successfully identified genes that contribute to proper cell wall placement (*tangled1*; Fig. 4h; Smith et al., 1996), maintenance of cell expansion, (*warty1*; Fig. 4f; Reynolds et al., 1998), organization of stomatal complexes (*brick*, *discordia* and *pan;* Fig. 4b, c), and intercostal cell shape morphogenesis (*brick;* not shown but similar in phenotype to *pan* in Fig. 4c), among others (Fig. 4e). Further advantage of the epidermis is its potential for *in vivo* investigation of dynamic processes. The next section considers specific cell biological questions about mechanisms of cell division and expansion in the epidermal model and insights derived from analysis of mutants.

4 Cell Division

Cell division is accomplished by chromosome segregation during mitosis followed by formation of a new cell wall during cytokinesis. Although the mechanism of division and cytokinesis are likely shared among all plants, most information has been derived from study of plants other than maize. These studies show that both actin and microtubule cytoskeletons participate in all aspects of mitosis and cytokinesis, and are organized into the arrays depicted in Fig. 5. Mitosis and cytokinesis are of particular interest in plants because spatial control of new wall placement is important for maintaining proper developmental patterns.

4.1 Spatial Control of Cytokinesis

Cytokinesis in maize leaf cells, as in other somatic plant cells, is achieved via construction of a new cell wall between daughter nuclei after completion of nuclear division. A cytoskeleton-based structure called the phragmoplast, which is unique to plant cells but structurally and functionally related to the midbody of animal cells (Otegui et al., 2005), plays a central role in formation of the new cell wall (called a cell plate while forming). The phragmoplast assembles from the remnants of the mitotic spindle and is composed of two opposing disks, each containing relatively short, parallel microtubules and actin filaments (Figs. 3c and 5). Cell plate formation is initiated at the interface between the two disks. Microtubules

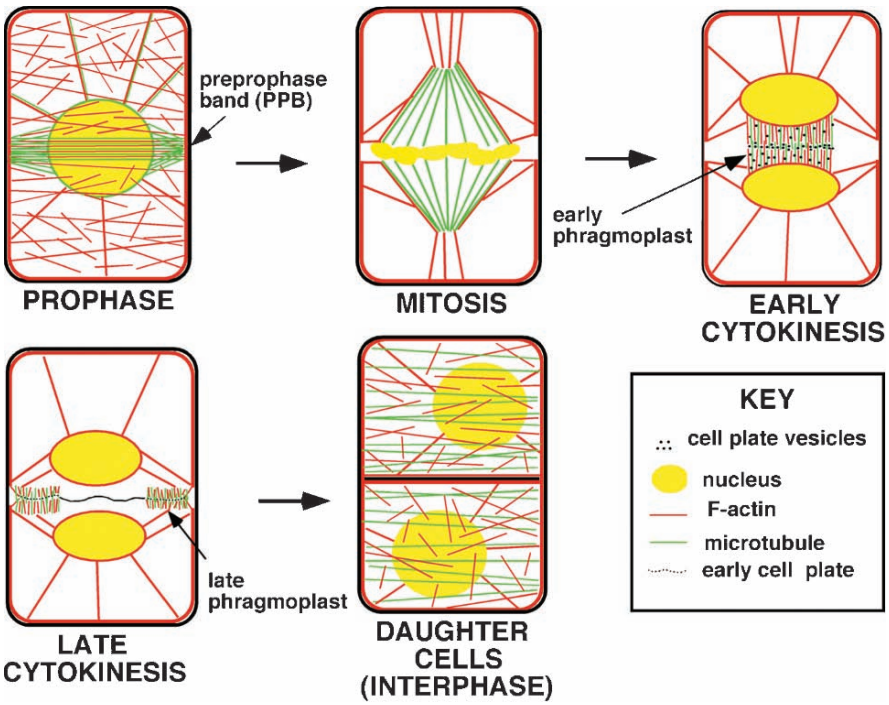

Fig. 5 Schematic illustration of cytoskeletal arrays involved in plant cell division. For cells in prophase and interphase, both surface and internal structures are illustrated. For clarity, only internal structures are illustrated for cells at other stages

are essential for cell plate formation and are thought to transport Golgi-derived vesicles carrying cell wall components and membrane to the phragmoplast equator for cell plate construction; the role of actin filaments within the phragmoplast is less clear (Staehelin and Hepler, 1996). Vesicles fuse with one another at the phragmoplast equator, initiating an intricate membrane remodeling process that transforms a collection of discrete vesicles into a flat, membrane-bound sheet of cell wall material perforated by channels that will become plasmodesmata, with endoplasmic reticulum running through these channels (Samuels et al., 1995; Segui-Simarro et al., 2004).

The phragmoplast arises between daughter nuclei after mitosis, initiating the cell plate in isolation from the mother cell wall and plasma membrane. As cell plate formation progresses, microtubules and actin filaments depolymerize at the center of the phragmoplast and reassemble at its periphery, bringing about a lateral expansion of the phragmoplast (Jürgens, 2005). The cell plate expands centrifugally along with the phragmoplast, eventually fusing with the mother cell wall and plasma membrane (Fig. 5). Interestingly, the location at which the cell plate will fuse with the mother cell wall is predicted much earlier in the cell cycle by a

cytoskeletal array called the preprophase band (PPB). As illustrated in Fig. 5, the PPB is a belt of cortical microtubules and actin filaments, which forms during late S or early G2 phase of the cell cycle and persists throughout prophase, but is disassembled as the mitotic spindle forms (Mineyuki, 1999). The cortex is essentially devoid of microtubules during the remainder of mitosis and cytokinesis, but cortical actin remains everywhere except the former PPB site, where the actin component of the PPB is removed along with the microtubule component at the prophase–metaphase transition to create an actin depleted zone (ADZ) of the cell cortex (Fig. 5; Cleary et al., 1992; Liu and Palevitz, 1992; Sano et al., 2005). The ADZ persists throughout mitosis and cytokinesis, negatively marking the division plane, but the significance of this actin arrangement remains unclear. Although few studies of plant cytokinesis have focused on maize leaves, epidermal cells of maize leaf primordia have been shown to exhibit all three classes of mitotic microtubule arrays: PPBs, spindles and phragmoplasts (Fig. 3), and also ADZs (Cleary and Smith, 1998). These mitotic cytoskeletal arrays are found in both transverse and longitudinal orientations consistent with the transverse and longitudinal cell divisions in the epidermis.

4.2 Case Study: Tangled

A poorly understood aspect of plant cell division is how the phragmoplast is guided to the former site of the PPB. The *tan* gene of maize was shown to be required for this process in the epidermal layer of the maize leaf primordium (Fig. 4h; Smith et al., 1996; Cleary and Smith, 1998). TAN protein is highly basic and lacks recognizable functional domains or strong homology to other proteins of known function, but is distantly related to the basic, microtubule binding domain of the human adenomatous polyposis coli (APC) tumor suppressor protein (Smith et al., 2001). Like the basic domain of APC, it can bind to microtubules *in vitro*. Proteins recognized by anti-TAN antibodies were found to be associated with PPBs, spindles, and phragmoplasts in dividing cells and are also present in the cytoplasm throughout the cell cycle (Smith et al., 2001). However, these antibodies were not completely specific for TAN, also recognizing proteins encoded by *tan*-related genes that have been identified via EST and genomic sequencing, so TAN localization has not been definitively determined (Smith et al., 2001). More recently, the *Arabidopsis* homolog of TAN was shown to colocalize as a YFP fusion protein with PPBs, and then to persist as a cortical ring after PPB disassembly, positively marking the division plane throughout mitosis and cytokinesis (Walker et al., 2007). Like maize TAN, *Arabidopsis* TAN plays a role in guidance of phragmoplasts to former PPB sites during cytokinesis, supporting the conclusion that this protein is a functional marker of the division plane that helps to orient expanding phragmoplasts (Walker et al., 2007). Future work will be required to determine whether maize TAN, like its *Arabidopsis* homolog, positively marks the division plane after PPB disassembly.

4.3 Case Study: Stomatal Complex Formation

Many studies of cell division in the leaves of grasses including maize have focused on the unique sequence of divisions underlying stomatal complex formation, which are among the last divisions to complete in developing maize leaves (Sylvester et al., 1996). The first asymmetric division is oriented transversely, and the smaller apical daughter of this division is called a guard mother cell (GMC) because it will later divide to form a guard cell pair (Fig. 6; Stebbins and Shah, 1960; Giles and Shehata, 1984). Asymmetric, GMC-forming divisions occur in files where cells are more square in shape compared to neighboring cell files (Fig. 6) and where every cell undergoes this division, producing a linear chain consisting of GMCs (and ultimately stomata) alternating with interstomatal cells (Stebbins and Shah, 1960; Giles and Shehata, 1984). These chains tend to lie in between underlying vascular bundles, which form earlier in development, suggesting that vascular bundles may be the source of a signal that inhibits formation of GMCs in overlying epidermal cells (Sylvester et al., 1996). Early work on stomatal patterning in monocots led to the proposal that linear files in which every cell produces a GMC represent clonal groups (Charlton, 1990; Chin et al., 1995). However, clonal analysis of the maize leaf epidermis showed that stomata within a single, linear chain are not necessarily more closely related than they are to other cells nearby, indicating that positional information rather than lineage relationships dictates which cells will undergo asymmetric, GMC-forming divisions (Hernandez et al., 1999).

An interesting feature of stomatal complex formation in grasses is that it appears to involve cell–cell communication. After formation of a GMC, both of the GMC's lateral neighbors (subsidiary mother cells or SMCs), which are not closely related to the GMC by lineage, divide asymmetrically to produce subsidiary cells (SCs) flanking the GMC on both sides (Figs. 6 and 7). Mature subsidiary cells participate in stomatal regulation by serving as a source and sink for potassium ions that are

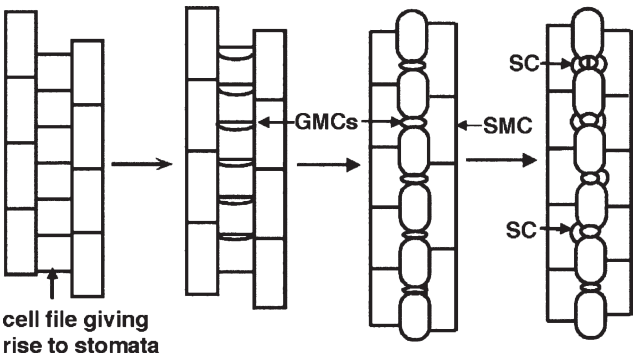

Fig. 6 Schematic illustration of asymmetric divisions giving rise to stomatal complexes in maize. *GMC* guard mother cell, *SMC* subsidiary mother cell, *SC* subsidiary cell. Reproduced from Hernandez et al. (1999)

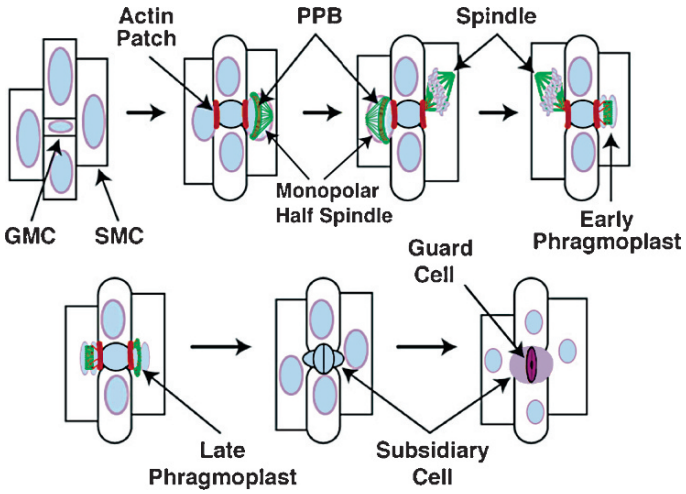

Fig. 7 Schematic illustration of key steps in asymmetric division of subsidiary mother cells in maize featuring cytoskeletal arrays. Green = microtubules; red = F-actin. *GMC* guard mother cell, *SMC* subsidiary mother cell, *PPB* preprophase band

transported in and out of the adjacent guard cells to regulate their turgor pressure and thus their degree of opening (Raschke, 1975). Circumstantial evidence strongly suggests that SMC division, and the orientation of this division, is determined by a signal emanating from the GMC (Stebbins and Shah, 1960). Perhaps the most compelling support for this conclusion is that in unusual cases where two GMCs share a common SMC, the SMC divides twice to produce two subsidiary cells, one flanking each of the GMCs (Stebbins and Shah, 1960).

Analyses of cytoskeletal rearrangements associated with subsidiary mother cell (SMC) divisions have revealed many interesting cytological features of this process, mostly described in other grasses (Pickett-Heaps and Northcote, 1966; Cho and Wick, 1989, 1990; Cleary, 1995; Cleary and Mathesius, 1996; Kennard and Cleary, 1997), but have since been observed in maize (Gallagher and Smith, 1999; Panteris et al., 2006, 2007). The asymmetric SMC division is preceded by migration of the premitotic nucleus to a position adjacent to the flanking GMC, and concomitant formation of a dense patch of cortical F-actin at the site where the polarized nucleus contacts the plasma membrane (Fig. 7). Subsequently, an asymmetric PPB forms in the cell cortex, as usual predicting the future, asymmetric division plane. Nuclear polarization in premitotic SMCs requires F-actin, and is thought to involve cytoplasmic F-actin cables that link the migrating nucleus to the plasma membrane (Kennard and Cleary, 1997; Panteris et al., 2006). Once it arrives at the actin patch site, the nucleus becomes physically attached there, as indicated by centrifugation studies performed on wheat SMCs (Pickett-Heaps, 1969). The cortical F-actin patch may anchor the polarized nucleus (e.g., Gallagher and Smith, 1999), but recent work suggests that an unusual microtubule array found in prophase SMCs of

maize may also contribute. Panteris and colleagues have described a "monopolar half spindle," consisting of a cone-shaped array of microtubules linking one side of the prophase nucleus (the side opposite the actin patch) to the PPB (Fig. 7; Panteris et al., 2006). Drug studies support the conclusion that this structure helps to anchor the polarized nucleus during prophase (Panteris et al., 2006). Upon entry into mitosis, the PPB is disassembled and a bipolar mitotic spindle forms. Following nuclear division, a phragmoplast forms, subsequently wrapping itself around the daughter nucleus in contact with the F-actin patch to form a subsidiary cell adjacent to the GMC (Fig. 7). The spindle and the early phragmoplast are continuously linked to the actin patch via actin cables, which are thought to help maintain the asymmetric location of the dividing nucleus (Gallagher and Smith, 1999).

Isolation of mutants disrupting various features of the asymmetric SMC division has identified a few genes that contribute to this process. The *brick1* (*brk1*) gene of maize is required for actin patch formation and proper premitotic polarization of SMCs, and encodes a protein that promotes F-actin polymerization through the WAVE/SCAR-ARP2/3 complex pathway (Frank and Smith, 2002; Smith and Oppenheimer, 2005). *brk2* and *brk3* mutants have the same phenotypes as *brk1* mutants (Frank et al., 2003). The molecular identities of *brk2* and *brk3* are not yet known, but these genes act in the same pathway as *brk1*, and are likely to encode subunits of the WAVE/SCAR or ARP2/3 complex (Frank et al., 2003). Similar to the *brk* genes, the *pangloss* genes (*pan1* and *pan2*) are required in SMCs for normal F-actin patch formation (Gallagher and Smith, 2000; Cartwright and Smith, unpublished). The identity of *pan2* is not yet known, but *pan1* encodes a leucine-rich repeat, receptor kinase-like protein that accumulates asymmetrically in SMCs at the site of the actin patch (Cartwright et al., unpublished). Thus, PAN1 may function in reception of a GMC-derived signal that polarizes the SMC in preparation for its asymmetric division. In contrast to *brk* and *pan* mutants, early steps in the asymmetric SMC division occur normally in *discordia* mutants (*dcd1*, *dcd2,* and *dcd3*), but a defect in phragmoplast expansion causes the SMC division to be misoriented in these mutants (Gallagher and Smith, 1999). The molecular identities of *dcd2* and *dcd3* remain unknown, but *dcd1* encodes a maize homolog of *Arabidopsis* TON2 (Wright and Smith, unpublished). TON2/DCD1 is related to B subunits of PP2A phosphatase complexes, and are thus likely to be involved in dephosphorylation of as yet unidentified substrates (Camilleri et al., 2002).

5 Cell Expansion

Cells spend a considerable portion of their time during the cell cycle in interphase. Many shared processes occur during this time for all organisms, including DNA replication, protein synthesis, regulation of cell cycling, etc. For plants, however, cell expansion is a significant and somewhat unique activity that occurs during interphase. During expansion, cell fates are manifest as plant cells begin to acquire shapes essential for specialized function. Also during this time, anisotropic

growth of individual cells contributes to the general anisotropic growth of tissues (Baskin, 2006). Cell expansion is mediated by interacting mechanical and biochemical factors, including turgor pressure and cell wall synthesis. To generate new cell walls, cellulose synthesis, and vesicle trafficking are processes that occur in distinct cellular compartments and require guidance by the cytoskeleton. Initial cell expansion is also closely followed by the specialized patterns of cell morphogenesis and both processes require coordinated wall growth. In the remaining section, we discuss control of cell expansion in the maize leaf epidermis and consider the mechanisms of compartmentalizing and orienting wall deposition in expanding cells.

5.1 Cell Wall Synthesis

During cell expansion, cellulose is synthesized at the plasma membrane by a complex of at least 36 related proteins that comprise cellulose synthase(CeSA), as identified in *Arabidopsis* through mutational and functional study (Somerville, 2006). Approximately 12 CeSA genes have been identified from maize to date (Appenzeller et al., 2004), with more on the horizon as the sequence of the maize genome emerges. The cellulose synthase complex is highly conserved and serves to catalyze glucan polymerization, forming the cellulose molecule. The complex also facilitates hydrogen bonding of adjacent glucose residues into the final microfibril by an as yet unknown mechanism (Somerville, 2006). Several hundred other proteins contribute to the matrix, a mix of proteins, glycoproteins and polysaccharides in the wall that hold the cellulose microfibrils in place and also facilitates wall loosening and signal transduction (Yong et al., 2005). Most of these wall matrix compounds are synthesized in the cytoplasm and are sorted, then trafficked and secreted to the wall space. For example, in maize, β glucans are characteristic of expanding coleoptile walls and have been shown to integrate into cellulose microfibrils (Carpita et al., 2001). These unbranched glucans are synthesized then packaged into Golgi-derived vesicles, trafficked to the plasma membrane where the contents are secreted to intercalate and possibly knit together nascent microfibrils (Urbanowicz et al., 2004). Clarifying how synthesis and vesicle trafficking couple to growth signals is of considerable interest to understand how expansion is integrated into growth of the whole organism.

5.2 Cytoskeleton and Cell Expansion

The cytoskeleton is necessary for successful cell expansion. It has long been known that pharmacological disruption of the microtubule cytoskeleton causes cells to expand more isotropically (equal in all dimensions) than normal. More recently,

mutations in genes encoding tubulin or microtubule-binding proteins have been shown to alter cell growth patterns as well (Smith and Oppenheimer, 2005). These observations have established that microtubules play an important role in control of cell growth pattern. In postmitotic, expanding cells, microtubules are confined to the cell cortex where they tend to be aligned in an orientation perpendicular to the major axis of cell expansion (Cyr, 1994; Sylvester, 2000). Cortical microtubule patterns typically mirror those of cellulose microfibrils, which are deposited into the cell wall by cellulose synthase complexes residing in the plasma membrane (Somerville, 2006). Although few studies on the role of microtubules in cell growth have focused on maize leaves, transverse alignment of cortical microtubules in elongating maize leaf cells has been documented (Hogetsu, 1989). Work dating back to the early 1960s has led to the hypothesis that cortical microtubules guide the deposition of cellulose, and that cellulose patterns constrain cell expansion under the force of turgor pressure, favoring growth in an orientation perpendicular to that of cellulose alignment (Green, 1984; Paredez et al., 2006a). Although some observations contradict this hypothesis, recent observation of a YFP-tagged cellulose synthase complex subunit tracking along cortical microtubules in living, expanding cells provides compelling evidence of a direct role for microtubules in guiding the deposition of cellulose into the wall (Paredez et al., 2006b). Mechanisms governing the microtubule-cellulose synthase interaction remain to be elucidated.

F-actin also plays an important role in plant cell expansion. Studies involving gross pharmacological and genetic perturbation of actin filaments suggest that actin promotes cell growth. However, more subtle perturbations of the actin cytoskeleton reveal roles for actin in patterning of cell growth as well (Smith and Oppenheimer, 2005). Unlike microtubules, actin filaments are found both in the cytoplasm of expanding cells and in the cortex, where coalignment with microtubules can sometimes be observed (e.g., Blancaflor, 2000), but alignment of cortical F-actin is generally less pronounced than that of microtubules in expanding cells. While the role of F-actin in promotion and patterning of cell growth is poorly understood, a leading hypothesis is that F-actin functions in part by guiding vesicle delivery to and/or fusion at the cell surface, thereby influencing the deposition of secreted wall components (Smith and Oppenheimer, 2005).

5.3 Case Study: Vesicle Trafficking in Warty

Normal vesicle trafficking is essential for coordinated cell growth. Recent studies show that mutants that lack normal trafficking can have altered cell patterns in the leaf, as observed clearly in the leaf epidermis (Sylvester, 2000; Zhang et al., 2007). The *warty1* (*wty1)* mutant was found to cause disordered cell expansion in the maize blade producing a rough, wart-like texture to the leaf and abnormal cell sizes (Fig. 4f; Reynolds et al., 1998). The gene encodes RAB2A (Sylvester et al., unpublished), a highly conserved small GTPase involved in trafficking vesicles primarily between nuclear-associated ER and the *cis* Golgi face in mammalian systems,

yeast, and some dicots (Zhang et al., 2007). RABs insert into specific endomembranes and are activated by GTP binding to interact with diverse protein effectors: the activated complex in turn facilitates vesicle sorting and membrane cycling (Vernoud et al., 2003). RABs have been identified in other grasses in addition to maize, and functional studies are underway (Zhang et al., 2006). Evidence for wall directed vesicle transport in live cell imaging of YFP-tagged RAB2A in maize suggests RAB2 is not restricted to the perinuclear membrane compartment, as predicted from RAB2 localization in mammalian and yeast systems (Sylvester et al., unpublished). These results suggest that the protein, despite highly conserved identity with other eukaryotic RAB2 proteins, may have acquired new subcellular transport functions, but further work is needed to confirm this hypothesis.

5.4 Case Study: Generation of Lobed Cell Shapes in Brick

Recent study has begun to clarify how the lobed shapes of epidermal and mesophyll cells are generated in various plant species including maize. Lobing is an almost universal feature of unspecialized leaf epidermal cell ("intercostal" or "pavement cell") shape. In dicots, pavement cell lobes tend to be large and irregular, with adjacent cells interlocking to produce a jigsaw puzzle-like cellular pattern. In grasses such as maize, pavement cells are rectangular in their overall shape but bear narrow, finger-like projections on their long sides, as described earlier (Fig. 1e). The projections from neighboring cells interdigitate to form a zipper-like interface. The functional significance of epidermal cell lobes is not well-established, but they may serve to enhance intercellular adhesion via physical interlocking of cells and/or by increasing the area of intercellular contact. In developing maize leaves, lobes begin to emerge at approximately the same time that stomatal divisions are concluding and grow out steadily as cells simultaneously enlarge their surface areas three to fourfold through postmitotic expansion (Frank and Smith, 2002). Mesophyll cells in maize are also lobed, as in many other species, but mesophyll lobes are more rounded and do not interdigitate, thereby creating air spaces within the mesophyll. This is believed to facilitate photosynthesis by increasing the surface area available for gas exchange.

Cytoskeletal rearrangements associated with leaf cell lobe formation have been studied in a wide variety of plant species including maize. Initial studies focused mainly on microtubules, revealing that lobe formation in both epidermal and mesophyll cells is preceded by the rearrangement of cortical microtubles into bands, which are associated with the formation of local, cellulosic wall thickenings (for maize see Apostolakos et al., 1991; Frank and Smith, 2002; Frank et al., 2003). Lobes subsequently emerge between these microtubule bands/wall thickenings (Fig. 8). This observation has led to the hypothesis that periodic, microtubule-dependent wall reinforcements locally constrain cell expansion so that growth occurs mainly in between these reinforcements to produce lobes (Panteris et al., 1994; Panteris and Galatis, 2005). Evidence of a role for microtubules in generating

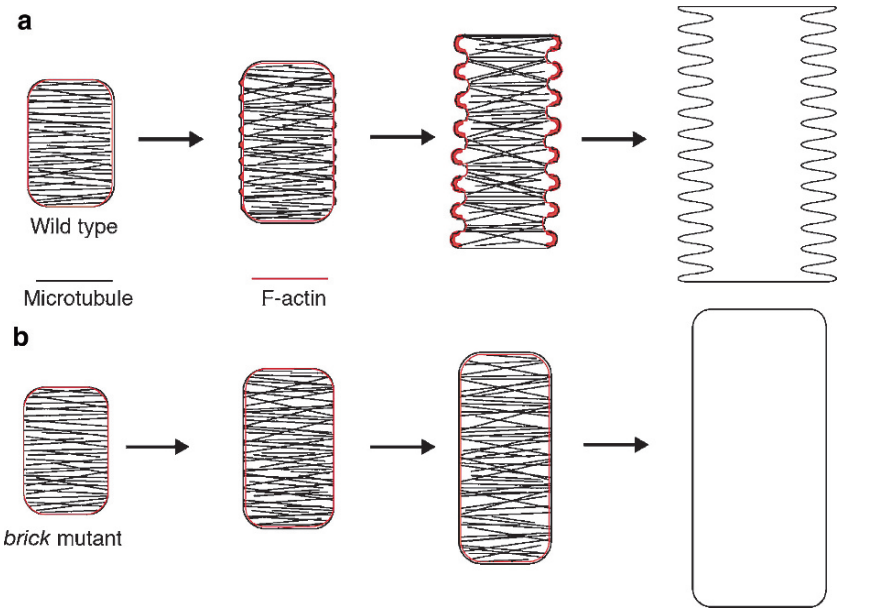

Fig. 8 Schematic illustration of epidermal pavement cell expansion in wild type (**a**) and brick mutants (**b**) of maize illustrating microtubules (black) and cortical F-actin (red). Reproduced from Smith (2003)

lobed cell shapes in the *Arabidopsis* epidermis has come from analysis of mutations disrupting microtubule-interacting proteins that reduce or abolish lobe outgrowth (e.g., Burk et al., 2001; Fu et al., 2005).

An important role for F-actin in epidermal lobe formation in maize and *Arabidopsis* has also been established recently through molecular genetic studies. In both *Arabidopsis* and maize, cortical F-actin is locally enriched at sites of lobe emergence, although cortical F-actin enrichment is more pronounced and more tightly associated with lobe formation in maize than it is in *Arabidopsis* (Fig. 8; Fu et al., 2002; Frank and Smith, 2002; Frank et al., 2003). In maize *brick* (*brk*) mutants (*brk1*, *brk2,* and *brk3*), which were mentioned earlier in connection with their stomatal division defects, epidermal cells expand to achieve a normal overall size and rectangular shape, but completely lack lobes. Interestingly, in all three *brk* mutants, the cortical microtubule bands normally associated with lobe formation are still present, but local F-actin enrichments are completely absent (Fig. 8; Frank and Smith, 2002; Frank et al., 2003).

Homologs of all subunits of the WAVE/SCAR and ARP2/3 complexes have been identified in *Arabidopsis*. Mutations disrupting several of these proteins (reviewed in Smith and Oppenheimer, 2005) including *Arabidopsis* BRK1 (Djakovic et al., 2006) have shown that the WAVE/SCAR and ARP2/3 complexes are required for normal trichome (epidermal hair) morphogenesis in *Arabidopsis* and also promote epidermal lobe outgrowth, although they are not absolutely

required for lobe outgrowth or for the formation of localized cortical F-actin enrichments in expanding pavement cells as in BRK1 in maize. Thus, analysis of maize *brk* mutants strongly supports the conclusion that local cortical F-actin polymerization is essential for lobe outgrowth in maize. Interestingly, *brk* mutations have no obvious effect on the lobing of mesophyll cells (Frank and Smith, 2002), suggesting that lobe formation in maize epidermal cells utilizes an actin-dependent mechanisms that is not essential for normal shaping of mesophyll cells. Further work will be needed to understand how local cortical F-actin enrichments promote lobe outgrowth in epidermal cells. Another interesting question to address will be what determines the sites at which microtubule bands and local F-actin enrichments form and how these sites are coordinated in neighboring epidermal cells to produce complementary lobing patterns.

6 Future Prospects: Emerging Tools and Analytical Methods

Cytokinesis, cell expansion, intracellular compartmentation, control of vesicle trafficking, and cytoskeletal dynamics are among the many cell biological processes that can be best studied in developing maize leaves. Changes in cell dimensions reflect significant rearrangement of cytoplasmic contents during cell wall deposition, membrane growth, and vacuole enlargement – all processes that are anisotropic and controlled together. Subcellular compartments change significantly as vacuoles enlarge to fill the expanding cell volume. Since cellulose synthesis and wall matrix synthesis is spatially segregated, vesicle secretion must be coordinated with new wall formation and cell growth, necessitating regulated vesicle trafficking. Coordinating these growth events are cytoskeletal arrays, including microtubules and actin filaments, which direct and help compartmentalize function during the cell cycle. All these processes can be ideally studied within the simple cellular architecture of the maize leaf.

A full toolbox for cell biological investigation of maize is on the horizon, as the first draft sequence of the maize B73 genome is completed and annotated. As genomic resources increase, the well-established genetic resources of maize can be even more fully exploited. New whole genome sequences also tend to activate technological advances for studying gene and protein function: tools for cell biological study are now developing rapidly. To address questions of the control of spatial patterning and intracellular compartmentation, live cell imaging of protein behavior is informative and in same cases essential to detect subtle protein activities lost to fixation or to experimental procedures. Proteins tagged with visual or biochemical markers thus provide access to new tools for studying protein dynamics within individual cellular compartments (Mohanty et al., in press). Coupled with availability of mutants, these tagged lines will be able to further our understanding of the controls of patterning in the unique cellular environment of the maize leaf.

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Light Signal Transduction Networks in Maize

Patrice G. Dubois and Thomas P. Brutnell

Abstract Light signal transduction networks integrate environmental signals with endogenous developmental programs. Several photoreceptors, including phytochromes, cryptochromes, and phototropins as well as some of their signaling partners have been characterized in higher plants. Recent studies in maize have revealed the importance of phytochromes in the regulation of several agronomically important traits, indicating that the manipulation of light response may prove fruitful in enhancing maize yields. However, little is known of the molecular components of light signal transduction pathways in maize, making it difficult to dissect the pathway using reverse genetic or association mapping techniques. Here, we summarize our current understanding of light response in maize and discuss strategies for enhancing agronomic performance through the manipulation of light signal transduction pathways.

1 Introduction

In the absence of light, a germinating seedling undergoes a period of skotomorphogenic development fueled by its seed reserves. Shoot and root growth are guided primarily by gravity (gravitropism) and touch (thigmatropism). The shoot apex, enclosed in its protective coleoptile, is pushed toward the soil surface by the elongation of the mesocotyl. Nearing the soil surface, the seedling perceives light and the transition to photomorphogenesis begins (Smith, 1982). In the model dicot *Arabidopsis thaliana*, this developmental switch is associated with global transcriptional change (Jiao et al., 2005; Parks et al., 2001; Tepperman et al., 2001) that is mediated through the relocalization (Jiao et al., 2007; Kircher et al., 1999; Sakamoto and Nagatani, 1996), phosphorylation (Al-Sady et al., 2006; Ryu et al., 2005) and degradation (Vierstra, 2003) of light response regulators. While it is likely that many of these early signaling events are conserved, few studies of light signal transduction have been conducted in maize. The morphological alterations observed in both monocots and dicots include a reduction of elongation growth, an enhancement of root growth and the activation of photosynthetic development (Fig. 1). Throughout its life cycle, the integration of exogenous light cues with endogenous genetic

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Fig. 1 Eight-day-old maize seedlings grown in darkness (D) or under greenhouse conditions (W). In the D (left), seedling tissues including the coleoptile and mesocotyl elongate and photosynthetic development is retarded (skotomorphogenesis). Under W (right), mesocotyl elongation is inhibited, leaves expand, and photosynthetic differentiation is initiated (photomorphogenesis). Significant variations in both skotomorphogenic and photomorphogenic development are often observed among inbred lines of maize. (**a**) B73 and (**b**) W22 inbred seedlings

programs allows the maize plant to constantly optimize its developmental program in response to environmental change (Quail et al., 1995; Smith, 1995).

Plants use multiple mechanisms to sense and respond to their light environment. The fluence (measured in μmol of photons m^{-2}), fluence rate (also called irradiance, measured in μmol m^{-2} sec^{-1}), spectral quality (measured in wavelength, nm), periodicity, and direction of radiation are all monitored (Bjorn and Vogelmann, 1994). Wavelengths perceived by plants are typically classified into UV-B (280–320 nm), UV-A (320–380 nm), blue (B, 380–495 nm), green (G, 495–570 nm), yellow/orange (570–620 nm), red (R, 620–690 nm), and far-red (FR, 690–800 nm). The photosynthetically active radiation (PAR) encompasses a spectrum that can be used for photosynthesis and is generally defined as wavelengths between 400 and 700 nm (white light, W). Chlorophylls have absorption maxima in the B (430–455 nm) and in the R (640–660 nm) and carotenoids absorb light primarily in the B (400–500 nm). Much of the G, and most of the FR are either transmitted through green tissues or reflected by the plant, and thus have the potential to serve as cues in sensing the vegetative environment (Fig. 2; Folta and Maruhnich, 2007; Halliday and Fankhauser, 2003).

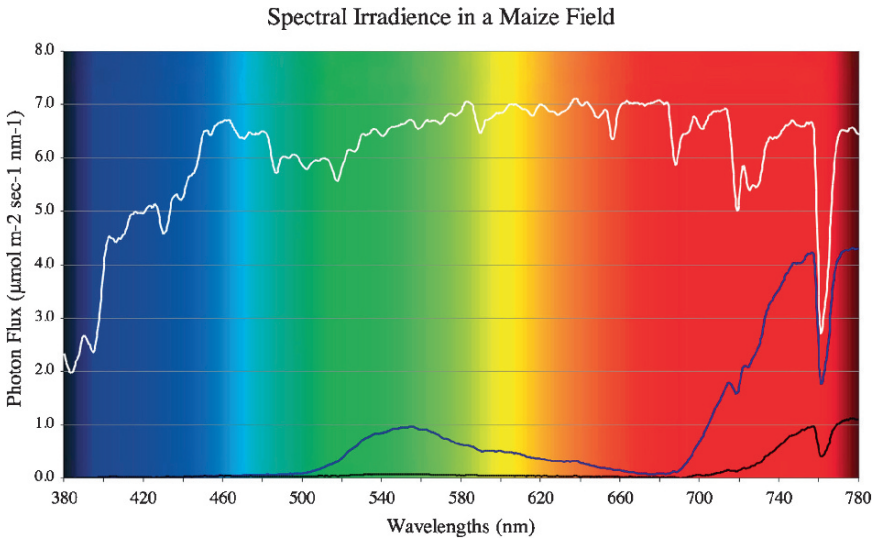

Fig. 2 Three spectroradiometer readings taken in a maize field plot (Emerson Garden, Cornell University, 09/08/2006). The white trace shows the light spectrum recorded above the maize canopy on a sunny day. The light transmitted through a maize leaf (blue trace) is enriched in G and FR regions of the spectrum. The black trace is the light spectrum at ground level under the maize canopy (row spacing of 18 in). A spectral shift cause the ratio of R to FR (R:FR) to be reduced from ~1.22 above the canopy to ~0.08 at ground level. The drop in irradiance around 760 nm is due to the absorption of these wavelengths by atmospheric water vapor

Although early studies of photomorphogenesis, most notably by Charles Darwin, focused on monocot grass seedlings such as maize and oat (Whippo and Hangarter, 2006), the vast majority of our current molecular understanding of light responses has come from studies of Arabidopsis. Through molecular and genetic characterizations, several photoreceptors and downstream components of light response networks have been defined (Christie, 2007; Jiao et al., 2007; Mathews, 2006). These studies have also begun to reveal the complex interplay between light, hormonal signaling pathways (Vandenbussche et al., 2003) and the circadian clock (Salter et al., 2003). Despite our detailed understanding of these networks in this dicot, there are many reasons to revisit species such as maize and related grasses. Comparisons of light signal transduction networks between evolutionarily distant monocot and dicot species has provided insight into the function of the photoreceptor gene family members (Childs et al., 1997; Izawa et al., 2000; Sawers et al., 2002; Sheehan et al., 2007; Takano et al., 2001, 2005). In addition, a characterization of these networks in crop species will provide potential targets for agronomic improvement (Kebrom and Brutnell, 2007; Sawers et al., 2005). The goal of this chapter is to summarize our current understanding of light signaling in maize and related monocots and to discuss how the manipulation of these pathways can be integrated into yield-enhancing breeding strategies.

2 Red/Far-Red Signaling in Maize

Phytochromes are R/FR photoreversible chromoproteins composed of two apoprotein monomers, each bound to a tetrapyrrole chromophore synthesized from heme in the plastid (Mathews and Sharrock, 1997; Terry, 1997). Phylogenetic analysis indicates that three major phytochrome lineages (*PHYA*, *PHYB*, and *PHYC*) are present in all angiosperms (Mathews and Donoghue, 1999). In Arabidopsis, the family has expanded to five members: *PHYA*, *PHYB*, *PHYC*, *PHYD*, and *PHYE* (Clack et al., 1994). In monocot grasses such as rice and sorghum, only three members are present: *PHYA*, *PHYB*, and *PHYC* (Goff et al., 2002; Mathews and Sharrock, 1996, 1997). The absence of *PHYD* and *PHYE* from the monocots (Mathews, 2006), suggests that the expansion of the phytochrome gene family has been limited in the monocots. It is also possible that *PHYE* was lost soon after the divergence of monocot and dicot lineages. This divergence in phytochrome gene family structure has likely been accompanied by variation in downstream components. Indeed, studies of phytochrome signaling in maize, rice and sorghum have revealed significant divergence in both photoreceptor function and downstream response (Childs et al., 1997; Sheehan et al., 2007; Takano et al., 2001, 2005).

Phytochrome apoproteins are synthesized in the inactive Pr form (absorption peak at 668 nm for oat PHYA) and autocatalytically assemble with a linear tetrapyrrole chromophore (phytochromobilin (PΦB) in the case of plant phytochromes) in the cytosol (Lagarias et al. 1987; Lagarias and Lagarias, 1989; Terry, 1997; Terry et al., 1993). The chromophore prosthetic group is covalently bound to a conserved cysteine residue found in all plant phytochrome apoproteins in the N-terminal region via a thioester linkage. The chromophore and the chromophore binding cysteine residue are not conserved in the bacterial phytochromes. However, a three dimensional X-ray crystal structure for the N terminal region of a bacterial phytochrome (Wagner et al., 2007) revealed a chromophore binding pocket that is likely highly conserved between plant and bacterial phytochromes. Interestingly, the chromophore is in close association with several residues of the apoprotein backbone and suggests a mechanism for phytochrome activation. The photoconversion from Pr to Pfr by R results in photoisomerization of the C15-C16 double bond followed by a series of light-independent chromophore-protein relaxation steps (Andel et al., 1996; Rudiger et al., 1983). The conformational changes induced by the photoconversion to the Pfr form (absorption peak at 730nm for oat PHYA) allows its translocation into the nucleus where it interacts with downstream components of the pathway. Since Pr and Pfr absorption bands overlap, it is not possible to fully convert one form into another. The proportion of Pr and Pfr is modulated by the antagonistic effect of R and FR (Lagarias et al. 1987; Jiao et al., 2007).

The apoprotein is composed of two separable domains joined by a protease-sensitive hinge region: a 60–70 kDa N-terminal photosensory region and 55 kDa C-terminal regulatory region (Fig. 3; Rockwell et al., 2006). The N-terminus contains a chromophore binding GAF domain (Fischer et al., 2005; Wagner et al., 2005) and a Pfr-stabilizing PHY domain. The C-terminus contains PAS repeats that are necessary for nuclear import (Chen et al., 2004) and a histidine kinase-related domain

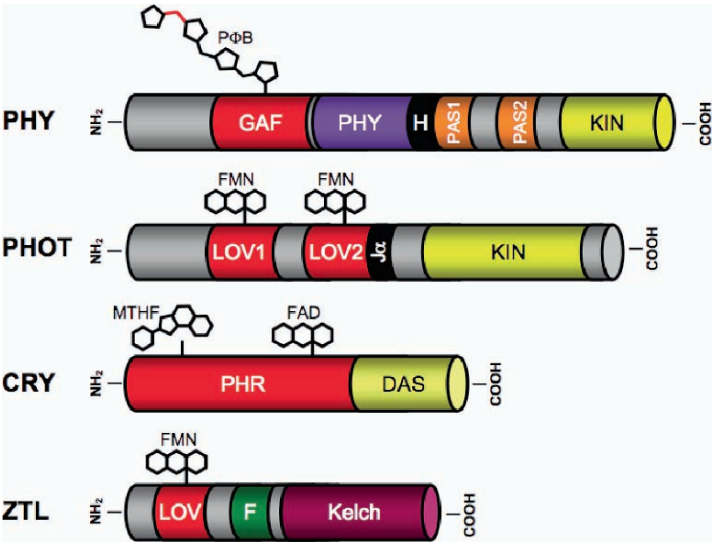

Fig. 3 Schematic representation of characterized photoreceptors. PHY: The holoprotein is composed of a chromophore-attachment region (GAF) where an isomerization of PΦB at C15 (in red) allows the conversion between Pr and Pfr forms, a PHY domain (Montgomery and Lagarias, 2002), a hinge region (H), a nuclear localization region comprised of two PAS domains involved in the phytochrome dimerization and protein interaction, and a kinase domain involved in autophosphorylation (KIN) (Rockwell et al., 2006; Sheehan et al., 2004). PHOT: Phototropin apoprotein domains includes: LOV1 and LOV2 (Light, Oxygen, or Voltage) sensing domains and their FMN chromophore attachment regions, a conserved α-helix domain (Jα), and a kinase domain (KIN) required for autophosphorylation in response to B (Christie, 2007). CRY: The cryptochrome apoprotein domains include: a photolyase homology region (PHR) where both MTHF (methenyltetrahydrofolate) and FAD (flavin adenine dinucleotide) cofactors are bound, and a DQXVP-acidic-STAES conserved motif (DAS) (Klar et al., 2007; Lin, 2002). ZTL: The recently discovered ZTL/ADO family have three major domains: a B/UV-A sensing LOV domain, an F-box for the degradation of protein targets, and a C-terminal Kelch domain involved in protein-protein interaction (Banerjee and Batschauer, 2005)

required for autophosphorylation of the holoenzyme following light activation (Yeh and Lagarias, 1998). The N-terminal domain is sufficient for activity in Arabidopsis when dimerized and translocated to the nucleus, suggesting that the C-terminus largely functions to attenuate phytochrome responses (Matsushita et al., 2003).

Phytochromes mediate responses to the intensity and duration of light through three distinct modes of action (Mancinelli, 1994). Plants respond to both the fluence, measured as the total light exposure, regardless of the time or intensity of light and fluence rate, defined as the flux of electrons that contact a surface. These responses include very low fluence responses (VLFR) where physiological effects can be achieved with fluences as low as 0.0001 μmol m^{-2}, low fluence responses (LFR) requiring fluences ranging from 1 up to 1000 μmol m^{-2} and high irradiance responses (HIR) requiring prolonged or continuous exposure at high fluence rates. Both VLFRs and LFRs obey the law of reciprocity where the magnitude of the response is a function of both the fluence rate and the time of exposure. Only LFRs

display the "classic" R/FR photoreversibility by which phytochrome was originally discovered (Borthwick et al., 1952). HIRs are proportional to both the fluence rate and the duration of irradiation. The same physiological response may be mediated by phytochromes acting in more than one response mode. For instance, seed germination in Arabidopsis is mediated both by phyA acting in the VLFR mode and by phyB acting in the LFR mode (Shinomura et al., 1996).

2.1 Maize Phytochrome Apoprotein Family

In maize, an ancient allopolyploidization event expanded the phytochrome family number to six: *PhyA1, PhyA2, PhyB1, PhyB2, PhyC1,* and *PhyC2* (Sheehan et al., 2004). *PhyA1*, *PhyB1*, and *PhyC1* are located on chromosome 1 and likely derived from one ancestral genome whereas *PhyA2*, *PhyC2* (chromosome 5) and *PhyB2* (chromosome 9) are located in syntenic regions on homeologous chromosomes and thus likely derived from the other ancestral genome. All six maize genes are predicted to encode functional apoproteins and are actively transcribed (Sheehan et al., 2004). Transcripts for *PhyA*, *PhyB* and *PhyC* accumulate to higher levels in many seedling tissues of plants grown in the D relative to W. In the D, transcripts of *PhyA* are the most abundant, suggesting that PHYA may predominate in mediating the transition from D- to W-growth whereas all three phytochromes may contribute to light responses. Highly similar expression patterns for *PhyA* homeologs indicate that the encoded products are largely redundant in function, whereas transcripts from *PhyB1* and *PhyC1* are more abundant over their respective homeologs in all seedling tissues examined (Sheehan et al., 2004).

2.2 elm1, a Chromophore-Deficient Mutant

A series of elegant phenotypic screens using EMS-mutagenized Arabidopsis populations allowed the identification of the first phytochrome mutants based on their etiolated development under W (Koornneef et al., 1980). A similar approach using transposon-mutagenized populations was used to identify the first photomorphogenic mutant of maize. The *elm1* (*elongated mesocotyl 1*) mutant was initially identified in a sand bench screen as a pale green plant with an elongated mesocotyl (Sawers et al., 2002). The mutants fail to accumulate spectrophotometrically detectable pools of phytochrome due to a block in chromophore biosynthesis. Cloning of *Elm1* revealed that it encodes an enzyme with phytochromobilin synthase activity, the last enzymatic step in the chromophore biosynthetic pathway. The lesion in *elm1* was mapped to a single base pair substitution at the 3' splice junction of intron III (Sawers et al., 2004). This lesion results in a greatly reduced pool of in-frame transcripts capable of directing the synthesis of a full-length phytochromobilin synthase protein.

As all phytochrome apoproteins likely bind the same chromophore (Terry, 1997), characterization of the *elm1* mutant has provided an opportunity to examine the role

of phytochromes in seedling and mature plant development (Sawers et al., 2002). Mesocotyl lengths of R- and FR-grown *elm1* plants were not significantly different from D-grown plants, indicating that phytochromes are essential for the suppression of mesocotyl elongation under R and FR. Chlorophyll and carotenoid content are also reduced in *elm1* mutants relative to wild-type (WT) seedlings. Characterization of photosynthetic gene expression in *elm1* mutants further defined a role for phytochromes in regulating both nuclear (*Cab* and *RbcS*) and plastid (*psbA* and *rbcL*) gene expression under R. However, under W *rbcL* and *psbA* expression was similar in WT and *elm1*, suggesting that B photoreceptors can largely compensate for a loss in phytochrome function in directing plastid transcript accumulation. At maturity, *elm1* plants are taller than their isogenic WT siblings, slightly pale green, often lodge and flower early (Markelz et al., 2003; Sawers et al., 2002). These phenotypes are consistent with reduced light responses that persist throughout development.

Unfortunately, the interpretation of *elm1* mutant phenotypes is problematic due to the residual accumulation of full-length *Elm1* transcripts. It is likely that low levels of phytochromobilin accumulate in mutant tissues that are below the limits of detection for spectrophotometric assays (Sawers et al., 2004). Thus, some active phytochrome pools may allow for a limited response to R and FR. For instance, *Cab* and *RbcS* gene expression increases slightly when *elm1* mutants are exposed to R, suggesting that phytochromes are functional (Sawers et al., 2002). When the light-dependent degradation of sucrose synthase (SUS) was examined in wild-type tissues, high levels of SUS were present in the D but decreased dramatically under R (Qiu et al., 2007). The degradation of SUS in light also occurred in the *elm1* mutant (Steve Huber, personal communication), suggesting that phytochromes do not mediate the light-dependent degradation of SUS. Alternatively, the low levels of phytochrome that accumulate in *elm1* tissues may be sufficient to mediate degradation of SUS. Such caveats illustrate the challenges associated with the interpretation of any phenotype associated with a weak mutant allele.

2.3 Phytochrome Apoprotein Mutants

The first phytochrome mutant described in monocots was a *phyB* mutant of sorghum (ma_3^R; Childs et al., 1997). Reverse genetic screens have been successfully utilized in rice to identify mutants in each of the three genes encoding the phytochrome apoproteins (Jeong et al., 2007; Takano et al., 2001, 2005). To date, only *phyB1* and *phyB2* single and double mutants have been characterized in maize revealing both overlapping and non-redundant roles for the two PHYB homeologs (Sheehan et al., 2007). In seedling tissues, both *PhyB1* and *PhyB2* contribute to the degradation of *PhyA2* and the accumulation of *PhyC1* transcripts under W. In mature plants, both *PhyB1* and *PhyB2* contribute to the control of plant height, ear node height, stem diameter, and leaf sheath-internode ratio. However, mesocotyl elongation is regulated by *PhyB1* whereas *PhyB2* predominates in the regulation of flowering time. Thus, it appears that *PhyB* homeologs of maize have undergone

Table 1 Characterized phytochromes of maize, rice, and sorghum

PHY	Roles/Traits	References
Maize PHYB1	Plant and ear node height, culm diameter, leaf sheath and internode lengths in mature plants. Constant W (Wc), Rc, and Bc: mesocotyl elongation. *PhyA* and *Cab* transcript levels. Delays flowering under short day (SD) and long day (LD).	Sheehan et al., 2007
Maize PHYB2	Plant height, ear node height, culm diameter, leaf sheath and internode lengths in mature plants. *PhyA* and *Cab* transcript levels. Delays flowering under LD conditions.	Sheehan et al., 2007
Rice PHYA	FR-HIR: mesocotyl and internode elongation, crown root growth orientation. FR-VLFR and FR-HIR: coleoptile elongation. Rc control of leaf and internode length. Bc control of leaf angle. R-LFR- and FR-mediated *Lhcb* and *RbcS* transcript levels. Flowering time regulation under SD and LD conditions.	Takano et al., 2001, 2005; Xie et al., 2007
Rice PHYB	R-HIR: coleoptile and first leaf elongation. Brassinosteroid regulation of leaf angle and coleoptile elongation in seedlings. R-mediated *Lhcb* and *RbcS* transcript levels. Perception of night break. Mediates *Os*CRY2 degradation. R-LFR and B/FR reversible coleoptile growth inhibition. Flowering time regulation under SD and LD conditions.	Hirose et al., 2006; Ishikawa et al., 2005; Jeong et al., 2007; Takano et al., 2005; Xie et al., 2007
Rice PHYC	FR-HIR: coleoptile, 1st leaf and 2nd internode elongation. R- and FR-mediated *Lhcb* and *RbcS* transcript levels. Flowering time regulation under SD and LD conditions.	Takano et al., 2005
Sorghum PHYB	Seedling elongation. Plant height, height to the ligule, leaf sheath, and sheath/blade ratio in mature plants. Repress *SbTB1* (suppressor of axillary bud outgrowth). Regulation of circadian ethylene production. Delays flowering under SD and LD conditions.	Finlayson et al., 1998, 1999, and 2007; Kebrom et al., 2006; Pao and Morgan, 1986

some subfunctionalization but maintain many overlapping functions as well. Characterizations of the phytochrome mutants in monocots have revealed diverse roles for phytochromes in both seedling and mature plant growth and are summarized in Table 1.

3 Blue Light Signaling in Maize

Although several B light photoreceptors have been characterized in Arabidopsis (see Fig. 3), none have been molecularly characterized in maize. Nevertheless, several classic physiological studies have been performed in maize. One of the best

characterized B responses in plants is phototropic curvature (Fig. 4). Studies by Charles and Francis Darwin first showed that light is sensed by the tip of the grass coleoptile (Darwin, 1880). Further characterizations, including detailed kinetic measurements in maize, revealed that B was most effective in mediating this response and required the lateral transport of auxin (Iino, 1990). Although these studies have clearly shown a relationship between auxin synthesis and transport to phototropic curvature in maize, the molecular mechanisms have remained elusive. Recently, an auxin-induced K^+ channel has been implicated in mediating the differential growth of coleoptiles in response to B (Fuchs et al., 2003; Philippar et al., 1999). K^+ channels have also been implicated in the function of maize guard cells to regulate stomatal movement (Buchsenschutz et al., 2005), another phototropin-mediated response (Christie, 2007). A role for auxin in controlling elongation of stem tissues was revealed by the characterization of the semi-dwarf *brachytic 2* (*br2*) mutant. The *Br2* gene of maize encodes a P-glycoprotein required for light-dependent polar auxin transport (Multani et al., 2003). Interestingly, the sorghum ortholog of this gene appears to have been a target of selection for plant height in breeding programs. In Arabidopsis, mutations in closely related *br2* homologs result in the mislocalization of the auxin efflux carrier PIN1, increased lateral auxin transport and hypertropic bending (Noh et al., 2003). A characterization of phototropic-insensitive mutants of maize (Fig. 4), may help to further define components of this pathway (Baskin et al., 1999; Tobias Baskin, personal communication).

Progress on identifying additional components of B-mediated growth have been limited in maize. As shown in Fig. 3, a number of B photoreceptors have been

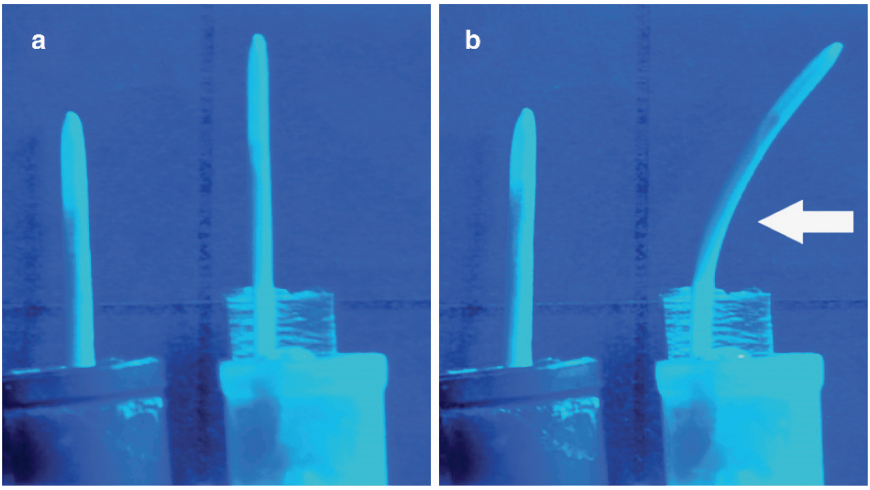

Fig. 4 The phototropic curvature of maize seedlings in response to unidirectional B. A non-responsive *blueless* mutant (left) and WT B73 inbred on the right (**a**) prior to and (**b**) after a 4 h B exposure

identified in Arabidopsis. Although a putative phototropin has been identified in maize (GenBank accession: AF033263), no mutations have been reported in this or other potential B photoreceptors.

4 Light Regulation of C4 Photosynthetic Development

Maize, like many semi-tropical and tropical grass species, utilizes C4 photosynthesis (Edwards and Walker, 1983) to efficiently capture CO_2 under hot, dry conditions (Sage and Monson, 1999). This specialized form of photosynthesis is achieved through the partitioning of photosynthetic activities between two morphologically and biochemically distinct cell types. Maize leaf blades display Kranz anatomy where files of photosynthetic bundle sheath (BS) cells surround the vasculature and are themselves surrounded by a layer of mesophyll (M) cells. As CO_2 diffuses into the leaf through the stomata, it is first fixed into a C4 sugar in M cells that diffuses to the BS. Once inside the BS plastid, the sugar is decarboxylated and the CO_2 fixed by Rubisco in the Calvin cycle. By sequestering Rubisco in the CO_2-rich environment of the BS, photorespiration is greatly reduced (Hatch, 1971; von Caemmerer and Furbank, 2003). Despite the increased energy required for generating this CO_2 gradient, C4 photosynthesis is still more efficient than C3 photosynthesis at high temperatures due to the enhanced oxygenase activity of Rubisco in C3 plants (Jordan and Ogren, 1984; Sage, 2004).

Decades of research have provided us with a detailed understanding of the biochemistry underlying C4 photosynthesis (Sage and Monson, 1999). However, our understanding of the networks mediating the differential expression of suites of genes and proteins is much less complete (Sawers et al., 2007). Light is one factor that regulates the abundance of many photosynthetic enzymes and initiates the cell-specific accumulation of transcripts (Langdale et al., 1988; Sheen and Bogorad, 1986, 1987, 1988). Cis-acting elements have been defined for a number of genes that appear to control the differential accumulation of photosynthetic transcripts in response to light (Sheen, 1999). For instance, the *Rbcs* gene contains both constitutive and light-responsive promoter elements, and sequences within the 5′ and 3′ UTRs that drive cell-specific expression (Bansal et al., 1992; Schaffner and Sheen, 1991). The induction of *Rbcs* in BS is dependent on a R signal, whereas repression of *RbcS* transcript accumulation in the M cells is dependent on a B signal (Purcell et al., 1995). Both 5′ and 3′ non-coding sequences appear to be required for light induction and repression of *Rbcs* transcript accumulation (Viret et al., 1994). Some trans-acting factors have also been identified that regulate cell-specific accumulation of photosynthetic enzymes, including a YY1-like suppressor of *Rbcs* accumulation (Xu et al., 2001) and a *Dof1* transcription factor that may regulate light-induced expression of PEPCase (Yanagisawa, 2000). UV-B also plays a role in the regulation of malic enzyme transcript (*Me1*) and protein accumulation, suggesting a role for C4 enzymes in the repair of UV-induced damage (Casati et al., 1999; Drincovich et al., 1998). Several mutants have been identified in genetic

screens that disrupt the cell-specific accumulation of photosynthetic enzymes (Brutnell et al., 1999; Hall et al., 1998a, 1998b; Roth et al., 1996). However, these mutations do not appear to alter cell fates, but rather a subset of photosynthetic enzymes or transcript accumulation patterns. Together, these studies suggest a complex interplay between light and developmental regulators during C4 differentiation.

To examine the contributions of phytochrome to C4 differentiation, transcripts encoding *Ppc*, *RbcS2* and *Me1* were examined in the *elm1* mutant (Markelz et al., 2003). In W, the transcript levels of each of these three genes accumulates to similar levels in the mutant tissues as in WT and the cell-specific patterns of expression are maintained. When plants are shifted from W to D, levels of *Ppc* and *RbcS2* decline more rapidly in the *elm1* background than in the WT. *Me1* transcript levels declined slowly over a 72 h period with a similar profile in both the *elm1* mutant and WT. When W-grown plants were shifted to B, R, or FR, the cell-specific patterns of gene expression were maintained in the *elm1* mutant. These results suggest a limited role for phytochromes in maintaining C4 photosynthetic gene expression under W, but imply a role for phytochromes in regulating the levels of some photosynthetic genes in the D (Markelz et al., 2003). As previously mentioned, low levels of chromophore likely accumulate in the *elm1* mutant, thus it is possible that small active pools of phytochrome are sufficient to induce a C4 photosynthetic state. With the recent identification of complete loss-of-function alleles of *phyB1* and *phyB2* (Sheehan et al., 2007), it should now be possible to directly address the role of *PhyB* in regulating C4 differentiation.

5 Light Regulation of Anthocyanin Biosynthesis

Flavonoid biosynthesis (including anthocyanins) is arguably one of the most well-characterized pathways in maize (Dooner et al., 1991). The structural genes encoding the biosynthetic enzymes and regulatory genes encoding transcription factors were some of the first genes cloned in plants (Cone et al., 1986; Kreuzaler et al., 1983; Paz-Ares et al., 1986). Thus, it is perhaps surprising how little is known of the molecular networks underlying light regulation of this pathway in maize. This is, in part, due to the lack of well-characterized light signaling mutants of maize until recently (Sawers et al., 2004; Sheehan et al., 2007). In addition, many of the early studies of anthocyanin accumulation were likely confounded by the segregation of regulatory elements that mediated both light-dependent and -independent accumulation of anthocyanins (Beggs and Wellman, 1985). Nevertheless, several studies have shown that light is necessary for the expression of both regulatory and structural genes in the pathway (Cone et al., 1993; Gavazzi et al., 1990; Petroni et al., 2000; Pilu et al., 2003; Procissi et al., 1997) as well as the subcellular localization of the anthocyanins (Grotewold et al., 1998).

The accumulation of anthocyanins upon UV-B exposure has been suggested to serve a photoprotective function (Casati and Walbot, 2003; Stapleton and Walbot, 1994). However, the photoreceptor(s) that mediate this response in maize have

remained elusive. A detailed analysis of the specific wavelengths necessary for the induction of regulatory and enzyme-encoding genes indicates that B and UV-B are most effective in mediating anthocyanin accumulation in young seedling tissues (Piazza et al., 2002). However, R and UV-A also contribute to the response. In endosperm tissues, both B and R contributed equally to accumulation of anthocyanins, though neither treatment on its own conditions as high a level of anthocyanins as W, suggesting a synergism between B and R signal transduction pathways (Piazza et al., 2002). These findings are consistent with observations that anthocyanin accumulation is greatly reduced in the aleurone of the *elm1* mutant (Thomas Brutnell, unpublished data), suggesting that both R- and B-induced accumulation of anthocyanins in the aleurone may require active phytochrome pools.

6 The Shade Avoidance Syndrome

Historical data on U.S. maize production shows an uninterrupted growth in average yields (see chapters by J. Holland and F. Troyer). In the 1930's grain yields averaged ~1,500 kg/ha whereas current grain yields are now greater than 10,000 kg/ha (Tollenaar and Wu, 1999; Troyer, 2006). One key factor that has contributed to these gains have been a steady increase in planting densities (O'Bryan et al., 2006; Troyer, 1996) while maintaining relatively constant per plant yields (Duvick, 1997).

The selective absorption of R and B by the chlorophyll results in a decrease in the ratio of R to FR (R:FR) of both transmitted and reflected light. This spectral shift is perceived by the plant as an indication of the presence of neighboring vegetation (Franklin and Whitelam, 2005; Smith, 2000). This proximity detection occurs before canopy closure, thus allowing the plant to anticipate a competitive threat and adjust its growth accordingly (Ballare, 1999; Ballare and Casal, 2000; Ballare et al., 1990). In response to both neighbor proximity (low R:FR) and vegetative shade (low R:FR and reduced PAR), many plants display a series of morphological changes referred to as shade avoidance responses or the shade avoidance syndrome (SAS; Smith, 1995).

In maize, the SAS is characterized by a decrease in chlorophyll content, an increase in plant height, a reduction in the number of tillers, thinner, longer and more erect leaf blades, an elongation of internodes and leaf sheaths and a reduction in root development (Andrieu et al., 2006; Kasperbauer and Karlen, 1994; Maddonni et al., 2001b). Upward reflected FR from low-lying weed vegetation can also impair maize development in its early stages. The resulting increase in leaf sheath extension is made at the expense of root development, suggesting that the reduction in yield due to weed competition is caused by the SAS rather than a limitation in water availability. These observations reiterate the importance of early season weed control to achieve optimal yield (Rajcan et al. 2004). Prolonged exposure to low R:FR also impairs reproductive development, causing an acceleration of flowering, a decrease in kernel number per plant and grain yield per plant (Borras et al., 2003; Hashemi et al., 2005; Maddonni and Otegui, 2006). The most

striking example of the SAS in a modern field setting can be seen at borders where higher R:FR and PAR affect plant growth (Fig. 5). This also demonstrates that a significant SAS is still operational in modern hybrids, despite breeding efforts that have likely attenuated many shade avoidance responses (Ballare and Casal, 2000; Maddonni et al., 2001a, 2002).

The molecular mechanisms underlying the SAS have been most intensively investigated in Arabidopsis (Franklin and Whitelam, 2005; Vandenbussche et al., 2005). Through the use of genetic screens (Cerdan and Chory, 2003), expression profiling (Devlin et al., 2003; Roig-Villanova et al., 2006; Salter et al., 2003) and physiological studies (Steindler et al., 1999), components of the SAS pathway are being defined. The low R:FR associated with canopy shade is transduced by the phytochromes through a modulation of the amount of active Pfr. In Arabidopsis, phyB predominates in mediating many of these responses but additional phytochromes are also involved (Devlin et al., 2003; Franklin et al., 2003; Smith and Whitelam, 1997).

In maize, much less is known of the molecular components of the SAS. The *elm1, phyB1* and *phyB2* single and double mutants each display several traits associated with shade response at maturity, including increased plant height and early flowering (Sawers et al., 2002; Sheehan et al., 2007), suggesting that PHYB may modulate many of the shade avoidance responses in maize. However, *PhyA* has also been implicated in mediating the SAS in sorghum (Finlayson et al., 2007). In Arabidopsis, a family of bHLH (basic helix-loop-helix) transcription factors have been defined that interact with phytochromes (Duek and Fankhauser, 2005; Monte et al., 2007). These phytochrome-interacting factors (PIF) and PIF-like factors

Fig. 5 Shorter plants can often be observed at the periphery of a maize field. This reduction in plant height is correlated with higher R:FR and PAR at the edges. Other factors including field topology and soil composition also influence plant stature (Upstate NY, 08/17/2007).

(PIL) act as both positive and negative regulators to mediate changes in gene transcription; (Huq et al., 2004; Huq and Quail, 2002; Kim et al., 2003; Martinez-Garcia et al., 2000; Oh et al., 2004, 2007; Shin et al., 2007), including responses to vegetative shade (Salter et al., 2003; Sessa et al., 2005). Recent studies in rice (Nakamura et al., 2007) and maize (Matthew Hudson, personal communication) suggest that a related class of proteins may also be functional in the monocots to mediate phytochrome responses. Other proteins that may mediate responses to vegetative shade belong to the homeodomain-leucine zipper (HD-zip) class and include ATHB-2 and ATHB-4 in Arabidopsis. These genes are regulated by R:FR and overexpression of ATHB-2 results in a phenotype that mimics the SAS (Carabelli et al., 1996; Steindler et al., 1999). Several plant hormones including auxins, gibberellins, brassinosteroids, and ethylene as well as some herbivore-induced phenolics have been defined as components of the SAS (Finlayson *et al.,*1999; Izaguirre et al., 2006; Morelli and Ruberti, 2000; Pierik et al., 2004; Reed et al., 1996; Vandenbussche et al., 2005).

7 Dissecting the Light Signal Transduction Networks

How do we further define the components of the light signal transduction pathways in maize? Several reverse genetic programs have recently been developed for maize (see chapters by D. McCarty and C. Weil). These programs will greatly facilitate targeted approaches that are based on our current understanding of light signal transduction in model plants. Another means to define components of light signaling networks is through exploitation of genetic diversity. Quantitative trait loci (QTL) analysis and genome-wide association mapping techniques both afford advantages to mapping quantitative variation (see chapters by R. Tuberosa and A. Rafalski). Mapping populations where elements of both bi-parental populations and association analysis are combined can overcome some of the limitations of both techniques, such as population structure (association mapping) and low resolution (QTL analysis) (Yu et al. 2008). A survey of light response in maize conducted with a panel of diverse maize germplasm revealed that North American temperate inbreds are less responsive than tropical and semi-tropical inbreds lines. This result suggests that light response may have been a target of selection (Markelz et al., 2003) and that QTL and genome wide association mapping techniques may prove fruitful in defining novel variation.

8 Manipulation of Light Signaling Pathways

Overexpression of photoreceptors has been widely used as a method to alter plant stature and yield (Hudson, 2007). However, these experiments have met with mixed success. In tobacco, overexpression of an oat *PhyA* using the CaMV-35

promoter resulted in a reallocation of resources to the leaf rather than the stem, thus increasing harvest index (Robson et al., 1996). It is likely that increased pool of active *PHYA* beyond the seedling stage enhanced FR-HIR and attenuated the *PhyB*-mediated SAS. A similar approach was taken to engineer wheat through overexpression of oat phyA using the maize ubiquitin promoter (Shlumukov et al., 2001). Although the seedlings displayed some enhanced response to FR, the effects on field grown plants have yet to be determined. In rice, two similar studies were conducted using the same transgenic construct. The light-regulated and tissue-specific *rbcS* promoter was used to ectopically express the Arabidopsis *PHYA* gene. In an *Indica* variety, *PHYA* overexpression increased grain yield from 6% to 20% in greenhouse-grown plants (Garg et al., 2006), but in a *Japonica* variety it reduced tiller number and overall grain yield (Kong et al., 2004). These studies suggest that perturbation of light signaling networks may have quite varied consequences depending on the genotype of the plant and its intrinsic light transduction networks. Thus, a large number of transgenic events across a broad germplasm collection may be necessary to fully explore the consequences of manipulating light signal transduction pathways.

An alternate approach for manipulating the SAS in maize can be the utilization of photo-insensitive phytochrome mutants. A single amino acid substitution in Arabidopsis *PHYB* (Y276H) results in constitutive photomorphogenic development in the dark, a light-independent activation of gene expression and a R/FR insensitivity (Su and Lagarias, 2007). As suggested by the authors, such constitutive activation of phytochrome response may have applications in engineering the architecture of crop plants. However, as mentioned above, modern maize hybrids have retained some response to vegetative shade that are likely beneficial under high density. For instance, in addition to increase hyponasty, relatively higher R:FR present between rows causes an azimuthal reorientation of leaves that limits physical interactions between adjacent plants and maximizes leaf area index (Maddonni et al., 2001a, 2002). These results suggest that some plasticity in the SAS may be beneficial for plants to achieve optimal yields under high density plantings (Pagano and Maddonni, 2007). Thus, the engineering of shade response may require a more fine-tuned approach, perhaps through the manipulation of downstream components of the networks.

9 Conclusion

Despite a detailed understanding in Arabidopsis, our knowledge of light signal transduction networks in maize remains limited. Through the isolation and characterization of photoreceptor mutants, roles for phytochrome in several agronomically important traits have emerged. With the availability of a complete genome sequence, reverse genetic tools and sophisticated mapping populations, it will soon be possible to functionally define many components of light signaling networks in maize. The potential for engineering plant architecture through the manipulation of

photoreceptors and downstream components suggests that a more detailed understanding of these pathways in maize could lead to the improvement of this important food, feed and fuel crop.

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Maize Disease Resistance

Peter J. Balint-Kurti and Gurmukh S. Johal

Abstract This chapter presents a selective view of maize disease resistance to fungal diseases, highlighting some aspects of the subject that are currently of significant interest or that we feel have been under-investigated. These include:

- The significant historical contributions to disease resistance genetics resulting from research in maize.
- The current state of knowledge of the genetics of resistance to significant diseases in maize.
- Systemic acquired resistance and induced systemic resistance in maize.
- The prospects for the future, particularly for transgenically-derived disease resistance and for the elucidation of quantitative disease resistance.
- The suitability of maize as a system for disease resistance studies.

1 Introduction

Worldwide losses in maize due to disease (not including insects or viruses) were estimated to be about 9% in 2001–3 (Oerke, 2005). This varied significantly by region with estimates of 4% in northern Europe and 14% in West Africa and South Asia (http://www.cabicompendium.org/cpc/economic.asp). Losses have tended to be effectively controlled in high-intensity agricultural systems where it has been economical to invest in resistant germplasm and (in some cases) pesticide applications. However, in areas like Southeast Asia, hot, humid conditions have favored disease development while economic constraints prevent the deployment of effective protective measures.

This chapter, rather than being a comprehensive overview of maize disease resistance, highlights some aspects of the subject that are currently of significant interest or that we feel have been under-investigated. We outline some major contributions to disease resistance genetics that have come out of studies in maize and discuss maize as a model system for disease resistance studies. We discuss primarily resistance to fungal diseases; resistance to insects and viral diseases are dealt with in Chaps. 11 and 12.

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2 Types of Disease Resistance

Genetic resistance in plants is often divided into two major classes; Qualitative, or major-gene, resistance, is based on single major-effect resistance genes (R genes) and generally provides race-specific, high-level resistance. Quantitative resistance typically has a multi-genic basis and generally provides non-race-specific intermediate levels of resistance. Genomic regions (or loci) responsible for quantitative effects are known as quantitative trait loci (QTL). Qualtitative resistance, often associated with a rapid cell death called a hypersensitive response (HR) around the point of pathogen ingress, is generally quickly overcome when deployed in the field, though there are exceptions (e.g., Steffenson, 1992). Quantitative resistance tends to be more durable (Parlevliet, 2002). Qualitative resistance is generally effective against biotrophic pathogens (pathogens that derive their nutrition from living host cells), while quantitative resistance is more often associated with resistance to necrotrophic pathogens (pathogens that derive nutrition from dead cells). The mechanisms of quantitative resistance have not been well characterized, but are likely to be variable depending on the specific interaction.

While qualitative resistance is extensively used in the other major grass crops, wheat and rice, only a few major resistance genes, such as the *Ht* genes for resistance to northern leaf blight (Welz and Geiger, 2000) and the *Rp* genes for resistance to common rust (Ramakrishna et al., 2002), have been used in maize breeding. The vast majority of genetic resistance used by maize breeders is quantitative. It is interesting to speculate why this contrast exists. The major factor might be that maize is substantially more genetically diverse than wheat or rice, probably because it alone is an outcrossing species (Buckler et al., 2001). Maize breeders therefore have more diversity available to them within adapted germplasm than their wheat or rice colleagues and consequently are better able to bring together multiple small-effect QTL to achieve effective levels of quantitative resistance. While diverse germplasm is available in wheat, it is often available only from exotic, unadapted sources. In order to maintain agronomic performance, the breeder must minimize the amount of exotic germplasm that is introduced into adapted lines. Therefore, in wheat, single genes of large effect have been much more attractive to breeders. Another factor might be that there simply are fewer commercially important biotrophic pathogens of maize than there are of wheat. It is not clear why this might be, but one can speculate that it is because effective quantitative resistance to these pathogens is available and widely utilized.

3 Seminal Disease Resistance Genetic Studies in Maize

As is made clear in this book, maize has been an important model species for many aspects of plant genetics. Disease resistance is no exception. Some key discoveries in the area of disease resistance genetics and resistance gene function that have been made in maize are listed below.

3.1 The First Cloning of a Susceptibility Gene to Pathogens

Maize carrying Texas cytoplasm for male sterility (cms-T) was widely used for hybrid seed production in the 1950s and 1960s. A new race (race T) of the southern corn leaf blight pathogen *Cochliobolus heterostrophus* emerged in 1969 that was very pathogenic on cms-T maize, causing disease epidemics in 1970 and 1971 (Ullstrup, 1972). It was found that race T produced a family of linear long chain polyketides, collectively called the T-toxin (Pring and Lonsdale, 1989) which binds specifically to URF13, a peptide of 13 kDa that resides in the inner membrane of mitochondria as an oligomer and acts as a ligand-gated channel (Levings, 1990; Levings and Siedow, 1992). Binding of T-toxin to URF13 transforms this channel into a large pore, causing the membrane to become leaky and lose function, somehow leading to cell death, and enhanced colonization of the maize tissue by the pathogen. The *T-urf13* gene, which apparently arose by chance in the mitochondrial genome of cms-T maize (Wise et al., 1999) encodes URF13.

URF13 was also exploited by *Phyllosticta maydis* (*Mycosphaerella zea-maydis*), previously unknown to cause disease in the United States. (Pring and Lonsdale, 1989). Like *C. heterostrophus*, *M. zea-maydis* produces a polyketide of the T-toxin family (PM-toxin) that functions as a specific determinant of virulence/disease of this pathogen on cms-T maize (Wise et al., 1999). So susceptibility to disease in both cases was due to specific interaction of a fungal metabolite with the product of a unique susceptibility gene in the host, and it was averted by simply avoiding the use of cms-T germplasm containing this gene.

In addition to uncovering the first plant gene for disease susceptibility, studies on this disease had additional implications. First, it is still the classic example of the perils of monoculture (Wise et al., 1999), reminding us to that whenever novel germplasm, derived either through traditional or biotechnological means, is widely grown, it can expose the crop to unpredictable risks. It also provided the first example of the involvement of mitochondria in plant disease and the first clear indication of the importance of mitochondrial integrity in cell death control in eukaryotes, an area of biology that exploded in the 1990s.

Although URF13 is also responsible for the male sterile phenotype of cms-T cytoplasm, the mechanistic basis for this remains unclear. A number of nuclear genes, called fertility restorers (Rf), have been identified that suppress the male sterile phenotype of cms-T (Wise et al., 1999). These Rfs, however, failed to restore resistance to pathogens. This is intriguing because some of these Rf factors lower the level of URF13 in mitochondria (Wise et al., 1999).

3.2 The First Cloning and Characterization of a Disease Resistance Gene

The *Hm1* gene (Ullstrup, 1941, 1944) confers specific resistance against a leaf blight and ear mold disease of corn, caused by *C. carbonum* race 1 (CCR1). The exceptional virulence of race 1 on susceptible *hm1* maize is due to production of a

host specific toxin, HC-toxin, a cyclic tetrapeptide (Ciuffetti et al., 1983; Kawai et al., 1983). *Hm1* was cloned by transposon tagging and was found to be an NADPH-dependent HC-toxin reductase, which reduces a key carbonyl group on HC-toxin, thereby inactivating it (Johal and Briggs, 1992; Meeley et al., 1992). The case of HC-toxin offers an interesting contrast to that of T-toxin. For HC-toxin, plant resistance is an active function, requiring the production by the plant of an enzyme to detoxify a fungal pathogenicity factor. Contrastingly, in the case of T-toxin, plant susceptibility is an active function, requiring production by the plant of a toxin binding protein.

3.3 The Genesis of a Plant Disease and a Grass-Lineage-Specific Disease Resistance Gene

Studies with *Hm1* and *Hm2* (a duplicate gene at an unlinked locus) have shown that resistance to CCR1 conferred by these genes is almost ubiquitously present in maize germplasm, (Nelson and Ullstrup, 1964; Zhang et al., 2002). Characterization of both *Hm1* and *Hm2* in susceptible maize inbreds revealed that the disruption of both of these disease resistance genes was required for the susceptibility of maize to CCR1 (Multani et al., 1998). All other cereals contain orthologs of *Hm1* as well as the HCTR activity responsible for the resistance function of this gene and have been considered non-hosts for CCR1 (Meeley and Walton, 1993; Multani et al., 1998). This suggested an ancient origin and recruitment of the *Hm1* disease resistance trait in the grass lineage. Barley leaves in which expression of the *Hm1* homologs is down-regulated by virus-induced gene silencing become susceptible to infection by *C. carbonum* race 1, but only if the pathogen is able to produce HC-toxin (Johal et al., unpublished). Phylogenetic analysis has shown that *Hm1* evolved early and exclusively in grasses, presumably in response to HC-toxin-mediated virulence of *C. carbonum* race 1. These studies provide a rare glimpse into an ancient host-pathogen struggle that occurred at the origin of the grasses. They also have implications for the survival and evolution of grasses; were it not for the *Hm1* gene, grass evolution might have proceeded quite differently.

3.4 First Indications of the Complex Nature and Function of R Genes

Together with the flax/flax rust system, genetic studies of the maize/maize common rust system, specifically at the *Rp1* locus, have elucidated a number of general genetic and mechanistic features of the R genes:

(1) Studies conducted on the *Rp1* locus established that it likely had a compound structure, containing tandemly duplicated genes of different specificities or paralogs (Hu and Hulbert, 1994; Richter et al., 1995; Sudupak et al., 1993).

This structure was thought to be a general feature of R gene loci which contributed to the genesis and death of R genes (Hulbert et al., 2001) and their complex and unstable nature. The instability of the *Rp1* locus was thought to stem largely from two mechanisms; unequal crossing over at the locus and gene conversion (Hulbert, 1997). These hypotheses were largely confirmed with the eventual cloning of the *Rp1* locus and other R gene loci in the 1990s.

(2) It was first shown in maize, again at the *Rp1* locus, that recombination within R gene loci can lead not only to new R gene specificities but also to aberrant R genes which malfunction and trigger the hypersensitive response (HR) spontaneously, resulting in so-called disease lesion mimics (Hu et al., 1996). This was also the first indication that an R gene could have two separate functions: pathogen recognition and the activation of the HR response (Pryor, 1987). Recently we have shown that the disease lesion mimic phenotype conferred by the aberrant R gene *Rp1-D21* is quite variable in genetically divergent backgrounds and have mapped some of the loci responsible for this variation (Johal and Balint-Kurti unpublished), which likely are important in the wild-type *Rp1*-mediated defense response.

(3) The maize *Rp1* gene was the first disease resistance gene ever to be tagged with a transposon (Bennetzen et al., 1988). Although instability at the *Rp1* locus frustrated attempts at cloning, this research did uncover an important aspect of the biology of the HR cell death underlying a resistance response; it manifests in a cell autonomous fashion (Bennetzen et al., 1988).

4 The Genetic Architecture of Disease Resistance in Maize

More than 50 major R genes have been cloned in plants (Martin et al., 2003). With some exceptions (e.g., Johal and Briggs, 1992; Multani et al., 1998; Romer et al., 2007; Xiao et al., 2001), most of these genes are dominant and share certain conserved domains such as a nucleotide binding site (NBS) and a leucine-rich repeat (LRR) region (Martin et al., 2003; McDowell and Simon, 2006). A set of genes known as "R gene analogs" (RGAs) have been defined that, while they have no demonstrated function in disease resistance, share these domains. By analyzing the publicly available genomic sequences, 585 RGAs have been defined in the rice cultivar Nipponbare (Monosi et al., 2004) and 149 in Arabidopsis (Meyers et al., 2003). In maize, 228 RGAs have been identified (Xiao et al., 2007) using partial sequence data derived from several different maize lines. Once the complete genome sequence of the standard maize line B73 is available, a more complete analysis will be possible. In all these species, RGAs were found to be located all over the genome, often clustering with groups of three or more RGAs mapping to the same locus, mirroring the clustering of plant R genes such as at the *Rp1* locus described above (Smith et al., 2004). Plant RGAs are both highly divergent and rapidly evolving (Ellis et al., 2000), this fact, together with the high level of genetic diversity found within maize (Buckler et al., 2006), suggests that a huge diversity of RGAs, and therefore a huge array of recognitional specificities, is likely available within maize germplasm.

In maize, one recessive major gene for resistance has been identified, namely *rhm1* which confers resistance to race O of *C. heterostrophus* (Chang and Peterson, 1995; Zaitlin et al., 1993). It is not clear whether *rhm1* also confers resistance to race T, but it has been shown that the resistance it confers is associated with relatively few changes in gene expression or protein levels (Simmons et al., 2001) and that it is most effective at the seedling stage. In the adult plant *rhm1* confers a level of quantitative resistance (Thompson et al., 1987, Balint-Kurti; et al, unpublished). In contrast, *Hm2* and *Hm1A* confer adult plant resistance to *C. carbonum* race (Balint-Kurti et al., 2007) but are not effective at seedling stages (Nelson and Ullstrup, 1964).

Unlike qualitative resistance, little is known about the identity or function of disease resistance QTL in any plant species. Despite the apparent differences noted above between qualitative and quantitative disease resistance, one can make the argument that, in some cases at least, pure qualitative and pure quantitative disease resistance are two ends of the same continuum and that most resistance genes reside somewhere between these two extremes. This is exemplified by the *Rcg1* gene that confers resistance to anthracnose stalk rot disease of maize. In certain populations *Rcg1* segregates as a major gene for resistance (Badu-Apraku et al., 1987a), while in others it was identified as a QTL (Jung et al., 1994). Thus, in some cases it becomes a semantic question as to whether a gene is a qualitative R gene or a large-effect QTL. Interestingly, *Rcg1* was recently cloned and shown to be an RGA (Broglie et al., 2006; Wolters et al., 2006).

Wisser et al (2006) recently summarized 50 publications reporting the mapping of disease resistance genes in maize. These included 437 QTL and 17 major genes. It was determined that reported disease resistance QTL covered 89% of the maize genome. This high degree of coverage is partly reflective of the relatively low precision and accuracy of QTL mapping (in some cases the confidence interval of a single QTL covered one third of a chromosome). However, just as with the genome-wide distribution of RGAs, it also reflects the large number of different loci involved in identified plant pathogen interactions. Wisser et al. (2006) further showed that the distribution of disease resistance QTL in maize was non-random (i.e., they were clustered to some extent) and that often QTL for resistance to different diseases clustered together, mirroring the clustered distribution of R genes and RGAs in plants (e.g., Milligan et al., 1998; Monosi et al., 2004).

Other reports suggest that disease resistance QTL and R-genes in fact do share some phenotypic characteristics. These include several reports suggesting that specific disease resistance QTL are race-specific (e.g., Avila et al., 2004; Calenge et al., 2004; Qi et al., 1999; Talukder et al., 2004; Zhu et al., 2003), reports showing that "defeated" R genes sometimes provide a basal level of non-specific, quantitative resistance (Li et al., 1999; Stewart et al., 2003) and that some R genes do not provide complete resistance (Choi et al., 1989; Hammond-Kosack and Jones, 1993; Smith and Hulbert, 2005), and reports showing that disease resistance QTL often co-localize with R gene clusters (Gebhardt and Valkonen, 2001; Wang et al., 1994; Welz and Geiger, 2000; Wisser et al., 2005; Yun et al., 2005).

This implies that RGA's may underlie at least some disease resistance QTL in plants. However most maize disease resistance QTL confer resistance to necrotrophic pathogens and, for the most part, resistance to true necrotrophs relies on mechanisms distinct from the R gene-mediated mechanisms involved in resistance to biotrophs

(Glazebrook, 2005). Therefore, for a significant proportion of maize disease resistance QTL it seems unlikely that RGAs will turn out to be the causative genes. Other genes involved in a wide variety of processes, including for example epidermal structure (e.g., Bradley et al., 2003), stomatal opening or phytoalexin synthesis (Zhou et al., 1999) might play a role In rice, several gene families, including mitochondrial transcription termination factors and glutathione S-tranferases were reported to be statistically associated with quantitative resistance (Wisser et al., 2005). Other reports have also implicated glutathione metabolism as an important component of disease resistance (Dean et al., 2005; Levine et al., 1994; Parisy et al., 2007). A proline-rich rice gene of unknown function named *Pi21* has recently been identified that confers quantitative resistance to rice blast (Fukuoka, personal communication). The high diversity available within maize, together with the genetic resources available make it a compelling system through which to understand the genetic architecture of disease resistance in plants, particularly with respect to quantitative disease resistance.

5 The Genetic Bases of Resistance to Specific Maize Diseases

This section briefly covers the main disease types, focusing on the genetics of resistance and highlighting specific points of interest and diseases that are particularly important for economic or food security reasons. For a more comprehensive review of maize diseases, including nematodes, root rots and striga which are not covered here, it is suggested the reader consult a number of recent reviews (Pratt and Gordon, 2006; Smith and White, 1988; White, 1999).

5.1 Stalk and Ear Rots

Stalk and ear rots are the most economically damaging of the corn diseases and occur wherever corn is grown. While the damage caused by ear rots in terms of lost yield is significant, their most problematic aspect is the mycotoxins associated with several of their ear rot fungi. These toxins have been associated with a variety of diseases ranging from nausea to neurological conditions to cancer (Richard and Payne, 2002). Here we will briefly discuss the four major ear rot problems (Fusarium, Aspergillus, Diplodia and Giberella) and the four major stalk rots (anthracnose, Giberrella, Fusarium and Diplodia) affecting maize. A common feature of genetic studies of all these diseases, particularly the ear rots, is the relatively high environmental variation observed. This has significantly hampered both investigations of the genetic architecture underlying these traits and the breeding of lines with reliable resistance to these diseases.

The maize ear represents a unique structure within the plant kingdom, quite different from the teosinte ear from which it evolved by artificial selection ~10,000 years ago, a relatively recent event in evolutionary terms. (Doebley et al., 1997; Piperno and Flannery, 2001; Reeves, 1950). Plant-pathogen interactions are generally thought to

coevolve over evolutionary time (Clay and Kover, 1996). It is interesting therefore to speculate as to the how the ear rots of corn originated. The answer likely is in the fact that the causal agents of maize ear rots tend to be relatively unspecialized pathogens. The causal agents of three of the four ear rot diseases detailed here have wide host ranges – *Aspergillus flavus* infects peanuts and cotton seeds among others, and *Fusarium verticillioides* and *Gibberella zeae* infect the seeds of rice, maize, and wheat as well as other grasses (Richard and Payne, 2002). In addition, three of the causal agents, *Fusarium verticillioides, Gibberella zeae* and *Stenocarpella maydis* are also the cause of important stalk rots. It seems possible therefore that these generalist pathogens were able to form novel interactions with the maize ear in a relatively rapid fashion in evolutionary terms – coming either from phylogenetically closely related hosts or from other maize structures (Parker and Gilbert, 2004).

As mentioned above, three ear rot/stalk rot pairs – Gibberella, Fusarium and Diplodia ear/stalk rot – share a causal organism. Curiously, few data exist on the potential correlations of reactions to ear rots and stalk rots in different maize lines. One study reported that, among 25 inbred lines of dent maize, Diplodia ear rot resistance was not correlated with Diplodia stalk rot resistance and likewise Giberella ear and stalk rot resistances were not correlated (Hooker, 1956).

5.1.1 The Genetics of Stalk Rot Resistance

Nyhus et al (1989) demonstrated that recurrent selection for Diplodia stalk rot resistance led to improvements in anthracnose stalk rot resistance. One study of the inheritance of resistance for anthracnose stalk rot indicated that additive effects were most significant (Carson and Hooker, 1981), while another suggested additive, dominance and epistatic effects were important (Badu-Apraku et al., 1987b). Similarly, additive effects have been reported to be most significant for Diplodia stalk rot resistance (Kappelman and Thompson, 1966). A major gene for Gibberella stalk rot resistance has been reported on chromosome 6 (Yang et al., 2004; Pè et al., 1993) reported at least 4 loci providing moderate levels of resistance to Gibberella stalk rot. Jung et al (1994) reported one major QTL for resistance to anthracnose stalk rot, which explained over 50% of the variation in an $F_{2:3}$ population. As mentioned above this QTL has also been described as the major gene *Rcg1* (Badu-Apraku et al., 1987a) and encodes an RGA (Broglie et al., 2006; Wolters et al., 2006). Arguably, this discovery represents the first report of the sequence of a gene underlying a plant disease resistance QTL.

5.1.2 The Genetics of Ear Rot Resistance

Fusarium Ear Rot

Fusarium ear rot, caused by the fungi *Fusarium verticillioides* (Sacc.) Nirenberg (synonym *F. moniliforme*) and *F. proliferatum* (T. Matsushima), is extremely widespread and is associated with production of the fumonosin mycotoxin. QTL for resistance to ear rot have been mapped in four populations (Pérez-Brito et al., 2001;

Robertson-Hoyt et al., 2006) and for resistance to fumonisin contamination in just two (Robertson-Hoyt et al., 2006). In all cases many, relatively small-effect QTL were found scattered throughout the genome. Evaluation of fumonisin levels in kernels is expensive and time consuming relative to evaluating levels of ear rot in the field. It has been reported that although phenotypic correlations between levels of ear rot and fumonsin are moderate to low (Clements et al., 2003, 2004), the genotypic correlations (the correlation observed after partitioning out non-genotypic effects due to environment and other factors) are significantly higher. Relatively high heritabilities for both traits have also been reported (Robertson et al., 2006). This implies that breeding for resistance to fumonisin contamination can be achieved by selection in multiple environments for resistance to fusarium ear rot. The composition of the maize kernel is a major determinant of fumonsin accumulation. Very little fumonsin accumulation was detected in kernels with low starch or where the amylase:amylopectin ration was high (Bluhm and Woloshuk, 2005).

Aspergillus Ear Rot

The primary cause of Aspergillus ear rot is the fungus *Aspergillus flavus* Link:Fr., which produces a mycotoxin called aflatoxin. Several studies have reported QTL for resistance to aflatoxin accumulation (Brooks et al., 2005; Busboom and White, 2004; Paul et al., 2003) and to Aspergillus ear rot (Busboom and White, 2004). In all cases high genotype/environment variation was observed and heritablities were low. In general most of QTL identified were of low to moderate effect, though two explained at least 20% of the phenotypic variation and were found in multiple environments (Brooks et al., 2005). This work suggests that, though difficult, it is possible to make progress in breeding for resistance to Aspergillus ear rot and aflatoxin accumulation. Indeed, several lines with enhanced aflatoxin accumulation resistance have been released (Williams et al., 2003).

A recent study showed high correlations, both phenotypic and especially genotypic, between Fusarium ear rot, Aspergillus ear rot and accumulation of aflatoxin and fumonisin in a set of recombinant inbred lines (Robertson-Hoyt et al., 2007). This suggests that common resistance mechanisms may function for the two diseases, and, conceivably, that breeding for Fusarium resistance may lead to a correlated response for Aspergillus resistance or vice-versa.

Gibberella Ear Rot

Gibberella ear rot is caused by the fungus *Gibberella zeae* (Schwein.) Petch (synonym *Fusraium graminearum*), which also produces several mycotoxins (Richard and Payne, 2002) including DON (deoxynivalenol, also known as vomitoxin). It has been shown that genetic variation for Gibberella ear rot resistance does exist (Chiang et al., 1987; Cullen et al., 1983; Hart et al., 1984; Schaafsma et al., 1997). One study (Ali et al., 2005) reported identification of 11 QTL for resistance to this disease but they were mostly small in effect and environmentally dependant.

Diplodia Ear Rot

Diplodia ear rot (also known as *Stenocarpella* ear rot) is caused by *Stenocarpella maydis* (Berk.) (syn *Diplodia maydis* [Berk.]). Resistant lines have been reported (Rensburg et al., 2003) but no mapping studies of loci conferring resistance to this disease have been carried out. One report has suggested that non-additive genes are important for resistance (Olatinwo et al., 1999) while another suggested that both general and specific additive effects are significant (Dorrance et al., 1998). These contrasting results likely reflect the different germplasm under investigation in each study.

5.2 The Genetics of Resistance to Foliar Diseases

Of the major types of maize disease listed here, foliar diseases are by far the easiest to observe and to assess. This is likely the reason most of the maize QTL and genetic inheritance studies of disease resistance have used foliar pathogens. It also explains why breeders have made the most progress against foliar pathogens. QTL and inheritance studies for maize foliar disease resistance are too numerous to list here, but the reader is directed to two recent reviews (Pratt and Gordon, 2006; Wisser et al., 2006). Worldwide, the most damaging foliar diseases are probably gray leaf spot, northern corn leaf blight (also known as turcicum leaf blight) and southern rust.

5.2.1 Gray Leaf Spot

The causal agent of gray leaf spot is *Cercospora zeae-maydis* (Tehon and Daniels). This disease has increased in importance over the past 15 years due to increased practice of conservation tillage (Ward et al., 1999) which allows plant residue to remain on the soil surface and act as a spore reservoir. Gray leaf spot is probably the major foliar disease problem in the United States and in sub-saharan Africa. In general, resistance has been reported to be moderate to highly heritable and based largely on additive effects (Gevers et al., 1994; Gordon et al., 2006; Thompson et al., 1987). Several studies have identified QTL for gray leaf spot resistance (Bubeck et al., 1993; Clements et al., 2000; Gordon et al., 2004; Lehmensiek et al., 2001; Saghai Maroof et al., 1996). There is also a report of a major gene for gray leaf spot resistance (Gevers and Lake, 1994), but a subsequent study contradicted this (Gordon et al., 2004).

5.2.2 Northern Leaf Blight

The causal agent of northern leaf blight is *Exserohilum turcicum* (Pass) K. J. Leonard and E. G. Suggs (teliomorph: Setosphaeria turcica [Luttrell] Leonard and Suggs). It is found in most maize growing areas that have high humidity combined with moderate temperatures. It is a significant problem in the north eastern United States, in

sub-Saharan Africa and areas of China, Latin America and India (Adipala et al., 1995; Dingerdissen et al., 1996). The genetics of northern leaf blight resistance have been extensively studied and are the subject of a recent review (Welz and Geiger, 2000). Northern leaf blight is unusual among necrotrophic diseases in that several dominant or partially dominant qualitative genes have been described that confer race-specific resistance to it, including *Ht1*(Hooker, 1963), *Ht*2 (Hooker, 1977), *Ht*3 (Hooker, 1981), *Htn*1 (also known as HtN, Gevers, 1975) and *Ht*P (Ogliari et al., 2005). This anomaly might be explained by the fact that northern leaf blight is arguably a hemibiotroph rather than a straightforward nectrotroph. After leaf penetration, initial growth of *E. turcicum* is mostly intracellular (Knox-Davies, 1974) in the mesophyll and does not cause cell death. It is also noteworthy that, compared to most major resistance genes, the *Ht* genes seem to have unusually high environmental dependence, particularly with regard to light and temperature (Leath et al., 1987, 1990; Thakur et al., 1989a, 1989b) and they tend to confer delayed lesion development or sporulation phenotypes rather than complete resistance. It could be argued that the *Ht* genes are rather atypical plant major resistance genes and should be thought of as large-effect, race-specific QTL.

QTL for northern leaf blight resistance have been identified in at least nine different populations (Brewster et al., 1992; Dingerdissen et al., 1996; Schechert et al., 1999; Welz and Geiger, 2000; Welz et al., 1999a, 1999b; Wisser et al., 2006). They seem to be distributed throughout the genome and tend to be insensitive to light and temperature variations (Carson and Van Dyke, 1994). Combinations of qualitative and quantitative resistance genes are generally employed in breeding for resistance, with the emphasis now on quantitative genes, due to their higher phenotypic stability (Pratt and Gordon, 2006).

5.2.3 Southern Rust

Southern rust is caused by the biotrophic fungus *Puccinia polysora* Underw.. It is a significant problem in Brazil and South America and is an intermittent but increasing problem in the United States. *Rpp9* a major gene for southern rust resistance has been mapped to the short arm of chromosome 10 (Scheffer and Ullstrup, 1965). Other major genes for southern rust resistance have also been mapped to this region (Chen et al., 2004; Holland et al., 1998; Jines et al., 2006; Liu et al., 2003; Scott et al., 1984; Ullstrup, 1965) but their allelic relationships to *Rpp9* have not been established. QTL for southern rust have been mapped in three populations (Brunelli et al., 2002; Holland et al., 1998; Jiang et al., 1999) with no co-localization of QTL across studies (Wisser et al., 2006).

The infrequent occurrence of southern rust in the United States has resulted in difficulties in breeding for durable resistance and most probably the loss of minor resistance alleles (Davis et al., 1990). While major genes have been effective so far (Pratt and Gordon, 2006), it would appear that in the absence of constant disease pressure, the use of marker assisted selection (MAS) would be a feasible approach.

6 Systemic Acquired Resistance and Induced Systemic Resistance in Maize

Plants previously infected by a microorganism may become systemically more resistant to subsequent pathogen attack. Two types of systemic responses have been extensively characterized in dicotyledonous systems; systemic acquired resistance (SAR) and induced systemic resistance (ISR). SAR is a response to necrotizing pathogens and confers a broad-spectrum resistance. It is associated with induction of a number of pathogenesis-related (PR) genes and the *NPR1* gene has been shown to be a key regulator of the process (Grant and Lamb, 2006). Salicylic acid (SA) appears to be the local inducer of SAR and methyl salicylate (MS) has been implicated as the mobile signal which induces SAR systemically (Park et al., 2007). It is thought that MS is produced at the site(s) of infection, transported throughout the plant in the phloem and is converted to SA at the site of action. ISR is induced by symbiotic micro-organisms in the rhizosphere (Vallad and Goodman, 2004). It also confers broad-spectrum resistance but the pathway is regulated by jasmonate and ethylene rather than SA and PR-genes are not induced.

The SAR and ISR pathways have been extensively characterized in dicotyledonous systems but the presence of analogous pathways in monocotyledonous systems has not been conclusively proven. Still there is a significant amount of evidence to suggest that monocotyledonous responses similar to ISR and SAR do indeed exist. For instance, *NPR1* seems to function similarly in rice and Arabidopsis (Chern et al., 2001, 2005; Dong, 2004) and inducers of SAR function in several monocotyledonous species including maize (Gorlach et al., 1996; Kogel and Huckelhoven, 1999; Morris et al., 1998). SAR has also been shown to function in a field context on wheat (Calonnec et al., 1996). An ISR-like response has recently been documented in maize in response to the fungal root colonizing fungus *Trichoderma virens* (Djonovic et al., 2007).

On the other hand no change in disease responses was observed when the maize ortholog of *NPR1* was disrupted or over-expressed (Johal et al., unpublished), in contrast to the results in rice (see above). Similarly, whereas SA is a critical signal in *Arabidopsis* for expression of multiple modes of resistance, it seems to have little or no effect on the interaction of maize with pathogens, based on the analysis of both SA-deficient and SA over-accumulating transgenic maize (Johal and Yalpani, unpublished). Furthermore, in experiments in maize with common rust, prior or simultaneous inoculation of an incompatible race seemed to have no effect on the subsequent host reaction to a compatible race of the pathogen (S. Hulbert, pers.com).” In conclusion, it seems clear that while aspects of induced resistance responses are conserved between dicotyledonous and monocotyledonous systems, the responses are not the same. Furthermore it may be that the mechanisms associated with induced responses vary between monocotyledonous species.

7 The Future

7.1 Prospects for Genetically Engineered Plant Disease Resistance in Maize

Despite a substantial amount of effort over more than a decade, there are no commercially available transgenic plants conferring increased levels of fungal or bacterial disease resistance. While there are many examples of transgenic plants with increased disease resistance (e.g., Gao et al., 2000, 2007; Grison et al., 1996), none have become viable commercial products (Gurr and Rushton, 2005b; Hammond-Kosack and Parker, 2003). There are many reasons for this lack of success, including growth penalties conferred by the precocious or over-activation of defense responses, presumably due to the redirection of cellular metabolism towards defense and away from growth (Logemann et al., 1995). In other cases, regulatory or economic factors have been the main stumbling blocks.

New technologies and knowledge may eventually facilitate the production of commercially viable disease-resistant transgenic plants. More precise control of gene expression, through the genomics-based discovery and use of pathogenesis-inducible natural and synthetic promoters has some potential (Gurr and Rushton, 2005a). The use of gene-shuffling techniques to produce novel disease resistance specificities (Bernal et al., 2005) or to produce novel enzymes (Duvick, 2001) also holds great promise. Perhaps most excitingly, the use of RNAi to inhibit specific, genes in the pathogen offers an almost unlimited number of essential pathogenesis pathways to target. In these cases, a double-stranded RNA (dsRNA) molecule, complementary to a specific pathogen gene would be expressed in the host and transferred to the pathogen during infection to silence specific genes. The effective transfer of the dsRNA from host to pathogen remains the main problem but there are indications that this is not insurmountable, at least in some systems (Bailey et al., 2006; Lilley et al., 2007).

The sheer economic value of maize, makes it an attractive target for some of these approaches. In many crops the economic value of transgenic disease resistance would be derived from the resulting reduction in pesticide applications. In maize, with the exception of seed treatments, pesticide applications to control disease are uncommon due to the low margin of profitability (Smith and White, 1988). In this case, economic value would likely have to be derived from increased yield or quality. Diseases for which conventional breeding has not produced entirely adequate solutions would be the main targets, including Aspergillus and Fusarium ear rots (Duvick, 2001) and grey leaf spot. In 2007 an unprecedented proportion of maize acreage in the mid-western U.S.A, about 15–20%, was sprayed with foliar fungicides (Gary Munkvold pers.com). This was due to the high price of corn making such treatments economically viable and it is likely to prove the exception.

7.2 *Maize as a System for Disease Resistance Genetics Studies*

Maize was for many years at the forefront of plant disease resistance genetics research (see above). It has somewhat fallen out of favor as a model system in recent years as *Arabidopsis*, tomato and rice have been more utilized in the public sphere. Disease resistance research in maize has been pursued most vigorously in the private sector.

We feel that maize is again becoming an attractive system for plant disease resistance genetics, both as a model system and as a target in its own right. The maize genome sequence should be finished as this book is published and other resources for fine scale mapping, such as the intermated B73/Mo17 (IBM population (Balint-Kurti et al., 2007), and the 5000-line nested association-mapping (NAM) population (Holland, Buckler personal communication) are becoming available. Furthermore, systematic mutagenesis within maize should also be more feasible with the development of large collections of transposon insertion lines in a uniform genetic background (http://www.plantgdb.org/prj/AcDsTagging/) These new resources can be combined with the existing beneficial features of maize such as its wide genetic diversity and ease of crossing and with the genetic stocks and resources that have been built up over many years of research.

For some important diseases there is no appropriate surrogate system for studying maize's interaction with pathogens. Examples include ear and stalk rots of the ear and the stalk, maize smut that causes tumors on many parts of the plant, and crazy top disease (caused by a downy mildew pathogen) that interferes with maize's transition to flowering (Figure 1, Agrios, 1997). The fact that maize is such an

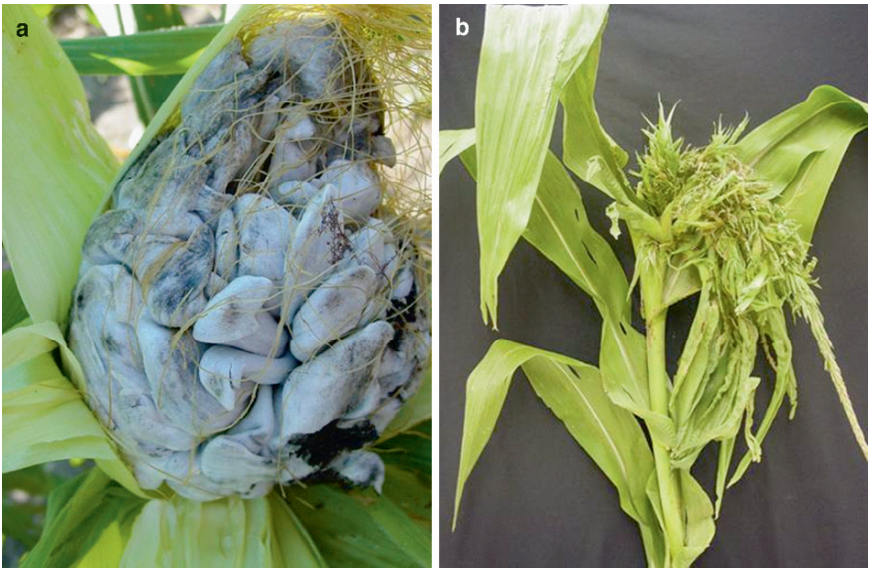

Fig. 1 Examples of diseases unique to maize, common smut (A) and crazy top (B)

important crop means that findings made in maize can be directly useful as well as providing a framework for disease resistance studies in other species. Its significance as a model plant may increase in the future, given that most energy crop plants proposed for biomass production are, like maize, C4 grasses.

In particular, maize is a superior system for the investigation of quantitative traits. In many cases quantitative disease resistance appears to be durable and the assumption is that it incurs few growth penalties- though this has not been rigorously tested (Brown, 2002). Furthermore, there is evidence that in some cases quantitative resistance confers resistance to a wide spectrum of pathogens (Mitchell-Olds et al., 1995, Balint-Kurti et al., unpublished). It may be that the cloning and manipulation of quantitative resistance genes will ultimately prove the most effective transgenic approach to disease resistance.

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Virus Resistance

Margaret G. Redinbaugh and Richard C. Pratt

Abstract Identification, characterization and deployment of virus resistant maize are complex tasks requiring multidisciplinary approaches. Insect transmission of viruses in nature and the potential presence of biologically distinct virus strains complicate screening for virus resistance. At least ten maize-infecting viruses cause damage worldwide, and naturally occurring resistance to each of these viruses has been identified in maize. Genes and quantitative trait loci for resistance to most of these viruses have been mapped in the maize genome, and regions on chromosomes 1, 3, 6 and 10 have been implicated in resistance to multiple phylogenetically unrelated viruses. Mechanisms associated with virus resistance and alternatives to naturally occurring virus resistance are discussed.

1 Introduction

Virus diseases cause crop losses in maize directly by damaging plants and reducing yields. Emerging viruses continually pose problems for seed and grain producers, and these are likely to become worse as acreage increases with the demand for biofuels and year-round planting of maize in the tropics. In the early 1960s, *Maize dwarf mosaic virus* (MDMV) and *Maize chlorotic dwarf virus* (MCDV) emerged very rapidly to become widespread pathogens in North America (Louie, 1999). The diseases caused by MDMV and MCDV are now controlled through planting of resistant hybrids and improved weed management (Duvick and Cassman, 1999; Louie, 1999). This pattern of an emerging virus "coming out of nowhere" to cause major disease problems occurred in more recent years with Wheat mosaic virus (WMoV, formerly High Plains virus) from the northern plains of the U.S. and *Mal de Rio Cuarto virus* (MRCV) in Argentina (Jensen et al., 1996; Lenardon et al., 1998), two previously unknown viruses that quickly produced significant disease problems on sweet corn/wheat and maize, respectively. In addition, changes in cropping patterns or germplasm can result in disease outbreaks. For example, the spread of MDMV and the related potyvirus *Sugarcane mosaic virus* (SCMV) in Europe (Kuntze et al., 1997) and China (Jiang and Zhou, 2002) is associated with increased maize acreage and planting of susceptible hybrids. Similarly, *Rice black*

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streaked dwarf virus (RBSDV), which historically caused disease on rice in Asia, is responsible for recent epidemics in China (Fang et al., 2001).

Like all viruses, plant viruses are obligate intracellular parasites that cannot replicate outside their host (Hull, 2002). They have relatively small genomes with two to about 20 genes. The genomes can be single or double stranded RNA or DNA, and single stranded virus genomes can be positive or negative sense, or both (ambisense). Most plant-infecting viruses consist of nucleic acid and protein, although viruses in a few families have an outer lipid bilayer. In nature, vectors transmit nearly all plant viruses. Arthropods are the vectors for the vast majority of viruses, with a few virus genera being transmitted by fungi or nematodes. Vector transmission complicates disease epidemiology, essentially taking the classic "plant disease triangle," in which disease development requires a virulent pathogen, a susceptible host and the right environmental conditions, and making it into a tetrahedron in which disease requires interactions among a competent vector, the virulent pathogen, a susceptible host and a suitable environment (Francl, 2001) (Fig. 1).

At least ten viruses from eight families have caused significant agronomic losses in maize world-wide (Louie, 1999): MDMV, SCMV, MCDV, MRCV, RBSDV, *Maize streak virus* (MSV), *Maize mosaic virus* (MMV), *Maize rough dwarf virus* (MRDV), *Maize chlorotic mottle virus* (MCMV), *Maize white line mosaic virus* (MWLMV), *Maize stripe virus* (MStV) and *Maize rayado fino virus* (MRFV) (Table 1). WMoV and *Wheat streak mosaic virus* (WSMV) can cause disease in wheat, sweet corn and some breeding materials; however, most maize hybrids are resistant to them. *Maize necrotic streak virus* (MNeSV) and *Maize fine streak virus* (MFSV) are emerging maize viruses that are not known to cause disease problems (Louie et al., 2000; Redinbaugh et al., 2002).

Virus disease control can come from breaking any of the interactions in the disease tetrahedron. For example, removing alternative weedy virus hosts can reduce the source of the pathogen, and widespread control of johnsongrass (*Sorghum halpense*), a weedy reservoir of MCDV, provided disease control (Waterworth and Hadidi, 1998; Vangessel and Cobel, 1993). Quarantines can be used to control disease by preventing geographic spread of viruses and their vectors. For viruses that are semi-persistently or

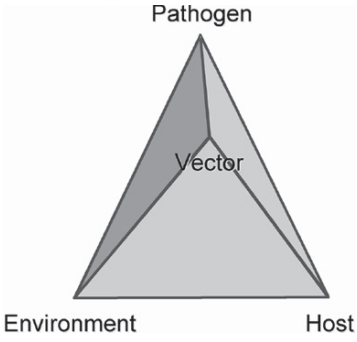

Fig. 1 Plant virus disease tetrahedron. The tetrahedron emphasizes the importance of vector interactions with the pathogen, host and environment

Table 1 Viruses of maize discussed in this chapter

Acron[a]	Virus	Family	Dist[b]	Trans[c]	Vect[d]
MDMV	*Maize dwarf mosaic virus*	Potyviridae	WW	NP/M	A
SCMV	*Sugarcane mosaic virus*	Potyviridae	WW	NP/M	A
WSMV	*Wheat streak mosaic virus*	Potyviridae	WW	NP/M	M
MCDV	*Maize chlorotic dwarf virus*	Sequiviridae	USA	SP/I	LH
MSV	*Maize streak virus*	Geminiviridae	Africa	P/I	LH
MMV	*Maize mosaic virus*	Rhabdoviridae	Carrib.	P/I	PH
MFSV	*Maize fine streak virus*	Rhabdoviridae	USA	P/I	LH
MNeSV	*Maize necrotic streak virus*	Tombusviridae	USA	I	unk.5
MCMV	*Maize chlorotic mottle virus*	Tombusviridae	USA	M	unk.
MWLMV	*Maize white line mosaic virus*	Tombusviridae	US/Eur.	I	unk.
MRFV	*Maize rayado fino virus*	Marafiviridae	Carrib.	P/I	LH
MRDV	*Maize rough dwarf virus*	Fijiviridae	Eur./Is.	P/I	PH
MRCV	*Mal de Rio Cuarto virus*	Fijiviridae	SA	P/I	PH
RBSDV	*Rice black streaked dwarf virus*	Fijiviridae	Asia	P/I	PH
MSpV	*Maize stripe virus*	Tenuiviridae	WW	P/I	PH
WMoV	*Wheat mosaic virus*	unk.	USA	unk./I	M

[a]*Acron*. virus acronym
[b]Distribution of the virus. *WW* world-wide, *Carrib*. Carribean, *Eur./Is*. Europe and Israel, *SA* South America, *USA* United States
[c]Transmission of the virus. Transmission by the insect vector occurs in a non-persistent (*NP*), semi-persistent (*SP*) or persistent (*P*) manner. "M" indicates the virus is mechanically transmissible by leaf rub-inoculation. "I" indicates obligate vector transmission
[d]Type of insect that transmits the virus in nature: *A* aphid, *M* mite, *LH* leafhopper, *PH* planthopper
[e]*unk* unknown

persistently transmitted by their insect vectors, insecticides can provide some disease control (Perring et al., 1999). In some cases, altering planting dates can allow crops to escape exposure to viruliferous vectors (Gregory and Ayers, 1982; Waterworth and Hadidi, 1998; Fritts et al., 1999; Itnyre et al., 1999; Vasquez and Mora 2007). However, the most economical, environmentally sustainable and effective means for controlling viral diseases of maize is to deploy resistant germplasm (Kang et al., 2005).

2 Identification and Assessment of Virus Resistance

2.1 Virus Transmission

Virus diseases are sporadic in nature, because establishment requires interplay among virus, viral vector, virus-susceptible germplasm and environmental conditions (Fig. 1). This year-to-year inconsistency in viral disease pressure complicates identification and development of resistant maize using naturally occurring disease. Artificial inoculation techniques are most often used to overcome fluctuations in disease pressure. For viruses that are easily mechanically transmitted (e.g., MDMV and SCMV), inoculation technologies are relatively straightforward and economical (Louie, 1986; Scott and Louie, 1996). For the obligately vectored MSV and MCDV, field grown

plants were exposed to viruliferous leafhoppers, allowing for 16–100% infection of susceptible maize (Rosencranz and Scott, 1987; Efron et al., 1989; Pratt et al., 1994; Welz et al., 1998; Kyetere et al., 1999). A highly efficient vector-based protocol for greenhouse transmission of MCDV (Louie and Anderson, 1993) was a key development for the identification and characterization of highly resistant germplasm (Louie et al., 2002; Jones et al., 2004). While very effective, these protocols are expensive because they are labor intensive and require specialized expertise, equipment and materials for rearing insects. A potential inexpensive alternative to vector mediated transmission of viruses to maize is vascular puncture inoculation (VPI) (Louie, 1995). All maize viruses tested can be transmitted to germinating maize kernels with this technique, including those that normally require transmission by insects or other vectors (Table 1). However, improvements to the technique are needed to raise transmission of phloem-limited viruses to the high levels required for resistance screening.

2.2 Viral Inocula

The source and makeup of viral inocula are important for identifying robust resistance. Phenotypic analysis is easier if strong, distinctive symptoms are incited in susceptible hosts. For example, although disease incidence was similar in maize inoculated with MCDV-type and MCDV-severe, the distinct vein banding incited by MCDV-severe made it a better choice for characterizing resistance (Pratt et al., 1994; Jones et al., 2004). In Africa, maize streak disease is incited by at least five distinct strains of MSV that differ in their degree of sequence identity, serological reactivity and in the severity of symptoms produced in maize (Pinner and Markham, 1990; Mesfin et al., 1992; Konate and Traore, 1994; Martin et al., 1999). In general, the responses of resistant germplasm were consistent with the severity of the isolate inoculated (Martin et al., 1999; Pernet et al., 1999a; Mawere et al., 2006). That is, the most severe symptoms were found on the most susceptible maize, and the least symptoms were found on the most resistant maize, regardless of the isolate used. However, to ameliorate the possible impact of strain differences on screening for resistant genotypes, a mixture of isolates has sometimes been used for screening (Kyetere et al., 1999). A similar differential response of maize lines to inoculation with MDMV isolates was found (Louie, 1985).

Another consideration for development of inocula is that related viruses in the same genus or family can give differential responses to the same resistant germplasm. For example, several species in the family *Potyviridae* infect maize: MDMV, SCMV (formerly known as MDMV-B), *Johnsongrass mosaic virus* (JGMV, formerly known as MDMV-O), *Sorghum mosaic virus* (SrMV), and WSMV (Shulka et al., 1994). These viruses share the same overall genome organization and have significant sequence similarity (Kong and Steinbiss, 1998; Stenger et al., 1998; Chen et al., 2002), but maize genotypes have differential responses to these virus species (Kovacs et al., 1998; Lubberstedt et al., 2006; Jones et al., 2007). Because most of these viruses have worldwide distribution and incite similar symptoms (Shulka et al., 1994), serological and/or molecular characterization of virus isolates prior to screening is needed.

2.3 Identifying Resistant Germplasm

Identification of virus resistance generally involves inoculating a genetically diverse array of germplasm with virus and identifying plants that show either no or reduced infection. Most frequently, infection is scored by the presence and severity of virus symptoms. However, it is useful to ensure that the resistance is associated with reduced virus titer using serological or molecular techniques, since asymptomatic plants with high virus titer (i.e., virus tolerant plants) could serve as a source of pathogen for further disease spread. Qualitative virus resistance can be dominant or recessive, although all qualitative resistance described in maize thus far is dominant. Quantitative or partial resistance allows some development of disease (Heitefuss, 1997) and this may be associated with oligogenic or polygenic resistance. Quantitative resistance may be associated with decreased incidence of infection, decreased symptom severity or both (Pratt et al., 1994). Both types of virus resistance have been identified in maize, characterized and deployed successfully.

3 Genetics of Virus Resistance

3.1 The Potyviridae

Resistance to three viruses in the family *Potyviridae*, MDMV, SCMV and WSMV, has been characterized in some detail. For all three viruses, there is a major resistance locus on the short arm of chromosome 6 near the *nucleolar organizer region (nor)*. *Mdm1* (McMullen and Louie, 1989; Simcox et al., 1995), *Wsm1* (McMullen et al., 1994; McMullen and Louie, 1991; Marçon et al., 1999) and *Scm1* (Melchinger et al., 1998; Xia et al., 1999; Xu et al., 1999; Dussle et al., 2000, 2003) are genes on the short arm of chromosome six that confer resistance to MDMV, SCMV and WSMV, respectively. All three loci co-segregate with the marker(s) *umc85* and/or *bnl6.29*, and are dominant genes in the U.S. lines Pa405 and B73, and the European lines D21, D32 and FP1360A. Although there have been efforts to produce high-resolution genetic maps for virus resistance in this region (Simcox et al., 1995; Xu et al., 1999; Dussle et al., 2003; Yuan et al., 2003), it has not yet been possible to separate the three resistance genes. For SCMV and WSMV, multiple major resistance loci have been identified. *Scm2* and *Wsm2*, for SCMV and WSMV resistance, respectively, map to the interval flanked by the RFLP markers *umc92* and *umc102* near the centromere of chromosome 3 in European lines (Xia et al., 1999; Dussle et al., 2000, 2003), Pa405 (McMullen et al., 1994) and Mo17 (Marçon et al., 1999). A third gene for WSMV resistance (*Wsm3*) maps to the long arm of chromosome 10 between *umc163* and *umc44* (McMullen et al., 1994). Interestingly, a minor QTL for SCMV resistance is also located in this region (Xia et al., 1999). Potyvirus resistance apparently arose more than once, because the RFLP markers (*umc85* and *bnl6.26*) near *Scm1/Mdm1* have different banding patterns in European and U.S. lines (Xu et al., 2000).

Resistance to MDMV has been identified in a variety of germplasm (Louie et al., 1990; Brewbaker et al., 1991; Scott and Louie, 1996, Kuntze et al., 1997). Segregation analyses indicated that more resistance loci (Scott and Rosenkranz, 1981; Mikel et al., 1984) or genetic modifiers of *Mdm1* activity might be present in Pa405 (McMullen and Louie, 1989). Evidence supporting both the universality of MDMV resistance associated with *Mdm1* and the implication of other loci in MDMV resistance came from analysis of F_2 populations derived from 43 MDMV resistant inbred lines (Jones et al., 2007). Markers on chromosome 6 were associated with resistance in 42 of the 43 lines. Markers flanking *Scm2*/*Wsm2* and *Wsm3* were also associated with MDMV resistance in 8 and 7 lines, respectively, indicating that resistance alleles at these loci may also contribute to MDMV resistance. In one inbred line (ICA L210), MDMV resistance was not associated with *Mdm1* (Scott and Louie, 1996; Jones et al., 2007). Here, resistance was associated with chromosome 3 markers (*Scm2*/*Wsm2*).

3.2 Maize Streak Virus

Maize streak is the most important and widespread disease of maize in Africa (Bosque-Pérez, 2000; DeVries and Toenniessen, 2001). Devastating epidemics occur across sub-Saharan Africa and the adjacent Indian Ocean islands, and the disease has been reported in Egypt and Yemen (Thottappilly et al., 1993). The disease is caused by MSV, a geminivirus transmitted by leafhoppers in the genus *Cicadulina*. Several factors complicate the identification of resistant germplasm and mapping of resistance genes: the diversity of MSV strains discussed above, obligatory vector transmission, and biosafety concerns that limit field testing to Africa. Nonetheless, resistant germplasm has been identified by several international maize breeding programs (Barrow, 1992; Welz et al., 1998; Pernet et al., 1999a, 1999b). A major QTL (*msv1)* for resistance was identified on the short arm of chromosome 1 in bin 1.04 or 1.05 depending on the population (Welz et al., 1998; Kyetere et al., 1999; Lu et al., 1999; Pernet et al., 1999a, 1999b). Alleles at this locus are partially dominant. Although *msv1* explained most of the resistance in all studies, population-specific minor QTL were found on chromosomes 2, 3, 4 (Welz et al., 1998). Minor QTL on chromosomes 2S, 9S and 10L were consistent across at least two populations (Kyetere et al., 1999; Lu et al., 1999; Pernet et al., 1999a, 1999b), and the QTL on 10L maps to a location similar to *Wsm3* (Pernet et al., 1999a).

3.3 Maize Chlorotic Dwarf Virus

MCDV (family *Sequiviridae;* genus *Waikavirus*) has a single stranded RNA genome that encodes a large, posttranslationally processed polyprotein (Hull, 2002). MCDV causes significant problems in the southern and southeastern parts of the U.S. where the ranges of its vector, the black-faced leafhopper (*Graminella nigrifrons* Forbes),

and its perennial reservoir host, johnsongrass, overlap (Gordon et al., 1981). Early efforts to identify and characterize MCDV resistance were hindered by incomplete virus transmission and the confounding effects of MDMV-infected plants (Guthrie et al., 1982; Rosenkranz and Scott, 1986, 1987; Louie et al., 1990). After a single inoculation with viruliferous *G. nigrifrons* under greenhouse conditions, Rufener et al. (1996) identified resistance QTL on chromosomes 1, 3, 5, 7, and 10 in the MCDV tolerant maize inbred Mp705. With an improved method for virus transmission (Louie and Anderson, 1993; Pratt et al., 1994) and F_2 progeny derived from the highly MCDV resistant inbred Oh1VI (Louie et al., 2002), two major QTL for MCDV resistance were associated with markers on chromosome 3 near *umc102* (*mcd1*) and chromosome 10 near *umc44* (*mcd2*) (Jones et al., 2004), markers that are also associated with *Wsm2* and *Wsm3* (Table 2). These findings suggest the two QTL on chromosomes 3 and 10 may be in common between the different resistance sources.

3.4 Maize Mosaic Virus and Maize Fine Streak Virus

Two phylogenetically distinct maize-infecting rhabdoviruses, MMV and MFSV, have been characterized (Jackson et al., 2005). The viruses are transmitted by the planthopper *Perigrinus maidis* and the leafhopper *G. nigrifrons*, respectively. MMV causes disease in tropical and sub-tropical areas worldwide including Africa, South America, Hawaii and Australia (Gordon et al., 1981; Greber, 1981; Thottappilly et al., 1993), but widespread disease has not been associated with MFSV. A major QTL for MMV

Table 2 Clustering of virus resistance genes and QTL in the maize genome

Chr.	Bin	Virus	Locus	Type	Reference
1	1.03	MRCV	–	Major QTL	Di Renzo et al., 2004
	1.05	MSV	*msv1*	Major QTL	Welz et al., 1998; Kyetere et al., 1999
3	3.05	WSMV	*Wsm2*	Gene	McMullen et al., 1994
		SCMV	*Scm2*	Major QTL	Xia et al., 1999; Xu et al., 1999
		MMV	*Mv1*	Major QTL	Ming et al., 1997
		MCDV	*mcd1*	Major QTL	Jones et al., 2004
		WMoV	–	QTL	Marçon et al., 1999
		MSpV	–	Major QTL	Dintinger et al., 2005b
	3.06	MSV	–	Minor QTL	Welz et al., 1998
6	6.01	MDMV	*Mdm1*	Gene	McMullen and Louie, 1989
		SCMV	*Scm1*	Gene	Xia et al., 1999; Xu et al., 1999
		WSMV	*Wsm1*	Gene	McMullen et al., 1994
		WMoV	–	Gene	Marçon et al., 1997b, 1999
	6.02	MRDV	*Mrdd1*	QTL	Wang et al., 2007
10	10.05	WSMV	*Wsm3*	Gene	McMullen et al., 1994
		MCDV	*mcd2*	Major QTL	Jones et al., 2004
		MSV	–	Minor QTL	Pernet et al., 1999a, b
	10.06	MSpV	–	Minor QTL	Dintinger et al., 2005a

QTL quantitative trait loci

resistance, *Mv1*, maps close to the chromosome 3 centromere in the inbred line Hi31 (Ming et al., 1997). This locus was recently introgressed into 27 parental lines (Brewbaker and Josue, 2007). *Mv1* maps to the same location as *Scm2*, *Wsm2* and *mcd1* (Table 2). MFSV resistance has not yet been mapped, but lines resistant to MMV (i.e., Hi31, Hi34 and Oh1VI) are also resistant to MFSV (Redinbaugh et al., 2002).

3.5 Wheat Mosaic Virus

The mite (*Aceria tosichella* Keifer) transmitted WMoV is an emerging viral pathogen that causes disease in maize and wheat (Jensen et al., 1996; Skare et al., 2006). *A. tosichella* also transmits WSMV, and contamination of experimental mite colonies with WSMV has complicated analysis of WMoV resistance (Marçon et al., 1997a). Several maize lines are highly resistant to WMoV, and resistance in B73 is associated with the marker *umc85* and *Wsm1* (Marçon et al., 1997b). In RIL derived from B73 x Mo17, resistance from a Mo17 allele on chromosome 3 near *umc102* and *Wsm2* was also identified (Marçon et al., 1999). In near isogenic lines derived from Pa405 x Oh28, lines carrying the *Wsm1* locus were highly resistant to WMoV, while those carrying the *Wsm2* locus were not (Redinbaugh, unpublished results). In this case, co-infection with WSMV was avoided by transmitting the virus using VPI.

3.6 Maize Stripe Virus

MSpV is a maize planthopper transmitted tenuivirus that is a threat to maize in tropical regions around the world (Falk and Tsai, 1998). The Reunion line Rev81 was identified as resistant to MSpV, and QTL controlling MSpV resistance in this line mapped to regions of chromosomes 2L, 3 (near the centromere) and 5L (Dintinger et al., 2005a, 2005b). The QTL on 2L affected both disease incidence and severity, while that on chromosome 3 QTL primarily affected incidence. The chromosome 5L QTL was mainly associated with severity.

3.7 Fijiviruses

Three planthopper-vectored virus species in the genus *Fijivirus*, MRDV, MRCV and RBSDV, cause disease problems in maize in Europe, South America and Asia, respectively (Marzachi et al., 1995; Lenardon et al., 1998; Fang et al., 2001; Bai et al., 2002). MRCV-resistant Argentine germplasm was identified, and resistance QTL from this line mapped to chromosomes 1S and 8L (Di Renzo et al., 2004). The QTL on chromosome 1S maps close to *msv1* (Table 2). For RBSDV, resistance has been identified in the Chinese line 90110, and preliminary reports indicate it is associated with QTL on chromosomes 6S, 7L and 8L (Wang et al., 2007). Although resistance to fijiviruses

from these two sources does not overlap, the RBSDV resistance locus on chromosome 6S is in the same region as potyvirus and WMoV resistance (Table 2).

3.8 Other Viruses

For a number of other maize viruses, resistant germplasm has been identified, but resistance loci or genes have not been mapped. MRFV is an agronomically important virus in Central and South America (Vasquez and Mora, 2007), and tolerant lines that had only mild symptoms and low virus titer were identified from locally adapted landraces (Bustamante et al., 1998). MNeSV is not known to be a disease problem in maize, but several maize lines with high resistance to virus infection were identified (Louie et al., 2000).

3.9 Clustering and Durability of Resistance Genes

Resistance genes have been found to occur in clusters within plant genomes (Hulbert et al., 2001). Consistent with this notion, resistance genes and QTL for multiple phylogenetically diverse viruses map to chromosomes 3, 6, 10 and possibly chromosome 1 (Table 2) (McMullen and Simcox, 1989, 1995; Redinbaugh et al., 2004). All four chromosomal regions also carry resistance loci for bacterial and fungal resistance, and the regions on chromosome 3 and 6 are rich in R genes.

Virus resistance durability under production conditions is a concern for maize breeders and geneticists, especially for alleles at single loci that are widely deployed for disease control such as *msv1*. This concern is rooted in knowledge that mutation and recombination rates during viral replication are high, resulting in a population of virus sequences within a single host (Roossinck, 2003). In practice, however, virus resistance is significantly more durable than resistance to cellular pathogens. For two-thirds of the virus-host systems analyzed, resistance breaking isolates have either not emerged or have not become prevalent (Garcia-Arenal and McDonald, 2003). At a molecular level, this can be related to the number and locations of genome sequence changes required for the virus to break resistance (Harrison, 2002). Resistance breaking isolates tend to be common if resistance can be overcome by a single base change in the virus, and to be rare if multiple simultaneous changes are required.

4 Breeding for Virus Resistance

The priority traits for improvement of maize in mechanized, high-input production systems in temperate regions are yield, stalk quality (“standability”) and grain moisture content at harvest. The development of resistant germplasm to manage

disease usually becomes a priority for the seed industry at about the time severe or repeated disease epiphytotics make headlines in the popular press. Seed companies respond by ensuring that their varieties are equal to, or better than, those of their competitors – although not necessarily highly resistant. In tropical and sub-tropical regions where the majority of the maize crop is used for food and the incidence of yield detracting diseases is generally higher than in temperate regions, resistance to pathogens is of ongoing, if not paramount, importance. In subsistence production systems, protecting what is likely to be only a modest or meager harvest under the most favorable conditions can mean the difference between an adequate diet and hunger. The consequences of a devastating epidemic are severe, but research, breeding, and extension are poorly resourced in most developing countries (Strange and Scott, 2005) and access to improved varieties is limited. Breeding for "defensive" traits such as host-resistance is perhaps less glamorous than selection for "offensive" traits such as high yield; it is nonetheless absolutely critical for the sustainability of all maize production systems. Host-resistance to crop-threatening pathogens is likely to remain the primary method of achieving effective and environmentally friendly disease management (Pratt and Gordon, 2006).

Maize breeders must prioritize the pathogens they consider most likely to cause economic damage. Some viral pathogens of maize are widespread, but the majority tend to be prevalent only in particular regions, or during certain seasons, due to environmental factors and the distribution of suitable insect vectors (Table 1). Many viruses occur only in a circumscribed region and hence may attract only limited research or product development efforts. A new virus, or virus strain, may also suddenly become a serious pathogen. Breeding for improved virus resistance also engenders major issues because insects transmit most viruses, and breeders frequently must confront the need for improving host-resistance without adequate information regarding the genetics of resistance or an understanding of vector biology. All of the requirements for resources and organization that complicate the identification and mapping of resistance are even more problematic for breeders because of the need for increasing scale while improving cost-efficiency in disease evaluations. The easiest approach for selection is to utilize disease nurseries in "hot spots," but frequently these are not sufficiently reliable to ensure positive gain through selection. Development of sites with consistent severe disease pressure allowed for effective MMV resistance evaluation (Ming et al., 1997), but this approach was not as successful for MSV or MCDV (Scott, 1983; Efron et al., 1989). Only an understanding of the "disease tetrahedron" can enable a breeder to appreciate the many ramifications associated with maintenance and increase of viral inoculum, inoculation procedures, timing and method of disease severity assessment, and recognition of typical symptoms. Cooperation with geneticists, and expert guidance from pathologists and entomologists is essential.

Even when inoculation methods reliably infect susceptible hosts, the timing of infection relative to the maturity of the host or environmental conditions can significantly impact the severity of expressed symptoms. The breeder must determine if incidence or disease severity assessment will be the more beneficial for selection purposes. In addition, genotype specific symptoms may be observed and one must

always assume that differential host responses to the insect vector are not in effect. These challenges are often addressed by the development of controlled inoculation procedures in the greenhouse or growth chamber as previously discussed. When such measures are taken, it then begs the question whether or not the genotypic responses obtained under controlled environmental conditions will predict the host responses observed when the plants are grown under field conditions. For example, an evaluation of resistance to MDMV and SCMV (then known as MDMV-A and MDMV-B, respectively) conducted in field and greenhouse tests indicated that the inbred Mo18W, which was resistant to MDMV in earlier field studies (Zuber, 1973), was susceptible to the when inoculations were made on younger plants in the greenhouse (Louie et al., 1990). Scott and Louie (1996) compared greenhouse and field resistance evaluations of the same lines using controlled inoculation procedures. Their study confirmed that lines from diverse breeding populations showing low levels of infection in the field were frequently rated susceptible in greenhouse assays. However, high levels of resistance of several breeding lines were also confirmed in the greenhouse assay. Pratt et al. (1994) assessed symptom severity in maize inbreds and hybrids after controlled inoculation with MCDV using a visual rating for three symptom types (vs incidence) in greenhouse and field experiments. In the field, host responses were not as severe as those as those displayed by plants grown in the greenhouse, although the visual rating protocol grouped genotypes into similar host response categories in both environments. During one season, symptom severity was correlated with both yield and plant height reduction of hybrids, suggesting that meaningful selection could be practiced in the greenhouse.

Single, major genes or QTLs are relatively easy to select in breeding programs, but these may not perform consistently in different genetic backgrounds or diverse environments, they may be linked to undesirable alleles for other traits, and their potential lifespan following deployment will always be a concern. “Conversion” of susceptible lines to resistance by successive backcrossing and phenotypic or marker-assisted selection of dominant major genes, such as *Mdm1*, should be relatively straightforward. *Mdm1* confers high levels of resistance, is highly heritable, and does not appear to have strong linkage to detrimental alleles for other important traits. Studies in this regard are limited, but field-resistance to either or MDMV or SCMV was not strongly associated with yield (<–0.30 genotypic correlation) and reduced incidence of virus infection was achieved during several cycles of recurrent selection of improvement for several agronomic traits, including yield (Jones et al., 1993).

To reduce the risk of “defeat” of genes such as *Mdm1* or *msv1* by pathogen mutations, breeders should be mindful of the need to pyramid multiple resistance genes whenever multiple genes or QTL are known or partial (quantitative) resistance has been identified and characterized. The need to select for multiple genes changes the approach to conducting a breeding program – especially by dramatically raising the number of progenies that must be screened to have a reasonable probability of identifying the few segregants that result from multiple, favorable recombination events that bring together resistance factors in an agronomically desirable individual (Pratt et al., 2003). Genetic engineering technology offers considerable promise for “direct” transfer of resistance constructs, but the evaluation

and successful integration of either constructs, candidate genes or QTL will always be more than a "numbers game" whereby genes are "piggybacked" into the myriad of traits that comprise a successful variety. The development of improved virus resistance will require effective selection pressure be maintained across cycles of selection – which implies that inexpensive and reproducible phenotyping can be achieved. Achieving this will require ongoing, multi-disciplinary cooperation.

5 Virus Resistance Genes and Mechanisms

To date, no virus resistance genes have been isolated from maize; however, a number of virus resistance genes have been cloned from other systems, particularly Arabidopsis and Solanaceous species (reviewed in Kang et al., 2005). Several genes that behave as dominant "R" genes were isolated using positional cloning or transposon tagging. Most of these genes encode proteins with coiled coil (CC), Toll/interleukin1 receptor (TIR) or leucine zipper (LZ) domains coupled to nucleotide binding site-leucine rich repeat (NBS-LRR) domains. However, the *RTM1* and *RTM2* genes for *Turnip mosaic virus* resistance in Arabidopsis encode jacalin-like proteins that are expressed only in phloem cells (Whitham et al., 2000; Chisholm et al., 2000, 2001). Resistance gene analogs (RGA) have been identified in maize using different approaches (Collins et al., 1998; Xiao et al., 2006), and RGA mapping near *Scm1* and *Scm2* were identified (Quint et al., 2002; Xiao et al., 2007). Although the RGA pic19 was identified as a candidate gene for *Scm1*, clusters of homologous sequences were found on chromosome 6S, complicating construction of high-density maps (Quint et al., 2003).

Because viruses rely on a myriad of host factors for replication, translation and movement, resistance can also arise from the lack of a required plant factor. Here resistance is generally recessive or quantitative (Kang et al., 2005). A well-characterized case involves the interaction between eukaryotic initiation factor 4e (eIF4e) and the potyvirus genomic protein that is required for translation of the viral polyprotein (Nicaise et al., 2003; Stein et al., 2005). Alleles of eIF4e that do not interact with the virus genomic protein act as recessive resistance genes. Interestingly, maize eIF4e (aka eIF6) maps to bin 3.05 in maize (Lawrence et al., 2007), co-localizing it with resistance to several RNA viruses (Table 2).

The hypersensitive response (HR) and extreme resistance (ER) are active virus resistance mechanisms in plants. Both involve pathogen recognition mediated by R genes, are associated with programmed cell death, and are similar to well-studied resistance mechanisms for fungal and bacterial pathogens (Heath, 2000, and Chap. 12). However, in maize active virus resistance associated with dominant or semi-dominant genes has been associated with a lack of systemic virus movement rather than an HR or ER. In several resistant lines and hybrids, including Pa405, MDMV can replicate but systemic movement is limited (Jones and Tolin, 1972; Lei and Agrios, 1986; Law et al., 1989). Preliminary results indicate that MDMV can enter and replicate in all cell types, including vascular cells after vascular puncture or leaf-rub inoculation, but that virus movement was blocked sometime after 3 days post inoculation (Redinbaugh, unpublished results).

Virus resistance in plants is associated with expression of defense response genes (Kachroo, 2006). Two early studies indicated that PR proteins are induced in maize after *Barley stripe virus* inoculation (Nasser et al., 1988, 1990). Shi et al. (2005b, 2006) identified transcripts differentially expressed in the responses of resistant and susceptible germplasm to SCMV using subtractive sequence hybridization (SSH) and microarray analysis. Defense response, transcriptional activation and metabolic genes were significantly up-regulated, with more differentially expressed genes being found at longer times post inoculation. Virus induced genes fell into all functional categories, with a major category being "unknown function." Gene expression in the MDMV and SCMV resistant line Pa405 had more in common with the susceptible lines than with European SCMV-resistant lines (Shi et al., 2005a). This suggests either different resistance mechanisms between the two types of lines or that resistance is associated with a small subset of differentially expressed transcripts.

6 Alternatives to Naturally Occurring Resistance in Maize

6.1 Genes from Closely Related Species

Introgression of genes from crop wild relatives has been used to develop disease resistant plants (Hajjar and Hodgkin, 2007), and the incorporation of virus resistance from closely related *Solanum* species was used to develop potatoes with extreme resistance to *Potato virus Y* (Flis et al., 2005). Although this approach has not been successful for introgression of traits into maize thus far, perennial teosintes (*Z. diploperennis* and *Z. perennis*) are resistant to several maize viruses (Nault et al., 1982). An attempt to develop MCDV resistance in backcross progeny from a *Z. diploperennis* x *Z. mays mays* cross was ultimately not successful (Findley et al., 1982).

6.2 Insect Resistance

For viruses that are transmitted by insects in a semi-persistent or persistent manner, the host plant is frequently colonized by the vector, and chemical control of the vector can effectively control virus infection (Perring et al., 1999). In contrast, no intimate interaction between vector and host is needed for non-persistently transmitted viruses such as potyviruses, as acquisition and transmission can occur within a few seconds or minutes. It is likely that host insect resistance would also provide virus disease control for persistently and semi-persistently transmitted viruses. Moderate field resistance to MStV and MMV could be overcome using a large number of viruliferous planthoppers (Dintinger et al., 2005a), suggesting resistance to the vector rather than the virus. In fact, MStV resistance QTL on chromosome 2 co-localized with various insect resistance loci (Dintinger et al., 2005b). Insect resistant lines also had field resistance to MSV (Bosque-Pérez, 2000).

6.3 Pathogen-Derived Virus Resistance

Pathogen-derived resistance (PDR), in which expression of viral transgenes in transformed plants interferes with virus replication, has been demonstrated for a number of viruses and in a wide range of plant hosts (Goldbach et al., 2003). PDR resistance varies among transformants, and does not always correspond to the number of insertion events, or to the accumulation of transgene RNA or protein. In some cases, expression of the viral protein appears to be important for resistance, but in many cases resistance is associated with RNA interference (Lindbo and Dougherty, 2005). A transgenic line expressing high levels of the SCMV coat protein had reduced incidence of infection and symptoms after inoculation with SCMV or MDMV (Murry et al., 1993), and maize transformed with the MRFV coat protein was virus resistant (Valdez et al., 2004). Maize and *Digitaria* transformed with constructs expressing a fragment of the MSV Rep gene were morphologically normal, fertile and MSV resistant (Shepherd et al., 2007a, 2007b). Because accumulation of the transgene RNA was associated with virus resistance, resistance appears to be associated with expression of the truncated Rep protein. Clearly, PDR can be effective for producing virus resistant maize; however, as with other crops, there may be political and environmental constraints to its widespread adoption (Pilson and Prendeville, 2004).

7 Concluding Remarks

Thus far, naturally occurring moderate to strong resistance to each of the economically important maize-infecting viruses, and genes or QTL associated with that resistance, have been identified in maize. Several alternative approaches, including vector resistance, PDR and wide species crosses, hold potential for developing virus resistant varieties should viral pathogens emerge for which no naturally occurring resistance can be identified. Little is known about the molecular mechanisms associated with virus resistance in maize or the molecular properties of genes and QTL that confer resistance. With continued rapid development of tools for investigation of the maize genome and isolation of specific maize genes, there is little doubt that information in these areas will be forthcoming.

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Genetics and Biochemistry of Insect Resistance in Maize

Michael D. McMullen, Monika Frey and Jörg Degenhardt

Abstract Insects are a major concern for maize production worldwide. Host plant resistance to insects involves a number of chemical and biochemical factors that limit, but rarely eliminate insect damage. Most chemical and many biochemical factors involved in resistance to insects are synthesized independent of the pest as phytoanticipines. These factors are stored in sequestered forms that are modified to active structures upon insect infestation and tissue damage. Because the genetic basis of varietal responses to insects for maize is quantitative in nature, quantitative trait locus analysis has been a standard approach to describe insect resistance. These studies often examined correlated biochemical traits to link genetic loci with biological mechanisms. Recently there has been a realization of the importance of herbivore enemies that are attracted by maize in response to herbivore damage. Upon infestation, maize releases volatile chemicals to actively recruit parasitic wasps or nematodes to combat the insect pests. In this review we examine the current state of knowledge of the biochemical, genetic and plant-insect tritrophic mechanisms involved in maize resistance to insect pests.

1 Introduction

Maize, like all other crop species, suffers damage from a large number of insect pests (Dicke, 1977). Insects are a particularly acute problem for maize production in tropical regions. Genetic differences in the host plant response of maize varieties to insects are almost exclusively quantitative in nature. In this review we will summaries the current state of knowledge of maize response to insect attack in three different areas, biochemical basis of resistance, genetic basis of resistance and the rapidly expanding knowledge on maize-insect tritrophic interactions. We will limit ourselves to native plant resistance and leave the extensive topic of transgenic resistance mediated by the expression of foreign proteins to other authors of these volumes (Vol. 2, Chap. 3).

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2 Biochemistry of Resistance

2.1 Chemical Defense

Plants produce hundreds of thousands of unique low-mass natural products, known as secondary metabolites. Secondary metabolites are distinct from components of primary metabolism in that they are generally non-essential for basic metabolic processes of the plant, but improve defense against microbial attack, herbivore predation and control allelopathic interactions. A common feature of many of these compounds is that they have a chronic rather than an acute toxicity on insects, and their effects are less dramatic than those of the synthetic insecticides. Maize host-plant resistance to corn earworm (*Helicoverpa zea*) (CEW) has been attributed to the presence of the secondary metabolites C-glycosyl flavone maysin (2”-*O*-α-L-rhamnosyl-6-*C*-(6-deoxy-*xylo*-hexos-4-ulosyl) luteolin) and the phenylpropanoid product chlorogenic acid in silk (see Sect. 3.3). The first brood of the European cornborer (*Ostrinia nubilalis*) (ECB) is controlled by high levels of the benzoxazinoid DIMBOA in seedlings and young plants (Klun et al., 1970). These secondary metabolites are produced independent of the presence of the pest in a tissue- and developmental-specific manner. In addition to secondary metabolites, ubiquitous phenolic acids, especially ferulic acid, may contribute to insect resistance in maize.

The biosynthesis of these defense-related metabolites has a common root in the shikimic acid pathway (Fig. 1). Maysin, chlorogenic acid and phenolic acids originate from the phenylalanine branch of the pathway, biosynthesis of benzoxazinoids shares intermediates with the tryptophan metabolism.

2.1.1 Benzoxazinoids

Natural benzoxazinoids were discovered in 1960 in rye when resistance against fungi was investigated (Virtanen and Hietala, 1960). Benzoxazinoids have been predominantly found in genera of the Gramineae. Outside the grasses, they have been isolated from several species of the Acanthaceae, Ranunculaceae and Scrophulariaceae. In rye and wild barley species DIBOA (2,4-dihydroxy-2*H*-1, 4-benzoxazin-3(4*H*)-one) is the predominant aglucone moiety, in maize and wheat it is the methoxy derivative DIMBOA (2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one) that prevails (Fig. 1). Benzoxazinoids are stored as D-glucosides. The glucosides are compounds of low toxicity that can undergo enzymatic and chemical degradation. In the intact plant cell, benzoxazinoid-glucosides and the specific ß-glucosidase are kept in two different compartments, the vacuole and the plastid, respectively (Babcock and Esen, 1994). In maize the enzymatic release of the aglucone by the ß-glucosidase after wounding followed by disintegration of the cell compartments, is completed within half an hour. In the case of benzoxazinoid synthesis, plants implement the concept of phytoanticipines: biosynthesis is independent of the presence of the pest, an alleviated product is stored and activated

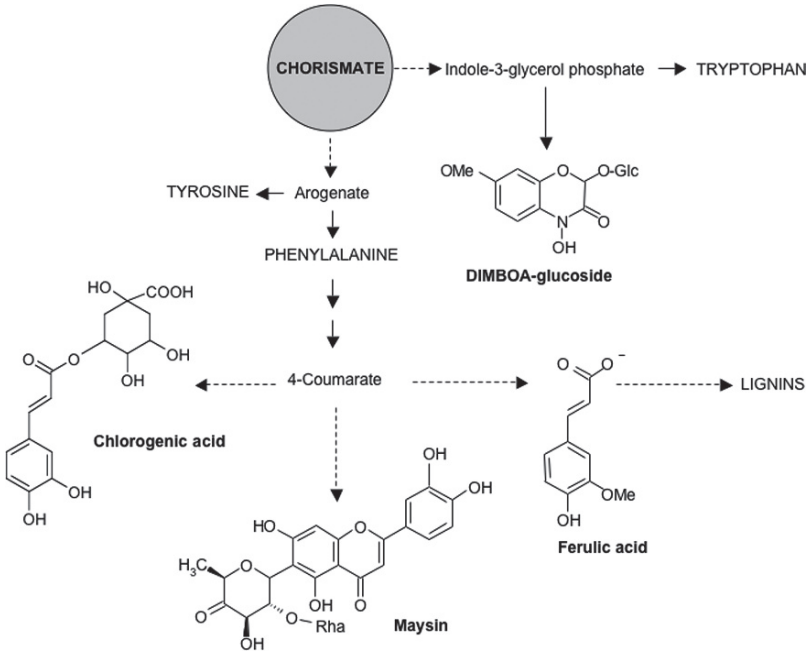

Fig. 1 Biosynthetic relationship between defense-related maize metabolities. Chorismate, synthesized by the shikimic acid pathway is the central intermediate. Chlorogenic acid, maysin and ferulic acid share the steps of the phenylpropanoid pathway to 4-coumarate and the tryptophan branch of aromatic acid biosynthesis. Ferulic acid is a general intermediate of the lignin biosynthesis, a primary biosynthetic pathway. Chlorogenic acid, maysin and DIMBOA are secondary metabolites. The primary metabolites are given in capitals

following infestation. DIMBOA-glucoside content in corn seedlings can reach concentrations up to 10 mmoles per kg of fresh weight (Long et al., 1975).

Benzoxazinoids control a wide range of pathogens and pests (bacteria, fungi, and insects) due to the general toxicity of the compound. DIMBOA is an enzyme inhibitor of α-chymotrypsin (Cuevas et al., 1990), aphid cholinesterase (Cuevas and Niemeyer, 1993) and plasma membrane H^+ -ATPase (Friebe et al., 1997). The hemiacetal DIMBOA undergoes an oxo-cyclo tautomerization. The aldehyde group of the oxo-form reacts with the ε-NH2 group of N-α-acetyl lysine, a model substrate for lysine residues in proteins (Perez and Niemeyer, 1989). The 7-MeO-group facilitates N-O bond heterolysis and hence the formation of a reactive, multicentered cationic electrophile (Hashimoto and Shudo, 1996). The presence of 7-MeO group might open yet another mode of action: it has been demonstrated that dehydration of DIMBOA generates a reactive formyl donor towards $-NH_2$, -OH, and -SH groups (Hofmann and Sicker, 1999).

The correlation between DIMBOA content and protection against insect feeding damage was assayed by two approaches: larval development and insect performance were analyzed on DIMBOA diets (Campos et al., 1989) and insect damage was monitored on high and low DIMBOA cultivars (Barry et al., 1994). These investigations demonstrated that DIMBOA can act as feeding deterrent and reduce the viability of insect larvae. The genetic basis of this resistance was demonstrated and the DIMBOA content was successively elevated in breeding programs (Klun et al.,, 1970, Grombacher et al., 1989). A *benzoxazinless* mutant defective in DIMBOA-biosynthesis was isolated in 1964 by Hamilton. The respective gene, *Bx1* was molecularly identified by directed transposon tagging using the *Mutator* transposon system of maize (Frey et al., 1997). *Bx1* is homologous to the alpha-subunit of tryptophan synthase and catalyzes the formation of indole. The sequence and gene structure of *Bx1* proves that this branchpoint gene of benzoxazinoid biosynthesis has its evolutionary origin in a duplication and subsequent modification of the *TSA* gene of the primary metabolism. In a separate study, BX1 enzyme was given the name indole synthase (Melanson et al., 1997). The introduction of four oxygen atoms into the indole moiety that yields DIBOA is catalyzed by four cytochrome P450-dependent monooxygenases *Bx2* through *Bx5* (see Vol 2, Genes and Gene Families, 3. P450s), a further oxygen function is introduced by *Bx6*, a 2-oxoglutarate dependent dioxygenase (Frey et al., 2003). Biosynthesis of the aglucone entity is completed by the *O*-methyltransferase *Bx7*. Two glucosyltransferases, *Bx8* and *Bx9* (von Rad et al., 2001) function to detoxify DIMBOA. All DIMBOA-glucoside biosynthesis genes are located on the short arm of chromosome 4, *Bx1* to *Bx6* and *Bx8* map within 6 cM. These biosynthetic genes may account for the major QTL for first brood ECB resistance at this position. Orthologs to the maize genes *Bx1* to *Bx5* have been isolated from wheat and a wild barley species (Nomura et al., 2003, 2005; Grün et al., 2005), and phylogenetic analysis reveals that benzoxazinoid biosynthesis in the grasses has a monophyletic origin. Regulatory genes of the pathway have not been identified.

2.1.2 Phenolic Acids and Cell Wall Components

Plants contain significant quantities of various polyphenolic acids, as well as their glycosides and esters. These compounds are implicated in two defense concepts, the phenolic fortification of cell walls and the deterrent effect of fiber content. Main components that strengthen the cell wall as mechanical barrier are (*E*)-ferulic and (*E*)-*p*-coumaric acid which are attached to hemicellulose through pentose sugars. Dimers of these bound phenolic acids can be generated enzymatically by peroxidase (5, 5'-diferulic acid) or through photochemical reactions (truxillic and trucinic acids). Formation of such dimers may increase the mechanical strength of the cell wall by the cross-linking of hemicellulose and hence reduce degradability (Bergvinson et al., 1995). Free phenols, mainly 4-coumaric and ferulic acid, were implicated as factors contributing to resistance of maize against ECB and the maize weevil (*Sitophilus zeamais*), and recently, to pink stalk borer

(*Sesamia nonagrioides*) (Santiago et al., 2006). Interestingly, the transgenic expression of wheat oxalate oxidase in maize significantly increased the phenolic concentrations. The highest increase was detected for ferulic acid. Field testing showed that the transgenic maize exhibited more resistance to ECB than the non-transgenic counterpart. Since a negative correlation between ferulic acid concentration in meridic diets and larval growth rate was found, it may be speculated that ferulic acid content is a main factor of resistance for these plants. The level of DIMBOA was decreased in the transgenic plant, possibly due to diversion of metabolism to the phenolics (Fig. 1). It is suggested that transgenic oxalate oxidase elicits defense responses by generation of H_2O_2 and activating jasmonic acid signaling (Mao et al., 2007).

The phenolic acid esters chlorogenic acid and the C-glycosyl flavone maysin have been implicated in CEW antibiosis (Sect. 3.3, Fig. 1). Both compounds were found to be feeding deterrents. For chlorogenic acid an inhibition of development and severe impairment of larval growth was demonstrated in feeding studies with *Spodoptera litura*. Foliar enzymes such as polyphenol oxidases and peroxidases increase the inhibitory effect by generation of chlorogenoquinone which in turn alkylates dietary protein and reduces its nutritional value. Covalent binding of ubiquinones to nucleophilic (-SH and -NH2) groups of proteins, peptides, and amino acids has been demonstrated and may account for deleterious effects of oxidized derivatives of chlorogenic acid (Stevenson et al., 1993).

2.2 Defense-Related Proteins

2.2.1 Maize Proteinase Inhibitor and Cysteine Proteinase

Protease inhibitors are synthesized and stored in seeds and tubers of plants. However, expression of some proteinase inhibitor genes is induced in response to mechanical wounding and insect damage. Local and systemic induction of expression of MPI, a maize protease inhibitor gene, was described by Cordero et al. (1994). MPI efficiently inhibits elastase and chymotrypsin-like activities from the larval midgut of *Spodoptera littoralis*. Hence, mode of action and expression profile suggests that MPI is a factor of maize insect resistance.

Representatives of tropical germplasm were found to exhibit resistance to Lepidoptera. In these lines, larval feeding led to the induction of a unique cysteine proteinase, Mir1-CP. Elevated Mir1-CP levels were found in the whorl in response to larval feeding (Pechan et al., 2000). Mir1-CP accumulates at the feeding site and is localized predominantly in the phloem of minor and intermediate veins (Lopez et al., 2007). Proteinase accumulation was correlated with a significant reduction in larval growth. A deleterious effect of Mir1-CP containing diet on the protective layer in the mid-gut of the larvae, the peritrophic matrix, has been detected. Consequently, nutrient utilization may be impaired and may account for the observed reduction in growth. Induction of gene expression by wounding is fast for

MPI and Mir1-CP, suggesting that signaling may be connected to the jasmonic acid pathway. In both cases induction levels are enhanced by damage caused by larval feeding, compared to mechanical wounding. An elicitor responsible for enhanced expression has not been identified.

2.2.2 Maize Ribosome-inactivating Proteins

Ribosome-inactivating proteins, (RIPs), share a site-specific RNA *N*-glycosidase activity and depurinate a universally conserved adenine residue of the large ribosomal RNA. RIPs impair susceptible ribosomes in translational elongation processes. RIPs are expressed by a wide variety of plants, an example of a highly toxic RIP is ricin from castor beans. Maize *Rip3:1* (old denomination *b-32*) is an abundant *Opaque-2*-regulated protein (>1 mg/g endosperm) associated with endosperm development. RIP3:1 requires proteolysis for activation. Proteolytic cleavage occurs during germination, but it has been shown that activation can also be performed by cysteine proteases found in several caterpillars and beetles. The association of increased insect susceptibility with RIP3:1 deficiency in *opaque-2* lines has led to suggestions that RIP can play a defensive role against insects. Effects of diets including activated RIP3:1 on mortality and weight gain of a wide range of caterpillars were assayed. Caterpillar susceptibility to the activated maize RIP appeared to be related to host adaptation, e. g. *Trichoplusia ni*, which does not feed on maize, was most severely affected (Dowd et al., 1998). The activated RIP protein is relatively stable to digestion by adversely affected caterpillar species. In a transgenic assay in tobacco, enhanced resistance to *Helicoverpa zea* was generated by constitutive expression of the activated maize RIP3:1 (Dowd et al., 2003). This finding provides support for the assumption that maize RIP plays a role in resistance to maize-feeding insects. Characteristics of RIP3:1, the developmentally regulated expression and synthesis of a non-toxic precursor, are reminiscent of the concept of phytoanticipine in the chemical defense strategy.

3 Genetics of Insect Resistance in Maize

3.1 QTL for Resistance to Tropical and Subtropical Maize Leaf Feeding Insects

Quantitative trait locus analysis has been used to examine the genetic basis of resistance in maize to the leaf feeding damage from tropical and subtropical insect pests of maize. The insects studied have included southwestern corn borer (SWCB), *Diatraea grandiosella*; sugarcane borer (SCB), *Diatraea saccharalis*; and the fall armyworm (FAW), *Spodoptera frugiperda*. SWCB and SCB were the focus of studies conducted at CIMMYT in collaboration with the laboratory of

A. E. Melchinger and SWCB and FAW by the USDA group at Mississippi State. The CIMMYT group used CML139 (Khairallah et al., 1998, Groh et al, 1998) and CML67 (Bohn et al., 1996, 1997; Groh et al., 1998) as the resistant parents to develop both F_2:F_3 (F_2-derived F_3 families) and RIL populations. A comparison of the QTL results across insects, populations and population structures (F_2:F_3 vs RIL) led to the following general conclusions. Within any particular experiment between 3 and 10 QTL of small to moderate effect were detected for a particular insect. There was often a good correspondence of QTLs between insects within a given experiment. Bohn et al. (1997) reported that in the CML131 × CML67 F_2:F_3 population, seven of 10 QTLs were shared for SWCB and SCB, likewise in the RIL population of the same cross all eight of the QTL for SCB were in common with the nine QTL for SWCB (Groh et al., 1998). In contrast to this high degree of pleiotrophy against the different insects, the commonality of QTL between population structures and resistant sources was much more limited. In comparing RIL to F_2:F_3 population structures for CML131 × CML67, four and five QTL were shared for SWCB and SCB (Bohn et al., 1997; Groh et al., 1998). For Ki3 × CML139 only one QTL for SWCB was shared between F_2:F_3 and RIL populations (Khairallah et al., 1998, Groh et al., 1998). In comparing resistant sources, for F_2:F_3 populations there were three shared QTL for resistance from CML139 versus CML67, located on chromosomes 5 (bins 5.06 and 5.07), and 9 (bin 9.05). In maize, the "bin" is a region of the genome of 15–20 cM bounded by specific markers (Gardiner et al., 1993). For the RIL populations only two QTL were in common, located on chromosomes 1 and 8. Groh et al. (1998) determined QTL for protein concentration and leaf toughness in the RIL populations. Although the genetic and phenotypic correlations of these traits with leaf feeding damage by SWCB and SCB were only low to moderate, a surprising number of common QTL positions were found suggesting that these traits may affect resistance. After re-evaluating all of these experiments by cross validation and independent sampling techniques, Bohn et al. (2001) expressed concern that the weaknesses in QTL detection across experiments and environment would limit potential to apply the results of these experiments by marker assisted selection (MAS). Willcox et al. (2002) conducted a pilot MAS experiment using CML67 as the resistance source and found that MAS was as effective as conventional selection (CS) and both selection procedures resulted in significant improvement in resistance over the susceptible parent, CML204, validating the prior QTL results.

Using the resistance sources Mp704 and Mp708, Brooks et al. (2005, 2007) mapped QTL for leaf feeding resistance to SWCB and FAW. These authors found that QTL on chromosomes 6, 7 and 9 were consistent for resistance to both insects and postulate that the candidate genes are the *mir* cysteine proteinase gene family (see Sect. 2.2) on chromosome 6 and the *Glossly15* gene on chromosome 9. *Glossly15* controls the adult to juvenile transition and adult leaves reduce FAW and SWCB growth and survival (Williams et al., 1998). Considering all of these studies on leaf feeding resistance, chromosomes 1, 5, 6, 7, 9 are commonly involved in resistance by what appears to be a number of different biological mechanisms.

3.2 QTL for Resistance to European Corn Borer

In the US Corn Belt there are two generations of ECB per season. Damage by the first generation ECB (1ECB) is predominately leaf-feeding on whorl stage plants. Damage by the second generation ECB (2ECB) is by sheath feeding and stalk and shank tunneling leading to stalk breakage and ear drop. In Europe there is one generation ECB that in Central Europe begins around anthesis and is similar to the second generation in the US. Schön et al. (1991) and Jampatong et al. (2002) mapped QTLs for resistance to the 1ECB using H99 and Mo47 as resistance sources. They reported common QTL in bins 1.06 and 6.02. Jampatong et al. (2002) also reported many QTL in the same chromosomal locations as QTL to the tropical leaf feeding insects suggesting a common genetic control. Jampatong et al. (2002) reported a large QTL in bin 4.01 consistent with the position of *bx* genes for DIMBOA synthesis. Resistance to 1ECB in temperate material is commonly related to DIMBOA levels in mid-whorl leaf tissue (Barry et al., 1994). Because of the established relationship of DIMBOA to 1ECB resistance, progress in selecting for 1ECB resistance has been relatively successful.

The issue of greater importance is selecting for resistance to the 2ECB stalk tunneling. This is reflected in the large number of QTL studies to determine the genetic basis of 2ECB resistance and to attempt to discover the biochemical basis of resistance. The sources of 2ECB resistance include the US temperate inbreds B52 (Cardinal et al., 2001, 2003; Cardinal and Lee, 2005) and De811 (Krakowsky et al., 2004, 2007), the European temperate line D06 (Bohn et al., 2000; Papst et al., 2004) and the temperate/tropical line Mo47 (Jampatong et al., 2002). Although each individual study generally identifies 3–10 QTL, some common themes have emerged. (1) Corresponding QTL positions are present among many of the studies, particularly on chromosomes 1, 5, 9. This result suggests that there may be some common biochemical basis for resistance among the different resistance sources. (2) QTL positions for tunnel length often overlap QTL for cell wall composition (CWC) traits. Cardinal and Lee (2005) reported that 10 of 13 QTL for tunnel resistance from B52 correspond with QTL for one or more of the CWC they studied. Krakowsky et al. (2007) found that nine of 10 QTL for tunneling resistance from DE811 co-localized with CWC QTL, although the direction of genetics effect often differed from that expected for resistance. Because of complex correlations among different CWC traits, it is difficult to identify specific biochemical constituents responsible for resistance. Papst et al. (2004) have proposed to use a candidate gene based association approach to attempt to clarify the resistance mechanisms controlled by the common QTL. (3) Many authors reflected on the overlap of QTL regions for tunneling resistance to ECB and leaf feeding resistance to tropical insects (Bohn et al., 2001; Cardinal et al., 2001; Jampatong et al., 2002) leading to speculation that leaf toughness traits for leaf feeding and CWC traits for 2ECB may share biochemical mechanisms. Flint-Garcia et al. (2003) demonstrated that MAS

using QTL for 2ECB can be as effective in selection as CS. Although the selection gains were relatively small, the results validate the QTL studies and suggest greater progress will be possible once the genes underlying the QTL are isolated and biochemical mechanisms elucidated.

3.3 Maysin and Corn Earworm Resistance

3.3.1 Genetic Regulation of Maysin Synthesis

The CEW is a major insect pest of maize in the Americas (Ortega et al., 1980). The adult CEW moths lay eggs on maize silks and the larvae access the kernels by feeding through the silk channel. Progress in understanding the chemical basis of native resistance to CEW began with the isolation of a C-glycosyl flavone, named maysin (Fig. 1), from silks of a CEW resistant Mexican landrace "Zapalote Chico" (Waiss et al., 1979, Ellinger et al., 1980). Snook et al. (1989, 1994) developed reversed phase high-performance liquid chromatographic procedures to quantify the amount of maysin and chlorogenic acid in maize silks. The biological effects of maysin, related flavones and chlorogenic acid on CEW growth and development were established through the development of artificial diet assays (Wiseman et al., 1992). The mechanism of antibiosis is an anti-nutritive effect (Summers and Felton, 1994).

The basic approach to understanding the genetic control of maysin synthesis was to develop F_2 or F_2:F_3 populations made by crossing maize lines known to differ in maysin levels and to conduct QTL analysis on the silk maysin concentrations or antibiosis as measured in the artificial diet bioassay. Traditionally, the interpretation of QTL studies has been limited by the lack of information on the metabolic pathways leading to most economic traits. Because the C-glycosyl flavones such as maysin are synthesized as a branch of the well characterized flavonoid pathway, a number of both regulatory and structural genes are known and can be applied as candidate loci in interpreting the QTL results. The synthesis of maysin and the genetic basis of antibiosis to the CEW became a model for understanding the genetic basis of agronomic traits.

The most common QTL for maysin and/or chlorogenic acid is in chromosome bin 1.03. This QTL is also often the largest QTL detected, commonly accounting for 40–60% of the variance in maysin concentration (Byrne et al., 1996, McMullen et al.,, 1998, Butron et al., 2001; Bushman et al., 2002). There is overwhelming evidence that the underlying gene is the *p* locus. The *p* locus encodes a Myb-homologous transcription factor (Grotewold et al., 1991). The *p* locus is complex and can contain two different but related *Myb* factors that were named *p1* and *p2* (Zhang et al., 2000). Zhang et al. (2000) demonstrated that while alleles of *p1* are expressed in pericarp, cob, tassel glumes and silks, *p2* is only expressed in glumes and silk. By studying deletions of *p1* and *p2*, Zhang et al. (2003) demonstrated that both *p1* and *p2* were capable of regulating maysin synthesis in maize silks.

Grotewold et al. (1998) and Bruce et al. (2000) demonstrated that expression of P1 protein in transgenic Black Mexican Sweet cells was sufficient to induce the synthesis of C-glycosyl flavones related to maysin and phenylpropanoids related to chlorogenic acid. A similar result was obtained with transformation of Black Mexican Sweet cells with the P2 protein expressed from a constitutive promoter (Zhang et al., 2003). Transformation of maize plants with a number of different *p1* alleles resulted in the accumulation of maysin in silks at levels sufficient for antibiotic effect (Cocciolone et al., 2005). The tissue specificity of expression of P1 in the transgenic plants was complex and indicative of epigenetic control of specificity. Szalma et al. (2005) conducted an examination of the relationship between *p* regulated phenotypes in maize pericarp, cob and silks, and the presence of *p1* and *p2* alleles in 76 maize lines. The phenotypes are given as a three letter designation as *p-XXX* with the first letter designating red or white pericarp (r or w), the second letter designating red or white cob glumes (r or w) and the third letter designating browning or non-browning silks (b or w). For 26 of 27 *p2* only lines the phenotype was *p-wwb*, that is white pericarps and cobs but presence of flavones in silks. Lines that were *p1* only or *p1* and *p2* could have a number of pericarp and cob phenotypes but all but one expressed the silk browning phenotype, that is, the lines were *p-wrb*, *p-rwb*, *or p-rrb*. Any population that is a contrast between functional and nonfunctional *p* alleles results in a major QTL at bin 1.03. Populations developed by crossing two functional, but distinct, *p* alleles can also result in a QTL in bin 1.03, but of much lower magnitude than the functional versus nonfunctional comparison (Meyer et al., 2007).

Chalcone synthase catalyzes the first committed step in flavonoid biosynthesis and is the branch point between the phenylpropanoid and flavonoid pathways. In maize, chalcone synthase is encoded by the duplicate loci *colorless2* (*c2*), located in bin 4.08 and *white pollen1* (*whp1*), located in bin 2.08. Therefore, the regulation and variation in expression of *c2* and *whp1* may be expected to be manifest as QTL for maysin synthesis. The chromosomal locations of *c2* and *whp1* have often been seen as containing QTL for maysin, related compounds and/or chlorogenic acid. Szalma et al. (2002) developed QTL populations incorporating mutant alleles of *c2* and *whp1* to test the role of each locus in maysin synthesis. Both the *c2* and *whp1* loci had an additive effect on maysin synthesis. Additional evidence that the QTLs in bins 2.08 and 4.08 are *whp1* and *c2* was obtained in gene expression studies by Meyer et al. (2007). In silks, both *whp1* and *c2* mRNA levels depended on *p1* genotype. A major regulatory point in maysin synthesis is P1 protein controlling the level of chalcone synthase, thereby controlling the amount of substrate entering the flavonoid pathway.

The maize anthocyaninless1 (*a1*) locus encodes NADPH dihydroflavonol reductase located in bin 3.09. The A1 protein is not involved in the synthesis of the flavones, but is required for anthocyanins and 3-deoxyanthocyanins which can also be produced in maize silks. It was therefore initially surprising that the *a1* locus was identified as a candidate QTL for maysin (Byrne et al., 1996). In maize silks the 3-deoxyanthocyanins are also regulated by the *p1* locus. McMullen et al. (2001) developed the (W23a1 × GT119)F_2 population to test if precursor shunting between

the 3-deoxyanthocyanin and flavone pathways in maize silks can be the basis of QLT effects for maysin. This population segregated for functional versus non-functional alleles at both *p1* and *a1*. The *p1* locus, *a1* locus and the *p1* × *a1* epistatic interaction were all highly significant QTL for both maysin and 3-deoxyanthocyanins. In the presence of at least one functional allele at *p1*, plants that were homozygous for the recessive *a1* allele accumulated twice the maysin as plants with functional *a1*. This result clearly demonstrated that controlling flux between alternative pathways can appear genetically as a major QTL effect. The basis of the epistatic interaction is also clear. Homozygous recessive *a1* can only induce enhanced maysin in the functional *p1* classes as both pathways must be active. Two sequence polymorphisms in the *a1* promoter were significant by association analysis for maysin suggesting that casual variation for the *a1* QTL may be transcriptional differences modulated by cis regulatory elements (Szalma et al., 2005). The QTL studies on maysin provide one of the best cases for the biological basis of epistatis for QTL effects for any plant system.

3.3.2 Maysin: How Much Is Possible? How Much Is Enough?

The level of maysin in maize silks in 2–3-day-old silks is generally in the range of 0.0–0.8% silk fresh weight (Snook et al., 1994), a very impressive accumulation for a secondary metabolite. Widstrom and Snook (2001) conducted recurrent selection in two populations, exotic populations of maize (EPM) and southern inbreds of maize (SIM), for high maysin levels. Selection was successful with an average 0.2% gain in maysin/per cycle of selection resulting in population means of ~1.5% fresh weight maysin. Meyer et al. (2007) investigated the genetic basis of the high maysin in inbreds derived from these selection populations and demonstrated that the major mechanism involved selection at alleles of *p*, *c2* and *whp1* resulting in increased flux into the flavone pathway. Meyer et al. (2007) also observed that a number of QTLs for high maysin differed between EPM and SIM. A new population was constructed by combining EPM and SIM. A single cycle of selection on this new population resulted in an increase population mean for maysin from 3.02% to 3.39% silk fresh weight (Meyer et al., 2007). There were individuals in the population with greater than 4% silk fresh weight maysin. This result shows how flexible cellular metabolism and physiology are in order to allow this high an accumulation of a "secondary" metabolite.

Based on these genetic studies we know the loci necessary to conduct MAS and develop lines with almost any desired level of silk maysin. Wiseman et al. (1992) demonstrated that 0.2% fresh weight silk maysin caused a 50% reduction in CEW larval weight gain in artificial diet feeding trials. So why has maysin not eliminated CEW as a pest on maize? The answer is that high maysin levels alone are not sufficient for field resistance (Rector et al., 2002). High maysin levels must be combined with tight and extended husk cover to force the CEW larvae to eat the silk rather than bypass the silk and move directly to feeding on kernels (Rector et al., 2002). This requirement for extensive husk cover removes maysin as a practical

control measure for CEW in the major feed grain producing areas of the US. Maize has been intentionally bred for short, open husks to permitted rapid drying of the grain in the field. The one market class of maize where maysin can play a viable role in host plant resistance to CEW is sweet corn, particularly the fresh market (Guo et al., 2001). Husk cover is desirable to protect the physical integrity of the kernels and can be used to force CEW and also FAW to ingest enough silk material to affect growth. Consumer preference for sweet corn is for a very clear pericarp and white cob. This preference would suggest that the best *p* alleles to use to engineer maysin into sweet corn would be *p-wwb* alleles (Szalma et al., 2005). Sweet corn lines with high maysin should gain consumer acceptance and help reduce the amount of chemical pesticides used to control CEW on fresh market sweet corn (Lynch et al., 1999).

4 Maize–Insect Tritrophic Interactions

Tritrophic interactions involving maize, herbivores, and enemies of the herbivores were first demonstrated two decades ago (Turlings et al., 1990). In these interactions, a blend of volatiles emitted from the herbivore-damaged plant attracts natural enemies of the herbivore. This phenomenon has been reported in more than 15 plant species (Dicke, 1999; Kessler and Baldwin, 2002; Meiners and Hilker, 2000). The attraction of herbivore enemies has been shown to benefit the plant by reducing subsequent herbivory and increasing reproductive fitness (Hoballah and Turlings, 1999; Van Loon et al., 2000; Kessler and Baldwin, 2001) although such advantages are not realized in all cases (Coleman et al., 1999). Therefore, these tritrophic interactions were also termed `indirect defense` of the plant (Dicke et al., 1990).

After damage of maize foliage by lepidopteran larvae, the plant releases a complex mixture of volatiles. The volatiles attract females of the parasitic braconid wasp *Cotesia marginiventris* (Hymenoptera), which oviposit on the larvae (Turlings et al., 1990). The parasitized lepidopteran larvae consume less plant material and will die upon emergence of the parasitoid, which can benefit the plant (Hoballah and Turlings, 1999; Hoballah et al., 2004). The composition of the lepidopteran-induced volatiles varies between different lines of maize and teosinte (Gouinguene et al., 2001; Degen et al., 2004) and is influenced strongly by abiotic factors like temperature, light intensity and nutritional status of the plant (Gouinguene and Turlings, 2002).

The maize volatile blend consists of indole, products of the lipoxygenase pathway, and a large number of mono- and sesquiterpenes (Turlings et al., 1990; Köllner et al., 2004). Attempts to identify the compounds which are crucial for the attraction of parasitic wasps have been hampered by the complexity of the blends and the difficulty of obtaining individual compounds with the correct chirality for bioassays (Turlings et al., 1991; D'Alessandro and Turlings, 2005, 2006). Fortunately, identification of genes involved in the biosynthesis of these volatiles has provided

molecular tools to demonstrate which of the compounds are attractive to the parasitic wasp. The major sesquiterpene volatiles of herbivore-induced maize are produced by the terpene synthase TPS10 which is strongly expressed after herbivory by Lepidoptera. TPS10 forms (*E*)-β-farnesene, (*E*)-α-bergamotene, and other herbivory-induced sesquiterpene hydrocarbons from the substrate farnesyl diphosphate (Schnee et al., 2006). Overexpression of TPS10 in *Arabidopsis thaliana* resulted in plants emitting high quantities of TPS10 sesquiterpene products identical to those released by maize. Using these transgenic Arabidopsis plants as odor sources in olfactometric assays showed that females of the parasitoid *Cotesia marginiventris* learn to exploit the TPS10 sesquiterpenes to locate their lepidopteran hosts after prior exposure to these volatiles in association with the host (Schnee et al., 2006). This gene-based dissection of the herbivore-induced volatile blend demonstrates that a single gene such as *tps10* can be sufficient to mediate the indirect defense of maize against herbivore attack. Furthermore, associative learning can also adapt parasitoids to alterations of the herbivore-induced volatile blend by plant species, age and tissue of the plant, and abiotic conditions (Takabayashi et al., 1994; De Moraes et al., 1998; Schmelz et al., 2003; Van den Boom et al., 2004). However, females of *Cotesia marginiventris* are also attracted to the full blend of maize volatiles without prior association, indicating that the blend contains additional attractive compounds that elicit an innate response (Hoballah and Turlings, 2005). Interestingly, the emission of volatiles after herbivore damage is not always beneficial for the maize plant since larvae of FAW, another lepidopteran species, use these volatiles to locate their food plants (Carroll et al., 2006).

Terpene-mediated interactions were not only observed in response to damage of the leaves but also in response to root-feeding herbivores. Larvae of the beetle *Diabrotica virgifera virgifera* (Western corn rootworm) are an important pest of maize. In response to feeding by the larvae, maize roots release a signal that strongly attracts the entomopathogenic nematode *Heterorhabditis megidis* (Boff et al., 2001; Van Tol et al., 2001). The signal released by the maize roots was identified as (*E*)-β-caryophyllene, a sesquiterpene olefin. Most North American maize lines do not release (*E*)-β-caryophyllene from the roots, whereas many European lines and the wild maize ancestor, teosinte, do so in response to *D. v. virgifera* attack. Field experiments showed a fivefold higher nematode infection rate of *D. v. virgifera* larvae on a maize variety that produces the signal than on a variety that does not. Spiking the soil near the latter variety with authentic (*E*)-β-caryophyllene decreased the emergence of adult *D. v. virgifera* to less than half (Rasmann et al., 2005).

In an agricultural setting, the value of indirect defense has been shown by the co-cultivation of maize with an African grass (*Melinis minutiflora*) that releases abundant volatile compounds (Khan et al., 1997, 2000). The proximity of this grass led to a significant reduction in damage to maize plants by lepidopteran larvae due to an increased parasitism by braconid wasps. These results suggest that the manipulation of volatile emission in maize may be a valuable strategy to attract herbivore enemies and thus minimize pest problems in an environmentally safe manner. This strategy might be aided by engineering of maize plants that emit strong, readily detectable volatile signals that match the preferences of particular enemy species

(Degenhardt et al., 2003; Turlings and Ton, 2006). The development of such plants is now feasible due to the elucidation of the pathways responsible for the biosynthesis of volatile compounds. The effectiveness of these tritrophic interactions is most likely in synergy with the direct defenses of the plant (e.g., toxins or feeding deterrents) which extend the time that herbivores remain vulnerable to attack from foraging enemies. Further studies of the interactions between maize, their herbivores and the enemies of their herbivores should provide more clues to facilitate the application of indirect defenses in the cultivation of maize.

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Chilling Stress in Maize Seedlings

Jörg Leipner and Peter Stamp

Abstract Maize is very sensitive to chilling especially during early autotrophic growth. Seemingly, photosynthesis is strongly affected due to the inhibition of certain enzymes of the C_4 and the Calvin cycle. Cold-induced perturbations of phloem loading may have negative feedback effects on photosynthesis, too. The reduced photosynthetic activity promotes dissipative mechanisms and affects the antioxidative defense in maize leaves. Although seedlings can withstand chilling stress without visual symptoms for few days, the development under such conditions results in irreparable damages with developing chloroplasts and the leaf meristem as the first targets. However, development at suboptimal temperature enables seedlings to better withstand further stress probably due to improved dissipative and antioxidative mechanisms. The causal physiological mechanisms for a better chilling tolerance remain still largely unknown; but recently, first QTLs for chilling tolerance of maize seedlings have been identified. Together with the growing amount of information from gene expression studies this may help to finally unravel the mechanism of chilling tolerance.

1 Introduction

As a tropical plant, maize is sensitive to low temperature, despite its origin at altitudes of about 2000 m.a.s.l. Low temperature prolongs growth duration, reduces crop growth rate, and thus weakens the seedling; at the end of the growth cycle frost may terminate grain filling prematurely. Consequently, grain yields are inconsistent and often lower in temperate climates or mountainous regions. Ensuring optimal conditions for germination have been recommended to alleviate the impact of cold on early crop development. At the same time, however, requirements to achieve sustainable soil protection by mulch or no-till systems, which decrease soil temperature and depend on increasingly chilling tolerant genotypes, are being enforced. Maize germinates at temperatures below 10°C. Therefore, a major improvement in low-temperature germination makes sense only if the seedlings are also chilling tolerant.

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Maize seedlings are most sensitive during the transition phase from heterotrophic to autotrophic growth. This indicates that C assimilation is often more affected by low temperature than metabolic processes. High chilling tolerance during autotrophic growth is accompanied by a high relative growth rate, sustained by a high net assimilation rate in spite of a low leaf area ratio. Thus, the emphasis must be placed on photosynthesis. To avoid misinterpretation of the results, it is necessary to define the types of chilling stress. Here *cold* refers to temperature below 5°C, when neither growth nor photosynthesis occur and the plant depends on defense mechanisms to avoid damage and to survive. *Chilling* occurs at 5°C to about 15°C, when plants are still capable of adapting developmental processes in order to survive more unfavorable temperature conditions like during cold spells. As in the literature, *optimal temperature* is the temperature between 20°C and 30°C at which plants develop rapidly; at *suboptimal temperature* (about 15°C) plants can acclimate rapidly but growth is retarded. Furthermore, the developmental stage of the plant must be defined. For example, during the early seedling stage, the leaf development depends strongly on soil temperature as the apex is located a few centimeters below the soil surface. Although cold spells lasting a few minutes to several hours have received a great deal of attention, it is now clear that chilling periods lasting a few days to weeks are more important during seedling establishment since farmers sow at soil temperatures above 8°C; at this time of the season, mean temperatures are at the acclimating level of about 15°C.

2 Physiological Effects of Short-Term Low-Temperature Stress

Plants sense temperature through changes in membrane rigidity, which cause an increase in the cytoplasmic concentration of Ca^{2+}, which functions as a second messenger in plant signaling. Investigations of the cold-induced Ca^{2+} signal in maize have revealed that, in comparison to cold-tolerant wheat, maize cannot quickly restore the cytoplasmic Ca^{2+} concentration, probably due to a decrease in Ca^{2+}-ATPase activity (Jian et al., 1999). A permanently high cytoplasmic Ca^{2+} concentration might lead to damage to the cell. Another sensor of low temperature is the redox state of the cell, which is strongly affected by photosynthetic activity. Disturbance of photosynthesis through low temperature causes the greatest harm because a chilling-induced slowdown of enzymes or reactions involved in photosynthesis affects the redox state and increases the risk of damage to the cell by reactive oxygen species.

2.1 Effects of Chilling on Photosynthesis and Down-Stream Processes

The most obvious response of maize seedlings exposed to low temperature is the reduction of photosynthesis. Several reactions, from the photosynthetic light reaction to the sink of assimilates, are considered to be the main effects of chilling stress.

Temperature *per se* seems to have little effect on the photosynthetic light reaction, as was demonstrated by infiltrating maize leaves with an artificial electron acceptor (Fracheboud and Leipner, 2003). As a result, the fluidity of the thylakoid membrane, known to be affected by temperature (Barber et al., 1984), does not seem to disturb the photosynthetic electron transport chain.

Low temperature affects plants primarily by decreasing the velocity of enzymatic reactions and, consequently, the photosynthetic dark reaction is potentially affected by cold. In maize, the reduction of photosynthetic capacity upon exposure to low temperature might be caused by a decrease in the activity of certain enzymes of the C_4 cycle and/or of the Calvin cycle. For example, Kingston-Smith et al. (1997) observe lower activity of NADP malate dehydrogenase and Rubisco in maize exposed to cold. In the C_4 plant *Sorghum bicolor* the maximum steady state rate of CO_2 fixation by the enzyme phosphoenolpyruvate carboxylase was limited by the re-synthesis of phosphoenolpyruvate (Laisk and Edwards, 1997). This re-synthesis, that is, the phosphorylation of pyruvate to phosphoenolpyruvate, is catalyzed by the enzyme pyruvate orthosphate dikinase (PPDK). Even when precaution was taken to maximize the recovered activity, it has been difficult to demonstrate a higher PPDK activity in leaf extracts from C_4 plants than the maximum rate of photosynthesis of those leaves (Usuda et al., 1984). This, combined with the fact that the enzyme is subjected to a complex mechanism of dark/light-based regulation, has led to the assumption that the PPDK reaction is a key site of regulation of C_4 photosynthesis. Other results indicate that the photosynthetic flux in C_4 plants is predominantly controlled by Rubisco and, to a lesser extent, by PPDK (Sage and Kubien, 2007). Rubisco does not seem to be particularly sensitive to low temperature, but the light-induced increase in its maximum catalytic activity was slowed down under cold conditions, suggesting that circadian- and light-regulated transcription and synthesis of Rubisco were impaired (Kingston-Smith et al., 1997). Transformation of maize with PPDK from *Flaveria brownii*, a C_4 plant, which is known to be more cold-tolerant than maize, resulted in a greater cold tolerance of extractable PPDK than in the wild type (Ohta et al., 2004). However, measurements of photosynthesis *in vivo* showed only marginal differences between transformants and the wild type (Ohta et al., 2006). This may be due to the fact that control of the photosynthetic flux seems to be shared by Rubisco and PPDK.

Although exposure to low temperature results in a decline in the assimilation rate, there was a strong increase in the sugar and starch contents after several days at chilling temperature (Sowinski et al., 1999), indicating effects of chilling downstream of the Calvin cycle. By means of radio labeling, Sowinski et al. (1999) indicated a slowdown in the transport of photoassimilates from the site of synthesis to the phloem. Furthermore, phloem loading is strongly affected by chilling temperature, especially in a chilling sensitive dent as compared to a chilling tolerant flint genotype (Sowinski et al., 1998). Difference in phloem loading capability at chilling temperature between ssp. *indentata* and ssp. *indurata* may be due to structural differences in the vascular bundles (Sowinski et al., 2001). Although phloem loading in maize is apoplasmic, the high number of plasmodesmata between donor cells and companion cells in seedlings of the dent type indicate that the companion cell/thin-walled sieve tube complex is not isolated symplasmically from the adjoining

cells. Since symplasmic transport is particularly sensitive to low temperature, the lower chilling tolerance of ssp. *indentata* was explained by structural differences (Sowinski et al., 2001). As well as the cold-induced slowdown of phloem loading, the transport speed of assimilates in the phloem also decreases when temperature is lowered. However, since the decrease in phloem transport was the same order of magnitude as the decrease in photosynthesis, the accumulation of photosynthates in the leaves does not seem to be due to a decrease in phloem transport (Sowinski et al., 1999).

It is obvious that chilling affects the photosynthesis of maize seedlings downstream of the photosynthetic light reaction. Wherever the location of this primary site is, a coordination between the photosynthetic electron transport and the activity of carbon metabolism is a prerequisite of minimizing the risk of the generation of reactive oxygen species (ROS). The chilling induced slowdown of the Calvin cycle, be it directly or indirectly by negative feedback mechanisms, results in a depletion of ADP and, consequently, in the formation of a large ΔpH across the thylakoid membrane. The lower pH in the thylakoid lumen triggers the down-regulation of photosystem II by the formation of non-photochemical quenching (*NPQ*) and by the de-epoxidation of violaxanthin *via* antheraxanthin to zeaxanthin (Szabó et al., 2005). In maize seedlings, exposure to cold in the light results in the rapid formation of *NPQ* followed by de-epoxidation of the xanthophyll cycle pool (Leipner et al., 1997). The importance of the xanthophyll cycle for photoprotection has been demonstrated in mutants lacking this pigment (Pasini et al., 2005). However, faster de-epoxidation of the xanthophyll cycle pool did not result in a greater cold tolerance of maize seedlings (Leipner et al., 2000a).

2.2 The Role of Antioxidants

The loss of metabolic homeostasis due to adverse environmental factors results in a greater production of reactive oxygen species (ROS) (Suzuki and Mittler, 2006). Exposure of maize seedlings to cold in the dark resulted in a transient increase in hydrogen peroxide (H_2O_2) (Prasad et al., 1994). Furthermore, the chilling induced reduction in photosynthetic activity increases the risk of ROS generation in the event that excess absorbed light energy cannot be dissipated as heat. Therefore, accumulation of ROS is thought to occur when maize seedlings are exposed to low temperature. Furthermore, it was observed that the accumulation of superoxide radicals (O_2^-) in maize leaves after exposure to light becomes more intense as temperature decreases (Ke et al., 2004). However, due to the high reactivity of ROS and, consequently, their short lifespan, the generation of ROS is difficult to quantify. Thus, our knowledge of this fundamental process is incomplete with respect to maize under chilling stress.

Plants possess non-enzymatic and enzymatic scavenging systems, which should keep ROS at a level that is not harmful. Scavenging enzymes operate as ROS scavengers or, rather, they are involved in recycling antioxidants. At the heart of the enzymatic scavenging system stays the so called water-water cycle (Asada, 1999).

In the water-water cycle, O_2^-, which can be generated by the reduction of molecular oxygen at photosystem I by means of the Mehler reaction, is detoxified by a pathway of enzymatic reactions. In the first step, superoxide dismutase catalyzes the dismutation of O_2^- to yield H_2O_2. In the chloroplast, H_2O_2 is detoxified by ascorbate peroxidase with ascorbate as the hydrogen donor. This catalytic removal of H_2O_2 results in the formation of monodehydroascorbate, which can be reduced enzymatically to ascorbate by monodehydroascorbate reductase, with both NADH and NADPH as electron donors. Alternatively, monodehydroascorbate can decay nonenzymatically by spontaneous disproportionation to ascorbate and dehydroascorbate. The enzyme dehydroascorbate reductase catalyzes the reduction of dehydroascorbate by the reduced form of glutathione (GSH) in the chloroplast stroma. The re-reduction of oxidized glutathione (GSSG) is catalyzed by glutathione reductase with NADPH supplying electrons.

A decrease in temperature affects the velocity of most of these reactions. In particular, the activity of glutathione reductase and especially that of dehydroascorbate reductase was lower under cold temperature (Jahnke et al., 1991). For glutathione reductase, a decrease in the assay temperature resulted in a strong decrease in V_{max}, while K_m was less affected (Hull et al., 1997). For ascorbate peroxidase, cold also resulted in a decrease in V_{max} but K_m increased considerably (Hull et al., 1997). At optimal temperature, the activity of glutathione reductase and ascorbate reductase are higher in chilling tolerant than in chilling sensitive genotypes or species of the genus *Zea* (Jahnke et al., 1991; Kocsy et al., 1996; Hull et al., 1997).

Ascorbate and glutathione are the two major antioxidants in plant cells. Whether or to which extent chilling stress affects maize seedlings by depleting or oxidizing these antioxidants seems to depend largely on the studied stress conditions and the developmental stage of the plant (Hodges et al., 1996; Leipner et al., 1997, 2000b). A clear correlation between the level of antioxidants in plants grown at optimal temperature or after a short-term chilling stress with the chilling tolerance of the genotype has not been found (Hodges et al., 1996). Although the amount of ascorbate is usually about ten times higher than that of glutathione in leaves of maize seedlings (Leipner et al., 1997) glutathione seems to play a central role in the redox system of the cell; the GSH/GSSG ratio may function as an ubiquitous regulatory signal (Foyer and Noctor, 2005). The importance of glutathione is supported by the observation that inhibition of glutathione synthesis reduced the chilling tolerance of maize seedlings (Kocsy et al., 2000) while an increase in glutathione synthesis by means of herbicide safeners resulted in a greater chilling tolerance (Kocsy et al., 2001). An artificial increase in ascorbate did not affect tolerance to cold-induced photoinhibition, although, at the same time, it accelerated the de-epoxidation of the xanthophyll cycle pool (Leipner et al., 2000a).

Due to the C_4 syndrome in maize, antioxidants are differentially localized between mesophyll and bundle sheath cells (Doulis et al., 1997). Bundle sheath cells are characterized by high amounts of superoxide dismutase as well as of ascorbate peroxidase. These enzymes are absent in mesophyll cells. On the other hand, mesophyll cells contain glutathione reductase and dehydroascorbate reductase, which are absent in bundle sheath cells. Consequently, the amount of reduced ascorbate and glutathione is higher in mesophyll than in bundle sheath cells. This lack of antioxidants might

explain the greater susceptibility of the bundle sheath cells to oxidative and chilling stress (Kingston-Smith and Foyer, 2000a). It is noteworthy that glutathione reductase transcripts are found in both types of cells but are translated to its protein only in mesophyll cells (Pastori et al., 2000b). However, *de novo* synthesis of glutathione can occur in both cell types (Foyer et al., 2002). After exposure to chilling, the content of GSSG increased in the mesophyll as well as in the bundle sheath cells, but the GSH/GSSG ratio decreased in the bundle sheath cells only (Kopriva et al., 2001).

3 Physiological and Developmental Effects of the Growth of Maize Seedlings at Suboptimal Temperature

The effects of a sudden temperature decrease on physiological processes of maize seedlings, especially on the photosynthesis and antioxidative systems, were investigated extensively under controlled growth conditions. In fact, under field conditions in temperate regions of Europe, maize is exposed permanently to suboptimal rather than to optimal temperature with the occurrence of sudden cold spells. With respect to the physiology and the development of the maize seedling, the effects of suboptimal growth temperature are quite different from the effects of a sudden short-term decrease to low temperature. Suboptimal growth temperature is considered to be a moderate stress under which growth occurs but nonetheless disturbs metabolism and development.

When maize is grown in the field, the efficiency of photosystem II (Φ_{PSII}) decreases during periods of low temperature (Andrews et al., 1995). Because phases of low temperature lead to a sustained reduction in photosynthesis (Stirling et al., 1993) and affect photosynthesis especially in young leaves (Stirling et al., 1991), chilling seems to have a strong effect on the development of the photosynthetic machinery.

3.1 Primary Sites Affected by Suboptimal Growth Temperature

The observation that fully developed leaves are less sensitive to chilling than developing leaves indicates differences in the response of the tissues to chilling, depending on the developmental stage. The shoot apex is very sensitive to low temperature, as shown by seedlings whose root zone was cooled down. As well as a negative effect on the growth rate and leaf expansion (Engels, 1994; Stone et al., 1999) a decrease in the temperature of the root zone also had a negative effect on the photosynthetic performance of maize seedlings, especially in chill-sensitive genotypes (Chassot, 2000; Hund, 2003). In other experiments, however, cooling of the shoot apex resulted in a delay in leaf development but did not significantly affect the efficiency of photosynthesis (Sowinski et al., 2005). The cold-induced growth retardation of the leaves seems to be caused by a strong increase in the duration of the cell cycle and by a reduction of cell production when seedlings are exposed to low temperature (Rymen et al., 2007).

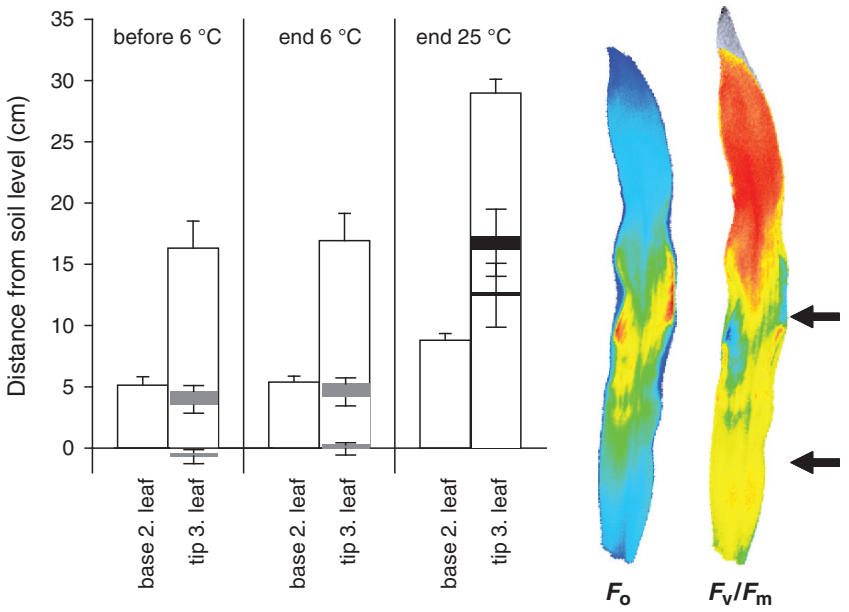

Fig. 1 Cold-induced damage to the third leaf of maize seedlings developed during and after 4 days at 6°C and 400 µmol m^{-2} s^{-1}. *Left:* Spatial location of chlorosis (black bars). The position of the chlorotic area, which became visible after recovery at 25°C, was used to localize the tissue that was most susceptible to chilling (gray bars). *Right:* Damage to the photosystem II reaction center determined by chlorophyll fluorescence images of the minimum fluorescence (F_o) and of the maximum quantum efficiency of photosystem II primary photochemistry (F_v/F_m) after recovery at 25°C. High values (red) of F_o and low values (blue) of F_v/F_m indicate damage to photosystem II. The cold-induced damage on the third leaf originated from the leaf tissue which was positioned, at the time of the cold treatment, at the height of the base of the second leaf (upper arrow) and at 1–2 cm above soil level (soil level indicated by lower arrow) (Leipner, unpublished results)

Spatial analysis of leaf blades, which were exposed to cold (6°C, 400 µmol m^{-2} s^{-1}) for 4 days, revealed two zones with chlorosis (Fig. 1). These areas could be tracked back to the meristem of the leaf and the region of the leaf that had just been exposed to light. It is concluded that (1) chilling induces damage during cell production, which prevents the development of fully functional cells, and (2) the combination of light and low temperature leads to the disturbance of chloroplast development.

3.2 Development of the Photosynthetic Apparatus Under Chilling Conditions

Suboptimal growth temperature results in a decrease both in the capacity and efficiency of photosynthesis (Nie et al., 1992), especially in chill-sensitive genotypes (Fracheboud et al., 1999). The weaker photosynthetic performance persists if seedlings that developed under suboptimal temperature are examined under optimal temperature

(Nie et al., 1992; Haldimann, 1996), indicating the occurrence of structural changes during the development of the photosynthetic machinery. Moreover, the photosynthetic activity of leaves that developed under suboptimal temperature and were afterwards exposed to optimal temperature for several days is lower than that of leaves developed continually at optimal temperature (Nie et al., 1995). In contrast to photosynthetic activity, which recovers only slightly after transfer to optimal temperature, the chlorophyll content of these leaves reached almost the chlorophyll content of leaves, which developed continually at optimal temperature. First conclusions about the nature in the changes of the photosynthetic machinery due to suboptimal growth temperature can be drawn from analyses of the pigment composition. Pigment analyses revealed a strong decrease in the chlorophyll *a/b* ratio at suboptimal growth temperature, especially in chill-sensitive genotypes (Haldimann et al., 1995, 1998). Since most of chlorophyll *b* is found in the antenna proteins, especially in the light harvesting complex II (LHCII), these results indicate that the ratio of LHCII to the reaction center (RC) increases at suboptimal growth temperature. A detailed analysis by means of sucrose gradient fractionation and SDS-PAGE confirmed this hypothesis (Caffarri et al., 2005). Furthermore, the number of minor antenna complexes decreased in comparison to LHCII when maize seedlings grew at suboptimal compared to optimal temperature but to a lesser extent than RCII. In particular, plastid-encoded proteins of the reaction centers fail to accumulate in the mesophyll thylakoids when seedlings develop at suboptimal temperature (Nie and Baker, 1991). This lack of certain thylakoid proteins was found in the chloroplasts of the mesophyll and the bundle sheath (Robertson et al., 1993).

The chill-induced reduction of the accumulation of certain thylakoid proteins is also reflected by the atypical ultrastructure of the mesophyll chloroplasts. Mesophyll chloroplasts from leaves that developed at suboptimal temperature are slightly bigger and more round in shape (Robertson et al., 1993; Kutík et al., 2004). Furthermore, extensive vesiculation occurs at low temperature, especially in maize genotypes sensitive to chilling, which results in the disruption of the granal array (Pinhero et al., 1999). Furthermore, growth at suboptimal temperature affects the fatty acid composition of the chloroplast membranes, with an increase in unsaturated fatty acids (Fracheboud, 1999).

With regard to the activity of enzymes from the C_4 cycle and the Calvin cycle, a lower content of Rubisco and phosphoenolpyruvate carboxylase was found in leaves that developed at suboptimal temperature, while the NADP malate dehydrogenase activity increased compared to leaves grown at optimal temperature more pronounced in a chill-sensitive than in a chill-tolerant genotype (Pietrini et al., 1999). However, the chill-induced decrease in the maximal Rubisco activity can be compensated by an increase in its activation state (Kingston-Smith et al., 1999).

Due to the C_4 syndrome, assimilates must be transported between the mesophyll and the bundle sheath cells. Leaf development at suboptimal temperature leads to a larger area between the cells of the mesophyll and the bundle sheath and to a higher number of plasmodesmata between these cell types (Sowinski et al., 2003). This acclimation seems to compensate for the chill-induced slowdown

of diffusion from cell to cell but was less distinct in chill-sensitive compared to a chill-tolerant genotype.

3.3 Consequences of Chill-Induced Changes in the Photosynthetic Machinery

Since pigment protein complexes as well as other components in the thylakoid membrane are not properly assembled when maize seedlings develop at suboptimal temperature, a safe operation of photosynthesis is more difficult. Similar to short-term chilling stress, growth at low temperature results, even at low light intensity, in an excess of absorbed light energy. Therefore, it is not surprising that growth at suboptimal temperature results in the accumulation of xanthophyll cycle pigments in the leaves (Haldimann et al., 1995). Furthermore, the xanthophyll cycle pool can be de-epoxidized to a greater extent in leaves grown at suboptimal than at optimal temperature (Leipner et al., 1997). The lower photosynthetic activity of chill-sensitive genotypes relies on a more efficient dissipation of the excess absorbed light energy as heat; therefore, these genotypes are characterized by higher amounts of xanthophyll cycle pigments (Haldimann, 1998).

It is assumed that suboptimal temperature increases the generation of ROS and, in consequence, causes damage to biological structures. Increasing amounts of H_2O_2 were found with decreasing growth temperature (Kingston-Smith et al., 1999). This increase in ROS was particularly pronounced in mesophyll cells, which are characterized by a higher H_2O_2 content compared to bundle sheath cells (Pastori et al., 2000a). Furthermore, under unfavorable growth conditions in the field, there may be a surplus of ROS (Fryer et al., 1998). However, chill-induced disturbance of the development of the photosynthetic apparatus under low-temperature conditions in the field are not necessarily associated with O_2^- production through the Mehler reaction (Leipner et al., 1999). Nevertheless, the increases in the amount of antioxidants and in the activity of scavenging enzymes indicate that seedlings are under an oxidative stress when they grow at suboptimal temperature. The contents of the three major foliar antioxidants, namely ascorbate, glutathione and α-tocopherol, increased when plants developed at suboptimal temperature (Kocsy et al., 1996; Leipner et al., 1997; Kingston-Smith et al., 1999). The situation is less clear for enzymes involved in the water-water cycle. Suboptimal growth temperature resulted in a higher activity of superoxide dismutase and glutathione reductase (Massacci et al., 1995; Hull et al., 1997; Leipner et al., 2000b). In another study, however, the activity of all the enzymes of the water-water cycle, with the exception of superoxide dismutase, was lower at a growth temperature of 14°C than at 18°C (Kingston-Smith et al., 1999). It is clear that the activity of catalase is largely lower in leaves that developed at suboptimal temperature (Kingston-Smith et al., 1999; Leipner et al., 2000b). The importance of ROS in the stress response is still not fully understood, especially since ROS, like H_2O_2, are important signaling molecules (Foyer et al., 1997) and seem to be essential for growth processes (Rodríguez et al., 2004).

The question also remains as to whether the weaker photosynthetic performance of chill-sensitive genotypes is reflected in a lower biomass accumulation compared to chill-tolerant genotypes. In a comparison of a chill-tolerant (Z7) and a chill-sensitive genotype (Penjalinan), Verheul et al. (1995) found an association between good photosynthetic performance and the relative growth rate in seedlings grown at suboptimal temperature. This was confirmed in a quantitative trait loci (QTL) analysis, which revealed a co-localization of a QTL for photosynthetic performance and a QTL for shoot dry matter in seedlings grown at suboptimal temperature (Fracheboud et al., 2004; Hund et al., 2004). However, this pleiotropic effect was not present when plants developed at optimal temperature. Furthermore, such a co-localization was not present under chilling conditions in the field, neither when seedlings developed under chilling conditions in early spring, nor when they grew later in the season at more optimal temperature (Jompuk et al., 2005). It seems that photosynthesis limits seedling growth only at low temperature combined with low to medium light intensity.

While the persistent depression of photosynthesis at suboptimal growth temperature can have a negative effect on the biomass accumulation of the maize seedlings, it has the beneficial effect that seedlings grown under such conditions are more tolerant to cold-induced photooxidative stress (Leipner et al., 1997) and recover faster from photoinhibition (Haldimann et al., 1996). Furthermore, in some genotypes, seedlings that develop at suboptimal temperature accumulate anthocyanins, which protect the leaves from photoinhibition without limiting photosynthesis (Pietrini et al., 2002). It is still not known which compound or reaction is primarily responsible for the improved chilling tolerance of the acclimated plants.

4 The Role of the Root System During Chilling Stress

When exposed to a sudden cold stress, maize seedlings exhibit symptoms of drought stress due to an imbalance between transpiration and water uptake (Aroca et al., 2003b). In particular, ineffective stomatal control is observed shortly after the onset of cold stress, which is the result of a decrease in the hydraulic conductance of the roots due to the greater viscosity of water at low temperature and to intrinsic characteristics of the root (Melkonian et al., 2004). The physiological activity of roots of chill-sensitive genotypes was marked by a stronger inhibition of water uptake and root respiration than chill-tolerant genotypes when exposed to chilling temperature (Sowinski and Maleszewski, 1989). The chill-induced change in the water status induced the generation of abscisic acid (ABA), especially in chill-tolerant maize genotypes as was demonstrated under controlled conditions (Janowiak et al., 2002) as well as in the field following a cold spell (Janowiak et al., 2003). Furthermore, it was shown that the inhibition of ABA synthesis decreased tolerance to chilling, while the application of ABA increased it (Janowiak et al., 2002). Similar effects were observed when maize seedlings were pretreated with drought; the drought-induced increase in the ABA content resulted in improved chilling tolerance (Pérez de Juan et al., 1997; Aroca et al., 2003a). The way in which ABA affects chilling tolerance, however, remains to be elucidated.

When seedlings develop at low temperature the shoot/root ratio decreases, mainly due to a higher root dry matter at that particular growth stage (Richner et al., 1997). Furthermore, seedlings with a large amount of plant dry matter after growth at suboptimal temperature are characterized by a lower shoot/root ratio and a smaller leaf area/root length ratio (Hund et al., 2007). Although root architecture seems to be a constitutive trait, it correlates with chilling tolerance. The root system of chill-tolerant genotypes tends to be heterogeneous (lateral roots of the primary root are longer than the lateral roots of the seminal roots); the roots of chill-sensitive genotypes, however, exhibit a homogenous root system with primary and seminal roots of similar length (Hund et al., 2007). This indicates that at suboptimal growth temperature, too, the uptake of water or nutrients is disturbed. The uptake of phosphorus in particular seems to be important because of its low mobility; therefore, efficient root growth is essential to achieve adequate chilling tolerance (Chassot and Richner, 2002). The chill-induced decrease in phosphorus uptake may also explain why the leaves of maize seedlings under cold stress often turn purple, which is characteristics of phosphorus deficiency.

5 The Genetic Basis of Chilling Tolerance

There is a large genetic variation in the chilling tolerance in maize. In particular, European flint and highland tropical material are characterized by greater chilling tolerance compared to dent material from the Corn Belt. Efficient breeding for improved chilling tolerance using these materials requires in-depth knowledge of the genetic basis of chilling tolerance. However, the inheritance of chilling tolerance is poorly understood. It is very difficult to deduce chilling tolerance of hybrid maize from the tolerance of its inbreds. The situation is complicated further by maternal effects in particular at early growth stages (Hodges et al., 1997).

5.1 The Genetic Basis of Chilling Tolerance Studied by QTL Analyses

QTL analysis is a useful tool for finding genomic regions responsible for chilling tolerance. Furthermore, it enables us to unravel the interaction of complex traits. Several QTLs for shoot fresh weight at early growth stages were identified under cool and moderately warm conditions (Presterl et al., 2007). Many of these QTLs are associated with leaf chlorosis but an obvious relationship was not found between the relative extent of the QTL effect and temperature conditions during growth. Focusing on photosynthesis-related traits, the main QTLs involved in the functioning of the photosynthetic apparatus were stable across cold environments (Fracheboud et al., 2004; Jompuk et al., 2005; Pimentel et al., 2005). Based on these QTL analyses, relationships were only frequently found between photosynthetic efficiency and greenness of the leaf. This indicates that the amount or the size of the photosynthetic units and their functioning are under different genetic control.

The association between low chlorophyll content and a reduction in photosynthetic efficiency may reflect a disturbance of the assembly of the photosynthetic apparatus, induced by low growth temperature. Consequently, potential candidate genes, which are located near the major QTLs for chilling tolerance of photosynthesis, are genes involved in the assembly of the photosynthetic apparatus or genes of enzymes playing an important role in carbon assimilation (Jompuk et al., 2005).

A disadvantage of QTL experiments is that the results are valid only for the studied population. However, analogies between different populations were found when plants were raised under similar conditions and when the traits characterizing the photosynthetic apparatus were considered, indicating to some extent a common genetic basis of chilling tolerance of photosynthesis. For example, on the short arm of chromosome 2, near the SSR marker *phi109642*, a common QTL was found for leaf greenness, carbon exchange rate and the quantum efficiency of photosystem II (Φ_{PSII}) in seedlings of the ETH-DL3 × ETH-DH7 population grown under suboptimal temperature in growth chambers as well as in the field (Fracheboud et al., 2004; Jompuk et al., 2005). QTLs for Φ_{PSII} were detected at the same position in two other mapping populations, derived from the hybrids B73 × Mo17 and ETH-EH3 × ETH-EL1, when both were grown at suboptimal temperature (Fig. 2). In this region but at some distance from the former QTLs, QTLs were found for the carbon exchange rate and Φ_{PSII} (Fracheboud et al., 2002) as well as for frost damage and fresh weight of seedlings grown under the temperate conditions of central Europe (Presterl et al., 2007).

5.2 Molecular Basis of Chilling Tolerance

Much of our knowledge of the molecular response during response to cold and of cold-acclimation is based on studies of *Arabidopsis thaliana*. Although some of this knowledge aids the better understanding of the chilling tolerance/sensitivity of maize, there are large phylogenetic, morphophysiological and ecophysiological

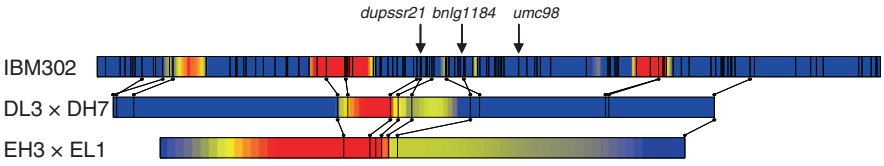

Fig. 2 Chromosome 2 with complete QTL raw data for the operating quantum efficiency of photosystem II (Φ_{PSII}) determined in seedlings of three mapping populations (IBM302, Reimer, unpublished results; ETH–DL3 × ETH–DH7, Fracheboud et al. (2004); ETH–EH3 × ETH–EL1, J. Leipner, unpublished results) grown at suboptimal temperature. The colors range from blue (low LOD score) to red (high LOD score). Homologue markers between populations are connected by lines. Positions of markers are indicated, which were found in the vicinity of QTLs for fresh matter yield (*dupssr21*) and frost damage (*bnlg1184*) in field-grown seedlings (Presterl et al., 2007) and for Φ_{PSII} in seedlings grown at suboptimal temperature (*umc98*) (Fracheboud et al., 2002)

differences between *Arabidopsis* and maize, which requires a detailed molecular biological analysis of maize as well. However, our knowledge of the molecular response of maize to chilling stress is rather rudimentary. Moreover, most of the information about cold-induced genes was gathered after a rapid decrease in temperature (usually from 25°C to 5°C), which poorly reflects the situation in the field. This type of experimentation is necessary, however, to unravel step by step the molecular response of maize to low temperature.

The increase in cytoplasmic Ca^{2+} seems to be an early response to exposure of plants to low temperature and may be triggered by cold-induced rigidification of the plasma membrane. In maize, the gene *ZmCDPK1*, coding a calcium-dependent protein kinase, is induced at low temperature (Berberich and Kusano, 1997), indicating that Ca^{2+} signals are important in the response of maize to cold stress. Time course experiments revealed that induction of *ZmCDPK1* precedes that of m*lip15*, another gene induced by cold, which codes for a DNA-binding protein of the basic region/leucine zipper (bZIP) type (Kusano et al., 1995). Therefore, *ZmCDPK1* might be located upstream of m*lip15* in the cold-stress signaling pathway. The presence of m*lip15* transcripts in senescent maize leaves and the cold induction of *ZmMAPK5*, transcribing a mitogen-activated protein kinase (MAPK) that is known to be involved in senescence, indicate that cold promote programmed cell death (Berberich et al., 1999).

As well as the induction of the bZIP transcription factor MLIP15, cold induces the transcription of ERF/AP2 type transcription factors in maize. One of these transcription factors, *ZmDREB1A*, was expressed during cold stress but almost no transcripts were found after ABA application (Qin et al., 2004), indicating an ABA independent pathway. Other genes for transcription factors that are induced in maize at low temperature, namely maize *DBF1* (Zheng et al., 2006) and *ZmDREB2A* (Nguyen, 2005; Qin et al., 2007), seem to be part of the ABA-dependent signaling since they are also induced by drought, salt or ABA application.

Other cold-induced genes are involved in DNA methylation (*ZmMET1*) (Steward et al., 2000) or in phytohormone metabolism: for example, genes for zeatin O-glycosyltransferase (Li et al., 2000) and 12-oxophytodienoic acid reductase (Nguyen, 2005; Zheng et al., 2006). The role of the fatty acid composition during acclimation to cold was supported by the differential expression of the ω-3 fatty acid desaturase genes, *FAD7* and *FAD8*, upon exposure to cold temperature (Berberich et al., 1998). Furthermore, the cold-induced expression of the *cat3* gene coding a catalase (Anderson et al., 1994) and of several genes of the anthocyanin pathway (Christie et al., 1991) underlines the importance of ROS defense and light protection, respectively, in the acclimation of maize seedlings to chilling.

Few attempts were made to increase the chilling tolerance of maize seedlings by transformation. Seedling were produced, which overexpressed Mn-superoxide dismutase (Van Breusegem et al., 1999; Kingston-Smith and Foyer, 2000b). Although changes in the antioxidative defense and in tolerance to methyl viologen-induced oxidative stress occurred, there was not a significant improvement of chilling tolerance. Similarly, the expression of the cold-tolerant pyruvate orthophosphate dikinase from *Flaveria brownii* in maize had very little effect on the chilling tolerance of

photosynthesis (Ohta et al., 2006). Enhanced tolerance to freezing was achieved by transforming maize with *NPK1*, a tobacco mitogen-activated protein kinase kinase kinase, which is assumed to be involved in an H_2O_2 signaling pathway (Shou et al., 2004). The plants in this study were characterized by a higher level of sugar and withstood a lower freezing temperature compared to the control plants. The application of glycinebetaine, which maize can synthesize only in small amounts if at all, improves chilling tolerance (Chen et al., 2000). Based on this finding Quan et al. (2004) transformed maize with the *betA* gene encoding a choline dehydrogenase from *Escherichia coli*. Transformants showed a higher level of glycinebetaine, which was related to better shoot growth, less cell injury, increased survival, a decrease in photoinhibition and higher photosynthetic activity at low temperature. Although the superiority of these transgenic plants has yet to be proven under conditions in the field, the results are very promising for a successful improvement of chilling tolerance by means of transformation.

6 Conclusions and Future Perspectives

Current knowledge of the complex physiological and molecular biological effects of low temperature on maize seedlings is based on extensive studies, most of which were performed in controlled environment. The seedling's response to low temperature was determined and genotypes with contrasting tolerance to cold stress were compared. These experiments have shown that, for a few days, maize seedlings can withstand temperatures as low as 5°C without showing signs of lasting damage, even though the effect on photosynthesis is considerable under such conditions. Tissue that develops under such low temperature, however, is irreparably damaged. The development of the photosynthetic machinery seems to play a crucial role in this effect. Nevertheless, the primary cause and site of this chilling-induced damage are still unclear. The recent development of molecular and quantitative genetics enables the detailed study of complex physiological processes. As well as a better understanding of the basic mechanisms of chilling tolerance, this approach may help to find molecular markers for marker-assisted breeding of stress-tolerant genotypes. The molecular biological studies will be challenging, because a main focus should be on the effect of chilling on developmental processes.

There is particular interest in chilling tolerance due to changes in farming practices, such as earlier or no-till sowing, both of which expose seedlings to colder soils. The current level of adaptation of maize to cold climates is generally based on avoidance mechanisms, for example, shortening of the plant vegetation to ensure a safe harvest in autumn. Nevertheless, these plants may be negatively affected by unpredictable spells of low temperature at the beginning of the season. Although current climate models forecast that temperatures will increase, in some regions this change may be accompanied by a considerable decrease in precipitation during the growing season. A possible strategy to overcome this inadequate water supply is to avoid the drought period in summer by sowing plants much earlier. Nonetheless,

these plants will be exposed to low temperature early in the season, so that a high chilling tolerance will be essential.

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Drought Tolerance in Maize

Jean-Marcel Ribaut, Javier Betran, Philippe Monneveux, and Tim Setter

Abstract Drought is the single most common cause of severe crop production shortage in developing countries, and global warming is predicted to further exacerbate drought's impact. This chapter describes how to best evaluate segregating germplasm under water-limited conditions, what are the secondary traits to be included in the selection and why is it important to identify key regulatory genes involved in selected metabolic pathways and signaling. The chapter also summarizes the current understanding of the genetic basis for drought tolerance. From this review, one can project that sustained progress in breeding for drought tolerance in maize is likely to entail the selection of plants with a reduced leaf area (especially in the upper part of the plant), short thick stems, small tassels, erect leaves, delayed senescence, smaller root biomass, and a deep root system with little lateral root branching. Tolerant genotypes are also expected to have robust spikelet and kernel growth at the cell-division and expansion-growth phases, with good osmotic adjustment to assist in cell retention of water during drought. In this way, root and ear growth is not completely inhibited, and leaf survival is enhanced despite limited water. Extensive genetic dissections of drought tolerance traits have been carried out in maize over the last decade, yielding numerous QTL involved in the determination of morphological traits and regularoty pathways. A better understanding of the physiological mechanisms and the genetic basis of the response of maize to drought should make it increasingly feasible and efficient to stack favorable alleles at key genes, so as to select for genotypes that will develop phenotypes described above. The final section of this chapter focuses on the genetic gains achieved in maize via an interdisciplinary approach, and includes an exploration of new strategies combining marker-assisted breeding and phenotyping selection to accelerate drought tolerance breeding in maize.

1 Background and Introduction

By 2025, it is expected that some 1.8 billion people will be suffering due to water shortage, and two-thirds of human kind will be affected by what the FAO defines as water stress. The FAO now considers drought to be the *single most common cause of severe food shortage* in developing countries, significantly outweighing other causes

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such as conflict, flooding and economic mismanagement. Irrigation is not an option for large numbers of farmers and there is limited potential for any expansion of irrigation in developing countries (World Bank, 2006). To make matters worse, the forthcoming changes in global climate are expected to produce unpredictable and extreme weather conditions, and in particular a rise in temperature (global mean temperatures are expected to rise 1.4–5.8 °C over the course of this century), which is predicted to impose water stress in hitherto unaffected and unexpected parts of the world (IPCC, 2001). The 2002–2003 drought in southern Africa resulted in a food deficit of 3.3 Mt, putting an estimated 14 million people at risk of starvation (WFP, 2003). Eastern Africa suffered a severe drought in 2005–2006. The value of food aid for Africa now ranges from US$500 million to over US$1 billion per year (WFP, 2007). Drought has been shown to have a devastating impact on the productivity of many crop species, in the South and in the North. The drought and heat wave that affected southern Europe in 2003 led to an estimated 20% fall in the productivity of maize (European Commission, 2004). The insurance industry estimates average economic losses from drought damage on German acreage at ~€200 million per year. Competition for water has become a reality and might affect the distribution of crop production around the globe as irrigated agriculture is a major consumer of water – in the US, more water is consumed by irrigation than by all other uses combined.

In the developing world, maize is the third most important food grain crop. By 2020, demand in developing countries is expected to outstrip that for wheat and rice, increasing the global annual requirement for maize by 837 Mt (Rosegrant et al., 2001). Much of this increased demand will have to be met by domestic supply, which can only be achieved through the intensification of production on existing arable land (Rosegrant and Cline 2003). Most tropical maize is rainfed, in situations where the alleviation of water stress through irrigation is seldom an option. Given the combination of an increasing competition for water, a trend toward higher global temperatures, and likely more extreme regional and seasonal climatic changes in some areas, the development of maize varieties with enhanced tolerance to drought stress and higher water use efficiency (WUE) has become a high priority goal for major breeding programs, both in the private and public sectors.

This chapter presents recent advances in enhancement of drought tolerance of maize, achieved by a complementary set of approaches and disciplines; reviews the range of breeding objectives appropriate for water-stressed conditions; sets out how to evaluate material under controlled field conditions; identifies the current and upcoming traits associated with drought tolerance; defines the state of knowledge with regards to key regulatory pathways; and summarizes the current understanding of the genetic basis for drought tolerance. The final section focuses on the genetic gains achieved in maize via an interdisciplinary approach, and includes an exploration of new strategies to accelerate drought tolerance breeding in maize. As plant science is rapidly becoming fragmented into specialized subdisciplines, there is a pressing need for a holistic consideration of drought tolerance in the cereals (and in maize in particular), using as a perspective the achievements already realized in the field (Blum, 2006).

2 Germplasm Evaluation

2.1 Definition of Breeding Targets

As both the occurrence of drought and its intensity are inherently unpredictable, screening and selection for drought tolerance under rain-fed conditions is always an unreplicable experiment. The use of irrigation and planting date to control the timing and severity of the water deficit in rain-free environments has proven to be an effective alternative (Campos et al., 2004). Both the genetic variance and the heritability of grain yield (and hence breeding progress) are lower under stressed than under optimal conditions. However, the correlation of grain yield for hybrids grown under well-watered (WW) and water stressed (WS) conditions is reduced with the degree of stress. Thus, despite enjoying a higher heritability, selection for yield in non-stressed environments is generally less effective in identifying the individuals which will perform best in low-yielding stressed environments than is direct selection under stressed conditions. Experiments in which the same set of genotypes has been evaluated under both stressed and nonstressed conditions have proven the ubiquity of interactions between genotype and the level of drought. Thus careful selection of evaluation sites representative of drought conditions prevalent in the target area, and those where the incidence and intensity of the stress can be controlled by irrigation is a critical issue (Fig. 1). Importantly, the genetic enhancement of yield under water limiting conditions does not necessarily imply the sacrifice of yield potential under favorable conditions (Jensen and Cavalieri, 1983; Edmeades et al., 1999), which is relevant, given the unpredictability of the incidence of natural drought.

Drought tolerance is best defined as the capacity to be more productive under drought stress. The expression of tolerance depends greatly on both the developmental stage during which water stress is imposed, and the intensity and duration of drought. Several strategies are available to mitigate drought damage. These include (1) drought escape, primarily by avoiding the coincidence of stress with flowering time; (2) increasing the crop's water use efficiency (WUE); and (3) increasing the plants' *per se* drought tolerance. As a consequence of the potential for drought escape by simply altering flowering date, minimizing the differences in maturity of populations under selection is critical to avoid confounding drought escape with drought tolerance *per se*. With respect to *per se* drought tolerance, three primary breeding targets can be distinguished – drought tolerance at flowering time, drought tolerance during grain filling, and yield stability across a range of environments (including drought stress). Maize is most susceptible to drought during flowering, suffering from sterility of the female gamete, floral asynchrony, nonreceptivity of the silk, tassel blasting, trapped anthers and embryo abortion (Westgate and Boyer, 1985). The physical separation between the male and female flowers, with anthesis occurring before pistil maturity, and the near-synchronous development of florets on a single ear make the species highly susceptible to moisture and temperature stress during flowering (Struik et al., 1986). Drought during this period also induces stomatal

Fig. 1 Effect of water-stress on maize. (**a**) Maize field trial at flowering under water-stressed conditions (right) and well-watered conditions (left). Field management to avoid water contamination of drought trials is critical; therefore, adjacent water regimes are separated by an empty row plus two border rows on each side of the empty row. (**b**) Drought at flowering combined with high temperature can generate a tassel blast phenotype. Such tassels are sterile and do not shed pollen, making accurate measurement of anthesis-silking interval impossible and confounding the isolation of factors responsible for drought susceptibility for grain yield under water stressed conditions

closure, and prolonged periods of drought cause wilting and rolling, premature senescence of leaves, and hence a reduction in the leaf area index and a fall in photosynthetic activity. Yield reduction in response to drought is most severe when the stress occurs during the 3-week period following flowering.

Genetic advances in the grain yield of commercial maize hybrids have been achieved for drought stress induced at various reproductive stages (flowering, early-, mid-, late- and terminal grain filling) as well as for well-watered conditions (Campos et al., 2004). Conventional selection improved drought tolerance most when the drought occurred at flowering or at early grain filling, rather than at mid- or late grain filling. There is therefore some potential to further increase the tolerance of both hybrids and open pollinated varieties to drought stress occurring during the late reproductive stages. However, to counteract the steady reduction in genetic gain over time which is inevitable when selection is continuously imposed on the same trait(s), it will be necessary to identify either alternative traits and/or novel strategies, as described in the following sections.

2.2 Evaluation of Segregating Populations Under Managed and Multilocation Drought-Stress Environments

The choice of the testing environment(s) is critical to the rate of achievable genetic gain (Cooper et al., 2006). Ideally, the selection environment should mirror the target environment in rainfall distribution, physical and chemical soil properties, water distribution profiles and potential evapotranspiration rates, otherwise significant genotype × environment (G × E) interactions will result in much of the gain achieved in the selection environment not being reproduced in the target environment. Soil analysis can help to avoid the use of sites where confounding factors, such as micronutrient deficiency, soil compaction, salinity, and the presence of damaging nematode and fungal pathogens, are likely to generate significant levels of G × E. It can also identify the extent of soil homogeneity. Spatial variability inhibits the detection of treatment differences in all field experiments, since it inflates the experimental error variance. Therefore the use of uniform soils, along with rigorous experimental design and appropriate statistical analysis will together maximize the precision of genotypic means (Gilmour et al., 1997). An important factor in the context of drought environments is temperature, since the occurrence of extreme temperatures during ear growth is known to confound the estimation of drought tolerance (Otegui and Andrade, 2000). High day and night temperatures can also have a negative effect on seed set, because the duration of ear establishment is decreased, thereby limiting the number of days for photosynthesis during this critical period, leading to reduced kernel set and subsequent kernel growth (Cantarero et al., 1999). Multi-location evaluation is necessary to estimate the importance of G × E. It is especially critical in the context of breeding for drought tolerance, where a consequence of lowered plant vigor is a higher responsiveness to environmental variation. Phenotype (P) is assumed to represent the sum of the

genotypic (G) and the environmental (E) effects, plus the interaction between them (G × E), or more formally P = G + E + G × E. The estimation of G, and to some extent G × E, has been more emphasized to date than E. However, the development of geographic information system tools and advanced statistical methods (such as spatial analysis and mixed models) has begun to create opportunities to characterize E, and to better understand what underlies the G × E component (Cooper et al., 2006).

Current means of estimating G have moved from a purely biometrical approach to one which exploits various assays of DNA sequence variation (Cooper et al., 2006). In particular, the G × E component has been broken down into its constituent QTL × E interactions, which has allowed for the development of models where aspects of a complex phenotype expressed under a stressed environment can be described in relation to molecular mechanisms. Factorial regression and mixed QTL models are particularly useful for this type of analysis, especially when the phenotypic data are derived from multi-environment (including both stressed and nonstressed conditions) experiments (Malosetti et al., 2004; Vargas et al., 2006). Multitrait multienvironment QTL models in particular serve to define the genomic regions associated with genetic correlations, whether these are the outcome of pleiotropy or genetic linkage, and can illustrate the dependence of genetic correlations on environmental conditions. Using multi-trait multi-environment data, several QTL for adaptation to drought stress in tropical maize have recently been identified (Malosetti et al., 2008). A better understanding of the contribution and characteristics of the G × E and QTL × E components to the phenotypic variance has the potential to provide a breakthrough in breeding under drought conditions.

3 Secondary Breeding Traits

3.1 The Use of Secondary Traits for Selection Under Drought Conditions

Secondary traits are those other than economic yield itself which can provide a measure of plant performance (Lafitte et al., 2003). An ideal secondary trait would be genetically correlated with grain yield in the target environment, genetically variable, have a high level of heritability, be simple, cheap, non-destructive and fast to assay, be stable throughout the measurement period and would not be associated with any yield loss under nonstressed conditions (Edmeades et al., 1997a; Lafitte et al., 2003). Under drought stress conditions, breeding progress is impeded by a significant level of G × E (both with respect to cropping season and location). Given the poor heritability of grain yield under drought stress conditions, genetic progress is hard to achieve via direct selection. However, because under drought, both the heritability of at least some secondary traits remains high (Bolaños and Edmeades, 1996) and the genetic correlation between grain yield and these traits increases significantly (Bänziger and

Lafitte, 1997), recourse to indirect selection becomes an attractive strategy. Selection based on secondary traits which reflect the direct effects of drought can improve the response, since it avoids the confounding effects of other stresses, such as poor soil fertility, micronutrient deficiency and pathogen presence (Monneveux and Ribaut, 2006). Application of this strategy has generated genetic gains under a range of environmental conditions (reviewed by Campos et al., 2004), and several of these success stories are described in the final section of the present chapter.

3.2 Traits Associated with Drought Tolerance

The physiology and inheritance of traits associated with drought tolerance in maize have been well documented in the literature (e.g., Bruce et al., 2002; Sawkins et al., 2006). The focus here is on the mechanism(s) by which the plant copes with stress, and preferably those which favor productivity as opposed to merely survival. The latter do of course have value and relevance to economic yield, such as when a young crop is exposed to drought stress.

Grain yield can be modeled as the product of the quantity of water transpired, the WUE and the harvest index (HI). Thus secondary traits can be classified on the basis of their contribution to any one or more of these determinants. Traits related to transpiration include root depth and health, leaf area, extent of leaf rolling, osmotic adjustment, stomatal conductance, canopy temperature, hydraulic conductivity, and ABA concentration. Canopy temperature depression (CTD) (the temperature difference between the leaf and the ambient air, as measured by infrared thermometry) is associated with evaporative water loss and hence access to water and stomatal conductance. Photosynthetic activity and high temperature tolerance both affect WUE, while the anthesis-silking interval (ASI), ear growth and barrenness are associated with assimilate partitioning during grain-filling, and thus ultimately affect HI. Grain yield under drought stress has been strongly correlated with traits related to HI, such as the number of ears per plant (EPP), the number of kernels per ear and a short ASI; and moderately correlated with leaf senescence, leaf chlorophyll concentration, and plant height. ASI, which is a measure of the asynchrony between anthesis and pistil receptivity, increases in response to water deficit, due to the inhibition of ear and silk growth (Bolaños and Edmeades, 1993b). Genotypes selected on the basis of a short ASI and high yield under drought generally expend less carbohydrate in the growth of tassels and vegetative organs, leaving a greater proportion for the growth of the ear (Edmeades et al., 1993).

Edmeades et al. (1997a), after ranking a series of secondary traits on the basis of heritability, association with grain yield, cost, genetic variability, and time required for recording, identified EPP and ASI as the best performing secondary traits. Both are associated with the HI of drought-stricken crops. Osmotic adjustment, dense and deep roots, and the ability to re-mobilize stem reserves have also been recommended as secondary selection traits in water-limited environments (Blum, 1997); however, most of these fail to meet the criteria of low cost and high

scoring speed, which are essential for realistic deployment in a maize breeding program (Chapman and Edmeades, 1999). Current photosynthesis, rather than remobilized reserves, is thought to underpin the formation of the developing ear under drought, but a decreased assimilate demand from other actively growing organs during the flowering period (particularly stems, tassels, and roots) could help to support the developing ear. Delayed leaf senescence (often referred to as "stay-green"), erect upper leaves, and reduced leaf rolling under stress have all also been proposed as stress-adaptive traits (Chapman and Edmeades, 1999; Blum, 1997). The remobilization of protein from senescing leaves is accelerated by water stress, and this inevitably decreases green leaf area, and thus photosynthetic activity and the assimilation of the sugars needed for grain fill (Thomas et al., 2002). A delay in the onset or progress of senescence during grain filling has been positively related to yield under drought (Bänziger et al., 2000), since photosynthesis can then be prolonged. Note, however, that this may be due to several indirect factors including nitrogen acquisition efficiency (Borrell et al., 2001), and some stay-green phenotypes are not associated with higher grain yield (Thomas et al., 2002). Leaf rolling is generally considered to have a negative effect on yield since it may reflect poor rooting or hydraulic conductance and it reduces the quantity of light intercepted by the canopy. However, Bolaños et al. (1993) could not establish any association between leaf rolling score and either the pre-dawn leaf water potential or any changes in leaf water status. Moreover, Ackerson (1983) noted that leaf rolling occurred at higher leaf water potential in a drought tolerant *latente* maize hybrid than in a normal commercial hybrid, suggesting that leaf rolling may be associated more with dehydration avoidance than with tolerance *per se*.

The expression of and genetic variation in grain yield and associated traits in maize vary with the level of imposed stress. While the heritability of grain yield declines under drought stress, those of ASI and EPP remain stable or may even increase as the severity of the drought rises (Bolaños and Edmeades, 1996). The utility of ASI and EPP for breeding stress tolerant hybrid maize has been illustrated by their genetic variability in drought stressed environments, good level of heritability, high correlation displayed between inbreds and hybrids allowing good prediction of hybrid performance from studies conducted on inbreds, and the strong correlation of those traits with grain yield. Thus, for example, a strong negative correlation was established between ASI and the grain yield of maize grown under drought stress (Bolaños and Edmeades, 1993b; Monneveux et al., 2006; Ribaut et al., 1997). ASI is without a doubt the most widely used secondary trait for improving drought tolerance of maize at flowering time (Beck et al., 1996; Edmeades et al., 2000; Troyer, 1983).

The contribution of ASI and EPP has been confirmed in a large-scale experiment conducted at CIMMYT using six segregating populations derived from four crosses, evaluated under 44 WS and 12 WW environments for grain yield and a set of eight secondary traits (including both EPP and ASI, see Fig. 2a, b). The stress was imposed around the time of flowering and was sufficient to cause a 40–90% reduction in grain yield, relative to the WW conditions. Pairwise genetic and phenotypic correlations among traits have been calculated for each individual trial, across trials of a single cross and across crosses using a matrix approach. EPP and

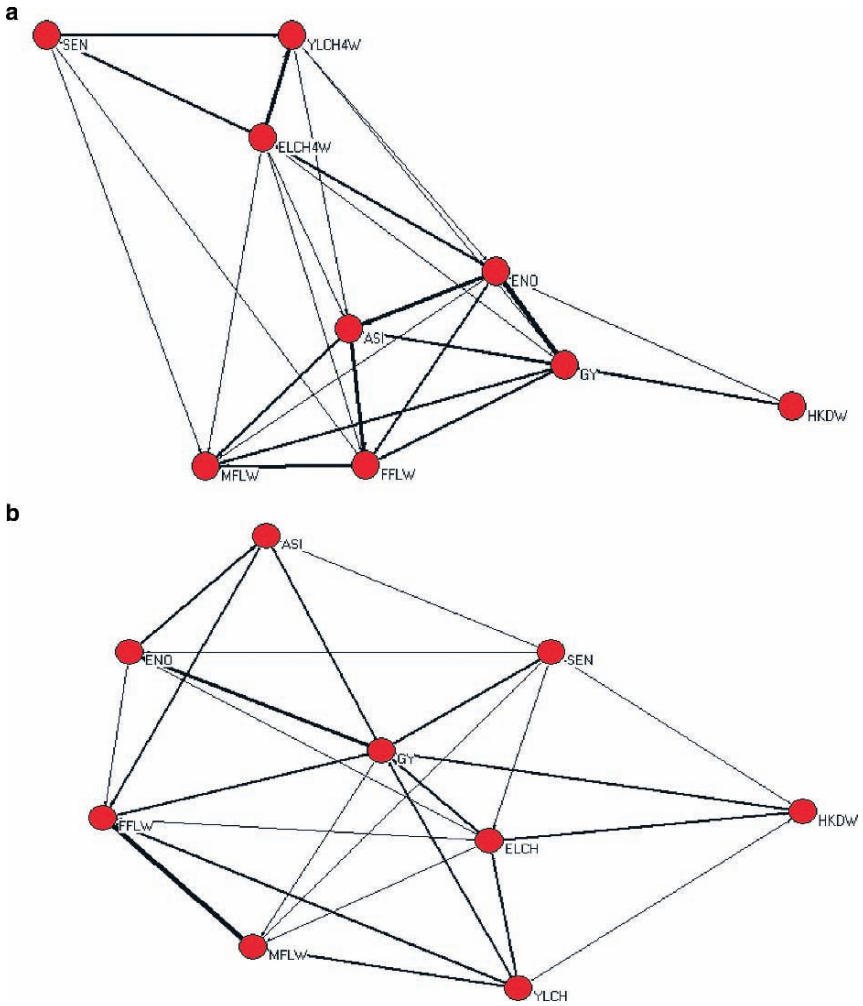

Fig. 2 Pairwise genetic correlations calculated among nine traits (*GY* Grain yield, *ENO* Ear Number, *HKDW* Hundred kernel dry weight, *ASI* Anthesis-silking interval, *FFLW* Days to female flowering, *MFLW* Days to male Flowering, *ELCH4W* Ear leaf chlorophyll content 4 weeks after the last irrigation, *YLCH4W* Young leaf chlorophyll content 4 weeks after the last irrigation, and *SEN* senescence) across 44 water-stressed environments (**a**) and 12 well-watered trials (**b**). Only correlations above a threshold of 0.3 are reported and the thickness of the line between 2 points is proportional to the level of the correlation

ASI presented the highest correlation with GY, and GY was significantly correlated with all other traits except for senescence when calculated under all 44 different WS environments (Fig. 2a, b). The level of correlation between traits was in general higher under WS compared to WW conditions. Such levels of correlation reinforce the importance of focusing on EPP and ASI to improve drought tolerance and

indicate that in addition to them, some of the other traits should also be considered for inclusion in the selection index. Although the parental lines for the segregating populations have been selected following different phenotypic characteristics, the low standard deviation obtained for the correlations calculated across crosses and environments (data not presented) underline the relevance across genetic background of the secondary traits selected here.

3.3 The Need for Further Secondary Traits

Continued selection for particular secondary traits generates a shift in their mean value, and hence lessens their genetic correlation with yield (McMillan et al., 1995; Edmeades et al., 1997a). Among drought tolerant hybrids generated in more recent years from crosses between elite (drought tolerant population) and La Posta Sequía lines, grain yield remained associated with the number of grains per ear and EPP (Monneveux et al., 2008), but there was no association between grain yield under drought and ASI. Byrne et al. (1995) postulated that once ASI has been sufficiently shortened in elite germplasm, its further reduction is unlikely to generate any significant genetic gain, and its segregation reduces significantly under WS conditions. In addition, yield and grain number are not just related to the overall flux of carbohydrate to the ear, but also to the moisture status of the ovary (Westgate and Boyer, 1986) and/or to impaired conversion of sucrose in the developing spikelet (Zinselmeier et al., 1995). As a consequence of the selection for short ASI, the relationship between grain abortion and ASI decreased over time (Monneveux et al., 2006). As selection progresses, the correlations between grain yield and either leaf senescence or leaf rolling fall, until becoming no longer significant (Chapman and Edmeades, 1999; Monneveux et al., 2006). Leaf senescence and leaf rolling consequently can be useful as secondary traits in a preliminary selection exercise among largely drought susceptible germplasm, but they become less informative as the level of drought tolerance improves. Thus an important priority lies in the identification of novel secondary traits, and many of these are likely to be involved with plant architecture.

Although there is reason to expect that the best performing lines across drought environments will have an extensive and deep root system, recurrent selection in tropical populations has actually led to a reduction in root biomass, while inbreds having poor early root development yield more under drought than inbreds with vigorous early root development (Bruce et al., 2002). Root traits have been only inconsistently associated with better drought tolerance in maize, largely because the improved redirection of scarce assimilate to ear and spikelet growth has been of overriding importance (Edmeades et al., 1999). A further possibility, however, is that although selection has decreased root biomass, the root system architecture may have changed to become deep, with reduced lateral branching. Consistent with this hypothesis is the observation that the root-ABA1a QTL on maize chromosome 2 affects both the extent of root branching and grain yield under water stress (Landi et al., 2007). In particular, homozygotes for the high-ABA allele produced extensive root branching close to the

surface and yielded rather poorly under water stress, whereas homozygotes for the alternative allele showed little surface rooting but yielded somewhat higher. However, the wide-scale evaluation of root characteristics under field conditions remains impractical, so the development of tagging markers is the preferred route to accelerate the manipulation of these root traits. QTL underlying root number (Guingo et al., 1998), root length, diameter and weight (Tuberosa et al., 2002b) and root pulling strength (Tuberosa et al., 2003) have also been identified.

Further progress in drought tolerance may be achieved by exploiting changes in aerial architecture, as this has a major impact on assimilate partitioning and/or radiation use efficiency. Increasing the supply of sugar to the growing ear can be obtained either by increasing HI, or by reducing competition between the reproductive organs (e.g., by selection for smaller tassels). In tropical maize, yield gains under drought have generally been associated with increases in HI (Edmeades et al., 1999; Chapman and Edmeades, 1999; Monneveux et al., 2006). Tropical maize varieties remain rather tall, with a HI below 0.50, still far short of what has been attained in wheat (Hay and Gilbert, 2001). An increase in HI can be achieved either by shortening crop duration to flowering and/or by a reduction in plant height and stem biomass (Edmeades et al., 1999; Lafitte and Edmeades, 1997). It can probably also be improved by inhibiting foliar development. Shading of the ear by a dense canopy lengthens ASI (Pagano et al. 2007). Light interception, which is critical to plant productivity, is related to leaf number, leaf blade area, leaf angle and tassel size. The effect of leaf angle on maize yield is well documented under high plant density conditions (Lambert and Johnson, 1978), but has been hardly explored under drought stress.

Yield increases resulting from tassel removal just before pistil maturity have been reported by Duvick and Cassman (1999), Hunter et al. (1969), and Mickelson et al. (2002). Competition between the tassel and the ear is greater under drought stress (Geraldi et al., 1985) as the shape of the tassel affects the quantity of radiation reaching the canopy, thereby affecting leaf photosynthesis, and the partitioning of assimilate for growth, thereby diverting some assimilate to tassels away from the developing grain. Aspects of tassel morphology show a moderate to high level of heritability (Mickelson et al., 2002), and are significantly correlated with both yield components and ASI (Ribaut et al., 2004). Although selection for smaller tassel size is limited by the need to retain sufficient pollen to ensure fertilization, this secondary trait could represent a viable selection criterion in tropical maize germplasm, which is characterized by large tassel size. Six tassel branch number QTL and one tassel branch angle QTL have been mapped to the same genomic region as a leaf angle QTL (Mickelson et al., 2002). Genes which appear to play a role in inflorescence architecture include *fasciated ear2* (*fae2*), *ramosa1* and *2* (*ra1*, *ra2*), *thick tassel dwarf1* (*td1*), *barren stalk1* (*bs1*), *barren inflorescence2* (*bif2*) and *branched silkless1* (*bd1*) (Volbrecht and Schmidt, Chap. 2).

Delayed leaf senescence during the grain filling period is positively correlated to yield under drought conditions, and has therefore been proposed as a suitable secondary trait for drought tolerance in maize (Bänziger et al., 2000). Senescing leaves are less effective in terms of radiation interception and photosynthesis (Wolfe et al., 1988), and although stay-green does enhance tolerance to late season stress

(Campos et al., 2004), it has not been much exploited as an indirect selection criterion. Selection for an increase in functional stay-green, in grain test weights and in mean kernel weight under conditions of late season drought stress could all contribute to further genetic gain. To date, the major emphasis in drought tolerance breeding in tropical maize has been the manipulation of HI. Remobilization of stem assimilate to the growing ear from thick stemmed genotypes may be a possibility, but remobilization efficiency has yet to be investigated. However, increased shoot diameter, as has been achieved in sorghum (Borrell et al., 2006), may favor assimilate storage and redistribution to the developing grain under water-limited conditions.

From those results and observations, one can project that sustained progress in breeding for drought tolerance in tropical maize is likely to entail the selection of plants having a reduced leaf area (especially in the upper part of the plant), short, thick stems, small tassels, erect leaves and delayed senescence. Lesser (or more impractical) traits include smaller root biomass, and a deep root system with little lateral root branching (Fig. 3). Tolerant genotypes are also expected to have robust

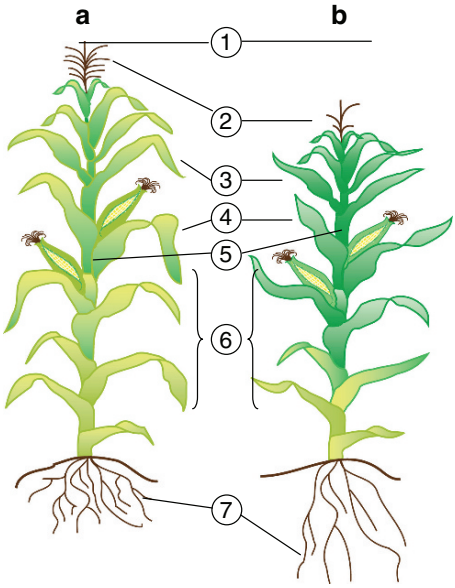

Fig. 3 Drought susceptible (**a**) and tolerant (**b**) maize phenotypes. Tolerant phenotype will have (1) shorter plant size (better light penetration into canopy, less C competition with ear and kernel development), (2) reduction of tassel size (less C competition with ear and kernel development, mainly for tropical maize) (3) smaller leaves above the ear (better light penetration into canopy, less C competition with ear and kernel development) (4) erect leaves (45°–60°) with lower average light flux density to (a) avoid light saturation and improve light penetration into canopy, thereby improving light use efficiency and (b) lower leaf temperature and improve WUE, (5) larger stem diameter (more C accumulation as nonstructural carbohydrate that can serve as a source of C for ear and kernel development during drought at kernel fill), (6) stay-green (more photosynthetic source of C for ear and kernel development), (7) deeper rooting with less lateral branching and somewhat less root biomass (better access to water, better use of scarce resources)

spikelet and kernel growth at the cell division and expansion–growth phases that has less tendency to be inhibited by water deficit and photosynthate deprivation such that kernel set is high and kernel abortion is low. They should also be characterized by good osmotic adjustment to assist in cell retention of water during drought such that root and ear growth is not completely inhibited and leaf survival is improved at low water potentials (Chimenti et al., 2006).

4 Selected Metabolic Pathways and Signaling

4.1 Resource Partitioning and Signaling Under Moisture Stress Conditions

4.1.1 At the Plant Level

In many cropping environments, drought stress increases in severity at the end of the growing season. In this situation, assimilate which has accumulated to a high level in the maize stem is re-mobilized, and contributes to yield (Jurgens et al., 1978). Under both well-watered and water stressed environments, the number of kernels per plant is strongly correlated with canopy-scale photosynthetic rate during flowering (Echarte and Tollenaar, 2006). The inhibition of the growth of the pre-fertilization ear in response to drought can be in part overcome by exogenous supply, via the stem, of sucrose, which is transported to the spikelets (Boyer and McLaughlin, 2007; McLaughlin and Boyer, 2004a; Zinselmeier et al., 1999). Thus the abortion of immature florets likely involves sensing of the plant's sugar status and is initiated when sugar status has fallen below a critical threshold. Pedicel and pericarp starch reserves are also used in times of assimilate scarcity, and the exhaustion of this reserve has been shown to coincide with a fall in grain set (Makela et al., 2005; Zinselmeier et al., 1999). Thus genotypes having a better ability to store and re-mobilize sugar and/or starch will probably be more productive in stressful environments.

Since nitrogen uptake by roots is limited during grain fill, especially in times of drought, the majority of nitrogen needed for growth of the grain is re-mobilized from vegetative organs (Paponov and Engels, 2005). Although the physiology and inheritance of carbon and nitrogen storage and remobilization in maize stems and leaves are largely unexplored, they are likely to play an important role in crop performance as drought and nitrogen deficiency often occur together.

4.1.2 From the Root to the Aerial Tissues

The action of ABA involves interactions with other growth regulators, particularly ethylene and reactive oxygen species (Spollen et al., 2000; Fan et al., 2006; Sharp, 2006). The analysis of ABA mutants and transgenic lines over- or under-expressing genes

in the ABA synthesis pathway has been unable to confirm any clear relationship between the content of either ABA or ethylene and leaf growth in response to water deficit (Voisin et al., 2006). Rather, leaf elongation under mild water stress is primarily limited by turgor (Bouchabke et al., 2006). A complication in these analyses is that in ABA mutants, the absence of an ability to synthesize ABA results in the failure of the stomata to control transpiration. In the transgenic studies, this problem was circumvented by evaluating growth rate during non-transpiring conditions (Voisin et al., 2006). Given the evidence that roots respond differently from leaves to water deficit and changes in ABA content, it will be valuable to use the maize ABA transgenics to study root responses.

4.1.3 In the Reproductive Organs

Although the concentration of total sugars in the pre-fertilization ear and spikelet tissues tends to remain high during water deficit (Setter et al., 2001), the levels of glucose nevertheless become depleted in the pedicel (McLaughlin and Boyer, 2004a). Glucose levels decline, in part because cell wall and soluble invertase activity is negatively affected by water deficit, thereby hindering hydrolysis of sucrose and sugar flux to growing cells (McLaughlin and Boyer, 2004b; Makela et al., 2005). While no sugar sensing or signaling factors have yet been identified, there is evidence that several genes are coordinately regulated, including those involved in abortion and senescence (Boyer and McLaughlin, 2007; McLaughlin and Boyer, 2004b).

4.2 Root Growth Responses to Water Deficit

The development of a root system capable of accessing water far down the soil profile is a valuable trait in drought-affected environments (Robertson et al., 1993). Many species, including maize, respond to water deficit by redirecting growth and dry matter accumulation away from the shoot to the root (Hsiao and Xu, 2000; Sharp et al., 2004). In maize this shift involves an increase in cell wall extensibility in roots, mediated by an increased expression level of expansins, xyloglucan endotransglucosylase/hydrolases and other wall-loosening factors at the root tip. This allows the root to maintain its growth in the face of decreased water potential, while shoot growth is inhibited (Ober and Sharp, 2007; Wu et al., 2001). At the root apex, cell division and growth-related transcripts predominate, while away from it, an expression profile prevails that is consistent with restrained growth and the allocation of limited carbohydrate to the root tip (Fan et al., 2006; Poroyko et al., 2007). The regulatory mechanisms responsible for these changes remain controversial, but mutation analysis and the exogenous application of hormones indicate that ABA plays a key role in the stimulation of root growth, while simultaneously inhibiting shoot growth (Sharp et al., 2004).

4.3 Osmotic Adjustment

Osmotic adjustment (OA) involves the active accumulation of solutes in the cell, and therefore represents a potentially valuable stress tolerance mechanism. This accumulation enables the retention of water during episodes of low external water potential, limiting turgor loss and damage from cell shrinkage. Under more prolonged or severe moisture deficit, these solutes also are implicated in the stabilization of various macromolecular structures. Maize has limited OA capacity compared to sorghum and millet (Inada et al., 1992). Germplasm surveys have revealed little evidence for genetic variation in OA, and since the trait shows low heritability, there is also little response to selection (Bolaños and Edmeades, 1991; Ribaut et al., 2004; Guei and Wassom, 1993). Nevertheless, selection for OA in materials derived from a cross between a pair of contrasting inbreds resulted in the identification of particularly high OA segregants (Chimenti et al., 2006). Under drought conditions, high OA selections generally developed deeper root systems, produced leaves which remained green longer, and generated higher yields and HI's than low OA ones. In maize and sorghum, a high proportion of the solutes contributing to OA are sugars. Both Ribaut et al. (2004) and Pelleschi et al. (2006) defined a number of QTL underlying the accumulation of sugar in the leaves of water-stressed plants. Both identified a chromosome 5 QTL involved in glucose accumulation which co-localizes with a gene for vacuolar invertase (*Ivr2*); this gene in turn is induced by ABA in the leaf (Trouverie et al., 2003). The coincidence of *Ivr2* with this QTL suggests the possibility that the former contributes to OA by catalyzing the hydrolysis of sucrose, thereby doubling its osmotic activity. These outcomes provide encouragement for the exploration of OA as a secondary trait for drought tolerance breeding in maize.

4.4 Stomatal Regulation

The stomata respond dynamically to changes in the environment and play a vital role in limiting water loss during drought. The signaling systems regulating the action of the stomatal guard cells are well researched (Fan et al., 2004). In particular, the maize gene *viviparous14* encodes a key rate-limiting and stress-regulated step in the synthesis of ABA, which initiates the signaling pathway for stomatal closure (Tan et al., 1997). Maize is an "isohydric" species, so called because the signaling and response system is sensitive to a decrease in water potential, limiting water loss in the early phases of water deficit, so that leaf water potential can be maintained at the well watered level until more advanced stages of drought (Tardieu and Simonneau, 1998). The sensitivity of maize stomata also protects xylem from cavitation (Cochard, 2002), and under high vapor pressure deficit, it curtails water loss in the middle of the day, so that photosynthesis and transpiration is restricted to the cooler morning periods when WUE is higher (Hirasawa and Hsiao, 1999).

5 The Genetic Basis of Drought Tolerance in Maize

5.1 The QTL Approach

Extensive genetic dissections of drought tolerance traits have been carried out in maize over the last decade, yielding numerous QTL involved in the determination of morphological traits, yield components, flowering traits and plant height (Agrama and Moussa, 1996; Austin and Lee, 1998, Veldboom and Lee, 1996a, b; Frova et al., 1999; Ribaut et al., 1996, 1997; Sari-Gorla et al., 1999; Tuberosa et al., 2002a, b, 2005; Li et al., 2003; Xiao et al., 2004). QTL underlying the accumulation of metabolites (carbohydrates, hormones, osmolytes, etc.), the regulation of water content, cell growth and organ elongation have been described elsewhere in the present chapter or in other cognate reviews (Ribaut, 2006). Phenotypic analysis combined with a modeling approach has also been a suitable alternative for characterizing genotypes and identification of QTL for drought parameters (Tardieu, 2006). The rationale is to "footprint" each genotype by a unique set of parameters that characterizes its development and its responses to environmental conditions. The basis for such rationale is establishment of stable relationships between environmental conditions and phenotypic traits. Using a modeling approach combined with field measurements, common QTLs were identified for leaf growth and ASI in a recombinant inbred line population evaluated under WS conditions. For all common QTL the allele conferring high leaf elongation rate conferred a short ASI, indicating a high silk elongation rate. This result suggests that the genetic determinants of "sources" and "sinks" could be partly common to those two organs (Welcker et al., 2007).

A broad set of QTL identified from various studies has been compiled on a single genetic map (Tuberosa et al., 2002a), an exercise that showed, perhaps unsurprisingly, that drought-related QTL are dispersed throughout the maize genome (Fig. 4). A large QTL mapping effort has been conducted at CIMMYT over the last 12 years, with a two-fold objective. The first was to demonstrate that standard phenotypic screening protocols and data analysis can be used to identify stable genetic regions which are involved in the expression of a particular phenotype across various genetic backgrounds and environments. The second was to compare their map locations in order to identify candidate regions for a marker assisted (MAS) approach to developing drought tolerant maize. The QTL analysis was first conducted individually on each of the 56 trials (44 WS and 12 WW) (Zeng, 1994), identifying 1080 QTL covering nine traits. No individual QTL accounted for more than 20% of the phenotypic variance (most ranged from 4% to10%). The total phenotypic variance for each trait explained by the set of statistically significant QTL lay typically between 25% and 50%, with a maximum of 65%. The genetic effects expressed by the individual QTL from each cross were quite diverse among crosses, depending on the allelic composition of the two parental lines at regulatory loci. Following this initial analysis, the trait data was pooled from all the WS environments and from all the WW environments for each population in turn in order to identify QTL which were stable across both types of environment. This resulted

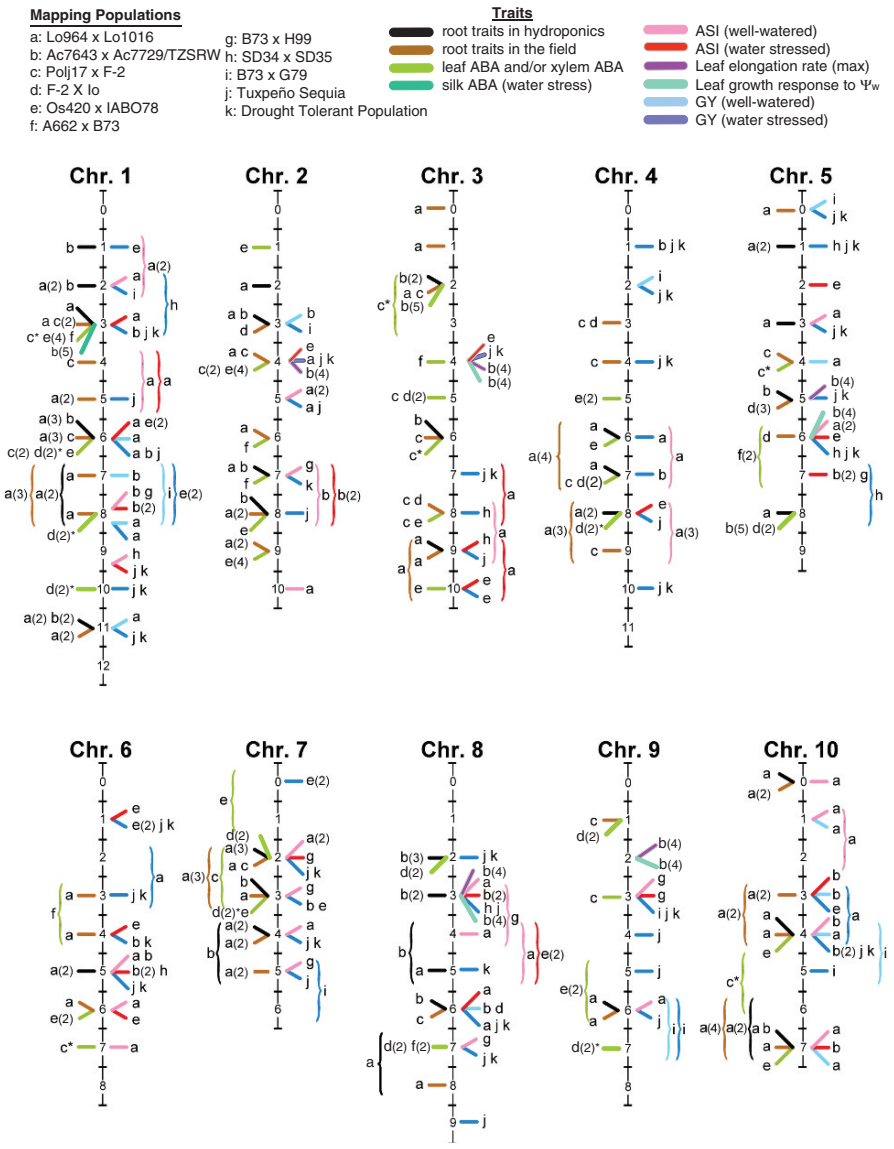

Fig. 4 Combined QTL map for drought related traits and grain yield in 11 maize populations (a–k). Colored bars indicate QTL bin location; vertical parentheses indicate that QTL position spans two bins. Numbers in parentheses indicate independent samplings and/or environments where a QTL was detected for the following: root traits in hydroponics (populations a and b), or in the field (populations a, c, and d); leaf or silk ABA concentrations (populations b, c, d, e, and f); ASI (populations a, b, e, g, and h); leaf elongation rate in well-watered conditions and slope of leaf elongation rate response to water potential (Ψ_w), and grain yield (populations a, b, c, e, g, h, i, j, and k). Asterisks indicate QTL for the concentration of ABA in the xylem sap. Figure modified, with permission, from Tuberosa et al. (2002a). Additional data from Welcker et al. (2007) (leaf elongation rate in population b[4]), Pelleschi et al. (2006) (leaf ABA in population d[2]), and Setter and Ribaut (unpublished data) (leaf and silk ABA in population b[5])

in the identification of, respectively, 372 and 145 loci. To compare QTL locations across populations efficiently, the genetic maps derived from each of the six populations were then aligned with the public maize reference map (IBM2 neighbors, www.maizegdb.org). Although this compilation is still ongoing, preliminary results show clearly that QTL-rich regions are present under both WS and WW conditions, and that some of these are common to both water regimes. An example of a QTL-rich region for stay-green traits identified under water-stressed conditions across a large number of environments on chromosome 10 is presented in Fig. 5. In general, WS QTL tend to be clustered in genomic regions related to drought tolerance, WW ones to plant performance, and common ones co-locate with genes for adaptation.

5.2 *Expression Profiles in Response to Water Stress*

The impact of water deficit on gene expression in maize has been studied by microarray analysis to: (1) provide a broad view of the categories of genes regulated by stress so that inferences can be made concerning the physiological, biochemical

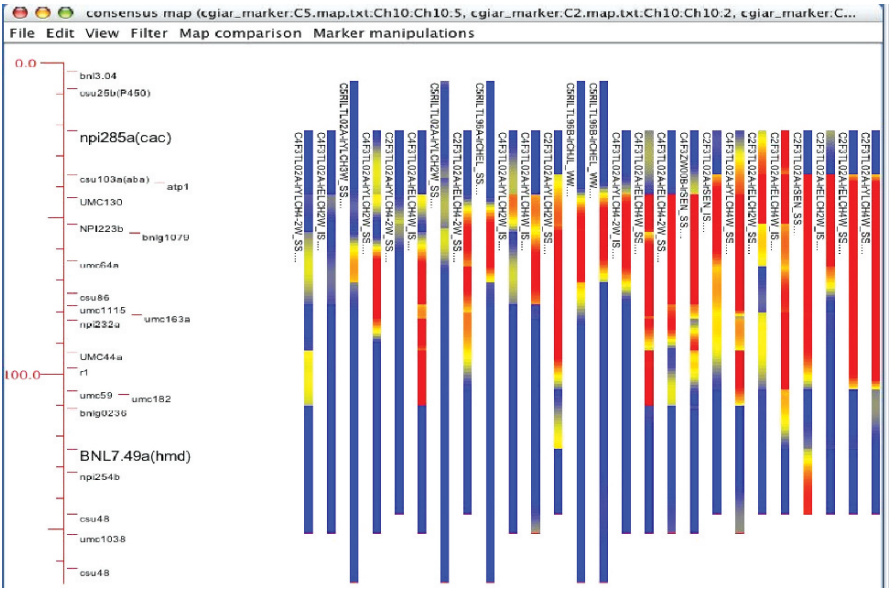

Fig. 5 Display of raw QTL LOD score data for stay-green traits (senescence and chlorophyll content) on chromosome 10 of a consensus map using the Comparative Map and Trait Viewer (CMTV, Sawkins et al., 2004). As the QTL data comes from different genetic maps, the length of QTL strips varies because the bracketing markers on the consensus map are not common to all maps. Each trait is represented as a single color bar or "heat strip." Associated with each heat strip is a text label describing the cross, trait, and environment. The color of the strip correlates with the level of LOD, red indicating significant level of QTL detection ($P > 95\%$), orange and yellow indicate modest QTL tendencies, while weak LOD scores are represented by blue and green

and regulatory changes involved in the response and the differences between tissues and genotypes; (2) identify highly responsive genes, which could represent candidates for cloning and in depth functional analysis; and (3) identify putative promoter motifs involved in the regulation of gene expression under water stress.

Zinselmeier et al. (2002) used a two-pronged approach to study gene expression in pre-fertilization ears: the first consisted of a 384-feature cDNA microarray populated with genes from metabolic pathways and regulatory systems hypothesized to play a role in the water stress response; and the second used a genome-survey (Affymetrix®) chip. Shade stress was used as a surrogate for the assimilate deficit induced by water stress. The major finding from the former experiment was that several starch synthesis genes were coordinately down-regulated, consistent with the known depletion of starch that occurs during stress. The latter confirmed that stress up-regulates a number of genes, such as ABA-responsive *Rab17* and *RIP-2* (Ribosome Inactivating Protein-2) (Bass et al., 2004), and identified several genes not previously associated with the stress response. A further set of microarray-based gene expression data has been generated by Yu and Setter (2003), who contrasted the post-fertilization pedicel/placenta tissue transcriptome of plants suffering from water deficit with that of the endosperm. Whereas numerous stress tolerance genes were up-regulated in the pedicel/placenta, in the endosperm, many genes associated with growth and development were down-regulated. These tissue-specific patterns were consistent with the physiological status of the two tissues. Several other microarray-based studies have been carried out on drought stressed maize (Yu and Setter, unpublished data), leading to the putative identification of multiple genes, including transcription factors and regulatory proteins, as being part of the drought response network. However, these studies have highlighted the problem of inconsistency and non-convergence between ostensibly similar experiments. There is now a recognition that microarray technology should be used only in conjunction with other approaches, rather than as a goal on its own, and that the full utilization of these data sets awaits the sequencing of the maize genome and the development of novel bioinformatic tools (Galbraith, 2006).

5.3 The Candidate Gene Approach

Thanks to rapid progress in biotechnology and genome sequencing, there is now a diverse choice of tools for the identification of candidates for genes involved in specific processes, including the response to drought. Many drought tolerance candidate genes have been documented in the literature (Bohnert et al., 2006a, b; Li et al., 2007; Talame et al., 2007), but few of them have been validated either via reverse genetics or transgenic approaches, or by the demonstration that directed selection under field conditions in diverse genetic backgrounds produces a clear genetic gain. QTL analysis can also provide a means of identifying putative candidates, on the basis of their colocalization with a gene of known or assumed function (Prioul et al., 1997, 1999). But for drought most studies stop at putative association based on

colocalization of candidate genes and QTL along genetic maps, but without strong evidence of causal effects. Due to the reduced genetic effect at most loci involved in drought tolerance, QTL cloning has not been successful so far to discover drought genes in maize. As Salvi and Tuberosa (2005) pointed out, although QTL analysis and cloning targeted at natural genetic variation can shed light on adaptation mechanisms, a mutagenesis and candidate gene validation strategy is much more likely to be productive in discovering the genes underlying QTL effects.

Genotype/phenotype associations show significant potential to identify the genes responsible for a given phenotype, and unlike populations derived from biparental crosses, can in principle evaluate the effects of multiple alleles (Buckler and Thornsberry, 2002). Although association genetics has frequently been used to elucidate the genetic basis of hereditary diseases in humans (Lander and Schork, 1994), the approach has only recently been applied to plant populations (Thornsberry et al., 2001; Camus-Kulandaivelu et al., 2006). In maize, due to its characteristically low level of linkage disequilibrium compared to other genomes, only those loci separated by a few hundred or thousand base pairs (pretty gene dependant) from a locus responsible for a particular phenotype will remain significantly associated with allelic variation for the trait. However, informative polymorphisms associated with contrasting phenotypes are generally well suited as MAS assays. Since association approaches are typically applied to diverse germplasm sets, informative markers are likely to be functional in multiple genetic backgrounds. Thus the combination of association genetics and genetic mapping should be of particular value for crop breeding. Currently, CIMMYT is collaborating with Cornell University and the Chinese, Kenyan, Thai and Zimbabwean national maize breeding programs, in an attempt to identify polymorphisms correlated with key regulators in the drought response and plant phenotypes from a panel of 350 tropical maize lines. The metabolite content (in particular sucrose, glucose, starch, ABA and ABA glucose ester) of leaves and reproductive organs under both WS and WW conditions are being assessed, alongside yield components and various secondary traits. The genotypic component of the association test comprises a haplotyping exercise at about 130 candidate genes chosen from the ABA and carbohydrate synthesis pathways, as well as genes involved in the drought tolerance response of maize and other plant species.

6 Genetic Gains

6.1 Improvement of Drought Tolerance Through Conventional Breeding

6.1.1 Population Improvement for Drought Tolerance in Tropical Maize

Improvement in drought tolerance at flowering has been accomplished at CIMMYT using recurrent selection under managed drought stress. Increases in grain yield of between 0.08 t ha^{-1} (3.8%) and 0.26 t ha^{-1} (12.6%) per selection cycle have been

achieved in this way (Edmeades et al., 1999; Bolaños and Edmeades, 1993a). These gains were associated with increases in both EPP and HI, and reductions in ASI, leaf senescence, plant height, stem biomass, time to anthesis and tassel primary branch number. A small, but significant, increase in grain yield, EPP, kernel number per fertile ear and individual kernel weight was also achieved under WW conditions. Most of those traits are indicators of an increased capacity to partition assimilate to the developing ear and of a higher ovary growth rate under drought stress (Westgate, 1997). The number of spikelets per ear was reduced after selection, but the remaining ones were vigorous, have a greater sink capacity and ultimately are more likely to set grain. Two further populations adapted to the lowland and mid-altitude tropics have been developed at CIMMYT using known sources of drought tolerance (Edmeades et al., 1997b), and deploying indirect selection based on a set of secondary traits. The development of these materials was associated with a yield increase of between 0.08 t ha^{-1} (3.8%) and 0.16 t ha^{-1} (14.3%) per cycle (Monneveux et al., 2006), which was associated with an increase in EPP, kernel number and HI, and a reduction in ASI (Table 1). These improvements in drought tolerance have also brought specific adaptation and improved performance under low fertility conditions, suggesting that tolerance to these two stresses involves common adaptive mechanisms (Bänziger et al., 1999). A common genetic basis between drought tolerance and better performance under low N conditions has been confirmed through QTL analysis, and QTL common for both abiotic stresses were identified for ASI and EPP (Ribaut et al., 2007).

6.1.2 Hybrid Improvement for Drought Tolerance in Tropical Maize

The genetic structure of the populations used to derive inbreds determines the extent of heterosis achievable in inter-inbred hybrids. The probability of obtaining a hybrid yielding 30–50% above the mean of all hybrids under drought stress in tropical maize was 3–6 times greater when the inbreds were selected from stress-tolerant source populations rather than from conventionally selected ones. The correlation between parental inbreds and hybrids, to predict hybrid performance from that of its inbred parents, depends on the trait and the environment. In general, the correlation is relatively high for some traits (e.g., plant morphology, ear traits, maturity) but is relatively low for grain yield which is consistently positive and significant but not high enough to predict hybrid performance. Drought stress factors may influence the correlation between inbred line and hybrid yield. The performance of a line under severe drought stress tends to be more strongly correlated with the performance of related hybrids under drought than with its performance under nonstressed conditions. Selection for a reduction in ASI, senescence and barrenness in the inbreds under stress can be used to predict drought tolerant related hybrids. Drought stress evaluations of lines may be justified in early generations, while at an advanced stage of inbreeding, the tolerance of lines is best evaluated in hybrid combinations to evaluate the dominance genetic effects responsible for heterosis (Betrán et al., 2003b).

A diallele experiment conducted in both WW and WS conditions led to the recognition that, under drought stress, general combining ability is more important than specific combining ability. Thus dosage effects are relevant, and the inference is that to obtain a

Table 1 Effect of selection for drought tolerance (carried out under drought conditions and based on selection for ears per plant, ASI, senescence and leaf rolling) on yield, yield components, ASI, kernel abortion rate, and indicators of dry matter partitioning, under drought and optimal conditions in the DTP1 population (6 cycles of recurrent selection) (from Monneveux et al., 2006)

	Grain yield (t ha^{-1})	Ears per plant	Number of grains per ear	1,000-Kernel weight	ASI	Kernel abortion rate	Tassel dry weight	Ear dry weight	Stem dry weight	Leaf dry weight	Husk dry weight	Above-ground biomass	Harvest index
Water-stressed													
Gain $cycle^{-1}$	0.16	0.03	12.76	−2.01	−0.58	−0.70	−0.39	3.58	−2.60	−1.02	0.46	−0.01	0.03
Gain $cycle^{-1}$ (%)	14.3	5.7	6.3	−1.1	−11.7	−7.1	−8.2	17.4	−6.8	−5.8	7.6	0.0	21.5
Gain significance	*	**	*	NS	***	*	*	*	**	*	NS	NS	***
Well-watered													
Gain $cycle^{-1}$	0.08	0.02	−8.00	4.58	−0.37	0.06	−0.24	4.32	0.91	−0.05	1.09	6.04	0.00
Gain $cycle^{-1}$ (%)	1.3	2.0	−2.1	2.1	−12.3	10.3	−6.7	4.4	2.7	−0.1	5.9	2.9	0.8
Gain significance	NS	NS	NS	***	**	NS	*	*	NS	NS	*	**	NS

Values within a column followed by the same letter are not significantly different
*Significant at $P < 0.05$; **Significant at $P < 0.01$; ***Significant at $P < 0.001$

tolerant hybrid, both parental inbreds should be drought tolerant in their own right (Betrán et al., 2003a). Greater heterosis was observed in hybrids evaluated under drought stress than under WW conditions, and the grain yield differential between hybrids and inbreds increased with the intensity of the drought stress (Betrán et al., 2003c). Bänziger et al. (2004) compared hybrids selected under managed drought environments with commercial hybrids developed conventionally in over 50 locations in eastern and southern Africa. The former were superior at yield levels ranging from 1 t ha^{-1} to 10 t ha^{-1}, and the yield differences were most pronounced in the lowest yielding environments. Thus it is clear that for hybrids as well as for conventional open-pollinated varieties, selection under controlled stress regimes is advantageous.

6.1.3 Hybrid Improvement of Drought Tolerance in Temperate Maize

The hybrid seed industry has succeeded by investing in extensive and efficient testing, improving mechanization, acquiring reliable phenotypic data, and imposing high selection intensities (Coors, 1999). The increase over time in commercial hybrid performance under drought stress has been attributed to the use of rain-fed nurseries which are occasionally prone to drought, a concentration on high planting densities and large populations, and the recycling of inbreds with proven high stability (Bruce et al., 2002). The evaluation of hybrids in multiple environments is designed to maximize adaptation and stability. These environments differ with respect to cultural practices (e.g., planting density, planting date, drought stress, fertilizer input, tillage, crop rotation), as well as climatically and edaphically. It has been suggested that much of the recent improvement in hybrid performance is due to a greater tolerance to abiotic stress, particularly in situations where high planting densities are used (Duvick et al., 2004; Tollenaar and Wu, 1999). Thus current hybrids are more drought tolerant than older ones, and the improved adaptive traits are productivity, ASI, rooting depth, water extraction capacity, and stay-green (Campos et al., 2004).

6.2 Molecular Breeding (MB) Approach

Despite the documentation of numerous QTL (www.maizegdb.org/qtl.php), including some involved in adaptation to WS conditions, the exploitation of QTL in public breeding programs has to date been limited, although it is not the case in the private sector. A few examples demonstrating the potential of these technologies are given below.

6.2.1 The Marker-Assisted Back-Cross (MABC) Approach

A successful MABC experiment, in which favorable alleles were introgressed at five genomic regions involved in the expression of yield under water-limited conditions, was described by Ribaut and Ragot (2007). Selection from MB BC_2F_3 families

were crossed with two testers and evaluated phenotypically. Under severe WS conditions (60–80% yield reduction compared to WW conditions), the best five MABC-derived hybrids yielded on average at least 50% more than control hybrids. Under mild stress (<50% yield reduction), no difference was observed between the MABC and the control hybrids. Importantly, no yield penalty was observed under WW conditions. Although this study demonstrated that genetic gains can be achieved by introgressing drought QTL such that favorable alleles are transferred from a donor to a recipient line, it was recognized that the approach can only deliver a limited level of improvement in drought tolerance because it is used to generate an improved version of an existing genotype. To overcome this limitation and take advantage of the presence of "good" alleles in two or more parental lines, other MB approaches, such as described below, are necessary.

6.2.2 The Marker-Assisted Recurrent Selection (MARS) Approach

Computer simulations suggest that MARS out-performs phenotypic selection in terms of the efficiency with which favorable alleles are accumulated in a single individual (Charmet et al., 1999; Van Berloo and Stam, 2001). The advantage of MARS over phenotypic selection is predicted to be larger, the more diverse the population, a situation which is the norm for tropical and subtropical maize breeding. Although the use of MARS has enjoyed only limited success in the public sector - probably largely because of inadequate facilities to conduct accurate phenotyping across multiple sites - several large-scale private sector MARS applications have delivered significant levels of genetic gain (Ragot et al., 2000; Johnson, 2004; Crosbie et al., 2006). For example, Ragot et al. (2000) identified QTL in a bi-parental maize population and then applied a genetic index involving agronomic performance (grain yield, grain moisture at harvest) and adaptation to abiotic stress (early vigor under cold conditions). Genetic values were computed for the index as well as for individual traits for all the MARS generations, including the parents of the population. Similarly Eathington (2005) demonstrated that the rate of genetic gain achieved through MARS was about twice that possible using phenotypic selection. Several recent accounts in which at least one of the parental lines of commercial maize hybrids was derived via MARS imply that MARS is being widely implemented in some private sector maize breeding programs.

6.3 The Transgenic Approach

Relatively few drought tolerant transgenics have been reported so far but research conducted in particular in the private sector appears to be very promising. Overexpression of a Nicotiana protein kinase (NPK1) with a putative role in oxidative stress response was found to enhance photosynthetic maintenance during drought, although yield was unaffected (Shou et al., 2004). Transgenic maize that overexpressed ASR, a transcription factor associated with stress, had an increase in foliar senescence under drought conditions (Jeanneau et al., 2002). Transgenics of

maize Rab17, a late embryogenesis protein that is associated with dehydration response, indicated that its phosphorylation status was an important determinant of its effects on stress response during seed germination (Riera et al., 2004). Overexpression of the stress-related maize transcription factor ZmDREB2A in Arabidopsis increased tissue survival upon dehydration, but it has not yet been tested in transgenic maize (Qin et al., 2007).

Considering the economical impact of drought both in the North and the South, the development of genetically modified drought tolerance crop, and maize in particular, has been a top priority for most private companies over the last decade. As a reaction to stress, plants activate defensive strategies in their cells. Of pivotal significance in the process is the so-called PARP enzyme, poly(ADP-ribose) polymerase. Expression of PARP results in the accelerated degradation of energy reserves and amplifies plant respiration. Consequently, free radicals form in the cells, permanently damaging the plants. Researchers at Bayer Corporation have down-regulated the genetic expression of the PARP enzyme to such an extent that defense to stress is still maintained, but without impairing the growth of the plant (De Block et al., 2005). Recently, a group from Monsanto Company reported that overexpression of the maize transcription factor, ZmNF-YB2, increased drought tolerance and yield under drought (Nelson et al., 2007). This transcription factor, which had not previously been associated with stress response, was isolated following high throughput transformation involving overexpression of 1,500 transcription factors in Arabidopsis followed by screening for stress tolerance. Based on a recent press release, Monsanto's drought tolerant corn may be ready for commercialization as early as 2010, and products from other companies should follow soon after (ACB, 2007).

6.4 Perspectives for New Segregating Populations and MB Strategies

The success of plant breeding is measured by a crop's performance in its target environment. The most critical determinant of the achievable level of genetic gain is the base population upon which selection is imposed. Two important characteristics of the base population are the genetic value of the segregating alleles present (which is governed by the choice of parental lines) and the size of the population itself. Several methods are available to identify critical genomic regions or genes to include in an MB scheme, and markers can be dove-tailed into a breeding program in a number of different ways (Fig. 3). Therefore, our thinking should no longer be limited to a consideration of what markers can do for conventional breeding. Rather, there is a need to explore alternative segregating populations and selection approaches which take advantage of our increasing ability to define which alleles are the most desirable at multiple target loci.

Novel approaches based on pre-existing genetic information, which a priori sidestep a need to identify QTL or gene-based markers, are particularly appealing. Thus, if a high value genomic region has been identified from a meta-analysis of diverse

germplasm, then there is a high probability that this same region will be involved in the determination of the target phenotype in a different genetic background. Exploratory MB experiments at CIMMYT aimed at improving the drought tolerance of tropical maize have generated promising results along these lines. The markers used were based on gene clusters involved in drought tolerance traits identified from previous studies. The two major limitations of using neutral markers for this mode of MAS are *linkage drag* (where deleterious alleles at loci linked to the selection markers are unintentionally co-selected) and the *lack of contrasting alleles* between a given pair of parental lines at (some of) the target loci. The next logical step will be to directly target the genes responsible for a desirable phenotype, seeking ways to increase allelic diversity at each of these loci (such as via the use of multi-parental crosses, Jourjon et al., 2005) and then screen sufficiently large populations (at least thousands of individuals) to maximize the probability of recovering critical recombinants. This strategy, which has been termed "breeding by design" (Peleman and van der Voort, 2003) is designed to enable the stacking of favorable alleles from the various parents at each of a large number of the loci under selection.

7 Conclusions

A multidisciplinary approach, which ties together breeding, physiology and molecular genetics, can bring a synergistic understanding to the response of maize to water deficit (Ribaut et al., 2004). Conventional breeding has improved the drought tolerance of temperate maize hybrids, and the use of managed drought environments, accurate phenotyping, and the identification and deployment of secondary traits has been effective in improving the drought tolerance of tropical maize populations and hybrids as well. The contribution of molecular biology will be to identify key genes involved in metabolic pathways related to the stress response, for example, the factors involved in kernel development. Functional genomics, reverse and forward genetics and comparative genomics are all being deployed with a view to achieving these goals. Armed with a better understanding of the physiological mechanisms and the genetic basis of the response of maize to drought, it should become increasingly feasible to identify, transfer and select key genes and alleles to build genotypes with much improved tolerance to drought.

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Responses to Oxygen Deprivation and Potential for Enhanced Flooding Tolerance in Maize

Chalivendra C. Subbaiah and Martin M. Sachs

Abstract Although plants release oxygen as a byproduct during the process of photosynthesis, they are obligatory aerobes requiring the gas for their survival, growth and productivity. Oxygen limitation, the predominant stress in flooded plants, dramatically affects the gene expression, development and productivity of maize. Serious efforts are being made to improve flooding-tolerance of the crop across the globe. Here, we present an overview of gene expression changes in response to oxygen deprivation. We also discuss the early cellular events that lead to altered gene expression and how the sub-cellular responses, in turn, may shape the organismal responses to flooding stress. Complete lack of O_2 (anoxia) leads to an immediate cessation of protein synthesis followed by a selective synthesis of about twenty anaerobic proteins in maize seedlings. Among these are enzymes involved in glycolytic-fermentative pathways needed for rescuing the cell from the resulting energy crisis and other genes that appear to function in longer-term responses, such as aerenchyma formation and root tip death. Although 'aerobic' proteins continue to be synthesized under hypoxia, the majority of the 'anaerobic' genes are transcriptionally and translationally induced even by a partial depletion of oxygen (hypoxia). This indicates the presence of an exquisite oxygen-sensing system in plants that may be optimizable for enhanced tolerance. Research in this area has shown that transient cytosolic Ca^{2+} perturbations are essential to trigger adaptive gene expression. Although glycolysis/fermentation enzymes are necessary for adaptation, their activities do not correlate with flooding tolerance. Instead, tolerance to prolonged stress seems to depend on the capacity to quickly restore cellular ionic homoeostasis and whole plant modifications for recouping O_2 supply. Current genetic or molecular breeding efforts are aimed at exploiting the genetic variability for flooding tolerance available in maize and its wild relatives. In addition, we suggest novel molecular strategies based on our understanding of early events and molecular responses.

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1 Maize Growth and Productivity Under Oxygen Deprivation

Oxygen availability is the primary limiting factor for plant growth in flooded soils. In 1993, ~20 million acres of corn (*Zea mays* L.) and soybean (*Glycine max* L.) were flooded in the Midwestern United States leading to heavy economic losses, as estimated by the United States Department of Agriculture, National Agricultural Statistics Service (Suszkiw, 1994). According to the CERES-Maize model (that predicts maize growth and development based on soil-atmospheric parameters), the probability of crop damage due to heavy precipitation and waterlogged soils in the corn-belt, i.e., the Midwestern US, could be 90% greater in the 2030s, and 150% more severe in the 2090s, compared to present levels (Rosenzweig et al., 2002). Early seedlings represent the most susceptible crop growth phase to flooding stress in maize (e.g., Zaidi et al., 2004) and are also most likely to experience drowning due to prolonged/heavy rainfall in wet springs. Therefore, research has been focused on the seedling responses to flooding. Later phases of maize development are also significantly affected by flooding stress (Yan et al., 1996; Zaidi et al., 2004; Yordanova and Popova, 2007). Flooding leads to oxidative damage, chlorophyll as well as protein loss and decreased rate of net photosynthesis in V3-V4 seedlings (Yan et al., 1996; Yordanova and Popova, 2007), while severely affecting plant size, anthesis, silking and eventually kernel development when the stress is imposed at later stages (Zaidi et al., 2004).

Even during normal development (i.e., under non-flooding conditions), plants suffer from oxygen limitation due to their bulky biomass and dependence on diffusion via intercellular spaces for their oxygen supply. The effects of atmospheric oxygen levels on reproductive development were shown to be agronomically important (e.g., Quebedeaux and Hardy 1975) and have received renewed attention (e.g., Kuang et al., 1998; Rolletschek et al., 2005).

2 Methods of Imposing Flooding Stress in Laboratory Studies

Anoxia has often been imposed by the complete submergence of 3-day-old dark-grown seedlings in a flooding buffer (5 mM Tris-Cl, pH 7.5 with 250 mg of amoxicillin plus 125 mg of ampicillin to minimize bacterial growth) in airtight containers. This treatment leads to a gradual depletion of oxygen, not an anoxic shock and thus, resembles the natural stress regime. The use of dark-germinated pre-emergent seedlings (where the leaves are still enclosed in the coleoptile) allows the imposition of strict anoxia, avoiding interference from light-dependent oxygen evolution manifested by green seedlings. In these experiments, flooding tolerance was measured as the percentage of seedlings that survive and can continue to grow following a period of submergence treatment (Lemke-Keyes and Sachs, 1989; Subbaiah et al., 1994b). Hypoxia was imposed by submerging only the root portion in the flooding buffer, while leaving the shoots exposed to the air (Saab and Sachs, 1996). Metabolic

labeling experiments were carried out in a homemade or a commercial 'anaerobic chamber', whose internal gas composition is maintained by a calibrated gas-mix pumped from outside (Sachs et al., 1980; Subbaiah and Sachs, 2001). Other researchers have used similar or minor variations of submergence/hypoxic stress treatments in their studies (e.g., Johnson et al., 1994).

3 Gene Expression Changes in Response to Oxygen Deprivation

Anaerobic treatment of maize seedlings drastically alters the profile of total protein synthesis. Under an anaerobic environment, 20 proteins that account for more than 70% of the total translation are selectively synthesized (Sachs et al., 1980). The coordinated synthesis of anaerobic proteins (ANPs) appears to follow the programmed transcription and co-regulation of genes that encode these proteins. This proposal is supported by gene expression profiling studies and analysis of upstream regulatory regions of genes that are responsive to low oxygen treatment in maize and other model plant systems (see below). Most of the ANPs identified were found to be enzymes of glycolysis or sugar-phosphate metabolism; such as aldolase (Kelley and Tolan, 1986), pyruvate decarboxylase (Laszlo and St Lawrence, 1983), enolase (Lal et al., 1998), glucose-6-phosphate isomerase (Kelley and Freeling, 1984), sucrose synthase (Springer et al., 1986), and alcohol dehydrogenase (Sachs and Freeling, 1978; Sachs et al., 1980). However, many genes not involved in glucose-phosphate metabolism (Vogel and Freeling, 1992; Peschke and Sachs, 1994; Saab and Sachs, 1995, 1996; Chang et al., 2001; Klok et al., 2002; Zhang et al., 2006) are also induced by anoxia. The genes and proteins induced by anaerobic stress in maize have extensively been reviewed (Sachs, 1993, 1994; Sachs et al., 1996; Subbaiah and Sachs, 2003).

Anaerobiosis results in alterations of gene expression in plants leading to the accumulation of the ANPs. These alterations occur at transcriptional, translational and post-translational levels (Sachs et al., 1980; Ferl et al., 1980; Rowland and Strommer, 1986; Bailey-Serres et al., 1988; Dennis et al., 1989; Webster et al., 1991; Russell and Sachs, 1989; 1992; de Vetten and Ferl, 1995; Manjunath and Sachs, 1997; Manjunath et al., 1999; Subbaiah and Sachs, 2001). At the level of translation, anaerobic treatment of maize seedlings disrupts polysomes (Bailey-Serres and Freeling, 1990) and leads to a redirection of protein synthesis (Sachs et al., 1980; Russell and Sachs, 1992). In the first five h of anaerobic treatment (transition period), there is a rapid increase in the synthesis of a class of polypeptides (~33 kDa; the transition polypeptides). After 90 min of anoxia, the synthesis of ANPs is induced. After 72 h, protein synthesis decreases concurrently with the start of seedling death (Sachs et al., 1980). The molecular basis of this selective translation is not yet fully understood. Specific structural determinants in the untranslated regions of mRNA (Bailey-Serres and Dawe, 1996; Branco-Price et al., 2005) and induction of specific RNA-binding proteins (Klok et al., 2002) are important in this process. In addition, post-translational regulation of initiation factors as well as ribosomal proteins by reversible phosphorylation appears to play a role (Webster et al., 1991; Perez-Mendez et al., 1993; Manjunath

et al., 1999). In addition to the protein synthetic machinery, post-translational changes involving ANPs occur under anoxia (e.g., Subbaiah and Sachs, 2001). Analysis of newly-synthesized proteins in the root tip indicated that several proteins undergo post-translational modifications under O_2 deprived conditions (Chang et al., 2001). This reprogramming of gene expression is accompanied by metabolic (e.g., switch to a fermentative pathway; Drew et al., 1985; Kennedy et al., 1992) and structural (e.g., aerenchyma formation, cell/tissue death; Drew et al., 1979) changes that define the organ/organismal tolerance to flooding.

Although, to date, there have been relatively few studies on the transcriptome and proteome changes in response to oxygen deprivation is in maize (Chang et al., 2001; Zhang et al., 2005, 2006), there have been a number of reports using microarrays and related technologies in *Arabidopsis* and rice (e.g., Klok et al., 2002; Agarwal and Grover, 2005; Liu et al., 2005; Loreti et al., 2005; Lasanthi-Kudahettige et al., 2007). Inferences from this data would immensely be useful in developing molecular strategies to improve flooding tolerance, e.g., in identifying novel candidate genes/gene networks, promoters of appropriate strength or specificity and post-transcriptional control mechanisms.

4 Calcium Perturbations Are Critical for Anoxic Gene Induction in Maize

The ANP genes are rapidly turned on even by mild hypoxia and rapidly turned off upon reoxygenation (Paul and Ferl, 1991; Klok et al., 2002). Such a response implicates a fast and precise O_2-sensing system operating in plant cells. Characterization of the pathway and the players involved would allow fine-tuning the system and enhancing stress tolerance. The work has identified a Ca^{2+}-mediated pathway of anoxic gene induction as well as post-anoxic seedling survival in maize cells and seedlings (Subbaiah et al., 1994a, b, 1998). The broad conservation of this pathway among plant species that possess diverse levels of flooding tolerance, e.g., rice and barley (Sedbrook et al., 1996; Chung and Ferl, 1999; Tsuji et al., 2000; Virolainen et al., 2002; Nie et al., 2006) allows the exploitation of species-specific signaling variations associated with the diversity in tolerance.

Confocal analysis using compartment-specific Ca^{2+} probes showed that the Ca^{2+} signal probably originates in mitochondria (Subbaiah et al., 1998). Since oxygen is more diffusive than any potential signal molecule that has to traverse the cellular membranes, anoxia may be first sensed at the mitochondrial electron transport chain, when O_2 levels limit electron transport. However, in view of the sensitivity of gene expression changes even to mild alterations in the O_2 availability (Paul and Ferl, 1991), i.e., the genes are induced at much higher concentrations than the K_m (O_2) of cytochrome a_3, a low affinity system, such as, the plasma membrane redox system or the mitochondrial alternative oxidase (AOX) may be a more likely sensor. Subbaiah et al. (2006b; unpublished) have recently shown that an inhibition of AOX activity in *Arabidopsis* suspension cells leads to an increase in mitochondrial ROS and the consequent induction of

ADH gene under normoxia. In *Arabidopsis*, O_2 deprivation stimulates a Rop- (a plant specific monomeric G-protein) signaling pathway. This, in turn, leads to the activation of a Ca^{2+}-dependent NAD(P)H oxidase located either on the plasma membrane or in mitochondria, resulting in H_2O_2 production and ADH gene activation (Baxter-Burrill et al., 2002). Subsequent work from this laboratory indicates that reactive oxygen species (ROS) production under low oxygen is primarily localized to the mitochondria of seedling roots (Branco-Price et al., 2006). While the role of plasma membrane redox systems and associated second messengers needs to be fully probed, these analyses highlight the centrality of mitochondria in oxygen sensing. It is likely that ROS may act as the trigger for the Ca^{2+} release from mitochondria under O_2 deprivation. The consequent cytosolic Ca^{2+} perturbations seem to link the oxygen deficit perceived in the mitochondrion with the chromatin changes that precede gene expression (Paul and Ferl, 1991). Consistent with this proposal, large anoxia-induced changes in the nuclear localized Ca^{2+} levels were observed in maize cells (Subbaiah et al., 1994a, 1998).

Oxygen deprivation is also accompanied by cytosolic acidification (Roberts et al., 1984). Cytosolic pH changes are potentially involved in ANP gene activation (Fox et al., 1995) and other responses such as anoxic down regulation of water uptake (Tournaire-Roux et al., 2003).

4.1 Ionic Homeostasis As an Integral Part of Adaptation to Anoxia

Cytosolic Ca^{2+} changes not only trigger downstream signaling components but also are involved in establishing ionic homeostasis. Under energy-deprived conditions, an imminent danger to cells (the organism, as well) is an unregulated traffic of ions. A continued elevation of Ca^{2+} or decline in pH, if unattenuated, is not only detrimental in the long term but may also impair the immediate capacity of cells to mount an adaptive response. Therefore, ionic homeostasis is undoubtedly a key component of the cellular adaptation to stress. In maize root tip cells, cytosolic pH sharply decreases in response to anoxia from pH 7.5 to 6.9 within the first 10 min, but then quickly stabilizes at 7.1 over the next 10–15 min (Saint-Ges et al., 1991; Fox et al., 1995). Mutants impaired in pH stabilization are acutely sensitive to anoxia (Roberts et al., 1984). A potential mechanism of preventing pH decline is the activation of proton-consuming enzymes, such as malic enzyme or glutamate decarboxylase (Roberts et al., 1984; Sachs et al., 1996; Ratcliffe, 1997), among other measures of pH stabilization (e.g., Xia and Roberts, 1994). The proposal that $[Ca]_i$ changes could be important in the establishment of the pH-stat was reinforced by the discovery that plant glutamate decarboxylases (GADs) interact with calmodulin (CAM; Snedden et al., 1995; Shelp et al., 1999). Furthermore, GAD activity in many species exhibits a sharp pH optimum of 5.8 with little activity at or near neutral pH in the absence of CAM (Shelp et al., 1999). Therefore, a pH- and CAM-dependent activation and its significant ability to consume protons (Snedden et al., 1995), make GAD a candidate regulator of cytosolic pH under anoxia. GAD activity

is induced in carrot protoplasts by a short hypoxic treatment (Carroll et al., 1994). We examined the role of GAD in pH homeostasis of anoxic maize roots. A rapid induction of GAD activity as well as an increased association of this activity with CAM-containing protein complexes was observed in maize roots, within minutes of anoxic treatment. Furthermore, Ca^{2+}/CAM antagonists abolished the activity *in vitro*, indicating that CAM-association may be needed for the activation of GAD under anoxia (Subbaiah and Sachs, unpublished). The kinetics of GAD activation with the onset of anoxia, coincided with the time course of pH stabilization in maize root tips (e.g., Fox et al., 1995). GAD transcript induction in O_2-deprived *Arabidopsis* root cultures also peaked during the first 30 min of low oxygen treatment (Klok et al., 2002). Several putative GAD clones were identified in the maize EST database (http://MaizeGDB.org/mgdp/est/), indicating that a gene family may encode this enzyme in maize. Sequence analysis indicated that GAD is encoded by at least three genes in maize (Subbaiah and Sachs, unpublished). Only one of them was inducible in maize roots during early anoxia, with root tip-specific expression (Subbaiah and Sachs, unpublished). We have also examined the distribution of ESTs among the source cDNA libraries ('e-northern analysis'). The majority of ESTs came from the libraries that were made from young and meristematic tissues (e.g., leaf and tassel primordia, early embryo, and anthers) that are metabolically-intense and rich in mitochondria (Subbaiah and Sachs, unpublished).

Efflux transporters (transporters that remove Ca^{2+} from the cytoplasm) play an important role in Ca^{2+} signaling, particularly in restoring Ca^{2+} levels to the resting levels. A CAP1 cDNA clone was isolated from anoxic maize roots. CAP1 encodes a Ca^{2+}-ATPase and is induced during the first 4–6 h of anoxia (Subbaiah and Sachs, 2000). This clone shares sequence identity with the animal Ca^{2+}-ATPases located on the endoplasmic reticulum (SERCAs or ER-type calcium pumps). However, maize CAP1 differs from other ER-type Ca^{2+}-pumps in that it has a calmodulin-binding domain at its carboxy-terminus, similar to the plasma membrane located Ca^{2+} ATPase of animal cells. The CAP1 cDNA complemented yeast mutants defective in Ca^{2+} pumps and supported CaM-stimulated Ca^{2+} transport (Subbaiah and Sachs, 2000). The CAP1 transcripts are of low abundance in maize seedling tissues (most abundant being in the root tip; unpublished) and mildly induced only during early anoxia (Subbaiah and Sachs, 2000). The low abundance of transcripts coincided with the scarce amounts of cognate protein in maize microsomes, indicating a tight regulation of CAP1 expression. Furthermore, calmodulin-regulation of Ca^{2+} transport suggests the involvement of CAP1 product in attenuating cytosolic Ca^{2+} rise by a feedback mechanism in the early hours of O_2 deprivation. On the other hand, suppression of CAP1 transcripts under prolonged anoxia indicates a loss of Ca^{2+} homeostasis and coincides with cell death initiation in the root tip (Subbaiah and Sachs, 2000).

5 Regulation of Sucrose Synthase (SUS) Under Anoxia

SUS is a unique enzyme with an ability to mobilize sucrose into structural (e.g., cellulose or callose biosynthesis), storage (starch synthesis) and metabolic (e.g., glycolysis) functions of plant cells (e.g., Ruan et al., 1997). Although invertase

is another key enzyme of sucrose utilization in plants, the pathway of sucrolysis by SUS is favored particularly under energy-limiting conditions due to the lower overall energy costs. This is reflected by the intricate regulation of the enzyme under O_2 deprivation (Zeng et al., 1999; Subbaiah and Sachs, 2001; reviewed in Koch, 2004). Maize SUS is encoded by three genes *sus1, sus2* and *sh1* (Chourey, 2006) and the corresponding enzyme isoforms are SUS1, SUS2 and SUS-SH1 (SH1). The *sh1* gene is expressed mostly in the developing endosperm, where as sus1 is expressed in many plant parts including the aleurone and basal part of the developing endosperm. The *sh1* gene is induced by anoxia both at transcriptional and translational levels (ANP87; Springer et al., 1986). The *sus1* and *sus2* gene are only mildly induced by anoxia. Although the *sh1/sus1* double mutants have been shown to be less tolerant to anoxia (Ricard et al., 1998), the contribution of SH1, i.e., the anoxia-inducible isoform, to anoxia tolerance had not been previously examined. Further analysis indicated that the differential regulation of the two genes at transcriptional and translational levels extends into the post-translational level, with potent effects on adaptation to anoxia (Subbaiah and Sachs, 2001).

5.1 Reversible Phosphorylation of SUS Under Oxygen Deprivation: A Mechanism of Carbon Flux Control?

One common mechanism that cells use to rapidly decipher and amplify the $[Ca]_i$ changes is reversible protein phosphorylation. Addition or removal of phosphate can lead to changes in the activation status, catalytic activity, or cellular localization of effector proteins (e.g., Kim et al., 1994; Huber and Huber, 1996). These changes, in turn, can lead to transient alterations in gene expression and metabolism or even long-lasting modifications in the plant form and function (e.g., Maurel et al., 1995; Fankhauser et al., 1999; Rashotte et al., 2001; reviewed in Huber, 2007).

The anaerobically induced SUS isoform, SH1, is also post-translationally regulated by phosphorylation, and this regulation is among the early responses that culminate in the death of primary root tip under prolonged anoxia (Subbaiah and Sachs, 2001). During prolonged anoxia, the protein was hypo-phosphorylated, and by 48 h, most of the protein existed in an unphosphorylated form. In seedlings submerged for two h or longer, a portion of SH1 became associated with the microsomal fraction (Subbaiah and Sachs, 2001). The membrane localization of SH1 increased with the duration of anoxia, but was confined only to the root tip. This preceded an extensive induction of callose and other symptoms of root tip death (e.g., nuclear DNA nicking; Subbaiah and Sachs, unpublished). Consistent with the Ca^{2+} dependence of SUS phosphorylation (e.g., Huber et al., 1996), EGTA addition to the submergence buffer led to an increased dephosphorylation as well as membrane localization of SH1 and greater callose accumulation. On the other hand, Ca^{2+} addition decreased the proportion of membrane-bound SH1 and callose deposits (Subbaiah and Sachs, 2001). In developing cotton fibers, sucrose synthase is shown to be associated with the glucan synthase complex

located in the plasma membrane (Amor et al., 1995). A redistribution of SUS activity with a concomitant increase in the content of cell wall carbohydrates has also been observed in O_2 deprived wheat roots (Albrecht and Mustroph, 2003). Furthermore, genetic analysis suggested that this response was isoform-specific in that *sh1* mutants maintained SUS phosphorylation and had low amounts of callose deposits in the root tip even under prolonged anoxia. This correlated with the superior anoxia tolerance of *sh1* mutants to that of the non-mutants (Subbaiah and Sachs, 2001). The work indicated a functional divergence of SUS isoforms under anoxia, in that, SUS1, existing mostly as a soluble form, may supply hexoses to glycolysis, while SH1, being distributed to both soluble and membrane fractions, contributes to cell wall biosynthesis as well. Such a dichotomy is consistent with the proposed roles of SUS isoforms in the developing maize endosperm (Chourey et al., 1998).

5.2 Organelle Distribution of SUS: A Signaling Role?

Sucrose synthase (SUS), may also have a signaling function in addition to its role as the major sucrolytic enzyme in plants (Subbaiah et al., 2006a). This inference is based on the following observations: (1) tissue-specific, isoform-dependent and metabolically-regulated association of SUS with mitochondria and nuclei and (2) isoform-specific and anoxia-regulated interaction of SUS with the voltage-dependent anion channel (VDAC; viz., mitochondrial porin), the major outer mitochondrial membrane protein. In addition, VDAC is also localized to the nucleus and is associated with SUS in this compartment as well. Phosphorylation of SUS seems to regulate its distribution between these two organelles, with increased nuclear accumulation under anoxia. Based on its increased accumulation in the nucleus and differential interaction with VDAC in anoxic cells, it was hypothesized that SUS and VDAC may be involved in inter-compartmental communication and the co-ordination of cellular energy metabolism (Subbaiah et al., 2007).

6 Cell Death Pathways Under Oxygen Deprivation

O_2 deprivation is an energy limiting stress that severely affects cell survival. However, even terrestrially-adapted plants survive complete submergence at least for a few hours, unlike their animal counterparts. One important feature that distinguishes multi-cellular plants from animals is their possession of multiple growing points, which endows plants a unique ability to eliminate superfluous cells/tissues/organs under stress and regenerate them if favorable conditions return. O_2-deprived maize roots exhibit two cell or tissue-death pathways. These two pathways are clearly distinct in their symptomology, oxygen requirement as well as the location within the root.

6.1 Lysigenous Aerenchyma Formation

Inner cortical cell layers of the primary or nodal roots are selectively killed under hypoxia (i.e., partial submergence, i.e., only when the roots were submerged), leading to aerenchyma formation (Drew et al., 2000). This selective cell death not only reduces the demand for O_2 but more importantly, enhances root porosity and facilitates oxygen diffusion from the exposed plant parts into the submerged ones. Aerenchyma formation requires the presence of oxygen and occurs 3–4 cm behind the tip (He et al., 1992). This allows the adaptation of root tip to the localized anoxia (Gibbs et al., 1995) and prolongs the survival of seedlings. The nature as well as regulation of cell death during aerenchyma formation has been examined in some detail (He et al., 1992; Drew et al., 2000; Gunavardena et al., 2001). These studies indicate that the aerenchyma formation is by a Ca^{2+}-dependent and genetically programmed cell death (PCD) pathway (Drew et al., 2000). Cyto-histological data indicate that the hypoxically-induced PCD may not be entirely similar to the canonical apoptic pathway of animal cells, but partly resembles the cytoplasmic or necrotic death (Gunavardena et al., 2001).

6.2 Root Tip Death

Under complete submergence, maize seedlings exhibit another cell death process that also appears to have an adaptive significance. Although prolonged anoxia ultimately kills the entire seedling, different tissues of an individual plant differ in their tolerance (Johnson et al., 1989, 1994; Ellis et al., 1999). Maize root tips that are not hypoxically-acclimated are very sensitive to anoxia and die within a few hours (Roberts et al., 1984; Johnson et al., 1989). Root tips are composed of tightly packed tissues with few, if any, intercellular spaces and consequently suffer from restricted oxygen supply. In flooded seedlings, root tip death may be a natural consequence of O_2-starvation and the attendant repression of substrate transport. Considerable attention has been paid to strategies/mechanisms that prolong the anoxia tolerance of the primary root tip in young maize seedlings, as the tip of the primary root is considered to be very important for seedling establishment (for a review, see Drew et al., 1994). On the other hand, when energy generation becomes extremely limiting under severe anoxia, the loss of metabolically intensive tissues such as the root tip might prolong the survival of the shoot and the root axis. The facilitated survival of these two organs (particularly the shoot tip: the only aerial meristem in non-tillering maize genotypes) during submergence may increase the chances of seedling recovery after reoxygenation. This proposal was tested and results indicate that the root tip indeed acts as a dispensable sink in anoxic seedlings (Subbaiah and Sachs, 2001; Subbaiah et al., 2000).

The time course of primary root tip death in submerged maize seedlings indicated that anoxia for 48 h or longer led to the death of the root tip (Subbaiah et al., 2000). However, cell death indicators such as callose development, DNA nicking and induction of hydrolytic activities were apparent much earlier than 48 h

(Subbaiah and Sachs, 2001; unpublished). These observations indicated that the death process, although initiated before 24 h, became irreversible at 48 h of anoxia. The necrosis extended into the root axis during post-anoxic recovery, leading to the mortality of 30–50% of the seedlings. Excision of the root tip (de-tipping) before anoxia led to a 30–40% greater recovery of seedlings from stress injury. Unlike in the case of intact seedlings with slow and progressive death of the root tip, de-tipped seedlings show a lesser extent of shoot- or root-damage resulting in a rapid emergence of leaves as well as lateral roots, after re-aeration (Subbaiah et al., 2000). Acceleration of seedling death when submergence buffer was reused indicated that the dying root tip of intact seedlings releases diffusible death inducing factors into the submergence buffer. Therefore, a reprogramming of root tip death to make it occur early during anoxia, may provide a definite adaptive advantage to maize seedlings to anoxic stress. In *Arabidopsis*, the whole root system is dispensable for hypoxic tolerance of the seedlings; in fact, de-rooted seedlings fared better under O_2 deprivation (Ellis et al., 1999). In maize, the primary root axis is helpful (in quickly generating a functional root system), if not essential, for the post-anoxic recovery of seedlings. However, the survival of the shoot meristem is critical for the post-anoxic re-growth and autotrophic life of the seedling.

6.3 Anoxia-Induced Protease (AIP) in Root Tip Death

Cysteine and serine proteases have been implicated in the cell death/injury induced by abiotic, biotic or developmental signals in plants (e.g., Stroeher et al., 1997; Solomon et al., 1999; Hatsugai et al., 2004; for a review, Bozhkov and Jansson, 2007). To identify potential regulators of the cell death process in the root tip, changes in protease activities were analyzed in the root tissues.

Different species of proteases, both soluble and membrane-bound, are induced or suppressed during different durations of anoxic stress and reoxygenation in roots of 3-day-old dark-grown maize seedlings (Subbaiah et al., 2000). The major aerobic proteases are suppressed after six h of anoxia and new enzymes are detected both in soluble and membrane fractions. Upon reoxygenation, the aerobic activities reappear, although the anoxic enzymes persist for at least 24 h post-anoxia. Studies were focused upon a soluble enzyme that becomes detectable after 12 h of anoxia. This enzyme increases with time, becoming the major proteolytic activity in roots of 48 h submerged seedlings (Subbaiah et al., 2000). Protein synthesis inhibitor studies indicate this to be a newly synthesized enzyme under anoxia (anoxia-induced protease: AIP). AIP activity runs as a 22–25 kDa complex in SDS-PAGE. Ca^{2+} is required for the renaturation and proteolytic activity of the enzyme. Inhibitor-sensitivities indicated that AIP is a cysteine protease. De-tipping caused a decrease in AIP activity. Thus, the appearance of AIP activity in the root tip before 24 h of submergence was spatially and temporally associated with the initiation of the root tissue death (Subbaiah et al., 2000). Identification of the cognate AIP gene may provide a handle to engineer a genetic de-tipping mechanism for enhanced post-anoxic recovery of maize seedlings (see below).

7 Mechanisms and Potential Strategies to Improve Flooding Tolerance

Significant attempts are being made to understand the mechanisms of plant survival under oxygen deprivation and, further, apply this information to enhance flooding tolerance in maize (e.g., Saglio et al., 1988; Parentoni et al., 1995; Subbaiah et al., 2000; Shiao et al., 2002; Mano et al., 2006; Qiu et al., 2007). There have also been new accomplishments in the submergence tolerance of rice, which are potentially translatable to maize (Fukao et al., 2006; Xu et al., 2006). In this section, we summarize the ongoing work and also present a few novel strategies to improve maize performance under O_2 deprivation.

7.1 Fermentative Pathway Enzymes and Flooding Tolerance

Fermentative pathway is indispensable for plant survival under anoxia, since the pathway regenerates NAD^+, which is needed for an uninterrupted operation of glycolysis and ATP generation. Oxidation of NADH is also important to prevent over-reduction of the cytoplasm under anoxia. PDC catalyzes the first step that drives carbon into fermentative pathway and is the rate limiting enzyme. ADH, the major terminal enzyme of fermentation in plants, is actually responsible for recycling NAD^+. Attempts to improve hypoxia tolerance have targeted these two enzymes. While some minimum level of ADH activity is required for any flooding tolerance (Schwartz, 1969; Lemke-Keyes and Sachs, 1989), natural variation observed in long-term seedling survival of anaerobic stress is not related to variation in the levels of ADH activity in maize (Lemke-Keyes and Sachs, 1989). Overexpression of ADH has no effect on flooding tolerance in several species (e.g., Ismond et al., 2003). Extractable PDC activity also showed no correlation with submergence tolerance in rice cultivars (Mohanty and Ong, 2003). However, overexpression of PDC enhanced the post-hypoxic survival in *Arabidopsis* (Ismond et al., 2003) and rice (Quimio et al., 2000), indicating that PDC levels may be rate-limiting for energy production under O_2 deprivation and, therefore, a potential target for engineering flood-tolerance.

7.2 Hemoglobin Overexpression Confers Anoxic Tolerance

Among many anoxia-inducible genes that have been tested for their effects on flooding tolerance, hemoglobin is the most promising candidate (e.g., Hunt et al., 2002; Dordas et al., 2003; Dordas et al., 2004). Hemoglobin (Hb), an oxygen-binding protein, is present in most organisms. In addition to the symbiotic Hbs of the root nodules that function as oxygen carriers (similar to the vertebrate homologs), most plant species express non-symbiotic Hbs in all tissues (Hill, 1998). Non-sym-Hbs are grouped into class 1 and class 2 Hbs, according to their

phylogeny, biochemical characteristics and expression profiles. Only class 1 genes are induced by hypoxia/anoxia. The cellular functions of most plant Hbs are still not fully characterized (Hill, 1998). There is some evidence to implicate non-sym-Hbs to (1) function as a terminal oxidase and facilitate glycolytic generation of ATP and (2) antagonize NO-induced cell death under hypoxic conditions (Hill, 1998; Dordas et al., 2003, 2004). Hb-overexpression enhances the post-anoxic survival of *Arabidopsis* plants (Hunt et al., 2002), maize cultured cells and alfalfa roots (Dordas et al., 2003, 2004). In addition to submergence tolerance, overexpression of Hb (even bacterial) has been shown to improve growth and biosynthetic capacity of transgenic cell cultures and plants (reviewed in Zhang et al., 2007).

7.3 Hypoxic Pre-treatment and Amelioration of Anoxic Injury

Acclimation of maize seedlings to mild hypoxia before imposing anoxia alleviates the stress injury and enhances their post-stress recovery (Saglio et al., 1988). Understanding the mechanisms of acclimation to anoxia may help to identify the genes involved and ultimately manipulate anoxia tolerance by genetic approaches. Several laboratories have investigated the biochemical basis of stress amelioration by hypoxic pre-treatment (HPT) in maize and other species (e.g., Johnson et al., 1989; Xia and Saglio, 1992; Xia and Roberts, 1994; Rivoal and Hanson, 1994; Bouny and Saglio, 1996; Germain et al., 1997; Albrecht et al., 2004; reviewed in Drew, 1997). A plausible mode of HPT's priming action is the induction of a lactic acid efflux pathway during pre-treatment and a substantial retardation of cytosolic acidosis during subsequent anoxic treatment (e.g., Xia and Saglio, 1992; Rivoal and Hanson, 1994; Xia and Roberts, 1994). However, the molecular machinery involved in lactic acid excretion has not been known. Recently, AtNIP2;1, a major intrinsic protein (MIP; aquaporin family) from *Arabidopsis*, has been identified to transport lactic acid across the plasma membrane to outside the cell. Transcript levels of AtNIP2;1 are rapidly but transiently induced in both roots and shoots upon hypoxia and anoxia, whereas other members of the *AtNIP* family are not responsive to flooding (Choi and Roberts, 2007). These data strongly implicate *AtNIP2:1* or its maize homologs as strong candidates for genetic engineering anoxia tolerance into flooding sensitive crops.

7.4 Modulation of Root Tip Death Under Anoxia

The essence of stress adaptation is redirecting scarce resources to the maintenance of essential sinks as well as activation of adaptive pathways, while disinvesting in non-essential sinks and pathways. As discussed above, selective cell death provides a distinct stress tolerance mechanism for multi-cellular plants. Most eukaryotes show two fundamentally different modes of death, namely apoptosis (termed as PCD, in plants) and necrosis. One highly relevant distinction between the two types of death is the early

preservation of membrane integrity in apoptosis, whereas a rapid release of intracellular constituents occurs in the case of necrosis. Therefore, necrosis can presumably be dangerous, while apoptic response is an adaptive mechanism to dispose cells without compromising the rest of the organism. Nevertheless, more and more evidence points out that PCD and necrosis represent just extreme ends of wide range of possible morphological and biochemical deaths. Root tip death is preceded by SH1 relocation, DNA nicking, induction of AIP as well as callose, indicating that the process, to some extent, is cell autonomous. On the other hand, the death of root tip cells is accompanied by the acidification of the cytosol (Roberts et al., 1984) as well as the external medium and release of diffusible cytotoxins (Subbaiah et al., 1999; unpublished data). Therefore, root tip death in nature may not be sufficiently cell-autonomous but more necrotic. De-tipping experiments suggest that accelerating the process and, more importantly, pushing it towards PCD would increase the post-anoxic recovery of maize seedlings (Subbaiah et al., 2000). Some maize genotypes appear to have evolved an accelerated root tip death as a genetically controlled flooding tolerance mechanism (Zeng et al., 1999).

7.5 Genetic Analyses and Prospects for Breeding Flooding Tolerance in Maize

Pre-emergent seedlings of most maize lines survive no more than three days of anoxia. Some inbred lines are even less tolerant, surviving no longer than two days of anoxia (Lemke-Keyes and Sachs, 1989). Among exotic germplasm, nine accessions survived five to six days of anaerobic treatment. The most tolerant accessions were Bajos De Arenal, Yodocono, Yosondua, and Chalcatongo de Hidalgo (Lemke-Keyes and Sachs, 1989). Crosses between these exotic accessions and an anoxia-sensitive inbred (Mo20W) or a 'normal' inbred line (B73Ht), showed that the tolerance loci are dominant and show simple segregation. This indicated that anoxia tolerance is encoded by one or two genes in each accession (Subbaiah and Sachs, 2003). However, the nature of these genes, i.e., whether they encode ANPs or participate in the anaerobic gene regulation, remains to be determined.

In Brazil, the EMBRAPA-Centro Nacional de Pesquisa de Milho e Sorgo (CNPMS) developed 'Saracura BRS-4154', a cultivar with a broad genetic base obtained after nine cycles of mass selection under high soil moisture conditions (Vitorino et al., 2001; Alves et al., 2002). The maternal parent makes an important contribution to the flooding tolerance of Saracura (Vitorino et al., 2001), indicating a possible role for organellar genomes in the anaerobic response. In a comparison with a flooding-sensitive cultivar BR107, submerged Saracura seedlings show retarded mesocotyl softening; i.e., cell wall degradation (Alves et al., 2002).

A flooding tolerant line, Hz32, was selected from the maize inbred Hz1 in China. A commercial hybrid was developed by the combination of inbreds Hz32 and Mo17 (Zhang et al., 2006). Morphological and molecular marker assays revealed extensive genetic differences between Hz32 and Hz1 (Qiu et al., 2007). Quantitative trait loci (QTL) associated with waterlogging tolerance at the seedling stage were identified

in an F2 population from a cross between HZ32 and a flooding-sensitive inbred, 'K12'. Several major QTLs determining waterlogging tolerance mapped to chromosomes 4 and 9. Secondary QTLs influencing tolerance were also located on chromosomes 1, 2, 3, 6, 7 and 10. These QTLs may prove to be useful in marker-assisted selection (MAS) and further genetic analysis (Qiu et al., 2007).

In Japan, maize is grown in upland rice paddy fields where drainage is often poor leading to severe flooding damage to maize seedlings. Mano and colleagues (Mano et al., 2005) have been testing teosinte (wild relatives of maize) germplasm that can potentially adapt to the rice paddies. Some accessions of *Zea nicaraguensis* have been found to be very tolerant to flooding (Bird, 2000; Iltis and Benz, 2000; Mano et al., 2005, 2006, 2007; Mano and Omori, 2007). These populations inhabit a geographically isolated coastal plain near the Gulf of Fonseca, Nicaragua and differentiated from *Zea luxurians* of southeastern Guatemala. Three primary factors seem to contribute to flooding tolerance in these accessions: (1) adventitious root formation (ARF) at the soil surface during flooding; (2) the capacity to form schizogenous root aerenchyma; and (3) tolerance to toxins (e.g., Fe^{2+}, H_2S) prevalent under reducing soil conditions. QTLs controlling ARF under flooding condition were identified in a F2 population from a cross between maize inbred B64 and a teosinte accession (*Z. mays ssp. huehuetenangensis*). ARF showed continuous variation in the F2 population. Interval mapping and composite interval mapping analyses revealed that the QTL for ARF was located on chromosome 8. The study suggests that waterlogging tolerance can be transferred to maize from *Z. mays* ssp. *huehuetenangensis* (Mano et al., 2005).

Mano et al. (2006) also investigated the capacity of aerenchyma development in drained soil and found that some accessions of *Z. nicaraguensis* and *Z. luxurians* form constitutive (schizogenous) aerenchyma, a potential adaptation against frequent flooding. Maize is not known to form aerenchyma in non-flooding or non-stressed conditions (but as discussed above, does form lysigenous aerenchyma induced by flooding). QTLs for schizogenous aerenchyma formation were located to two regions of chromosomes 1, 5 and 8. This trait could be introduced into maize using SSR markers linked to the QTLs (Mano et al., 2007). Additionally, *Z. nicaraguensis* develops soil surface adventitious roots and exhibits an excellent adaptability to flooding in soils with high reduction potential due to Fe^{2+} or H_2S. Using this genetic resource as a donor parent, it might be possible to find additional QTLs with larger effects on flooding tolerance (Mano and Omori, 2007).

8 Conclusions

Anoxia is one of the most important abiotic stresses encountered by plants in flooded soils. Our goal has been to understand how maize perceives the changes in external O_2 concentration and adapts its growth and metabolism in short and long time scales. Regulation of gene expression under anaerobiosis occurs at multiple levels. Several genes involved in the anaerobic-response have been characterized and some components of the signal transduction pathway have been unraveled.

The elucidation of gene expression changes and the events that lead to altered gene action offers opportunities to develop targeted molecular strategies to improve maize productivity under excessive soil moisture. Additionally, natural variation in flooding tolerance in maize and the excellent flooding adaptation of teosintes (especially *Zea nicaraguensis*) provide opportunities to further our understanding of plant responses to oxygen deprivation and develop maize varieties with increased tolerance to flooding-stress.

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Maize Al Tolerance

Leon V. Kochian, Owen A. Hoekenga, Jurandir V. Magalhaes, and Miguel A. Piñeros

Abstract Maize is one of the most economically important food crops grown on acid soils, where aluminum (Al) toxicity greatly limits crop yields. Considerable variation for Al tolerance exists in maize, and this variation has been exploited for many years by plant breeders to enhance maize Al tolerance. Currently, there is considerable interest in facilitating further improvements in maize Al tolerance via interdisciplinary efforts aimed at identifying the genes conferring tolerance and the associated physiological mechanisms in maize. This chapter describes recent research into the physiology, genetics and molecular biology of maize Al tolerance, and potential candidate Al tolerance genes in maize are also discussed.

1 Introduction

Al (Al) toxicity is the primary factor limiting crop production on acidic soils. At soil pH values below 5, the rhizotoxic Al species, Al^{3+}, is solubilized into the soil solution, inhibiting root growth and function and thus reducing crop yields. Acid soils limit agricultural productivity in many regions of the world, including significant areas of the United States. Approximately 30% of the world's total land area consists of acid soils, and it has been estimated that over 50% of the world's potentially arable lands are acidic (von Uexküll and Mutert, 1995). Significant portions of the land acreage used to produce important grain crops are also acidic, and maize is one of the most important grain crops grown on acid soils, with approximately 20% of all maize grown found on acid soils (von Uexküll and Mutert, 1995). A large proportion of the acid soils occur in developing countries in the tropics and subtropics; it has been estimated that the humid tropics account for 60% of the acid soils in the world. Thus, acid soils limit maize yields in many developing countries where food production is critical. Furthermore, in developed countries, high-input farming practices such as the extensive use of ammonia fertilizers are causing further acidification of agricultural soils. While liming of acid soils can ameliorate soil acidity, this is neither an economic option for poor farmers nor an effective strategy for alleviating subsoil acidity.

As seen in Fig. 1, there is considerable variability for Al tolerance in maize, and this variability has been utilized by maize breeders to enhance Al tolerance. In order to

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Fig. 1 Acid Toxicity Reduces Maize Yields. Growth of Al tolerant (left) and Al sensitive (right) maize lines on a highly acidic oxisol soil as part of a field study in Sete Lagoas, Brazil. Photograph is courtesy of Dr. Vera Alves of Embrapa Maize and Sorghum in Sete Lagoas, Brazil

facilitate further improvements in maize Al tolerance, it will be necessary to better understand the underlying molecular, genetic and physiological bases for this trait in order to identify the genes underlying tolerance. Because of the agronomic importance of this problem, this is an area that has attracted significant interest from a number of molecular biology and physiology laboratories around the world. Despite the interest from many researchers, the molecular basis for Al tolerance is still poorly understood. However, recent research progress is now allowing us to begin to identify and characterize both the genes and associated physiological mechanisms that condition Al tolerance in maize. This should, in turn, provide the necessary molecular tools to address a worldwide agronomic problem that is only exceeded by drought stress with regards to abiotic limitations to crop production (von Uexküll and Mutert, 1995).

2 Physiological Mechanisms Underlying Maize Al Tolerance

Plants avoid the phytotoxic effects of Al^{3+} by using a variety of physiological mechanisms aimed at excluding Al^{3+} from entering the root apex (Al exclusion) and/or by mechanisms that confer plants the ability to tolerate Al as it enters the plant symplasm. The reader is directed to Kochian et al. (2004, 2005) and references therein for discussion of earlier works on Al-toxicity and Al-tolerance. Here we will address the recent literature regarding Al tolerance in maize. Overall, the most compelling evidence

indicates that many plant species make use of a generalized exclusion mechanism based on the chelation of Al^{3+} by organic acid anions released from the growing root tip. The seminal work by Delhaize et al. (1993a, b) demonstrated that Al-tolerance in wheat was correlated with a strong Al-activated exudation of malate. Since then, Al tolerant genotypes from many plant species have been shown to share this mechanism of Al exclusion, with the identity of the organic acid released being the main difference among plant species (see Table I in Kochian et al., 2004). In the case of maize roots, Al tolerant genotypes exhibit an Al-activated citrate exudation upon exposure to extracellular Al. The physicochemical properties of $citrate^{3-}$ (a tricarboxylic acid anion) suggest it should chelate Al^{3+} more effectively than dicarboxylic (e.g. $malate^{2-}$) anions, potentially making it more effective at excluding Al from entering the root. There have been a number of studies implicating the Al activated citrate exudation response in maize roots as a potential Al-exclusion mechanism (Pellet et al., 1995; Jorge and Arruda, 1997; Ishikawa et al., 2000; Kollmeier et al., 2001; Kidd et al., 2001; Mariano and Keltjens, 2003; Piñeros et al., 2002, 2005, 2007).

With regards to the physiology of maize Al tolerance, a general pattern has emerged, as most studies agree on the fact that the degree of Al-tolerance consistently correlates with reduced Al accumulation in the root tip. These results indicate that an Al-exclusion mechanism most likely underlies much of the Al-tolerance in maize. Likewise, the degree of Al tolerance in a limited number of maize genotypes has been shown to be positively correlated with the onset of a rapid (detectable within 30 min) Al-activated citrate exudation, with the magnitude of the citrate exudation rate similar among the Al tolerant maize genotypes studied (SA3: Pellet et al., 1995; IAC-TAIUBA: Jorge and Arruda, 1997; ATP-Y: Kollmeier et al., 2001; CMS36: Mariano and Keltjens, 2003; Cateto-Colombia and Cateto 100-6: Piñeros et al., 2002, 2005, 2007). Likewise, Al-induced citrate exudation rates are positively correlated with the level of Al in the nutrient solution. Spatial analysis indicates that this exudation is not tightly localized to the root tip, but rather occurs over a large region of the root apex (as much as the terminal 5 cm of root).

Additionally, research has also identified membrane proteins that can potentially mediate the Al-activated organic acid transport described in intact roots. In agreement with the thermodynamic nature of this process, electrophysiological studies unveiled a novel type of plasma membrane anion channel that is specifically activated by extracellular Al^{3+}. These channels have been reported in protoplasts isolated from root tips of Al-resistant wheat (Ryan et al., 1997; Zhang et al., 2001) and maize (Kollmeier et al., 2001; Piñeros and Kochian, 2001; Piñeros et al., 2002). These channels are specifically activated by Al and are permeable to organic acids such as citrate and malate. The remarkable similarities between the functional characteristics of these channels and those observed for the Al-activated organic acid response in intact roots strongly suggests these membrane proteins are responsible for mediating the Al-activated transport (i.e. exudation) of organic acid. These electrophysiological studies have provided the conceptual foundation for subsequent molecular investigations that have resulted in the cloning of the first two Al tolerance genes, which are both Al-activated organic acid transporters. The first tolerance gene identified, *ALMT1* (for the *Al*-activated *m*alate transporter), is a novel transporter that was

isolated from wheat root tips (Sasaki et al., 2004). This initial finding, along with several subsequent publications, established that *ALMT1* is the major Al tolerance gene in wheat and it possibly encodes the Al-activated transporters characterized in the earlier electrophysiological studies with wheat root protoplasts (Delhaize et al., 2004; Raman et al., 2005; Yamaguchi et al., 2005). The identification of ALMT1 as an Al tolerance protein opened up subsequent work looking at homologs in other plant species that employ Al-activated malate release as a tolerance mechanism. Recently, *ALMT1* homologs in Arabidopsis (Hoekenga et al., 2006) and rape (Ligaba et al., 2006) have been shown to be major Al tolerance genes in these species.

However, in maize, Al tolerance is correlated with citrate and not malate release. Hence, the recent identification of a second plant Al tolerance gene, Alt_{SB}, which is a member of the MATE family of membrane transporters and is an Al-activated citrate efflux transporter in sorghum (Magalhaes et al., 2007), has led researchers to begin to look at Alt_{SB} homologs in maize. The role of this gene in Al tolerance in sorghum, a close relative of maize, will be discussed later in this chapter. Furthermore in maize, the *ALMT* homolog with closest sequence similarity to the wheat *ALMT1* gene has been studied in detail, and it does not appear to be involved in maize Al tolerance (Piñeros et al., 2007). Although ZmALMT1 is an anion-selective transporter, functional characterization of its transport properties in *Xenopus* oocytes suggests it is not an organic acid transporter. In addition, the low constitutive ZmALMT1 expression levels observed in root tips relative to the other plant tissues in both tolerant and sensitive maize lines, along with its functional properties suggest that ZmALMT1 is likely involved in mineral anion nutrition (NO_3^-, SO_4^{2-}, Cl^-), rather than in maize Al tolerance.

As discussed in next section on the genetics of maize Al tolerance, it has been shown that this is a complex quantitative trait. This raises the question as to whether multiple tolerance mechanisms might be operating in maize. More recent physiological investigations of maize Al tolerance support this speculation. In contrast to the strong correlation between Al tolerance and Al-activated root malate release observed with 36 different wheat cultivars (Ryan et al., 1995a, b), the correlation between Al tolerance and Al-activated citrate exudation in maize roots has usually been studied with a single Al tolerant genotype in comparison with one or two Al sensitive lines. Additionally, the only study comparative study using a broader panel of maize lines that capture the range of maize Al tolerance and differ significantly in their genetic background (investigating both Brazilian and North American genotypes), indicated that the degree of Al tolerance among the genotypes was not entirely correlated with the magnitude of Al-activated citrate release (Piñeros et al., 2005). Although there was a clear correlation between root tip Al-exclusion and Al tolerance, there was not a close correlation between differential Al tolerance and root citrate exudation for the maize genotypes studied. All of Al tolerant lines exhibited a high rate of Al-activated citrate release (as expected); however, several of the Al sensitive lines from Brazil and North America also exhibited rates of citrate release that were comparable to those seen in the tolerant lines.

Are multiple Al-tolerance mechanisms operating in maize? Both the genetics and some of the physiological investigations suggest this. Thus, it is possible that in very

tolerant maize genotypes, Al-activated root citrate exudation could be a basal mechanism which is combined with other, currently unidentified mechanisms to condiction the full range of Al tolerance. One could envisage a scenario where the initial response is a rapid citrate release mechanism activated by Al and mediated by an Al-activated channel such as a homolog of the Alt_{SB} gene in sorghum. This tolerance response could be accompanied by more slowly adapting mechanisms.

Thus, the question regarding what other Al tolerance mechanisms could be operating in maize remains unanswered. However, the consistent correlation between Al-tolerance and reduced root tip Al accumulation suggests that another Al exclusion mechanism is superimposed on the basal mechanism mediated by citrate release. These new mechanisms could include the root exudation of other currently unidentified Al-chelating ligands, Al-binding by mucilage secreted from the roots, or the less likely scenario where Al exclusion could be due to Al efflux from the root apex via some type of Al transporter. Among these scenarios, exudation of phenolic compounds is a possible candidate, as they have the ability to complex metals such as Al and also act as strong antioxidants in response to abiotic stress. As phenolic compounds form strong complexes with Al^{3+} at neutral pH, they have been implicated in internal Al detoxification in tea and other Al-accumulating species (Matsumoto et al., 1976; Ofei-Manu et al., 2001). However, their role as an Al exclusion mechanism in the rhizosphere is less clear, as under acidic conditions Al^{3+} and H^{+} compete for the metal binding sites, thus reducing their metal-binding capabilities when compared to most organic acids in acidic solutions. Nevertheless, their potential role in Al exclusion cannot be disregarded. In fact, one study has reported a better correlation between the rate of Al-stimulated root exudation of the flavonoids catechin and quercetin and differential Al tolerance in three maize genotypes, than with Al-activated organic acid exudation (Kidd et al., 2001). The authors suggested that Al-activated exudation of phenolics may play an important role in the detoxification of Al in the rhizosphere surrounding the root apex.

It is likely that a purely physiological approach will not be sufficient to identify novel Al tolerance mechanisms in maize, but will require an interdisciplinary approach integrating genetic, molecular, and physiological investigations.

3 Genetics of Maize Al Tolerance

There has been quite a large body of work on the genetics of Al tolerance, primarily from the applied genetic or breeding perspective. Less work has been done in the fundamental genetics of Al tolerance, either for marker assisted breeding purposes or to set the stage for identifying the individual genes responsible for Al tolerance differences observed between varieties. The point of this portion of the chapter is to identify a small number of particularly important milestones in maize genetic research into Al tolerance, rather than to assemble a comprehensive review of the complete field.

3.1 Applied Genetic Research

Most of the genetics-based research in the maize Al tolerance field can be categorized as germplasm surveys. These studies assess a variety of materials, such as inbreds, hybrids, and synthetic breeding populations, generally using a laboratory-based methodology rather than field-based observations. Some of these studies estimated general or specific combining abilities or otherwise approximate breeding potential. Perhaps the most important of the early germplasm surveys were those described by Magnavaca and co-workers in a trio of papers in 1987 (Magnavaca et al., 1987a, b, c). In the first study, several synthetic populations were evaluated in hydroponic culture together with a synthetic population developed in the field at Embrapa Maize and Sorghum (Magnavaca et al., 1987c). Other nutrient solution formulations had been developed before and after 1987; however, the solution developed by Magnavaca et al. has been adopted as the standard conditions by Embrapa Maize and Sorghum and the USDA-ARS group at Cornell University (see Ninamango-Cardenas et al., 2003, Pineros et al., 2005, as examples). The results in solution culture were well correlated with the observations made under field conditions, and so an alternate and perhaps more convenient screening tool was made available. In the second study, seven inbred lines with contrasting levels of Al tolerance were used to construct a series of populations that were evaluated with four different generations (F_1, F_2, BC_1, BC_2) (Magnavaca et al., 1987b). Thus, evaluation in hydroponic culture using the 24 families could rapidly estimate the inheritance of Al tolerance. In each case, the data supported the hypothesis that Al tolerance is a quantitative trait, as the frequency distributions were all continuous and unimodal (Magnavaca et al., 1987b). In the third study, experimental conditions were refined so as to focus attention on the effect of Al toxicity on root growth, minimizing the interference from phosphorous deficiency or other aspects of low pH stress (Magnavaca et al., 1987a). The effect of this last accomplishment cannot be underestimated – the lack of consistency between stress treatments chosen by different experimenters makes comparison of results between studies difficult or even impossible, a point raised by Magnavaca et al. and by others since (Magnavaca et al., 1987b; Kochian et al., 2004). Magnavaca led a research group at Embrapa Maize and Sorghum, and so all of the subsequent studies produced by that institute have followed these hydroponics protocols. This means that all of the body of work produced by Embrapa and others that likewise adopted these methods can be compared directly, which greatly simplifies making comparative and synthetic statements about relative levels of Al tolerance observed between accessions.

The next major advancement in applied genetic research on Al tolerance came from CIMMYT. Breeders working on two different campuses identified 192 genotypes to use as parents for a new synthetic population for improved yield on acid soils (Granados et al., 1993). The initial fieldwork was done on an Al-intoxicated field site in Colombia and 16 cycles of modified ear-to-row (MER) selection was performed under stress conditions. The substantive achievement came with the late rounds of fieldwork, as MER and full-sib selections were phenotyped on Al-intoxicated

and non-stressed conditions in Colombia, India, Indonesia and Thailand. This multi-location testing demonstrated the efficacy for breeding Al tolerant, tropically materials that could be shared among multiple national breeding programs (Granados et al., 1993). Given the current state of high through put genotyping and statistical methods such as association analysis, it could be highly informative to test allelic frequency on the genome-wide scale. This combination of high quality, field-generated phenotyping together with genomics-scale research could identify regions of the genome that have been selected through these many cycles of improvement and potentially locate the favorable genes that have been the targets of selection (as per Gallais et al., 2007, and others).

Al tolerance phenotyping moved from the field to the growth chamber quite early in these studies, where laboratory-based observations had strong correlation with those made in the field (Magnavaca et al., 1987a, b, c). However, it is reassuring to see re-validation of the assumptions made when the laboratory-based screening largely replaced field-based work. A pair of short papers by Giaveno and Miranda Filho helps to reassure the scientific community that laboratory-based screening is effective (Giaveno and Miranda Filho, 2002a, b). In the first study, trait data from hydroponic culture was compared with soil grown plants in the greenhouse and field. For the hydroponic studies, trait data were collected from seedling plants while grain yield was used as the field trait for comparison (Giaveno and Miranda Filho, 2002a). Grain yield data were collected from five different sites across a gradient of Al stress, from highly productive to highly stressed. Interestingly, the most significant and strongest correlations were made between the hydroponically grown plants and the most stressed field site, while the highly productive field had no significant correlations with any of the five seedling traits collected (Giaveno and Miranda Filho, 2002a). In their second study, Giaveno and Miranda Filho assessed the efficacy of selection of breeding materials under hydroponic and greenhouse pot culture conditions to predict outcomes under field conditions (Giaveno and Miranda Filho, 2002b). A single cycle of divergent selection under laboratory conditions was used to generate pools that were tested in the field in two seasons. Grain yield, plant height and days to male flowering were the full season traits assessed for sensitivity to Al. Both hydroponic and soil culture based phenotyping were effective at discriminating tolerant from sensitive varieties, although the differences were most pronounced under intermediately high and high levels of Al stress than at lower levels of stress and grain yield was better correlated with the seedling root traits than final plant height or days to male flowering (Giaveno and Miranda Filho, 2002b).

3.2 Basic Genetic Research

As demonstrated by Magnavaca and others, Al tolerance in maize is a quantitative trait controlled by a large number of genes (Magnavaca et al., 1987b). This fact, together with the difficulties of reproducible high throughput phenotyping, has limited

the number of basic genetic studies that have investigated the complexity of maize Al tolerance or sought to identify linked markers for marker-assisted breeding. Three studies are briefly discussed here to represent the progress made to date.

It is well known that regeneration of plants through tissue culture can induce somaclonal variants with altered properties relative to their parental stock. Somaclonal variation was used to create an Al sensitive mutant from the highly Al tolerant inbred line, Cat100-6 (Moon et al., 1997). A mapping population was then generated between these highly related materials and two loci (*Alm1* and *Alm2*) were identified that were responsible for the difference in Al tolerance, located on chromosomes 6 and 10 (Sibov et al., 1999).

Subsequent to this initial study, the Embrapa Maize and Sorghum group produced the first full QTL analysis for Al tolerance in maize, using parents from their maize improvement program (Ninamango-Cardenas et al., 2003). An Al tolerant inbred (L1327), commonly used as a tolerance donor in the breeding program, was crossed to a high market quality inbred (L53) with low tolerance; a population of 168 $F_{3:4}$ families was generated and screened in hydroponic culture (Ninamango-Cardenas et al., 2003). Five QTL were detected on chromosomes 2, 6 and 8 that could explain 60% of the variance in net seminal root growth. For all but one of the QTL, the tolerant allele was donated by the tolerant parent and indicates that relatively little transgressive segregation occurred in the $F_{3:4}$ families. Intriguingly, one of the chromosome 6 QTL was consistent with the map position of the *Alm2* locus identified by Sibov et al. Further research into these QTL will be facilitated by the construction of F_8 recombinant inbred lines, which will permit higher resolution genetic mapping and replicated testing under laboratory and field conditions C. Guimaraes, unpublished results).

Genetic mapping populations are often deliberately constructed between highly contrasting parental lines in order to separate additive or dominance-based variation. An alternative strategy is to employ parents that accomplish similar phenotypes by different pathways and thus maximize transgressive segregation to produce a more exaggerated phenotypic range among the mapping population. In the physiological study discussed above by Pineros et al. (2005), the North American maize lines B73 and Mo17 have similar levels of Al tolerance and can be classified as moderately sensitive (Pineros et al., 2005). However, it is clear that Mo17 has Al-activated citrate release rates in the whole root that are greater than either of the highly tolerant inbreds Cat100-6 and L1327 (Pineros et al., 2005). Equally puzzling is the observation that B73 has little or no citrate release and permits 2.5× or greater accumulation of Al into the root apex than seen in Mo17. Clearly, B73 and Mo17 possess different strategies to achieve similar levels of Al tolerance. An intermated recombinant inbred population has been derived from a B73 × Mo17 cross, and is commonly called the IBM (for **I**ntermated **B**73 × **M**o17) (Lee et al., 2002). QTL analysis for plants grown in hydroponic culture has been initiated and wide transgressive segregation has been observed (Mason, 2005). None of the QTL detected in the IBM population on chromosomes 4, 8 and 10 coincide with QTL detected by Embrapa. However, near isogenic lines are under construction to validate the initial QTL analysis and to permit dissection of the molecular and physiological bases for Al tolerance in this population (unpublished results, O. Hoekenga and L. Kochian).

3.3 Comparative Genomics-Based Research

The greatest number of Al tolerance QTL studies has been performed in rice (Wu et al., 2000; Ma et al., 2002; Nguyen et al., 2001, 2002, 2003). These studies, together with the well-annotated rice genome, are an excellent resource for comparative genomic research. In addition to rice, the sorghum genome was completed in 2007 and the maize genome will be completed in 2008. Given that three of the staple crops for acid soil regions will all have sequenced genomes, candidate gene selection for Al tolerance QTL will be greatly facilitated. This will be especially true for those QTL located in syntenic chromosomal segments, which presumes that homologous genes are at work in multiple species. Barley, wheat and rye, all members of the Triticeae, carry Al tolerance genes on the long arm of chromosome 4 (Kochian et al., 2004). Due to improvements in the annotation and presentation of the rice genome, notably by increased sequencing of older RFLP probes, three rice Al tolerance QTL can be placed on rice chromosome within the syntenic segment to Triticeae 4 (Nguyen et al., 2003; Kochian et al., 2004; Jaiswal et al., 2006). Neither of the initial maize studies implicate chromosomal regions syntenic to rice 3 at present (Ninamango-Cardenas et al., 2003; Mason, 2005). However, with the improved resolving power that will come by screening the RILs developed by Embrapa and the NILs derived from the IBM population, new QTL may emerge in the syntenic chromosomal intervals.

4 Sorghum Al Tolerance – Identification of a Novel Al Tolerance Gene

Sorghum is the fifth most important cereal crop grown worldwide (http://faostat.fao.org) and is among the most tolerant of the major cereals to a number of abiotic stresses. Of the major grain crops, it is the closest relative of maize and like maize, sorghum Al tolerance is associated with Al-activated root citrate exudation (Magalhaes, 2002). Therefore the recent discovery of the second plant Al tolerance gene using sorghum as a model system is potentially of importance to better understanding maize Al tolerance with regards to candidate tolerance genes. Magalhaes et al. (2007) used high resolution mapping to clone the gene responsible for the major sorghum Al tolerance locus, Alt_{SB}. This mapping approach identified two flanking markers that delineated a 25 kb region containing three putative open-reading frames (ORFs). Two of the ORFs (sucrose phosphate synthase and an unknown protein) have putative functions inconsistent with known physiological mechanisms of Al tolerance, while the third ORF is a member of the MATE or multi-drug and toxic compound extrusion family of membrane transporters involved in the efflux of small organic solutes, which would be consistent with a role in root citrate release. Subsequent work confirmed that this transporter is in fact the sorghum Al tolerance gene based on the following evidence: (1) The transporter gene is highly expressed in the root apices (the site of Al tolerance) of a tolerant NIL derived from a cross

between the tolerant (SC283) and sensitive (BR007) parents, but it is not expressed in the root apices of an Al sensitive NIL; (2) Expression of this gene is induced by Al only in the tolerant NIL; (3) Alt_{SB} expression increases significantly over a 3- to 6-day period of Al exposure, with a parallel increase of Al-activated root citrate exudation and Al tolerance over the same time period; (4) The other 2 ORFs are expressed solely in the shoot, and thus are not expressed in the site where Al tolerance mechanisms must operate (the root apex); (5) Transformation in Al sensitive *Arabidopsis* generated Al tolerant transgenic plants that showed increased citrate release.

Due to the large difference in Alt_{SB} expression observed between Al tolerant and Al sensitive NILs and the lack of polymorphisms in the coding sequence of the gene, it was hypothesized that the primary difference between the tolerant and sensitive lines was due to changes in regulatory regions of Alt_{SB}. Based on the premise that regulatory factors should be polymorphic between the Alt_{SB} alleles from SC283 and BR007 and that they would be located within the 25 kb region defined by our flanking markers, we scanned this entire region from these lines for polymorphisms. Several differences between these two alleles were identified, including a miniature inverted repeat transposable element insertion (MITE-type transposable element) in the promoter region of the gene at ~2.0 kb from the transcription initiation site, 6 single nucleotide polymorphisms (SNPs) and one insertion/deletion (indel) in the second intron of Alt_{SB}, and 2 SNPs/1 indel and a 19 bp indel located after the stop codon, in the 3′ region of the gene. It should be noted that the set of polymorphisms we described above was detected between two sorghum lines only, and that many more polymorphisms important for function might be found in a diverse and relatively large sorghum panel. In fact, we have recently identified another tolerance source that has the most effective Alt_{SB} allele we have identified to date, exhibiting the largest Al-activated root citrate release and greatest Al tolerance we have observed (Magalhães, unpublished results). This line harbors a non-conservative amino-acid substitution in the coding region of the gene, suggesting this amino acid may play a critical role in citrate transport. Altogether, these findings support the notion that identification of superior Alt_{SB} haplotypes can only be gathered through a comprehensive analysis on a relatively large and diverse sorghum panel.

Our sequence scanning was subsequently expanded into a broader investigation of Alt_{SB} diversity using a panel of 47 sorghum accessions of diverse origins. These studies indicate that the MITE containing region in the promoter is highly structured, with the MITE and flanking sequences repeated between 1 and 5 times. There is a significant and positive correlation between the number of repeats in this region, Alt_{SB} expression and Al tolerance, with most of the Al tolerant lines harboring larger number of repeats. However, a number of interesting outliers have also been found using NILs generated with different sources of Alt_{SB}. Significant allelic variation was detected with these NILs within a single MITE insertion class, suggesting that the degree of phenotypic expression may depend on interactions among polymorphisms in the 25 kb region.

As with the discovery of *ALMT1* in wheat, the discovery of a second Al tolerance gene from a different family of membrane transporters provides a new avenue of inquiry into candidate Al tolerance genes in other species. Thus as was described

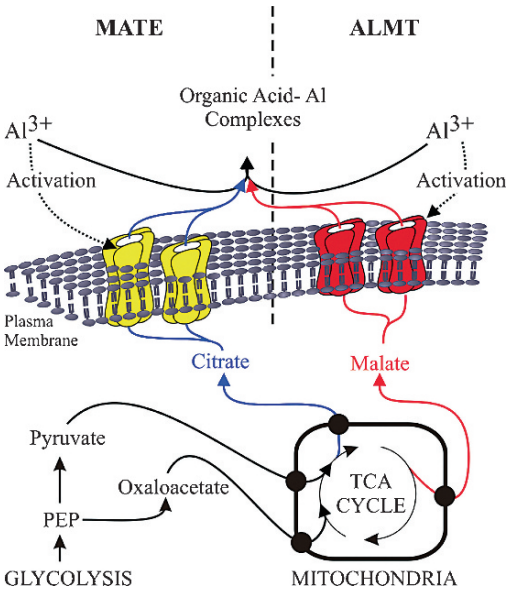

Fig. 2 Model illustrating a major Al tolerance mechanism based on Al-activated release of organic acids into the rhizosphere, where they chelate Al^{3+} ions and prevent their entry into the root tip. The model suggests that members of the ALMT family of membrane transporters mediate malate release in certain plant species, while MATE transporters are involved in Al-activated citrate exudation

previously regarding candidate maize Al tolerance genes that might be *ALMT* homologs, we currently are investigating the possibility that an Alt_{SB} homolog in maize may be an Al-activated citrate transporter underlying this mechanism of tolerance. Indeed, in two other plant species, *Arabidopsis* and barley, that exhibit Al-activated root citrate release, members of the MATE family that are closely related to Alt_{SB} have been shown to be involved in Al tolerance (Furukawa et al., 2007; Liu et al., in preparation). It is interesting that there is convergent evolution regarding a physiological mechanism of Al tolerance based on organic acid release in different plant species that is based on genes from two completely unrelated families of transporters. This is depicted in Fig. 2, where Al-activated malate and citrate release are shown to be mediated by members of the ALMT and MATE transporter families, respectively.

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Maize Under Phosphate Limitation

Carlos Calderón-Vázquez, Fulgencio Alatorre-Cobos, June Simpson-Williamson, and Luis Herrera-Estrella

Abstract Phosphorus is one of the least available macronutrient for plants in soils, and is therefore considered to be a major constraint for plant growth and crop productivity. As a consequence, plants evolved a number a biochemical and developmental adaptations to combat this deficiency. In maize, such adaptations are based on a wide spectrum of mechanisms needed to increase the P uptake, assimilation and use efficiency. These mechanisms frequently act in parallel with a morphological plasticity in root architecture. Such adaptive strategies have been reported in several phosphate efficient genotypes, identified and selected from the large natural and man-made diversity found within the maize species. Advances in research have now begun to identify at the molecular level the adaptations evolved by maize to cope with Pi limitation. In this chapter we summarize the current research on the development of tolerant genotypes and the physiological, biochemical and molecular adaptations associated with low phosphate availability. Such knowledge will allows us to identify putative targets for breeding and opens the possibility to improve nutrient acquisition and productivity in maize and other cereals.

1 Introduction

Inorganic phosphate (Pi) is an essential macronutrient with a fundamental role in most developmental and biochemical processes in plants. Pi is both a structural component of key molecules such as nucleic acids and phospholipids and a metabolic regulator that acts in signal transduction cascades and posttranscriptional modifications. Pi also participates as an intermediate in fundamental processes such as photosynthesis, energy transfer reactions and carbon and nitrogen metabolism. Despite the fact that total Pi in many soils may be relatively abundant, Pi availability is one of the most limiting factors for plant growth and productivity in both natural ecosystems and under agricultural conditions. Low Pi availability is mainly due to its low diffusion rate and to its rapid conversion into organic and inorganic forms not readily available for plant uptake (Marschner, 1995). Indeed, it is considered that at least one third of the world's arable land suffers from Pi limitation (Herrera-Estrella, 1999; Vance et al., 2003; von Uexküll and Mutert, 1995).

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1.1 Low-Pi Soils: Physical, Biological, and Agricultural Limitations

Pi-limited soils are widely distributed worldwide (Fig. 1). It is estimated that 5.7 billon hectares throughout the world contain levels of available Pi insufficient to sustain optimal crop production (Cakmak, 2002; Hinsinger, 2001). Low Pi soils are typically those with extreme pH (acid or alkaline soils) or with high concentrations of organic compounds. These soils occur in tropical and subtropical regions as well as in temperate climates. Regardless of the diversity and variability found among tropical soils with low Pi, the vast majority of them are classed as Oxisols and Ultisols (Rao et al., 1995; von Uexküll and Mutert, 1995). Both soil types are common in old land surfaces and have excellent physical properties. However, these soils are also characterized by a high loss of basic cations as well as a large content of iron (Fe) and aluminum (Al) oxides. In these soils, Pi availability decreases when the pH drops to 5 or below, since concentrations of soluble Fe and Al increase significantly and react with Pi to form insoluble compounds not easily assimilated by plants (Hinsinger, 2001; Rao et al., 1995; Schaffert et al., 2000; von Uexküll et al., 1995). Alkaline soils, particularly Calcisols, are located in arid and mediterranean climates. These soils are calcium carbonate-rich; therefore, Pi is readily precipitated by Ca ions and, to a lesser extent, by Mg ions. Although the formation of insoluble Pi compounds largely explains the low levels of free Pi in acid and alkaline soils, other factors can contribute actively to decrease the available Pi. For example,

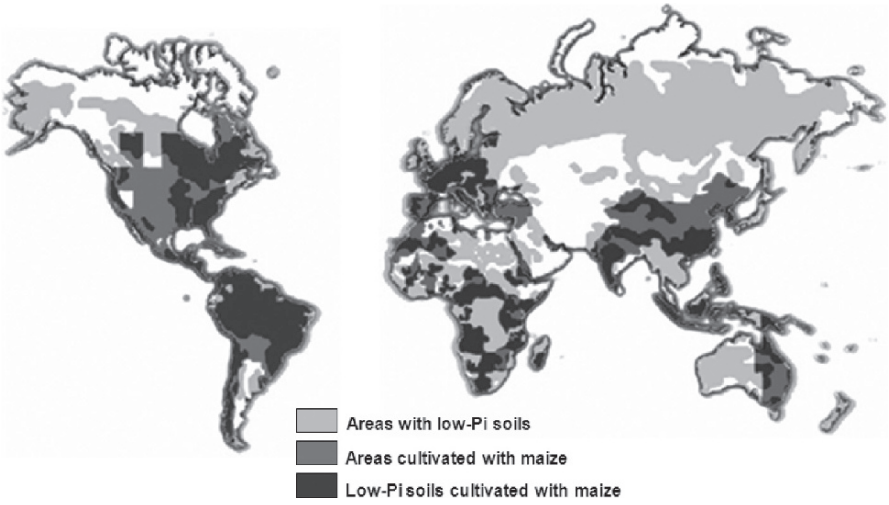

Fig. 1 World distribution of low-Pi soils and soils cultivated with maize. The map shows areas with Pi-low soils (Fairhurst et al., 1999) and where maize is grown (FAO, 2007). Overlay of them indicate areas where maize is grown under Pi deficiency

in some tropical soils, the contents of crystalline iron and aluminum oxyhydroxides can be significantly high, becoming a potential surface for Pi adsorption (Rao et al., 1995). In many calcareous soils Pi availability is also limited by soil texture and structure. In these areas the rainfall is often low and intensive agriculture depends on irrigation. However, applied water contains salts which following evaporation accumulates on the soil surface leading to a decrease in permeability. In shallow calcareous soils, the soil structure is a mix of carbonate fragments, weathered and unweathered minerals and organic matter (Weisbach et al., 2002). In this case, low Pi diffusion is reduced even more, exacerbating the Pi limitation and the effect on crop productivity.

Together with Pi limitation, acid and alkaline soils also exhibit deficiencies in other nutrients. Ca, Mg and Zn deficiencies are common in acid soils, whereas levels of Fe and Zn are low in calcareous soils. Another problem related to pH is Al toxicity which restricts plant growth and severely affects crop yields.

1.2 Plant Responses and Adaptation to Pi Limitation

Plants must acquire Pi from the soil in order to reach the intracellular Pi concentrations necessary for growth and reproduction. As a consequence of low Pi availability, both mono and dicotyledonous plants have evolved well-regulated systems for Pi acquisition and recycling. In general, these strategies involve (A) the release of soil Pi from organic and inorganic sources that are not readily available for plant uptake and the employment of high affinity Pi transporters, (B) the modification of root system architecture to increase the exploratory capacity and reach Pi rich patches in the soil, (C) recycling and mobilization of internal Pi as well as the optimization of the exploitation of a wide range of structural and metabolic compounds and (D) the establishment of symbiotic relations with arbuscular mycorrhizal (AM) fungi (Bucher, 2007).

In the first strategy, an increase in synthesis and secretion of organic acids, acid phosphatases and ribonucleases from roots is activated to optimize the release of Pi from soil sources and the activity of high affinity Pi transporters is also enhanced in order to increase Pi uptake (Baldwin et al., 2001; Bariola et al., 1994; Chiou et al., 2001; Daram et al., 1998; del Pozo et al., 1999; Li et al., 1997, 1998, 2002; Neumann and Römheld, 1999; Raghothama, 1999). The second strategy triggers postembryonic developmental modifications that alter the architecture of the root system and increase the exploratory capacity of the plant, particularly within the upper layers of the soil where Pi rich patches are commonly found. These alterations include an increase in the number and length of lateral roots and root hairs, a decrease in primary root length and changes in the angle of growth and diameter of lateral roots (Bonser et al., 1996; Hodge, 2004; Lopez-Bucio et al., 2002; Lynch and Brown, 2001; Williamson et al., 2001). In the third strategy the synthesis and activity of intracellular phosphatases is increased to mobilize Pi stored in the vacuole and different subcellular compartments of source tissues (Duff et al., 1994).

Photosynthates are differentially distributed between shoots and roots (Usuda and Shimogawara, 1991) and in carbon metabolism a series of bypass reactions are established in to avoid Pi- or ATP-dependent enzymes (Duff et al., 1989; Theodorou and Plaxton, 1993) in coordination with the degradation of phospholipids and their replacement by sulfo and/or galactolipids allowing the release of Pi from membranes (Andersson et al., 2003; Essigmann et al., 1998).

In the fourth mechanism, the association between the root system and the AM fungi produces structural changes in root epidermal cells in order to coordinate AM fungi colonization (Eckardt, 2005) in conjunction with the establishment of molecular communication, including the exudation of plant sesquiterpenes such as strigolactone (the called branching factor) which induces hyphal branching in AM fungi (Akiyama et al., 2005). Once symbiosis is established a series of membrane transport systems are activated to facilitate the exchange of metabolites. These systems include P-type H^+-ATPases and mycorrhizal-specific Pi uptake transporters. Such systems have been reported in several species including cereals (Glassop et al., 2005), but more commonly in legumes and solanaceous plants. Several studies have shown that mycorrhizal roots are able to assimilate Pi beyond their normal Pi-depletion zone. Recently new evidence has also supported the hypothesis that AM can also increase soil Pi availability by inducing acid phosphatase activity (Ezawa et al., 2005), which together with the excreted protons, hydroxyls and organic anions facilitates the release of organic and inorganic forms of Pi present in the rhizhosphere (Grant et al., 2005; Hinsinger, 2001). The mutualistic interaction increases the assimilation of not only of P but also K, S, N, Zn and Cu by the plant and provides photosynthates to the fungus from the plant (Bucher, 2007).

Knowledge of Pi metabolism under limiting conditions has been obtained mainly from model systems such as Arabidopsis and tomato and in the case of mycorrhizal symbioses from *M. truncatula* and *L. japonicus*. Although reports of tolerant genotypes and the identification of the associated biochemical and developmental strategies to combat Pi deficiency were published several decades ago, the study at the molecular level of responses to Pi limitation in *Zea mays* has only recently begun.

2 The Maize Crop Under Pi Limitation

The limited access to Pi fertilizer in developing countries and the excessive runoff and contamination from Pi fertilization in high throughput agrosystems have stimulated the development of maize genotypes with higher efficiency of Pi assimilation and utilization (Lynch, 1998). However, the high level of genetic diversity which enables maize to grow in a wide variety of soils remains largely untapped. Several inducible mechanisms which allow maize to more effectively cope with low-P availability have already been described (Da Silva and Gabelman, 1992; Fan et al., 2003; Gaume et al., 2001; Hajabbasi and Schumacher, 1994; Zhu and Lynch, 2004).

2.1 Growing Maize Under Pi Limitation: Soils and Fertilizers

Maize is probably the crop with the widest range of growing environments known. It is cultivated up to 58° N in Canada and Russia and up to 40° S in the Southern Hemisphere. It can grow below sea level in the Caspian plain and up to 4000 m above sea level in the Peruvian Andes (FAO, 1992). CIMMYT has developed a standardized system to classify these diverse geographic regions, defined as 4 mega-environments for maize production in terms of climatic factors (latitude, altitude and temperature): (1) Lowland tropics, (2) subtropics/mid-altitude zones, (3) tropical highlands and (4) temperate zones (Dowswell et al., 1996; Morris and López Pereira, 1999).

There is a strong correlation between the mega-environments and the occurrence of low Pi soils. Tropical and subtropical environments account for more than half of the area cultivated with maize worldwide (Pingali and Pandey, 2001) and are composed of mainly acid soils (Fig. 1). In contrast temperate maize is grown mainly in calcareous soils, for example, Mediterranean regions or in fertile soils such as the North American Corn Belt. Although maize in this latter area is cultivated in deep fertile soils with controlled fertilization regimes, the Pi levels in these soils are apparently decreasing due to over cropping during many decades (Fixen, 2002; Leakey et al., 2006).

Low Pi availability and the high Pi requirements of maize make the intensive application of phosphorus fertilizer in agricultural systems indispensable. In maize, Pi is critical in early developmental stages (Barry and Miller, 1989; Hajabbasi and Schumacher, 1994; Plénet et al., 2000a); very low P- concentrations in shoots in this period have been observed prior to a reduction in plant growth (Plénet et al., 2000a, 2000b). In tropical acid soils, aerial growth in maize is affected in two ways: (a) by delaying of the appearance of leaves and, (b) by a reduction in leaf area and leaf area index (Sierra et al., 2003). With regard to the effects of low Pi availability on grain yield in maize, long-term experiments carried out in the field with non-limiting nitrogen and potassium have shown that Pi limitation reduces grain yield mainly by decreasing grain weight (Plénet et al., 2000b).

Maize crops with an estimated grain yield of 10,000 kg ha^{-1} can absorb about 102 kg P_2O_2 ha^{-1}, from which about 76% is removed by harvested grains. This value must be considered as the amount of P that should be returned to soils by fertilization in order to maintain soil fertility (Fixen, 2003). Pi fertilizers are normally applied as water soluble granules which dissolve and enter the soil solution, where depending on the soil composition the Pi may react and precipitate or become immobilized by adsorption on reactive surfaces. Therefore solubility, method of application and application site are all important considerations. Maize requires a Pi fertilizer with a solubility of about 40–60% of total P_2O_2 content (Englestad and Hellums, 1992) that should be applied close to the root system. New fertilizer application techniques such as fertigation have proved successful in improving Pi uptake and Pi utilization in maize (Iqbal and Iqbal Chauhan, 2003).

2.2 Genotype Diversity in Maize

Currently, maize is the second most consumed cereal worldwide, and it is estimated that by 2020, the demand for maize will be higher than the demand for wheat and rice, implying a necessary increase in production (Pingali and Pandey, 2001). This challenge will have to be met by developing countries, where paradoxically population growth rates are higher and maize yields are lowest. In addition, around 70% of total maize production is localized in these countries, where maize is grown in poor or acid soils (Lafitte, 2001; Pingali et al., 2001). With this perspective, one of the strategies for improving maize production has been based on selecting and developing more nutrient efficient genotypes (Shenoy and Kalagudi, 2005; Vance et al., 2003). Genotypic diversity in Pi acquisition and utilization has been reported in maize (Da Silva and Gabelman, 1992; Kaeppler et al., 2000; Li et al., 2004, 2007; Rao et al., 1995).

Pioneering studies on genotypic variation in maize and response to low P soils were reported more than a century ago, however, breeding programs to develop standard maize cultivars and commercial hybrids more efficient in P assimilation and metabolism were only initiated in the early 90s by the Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) in Brazil. Currently, EMBRAPA in conjunction with CIMMYT are the front-runners in research to identify Pi efficient materials suitable for tropical climates and soil conditions in America and Africa (Schaffert et al., 2000). Most screenings have been conducted under field conditions or under controlled greenhouse environments and the selected materials have been used to produce commercial hybrids, open-pollinated populations and recombinant inbred lines (RILs) adapted to acid soils (Baligar et al., 1997; Fernández and Ramírez, 2000; Kaeppler et al., 2000) or calcareous soils where assimilation is strongly inhibited by the presence of $CaCO_3$ and Fe compounds (Ramírez, 2006).

Classic screening methods are based on evaluating two components of P efficiency: P acquisition and P utilization (Horst, 2000; Rao et al., 1995). P acquisition is directly related to the nutrient absorption capacity and hence P uptake kinetics. Root morphology and mycorrhizal associations are parameters often used in conjunction with biomass and/or economic yield/unit of P acquired which determines the P utilization efficiency. Several studies have shown that kinetic parameters such as maximal assimilation rate or influx (Vmax or Imax), Michaelis-Menten constants (Km) and minimal concentration at which net uptake of ions cease (Cmin) are good indicators to identify genotypes adapted to low-Pi concentrations (Jungk et al., 1990; Torres de Toledo Machado and Cangiani Furlani, 2004). Experiments carried out in Brazil with local and improved varieties of maize have shown that genotypes more efficient in P-uptake have low Vmax and Km under Pi deficiency (Torres de Toledo Machado and Cangiani Furlani, 2004), suggesting that these materials are adapted to low-Pi soils and have carrier sites for Pi with a high affinity when the nutrient is diluted.

The relationship between P assimilation efficiency and root morphology has also been analyzed in maize. Genotypes with a high efficiency for P uptake exhibit a modified root system, including enhanced crown, brace and lateral root growth (Alves et al., 2001; Gaume et al., 2001; Li et al., 2007; Zhu and Lynch, 2004). Anatomical adaptations to P stress such as increased aerenchyma formation have

also been associated with tolerance to low-P soils (Fan et al., 2003; Lynch and Ho, 2005).

3 Low-Pi Adaptive Traits in Maize Tolerant Genotypes

In the following section we will review the current knowledge on the genotypic variation in Pi efficient maize genotypes as well as the strategies evolved by maize to combat Pi limitation. Biochemical adaptations to Pi limitation in maize are depicted in Fig. 2.

3.1 Physiological Traits

3.1.1 Associations with *Arbuscular Mycorrhizal* Fungi

Maize is considered a low dependence, facultative mycorrizal plant (Howeler et al., 1987), however this cereal is efficiently and homogeneously colonized by AM

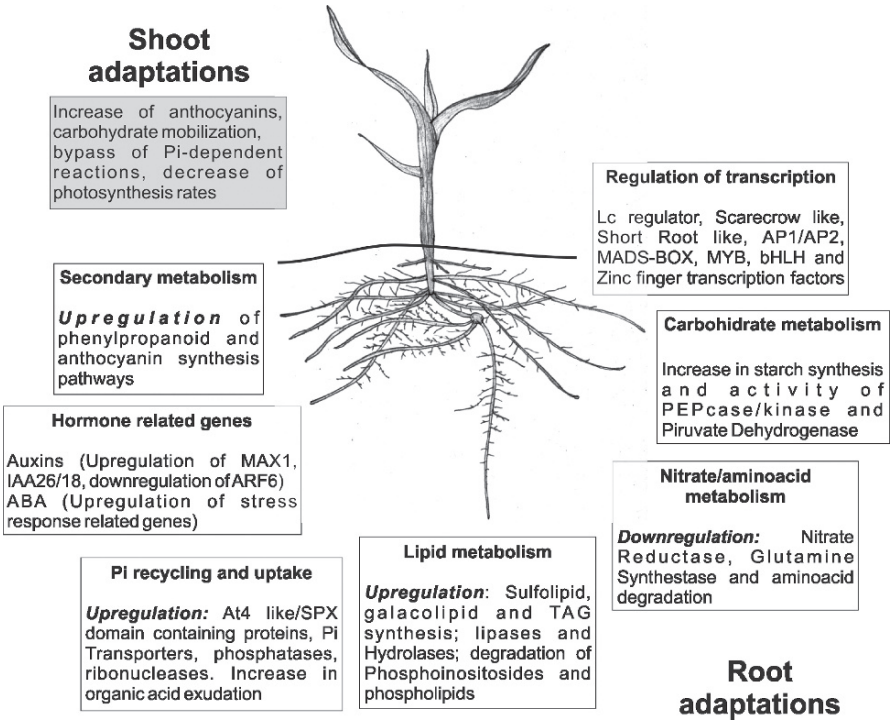

Fig. 2 Summary of molecular and biochemical adaptations to cope with Pi limitation in maize. The Shaded box corresponds to shoot adaptations

fungi (Paszkowski and Boller, 2002). Establishment of mycorrhizae, especially under low Pi-levels, can significantly increase maize shoot growth rate, tissue P content, dry matter accumulation and rate of photosynthesis (Gavito and Varela, 1995; Kaeppler et al., 2000; Kothari et al., 1990; Wright et al., 2005). These benefits seem to be greater when the colonization of roots by fungi occurs in the early stages of plant development (Gavito and Miller, 1998). The benefit to the plant can also vary depending on the fungal species, soil Pi status and the maize genotypes (Bressan and Vasconcellos, 2002; Gavito and Varela, 1995; Kaeppler et al., 2000; Wright et al., 2005). However, studies realized with maize inbred lines indicate that the ability of maize genotypes to respond to low Pi, is the most important factor and not the interaction with the mycorrhizal fungus as such (Kaeppler et al., 2000).

3.1.2 Photosynthesis

One of the most important physiological effects of phosphate deficiency on plant metabolism is the reduction in photosynthetic capacity. This reduction occurs through several mechanisms, involving morphological and metabolic changes in photosynthetically active cells (Jacob and Lawlor, 1991; Natr, 1992; Poirier and Bucher, 2002). Pi plays a central role in the ATP cycle in both the light and dark reactions of photosynthesis and the allosteric regulation of specific enzymes involved in carbon metabolism (Dietz and Harris, 1997), so it not surprising that under Pi deficiency these processes are altered.

When Pi deficiency occurs, the dynamic equilibrium in the Pi active pool between cytoplasm and chloroplasts is broken (Jacob and Lawlor, 1991). The reduction in the cytoplasmic level of Pi limits the efflux of triose-phosphates synthesized in the chloroplast promoting starch production. This conversion liberates Pi within the organelle allowing photosynthesis to continue (Nielsen et al., 1998; Pieters et al., 2001; Poirier and Bucher, 2002). Another metabolic impact of Pi starvation on maize photosynthesis is the reduction in the rate of the photosynthesis (Pn). The Pn of maize plants (36 umol m^{-2} s^{-1}) grown under relatively low light intensity (ca. 350 uEm^{-2} s^{-1}) is within the range of those found in typical C_4 plants. However this rate can be reduced to one third under long term low-Pi treatments (17–19 days after transplanting) and drops to only 6% at day 24 (Usuda and Shimogawara, 1991). The decrease in the rate of photosynthesis is thought to be caused by the inefficient regeneration of Ribulose-1,5-Bisphosphate (RuBP). Under optimal Pi, there is a non-limiting supply of RuBP and only the concentration and specific activity of Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) determines the carboxylation efficiency. In plants grown under Pi starvation, Rubisco content decreases, but the level of specific activity is maintained (Jacob et al., 1991; Pieters et al., 2001; Rao et al., 1989), suggesting that inefficient RuBP regeneration leads to the reduction in the rate of photosynthesis. ATP supply may limit RuBP regeneration either because (1) Low-Pi stress diminishes photosynthetic electron transport capacity or because (2) Insufficient Pi restricts the conversion of ADP to ATP (Rao et al., 1989). The second hypothesis explains the

low RuBP regeneration in maize. Plants grown under Pi starvation accumulate less Pi per unit of leaf area (Jacob and Lawlor, 1993; Jacob et al., 1991; Usuda and Shimogawara, 1991) with a 75% decrease in ATP, a 40% decrease in ADP and a 3% decrease in AMP (Jacob and Lawlor, 1993). Therefore Calvin Cycle activity as a whole is affected, including the conversion of NADPH to $NADP^+$ and the final electron acceptor steps in photosynthesis.

Stomatal function is apparently unaffected by Pi deprivation in maize since the rate of CO_2 diffusion does not change. However the number of cells per m^2 increases and total chlorophyll and soluble protein contents may fall drastically (Jacob et al., 1991; Usuda et al., 1991a).

It has been proposed that Pi starvation may affect C_4 and C_3 plants differently. In C_4 plants, such as maize, Pi is also required to export phosphoenolpyruvate from mesophyll chloroplasts (Huber and Edwards, 1975). However, this does not have a negative influence on photosynthesis during Pi stress. Instead, C_4 plants may reach higher net photosynthetic rates than C_3 plants under Pi deprivation by maintaining adequate levels of Pi in the bundle sheath cells. Although greenhouse experiments have shown that there were no significant differences in net photosynthetic rates under P starvation among maize and C_3 species such as sunflower, wheat and common bean (Halsted and Lynch, 1996; Jacob et al., 1991; Usuda et al., 1991a). Nevertheless genetic variation in the photosynthetic capacity of maize cultivars has been reported (Ming et al., 2006) and more efficient genotypes should be able to maintain a higher Pn in Pi deficient soils and to potentially produce higher yields.

3.2 Biochemical Traits

For some time Pi uptake has been an important research topic in maize. Determination of Pi content and assimilation showed that Pi influx is regulated and stimulated by the concentration of this mineral in the root (Anghinoni and Barber, 1980) and between genotypes in the kinetics of Pi uptake have also been observed (Nielsen and Barber, 1978). Based on biochemical studies (Nandi et al., 1987) assimilation mechanisms dependent on the transmembrane proton gradient have been established (Tu et al., 1990), leading to the conclusion that inducible mechanisms of Pi uptake similar to those in Arabidopsis are also present in maize. It is now known that plants possess at least two Pi uptake systems. A low-affinity system that works mainly under optimal growth conditions and distributes Pi into plant organs and a high-affinity system which directs most Pi uptake and allocation under limiting soil Pi concentrations (Raghothama and Karthikeyan, 2005). Pi transporters surely play a role in these mechanisms and several genomic and EST sequences encoding maize Pi transporters have now been deposited in public databases. Five Pi transporters in maize were described recently (Nagy et al., 2006). Their differential expression in different tissues and Pi conditions suggest distinct functions for these transporters including Pi uptake and translocation and a role in the symbiotic relation with mycorrhizae.

Some maize genotypes are able to release Pi from external organic and inorganic sources. Higher levels of acid phosphatase activities have been reported on the root surfaces of these genotypes when plants are grown under Pi starvation (Gaume et al., 2001; Sachay et al., 1991). Acid phosphatases can release Pi from organic compounds in soils and a high level of organic acids in the rhizosphere leads to increased P concentrations in shoots and roots (Corrales et al., 2007). This increase has been correlated with an increase in PEP carboxylase activity (Gaume et al., 2001). Interestingly, only maize genotypes tolerant to low Pi show increases in organic acid exudation, mainly in the form of citric and malic acids (Corrales et al., 2007; Gaume et al., 2001). Organic acids in the rhizosphere release Pi from insoluble Ca, Mg or Al compounds, commonly found in acid and alkaline soils.

In Arabidopsis as part of the recycling strategy to cope with the Pi starvation phospholipids are hydrolyzed to release Pi for cellular functions, in conjunction with an increase in the synthesis of non-P lipids (sulfo, mono- and digalactolipids; Hammond et al., 2004) which replace phospholipids and sustain the integrity and functionality of membrane systems. In maize roots, lipid composition is also modified upon Pi starvation and a 30 to 50% decrease in the content of major phospholipids (phosphatidylglycerol, phosphatidylethanolamine and phosphatidylcholine) was observed. In parallel, a two- to fourfold increase was reported for the sulfolipid sulfoquinovosyldiacylglycerol and the galactolipids monogalactosyldiacylglycerol and digalactosyldiacylglycerol (Calderón-Vázquez et al., unpublished).

3.3 Morphological Traits

The maize root system can be divided into an embryonic root system, which includes the primary and seminal roots, and a postembryonic root system comprising lateral roots formed from seminal roots and shoot borne roots ordered in whorls or phytomers (Hochholdinger et al., 2004).

Root plasticity in response to a heterogeneous nutrient supply was reported several decades ago. In order to adapt to such conditions, several maize genotypes have evolved root developmental programs which include the formation of branched and longer root systems, leading to an increase in exploration and absorption within a wider radius. Pi efficient maize genotypes usually have, among other characteristics, larger root systems with increased root dry weight and root to shoot ratios (Alves et al., 2001; Corrales et al., 2007; Gaume et al., 2001; Liu et al., 2004; Nielsen and Barber, 1978). Tolerant genotypes are able to sustain root growth rates at the onset of Pi deprivation (72–144 h), similar to plants under optimal conditions, in contrast to susceptible lines (Corrales et al., 2007). The well-documented increase in the root/shot ratio in response to Pi deprivation indicates an alteration in the distribution of photosynthates leading to an increase in root exploratory capacity. These changes in developmental programs associated with certain Pi efficient genotypes can be observed on at least three different levels: (1) modification of the density and length of lateral roots, (2) changes in the growth angle and length of

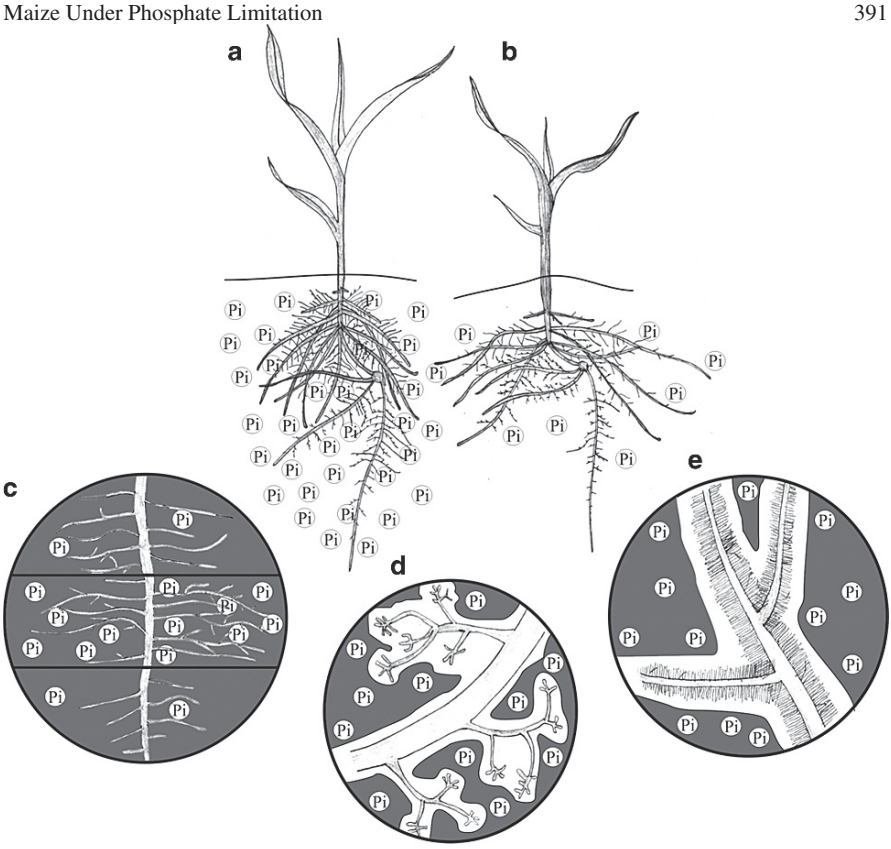

Fig. 3 Modifications of maize root architecture and growth under Pi limitation. **a** Maize growth under sufficient Pi. **b** Under Pi limitation, the root/shoot ratio increases as well as the length of crown, brace and lateral roots. Root shallowness is also a trait described in tolerant genotypes. Localized lateral root growth (**c**), association with AM fungi (**d**) and root hair plasticity (**e**) under Pi starvation enhance Pi uptake and soil exploration

crown roots and (3) an increase in root hair length (Fig. 3). However for other genotypes Pi assimilation efficiency is associated with Pi-uptake kinetics rather than modifications to root system architecture (Bhadoria et al., 2004).

It has been hypothesized that the root system modification response may be caused by the effect of Pi deficiency on the carbon budget of the maize plant. Field grown maize plants under Pi starvation as described above show a large and rapid effect on leaf expansion and rate of photosynthesis. These effects coincide with a delay in emergence from specific root phytomers, leading to a reduced number of adventitious roots (Pellerin et al., 2000). Hydroponically grown plants also showed similar responses to Pi starvation. Although root growth was slightly enhanced a few days after Pi starvation, long-term starvation reduced elongation of lateral roots and also the total number of auxiliary roots per plant. The authors of this study

suggest that such changes may be secondary effects caused by a rapid reduction in shoot growth which temporarily increases carbohydrate availability for root growth. Later however, the reduced leaf area of Pi deprived plants and their lower photosynthetic capacity severely reduces root growth (Mollier and Pellerin, 1999).

3.3.1 Lateral Roots

Lateral roots have a strong influence on root architecture (Lynch, 1995) and are responsible for the majority of water and nutrient uptake carried out by the plant (Hochholdinger et al., 2004; McCully and Canny, 1988; Varney and Canny, 1993; Wang et al., 1991, 1994). Cereals such as barley and maize respond to nutrient patches, high in nitrate, phosphate, ammonium or potassium, by stimulating an increase in both the weight and length of roots (Anghinoni and Barber, 1980; Granato and Raper, 1989; Stryker et al., 1974), reviewed in Robinson (1994).

Under low-P stress, root:shoot ratio, lateral root number and length as well as total root length is higher in Pi efficient genotypes (Fig. 3; Li et al., 2006; Zhu et al., 2005a; Zhu et al., 2004). The greater plasticity of these genotypes increases the number of lateral root meristems actively exploring new soil areas, allowing greater Pi acquisition and biomass accumulation (Zhu et al., 2004).

The maize mutant *lateral rootless 1* (*lrt1*), which lacks lateral roots under moderately low phosphate conditions, grows as wild type when AMF or Pi are added and develops some lateral roots, indicating that both AMF and high phosphate induce a developmental program independent of those related with the *lrt1* mutation (Paszkowski and Boller, 2002). Despite the lack of normal lateral roots under relatively low-P conditions, AMF symbiosis was able to fully restore *lrt1* aerial growth. Thus increased Pi assimilation by mycorrhizal *lrt1* plants may be due to fungal mediated nutrient absorption and to a lesser extent induced lateral root formation.

3.3.2 Crown and Brace Roots

Under Pi starvation, primary roots of Arabidopsis enter into an irreversible determinate root growth program where they cease elongation and mitotic activity, with the corresponding premature differentiation and loss of the apical meristem (Sanchez-Calderon et al., 2005). This phenomenon has been observed in primary maize roots under normal growth conditions. Interestingly, in some maize genotypes crown roots but not seminal roots increase their length under Pi stress (Fig. 3), although their emergence from the shoot is delayed, compared with their respective controls (Calderón-Vázquez, unpublished; Pellerin et al., 2000).

Aerenchyma formation may play an important role in the maintenance of apical growth through the release of Pi from senescing cortical tissue presumably for recycling and so reducing the metabolic costs of soil exploration (Fan et al., 2003). However, impeding the radial movement of water nutrients from soil in the root cylinder by the formation of aerenchyma may also be a disadvantage (Fan et al.,

2007) and further investigation is needed to clarify this situation. Root thickness is another characteristic that has been associated with low-Pi efficient genotypes and provide an advantage by increasing root penetration (Zhu et al., 2005b).

Recently, morphological characters such as root depth have also been proposed as important traits for phosphorus acquisition (Zhu et al., 2005a). Because nutrients are more abundant in upper soil strata than the subsoil, it is possible that genotypes with a well developed superficial root system are more efficient in Pi assimilation and hence improve biomass yield. Zhu and coworkers (Zhu et al., 2005a) analyzed several maize genotypes and reported significant differences in root shallowness between them when growing under low Pi and in the absence of AM fungi (Fig. 3). Materials with shallower roots showed greater Pi absorption and carbon assimilation rates, and hence greater total P content, relative growth rates, and biomass accumulation than genotypes with deeper root systems.

3.3.3 Root Hairs

It has been established that root hair length and density (Ma et al., 2001a, 2001b) are both characters regulated by Pi availability and that they play major roles in phosphate uptake in plants (Bates and Lynch, 1996). Arabidopsis mutants lacking root hairs have impaired phosphorus uptake and low competitiveness in soils with low Pi concentrations (Bates and Lynch, 2000a, 2000b). Therefore, root hairs under low Pi conditions make an important contribution in Pi uptake by increasing the total absorption surface of roots and also to the dispersion of exudates throughout the rhizosphere (Zhu et al., 2005b). In maize, genetic variation in root hair length has been associated with improved adaptation to low Pi availability (Zhu et al., 2005b). Maize genotypes showing root plasticity under low Pi conditions are able to increase root hair length up to 180% (Fig. 3). Furthermore, QTLs for this plasticity have been identified (Zhu et al., 2005b) thus reinforcing the knowledge that root hair development is under genetic control and identifying possible sources for maize breeding to improve Pi assimilation under field conditions. Three maize mutants with abnormal root hair development have been reported (Wen and Schnable, 1994). Surprisingly, only the mutant *root hair defective 1* (*rth1*, with defects in both root hair initiation and elongation) exhibits affected growth while *rth2* and *rth3* (with defects in root hair elongation) presented normal growth. These results indicate the need for further research in order to fully understand the physiological role of root hair growth in response to Pi limitation in maize.

3.4 Molecular Traits

Findings in Arabidopsis have revealed that adaptations to Pi starvation in plants are regulated mainly at the transcriptional level (Hammond et al., 2004). Microarray analysis and large scale sequencing projects have reinforced this idea

but have also widened our knowledge and uncovered new strategies exploited by plants to cope with Pi starvation and regulation and revealed how particular species have evolved specific mechanisms. Valuable genetic and genomic information has been obtained from *Arabidopsis thaliana*, *Oryza sativa*, *Phaseolus vulgaris*, *Lupinus albus* and also *Zea mays*, species in which large scale studies have permitted a detailed characterization of genetic responses to Pi starvation (Calderón-Vázquez et al., unpublished; Hernandez et al., 2007; Misson et al., 2005; Morcuende et al., 2007; Uhde-Stone et al., 2003; Wasaki et al., 2003, 2006). Although conserved regulatory patterns as well as common effectors that mediate the biochemical and developmental responses to Pi starvation have been determined depending on particular necessities and adaptations to particular growth conditions, specific responses have also evolved, and in the particular case of maize, the large natural diversity but also the manual selection of tolerant genotypes may have increased the complexity of the responses to Pi deprivation. For maize, a comprehensive catalog of Pi starvation responsive genes has recently been reported (Calderón-Vázquez et al., unpublished). Additionally, a recent study reported a proteomic analysis from root tips of maize plants subjected to Pi long-term starvation (Li et al., 2007). Molecular and biochemical adaptations to Pi limitation in maize are depicted in Fig. 2.

Development-related modification programs have been proposed as intermediates between Pi starvation and plant root responses. In the case of maize, quantitative trait loci (QTL) analysis of maize root responses to low Pi stress demonstrates that plasticity is genetically controlled under both high and low Pi availability (Reiter et al., 1991; Zhu et al., 2005b, 2005c,, 2006). Generation of recombinant inbred lines from a cross between B73 and Mo17 with contrasting responses to low phosphorus has been a valuable tool to discover determinants for low-Pi adaptation in maize. Genetic differences lead to variation in the phosphorus investment in lateral root elongation, the genotypes showing least investment being more efficient (Zhu et al., 2004). Zhu et al. (2005c) described a substantial genetic variation among recombinant inbred lines and identified five QTLs controlling lateral root length, one associated with lateral root number under high Pi and six under low Pi conditions. Interestingly no QTLs were detected for lateral root length plasticity. (Zhu et al., 2006) also reported one major QTL associated with seminal root length and three QTL's for seminal root number. These accounted for an important percentage of the phenotypic variations in seminal root length and number under Pi-deprivation however seminal root traits were weakly correlated with shoot biomass under low Pi growth conditions. Screening of recombinant inbred lines for root hair length, tap root length, root thickness and root biomass identified a QTL associated with root hair length plasticity, three with root hair length under optimal Pi and one under low-Pi conditions. In addition, six QTL's were associated with 53.1% of the total variation of seed phosphorus (Zhu et al., 2005b).

In Arabidopsis, Reymond et al. (2006) identified three QTL's controlling root growth responses to Pi starvation. A gene mapping to one of these QTL's, *Low Phosphate Root 1* (*LPR1*), encoding a Multicopper Oxidase, explained 52% of the variation associated with primary root growth. Differential allelic expression of

LPR1 in the root cap explained the nature of this QTL and provided strong evidence for the involvement of the root cap in sensing or responding to Pi deficiency (Svistoonoff et al., 2007). Thus QTL analyses are valuable tools for both marker assisted selection in crop species and also to analyze the molecular determinants of the response to Pi starvation in plants in general.

Research in Arabidopsis and to some extent in rice has shown that an important step in the response to Pi starvation is the regulation of gene expression at the transcriptional level and the posttranscriptional level mediated by miRNAs. A large number of transcription factors (TF's) responsive to Pi starvation have been reported (Wu et al., 2003; Misson et al., 2005, Morcuende et al., 2007), and for some, their function as specific regulators of Pi related genetic, developmental and biochemical processes has been documented (Chen et al., 2007; Devaiah et al., 2007a, 2007b; Rubio et al., 2001; Yi et al., 2005). From these studies, PHR1, a member of the MYB family of TF, was identified as a key component that regulates several Pi starvation responsive genes through binding to an imperfect palindromic sequence (Rubio et al., 2001). Subsequently, SIZ1 was identified as a SUMO E3 ligase that acts upstream of PHR1 (Miura et al., 2005). In addition PHO2, an unusual E2 conjugase and the micro RNA miR399 have been placed on a branch of the Pi signaling pathway, downstream of PHR1, thus defining a complex Pi signaling pathway in plants (Bari et al., 2006).

The maize genome contains at least five PHR1-like and four PHO2-like sequences, suggesting that the PHR1 signaling pathway may be common in both mono and dicotyledonous species. In maize, the transcriptional regulation of Pi-starvation responses is reflected by the regulation of 43 TFs. These include members of the bHLH, Zinc Finger and Leucine Zipper families. Notably, the TF LEAF COLOR (LC) was among the most differentially expressed (Calderón-Vázquez et al., unpublished). LC is known to regulate the expression of genes involved in anthocyanin biosynthesis (Dooner et al., 1991), a typical Pi starvation response. Furthermore, LC may be regulating additional Pi starvation responsive genes in maize since that its ortholog in rice, OsPTF1, was reported to be involved in tolerance to Pi starvation (Yi et al., 2005).

Development and hormone-related genes have also been reported for Arabidopsis as mediators of the changes in root architecture in response to Pi-deprivation (Lopez-Bucio et al., 2002; Ma et al., 2003). Homologs of TF's such as SHORTROOT and SCARECROW, which are involved in determining meristem identity and thus root morphology in both eudicots and monocots (Lim et al., 2005; Nakajima and Benfey, 2002), show altered expression patterns under Pi starvation in maize roots (Calderón-Vázquez et al.). Similarly, several homologs of ENHANCER OF GLABRA3, TRANSPARENT TESTA1, NAC, AP1, AP2, previously reported to be involved in root developmental processes such as lateral root emergence (Hardtke, 2006; Xie et al., 2000; Xie et al., 2002) are also responsive to Pi starvation. A set of transcripts responsive to both abscisic acid and auxins were also up or down regulated under Pi starvation. Among these, several genes involved in auxin signaling including the Auxin-Responsive Factors (ARF's) and the AUX/IAA protein families, previously reported as development related genes (Vieten et al., 2007), were

identified (Calderón-Vázquez et al.). The finding that expression of several development-related regulators and auxin responsive genes are modulated by Pi-availability in maize roots suggests that the proposed role for the development-related genes and for auxins in the plasticity of root system architecture in Arabidopsis (Lopez-Bucio et al., 2002) probably also applies to maize responsiveness.

As previously mentioned, metabolic adaptations as a response to Pi starvation in maize do occur. However, other major modifications are also reflected by the differential expression of nitrogen- and carbon- metabolism related genes. In maize roots, glycolysis is modified by bypassing reactions that require Pi. The induction of PhosphoenolPyruvate Carboxylase and PhosphoenolPyruvate Carboxylase Kinase genes is notable. Modifications in their expression may sustain C supply through the tricarboxylic acid cycle or provide C skeletons for continuing C metabolism (Calderón-Vázquez et al., unpublished). Similarly the modification of metabolic pathways can be seen at the protein level (Li et al., 2007a). In addition, pyruvate dehydrogenase complex proteins and tricarboxylic acid cycle-related proteins are also induced, possibly increasing the flow of C through the tricarboxylic acid cycle and consequently organic acid exudation (Li et al., 2007a).

N assimilation and amino acid metabolic pathways are also regulated by Pi-starvation. A significant decrease of Nitrate Reductase and Glutamine Synthetase was observed under moderate Pi limitation. The general down regulation of enzymes involved in amino acid degradation and the specificity for synthesis and/or degradation of some amino acids in conjunction with the fact that there were no up-regulated genes related to degradation of any amino acid suggests that a dynamic recycling of N metabolites to retain certain metabolic priorities is occurring during the Pi-deprivation process in maize roots.

Further Pi-starvation responses were revealed upon analyzing microarray data from maize roots under Pi starvation (Calderón-Vázquez et al., unpublished). A significant modulation of a large set of components of secondary metabolism, including lignin and anthocyanin synthesis-related genes was observed. Similarly, protein content of phenylalanine ammonia-lyase, O-methyltransferase, caffeate O-methyltransferase and UDP-glucosyltransferase BX9 was increased. In addition, the up-regulation of Pi-transport and Pi-recycling related genes was the most extensive and constant adaptation reported. The latter is mirrored by the up regulation of eight putative Pi transporters, 26 different phosphatases and four ribonucleases, hence, it seem that maize roots evoke complex and extensive genetic modifications that finally increase the viability and growth of maize plants under Pi starvation.

4 Conclusions

After Nitrogen, Phosphorus is the second most limiting nutrient for plant growth and crop production. Limitation is due to the scarcity of P in soils but also because of limited access to Pi fertilizers mainly for soils in the tropics and developing countries. In spite of these drawbacks, maize is the most widely cultivated crop in

the world and is able to grow in a wide variety of environments including Pi limiting soils such as acid or alkaline soils.

Maize has evolved Pi efficient genotypes in order to adapt to these environments with a wide spectrum of mechanisms to increase Pi availability both internally and in soil. These adaptations are accompanied by a phenotypic plasticity which is a valuable tool that allows maize to adapt and to reach a higher productivity even under Pi limitation.

Research has been carried out to identify the molecular processes that mediate low Pi adaptation for some plant species and recently, this research has been extended to include maize. These studies have revealed a large and complex regulatory system involving several traits that in conjunction act to produce changes in growth programs and metabolism. The search for the components of the signaling pathway which directs these changes is still in progress. At the level of regulation, it has been demonstrated that hormones such as auxins and auxin-signaling genes mediate the developmental changes in roots. Several transcription factors including PHR1 regulate the Pi-starvation responsive genes and miR399 mediates Pi homeostasis through posttranscriptional regulation. Several cis-regulatory elements have also been identified in the promoters of genes associated with Pi responses in Arabidopsis and rice. On the other hand, research in economically important crops such as maize has been focused mainly on the identification of tolerant genotypes and in marker assisted selection for breeding.

Recent advances on the molecular determinants of maize adaptations to low-Pi growth conditions have been due to powerful tools such as transcriptional profiling and proteomics. This has lead to the identification of both regulators and effectors in maize such as TF's and several genes and proteins that participate in Pi mobilization, uptake, recycling and the bypassing of Pi-mediated reactions. However, further research is needed to identify the specific roles of these elements and to correlate them with tolerant maize genotypes in an agronomic context through a continue collaboration between maize breeders and molecular physiologists. This will lead to the development of genotypes with enhanced low-P adaptation and to sustainable production in the soils where P-limitation is a principal constraint. For this task, metabolite profiling and analysis of isolated mutants or transgenic lines will be employed to indicate which molecular aspects of Pi starvation are affected.

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Agronomic Traits and Maize Modifications: Nitrogen Use Efficiency

Hartwig H. Geiger

Abstract Developing nitrogen (N) efficient cultivars is a major challenge of modern plant breeding. Globally, soil-N deficiency belongs to the most serious constraints of maize production. Improvement of N-use efficiency (NUE) would therefore significantly contribute to securing world food and feed production and raise agricultural incomes. In this chapter I will use the term NUE synonymous for 'adaptedness to low N supply'. NUE is a highly complex, polygenically inherited trait. Significant genetic variance for NUE and NUE-related traits has been found in various genetic materials from temperate as well as tropical growing regions. The genetic correlation between grain yields under low and high N supply (LN and HN, respectively) decreases from strong via moderate to weak as the stress caused by N deficiency increases. Mapping studies in different populations revealed consistent QTL for NUE on most chromosomes. The most important QTL regions seem to be located on chr. 2, 4, 5, and 8. Experimental evidence about putative candidate genes for NUE is still very meager. The only consistent results were obtained for gene *gln4* on chr. 5 encoding a cytosolic glutamine synthetase (GS). Divergent selection for grain yield under LN and HN leads to germplasm being specifically adapted to one or the other of these two growing conditions. However, in large-scale selection programs, breeders have also found genotypes being well adapted to both N-supply levels. The relative importance of N-uptake and N-utilization efficiency, the two constitutive components of NUE, largely depends on the genetic material. Genotypes with low grain protein concentration were found to excel in N-utilization and those with high concentration were superior in N-uptake efficiency. Several stress-indicator traits such as anthesis-silking interval, leaf chlorophyll concentration, ears per plant, and kernels per ear may be strongly correlated with NUE. Selection based on an index composed of grain yield and these indicator traits was shown to improve breeding progress considerably.

Dedicated to Prof. Dr. Dr. h.c. F. Wolfgang Schnell who passed away on December 29, 2006, at an age of 93 years.

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1 Introduction

Nitrogen (N) fertilizer is a major input variable in modern agriculture. The enormous gains in productivity of crop plants during the twentieth century were strongly correlated with the amount of N fertilizer applied by farmers. However, aspects of ground water protection, energy saving, and production costs are meanwhile restricting the ecologically and economically justifiable N input (Presterl et al., 2003). Moreover, in most developing countries, limited availability of fertilizer, inadequate infrastructure, and/or low purchasing power constantly lead to severe N-deficiency stress (Ribaut et al., 2007). Thus, developing plant varieties that more efficiently make use of the available soil N is a major challenge of modern plant breeding.

According to Moll et al. (1982), N-use efficiency (NUE) is defined as the amount of dry matter produced relative to available soil N. It is the product of N-uptake efficiency and N-utilization efficiency. The first component, N-uptake efficiency, refers to the amount of N absorbed by the plant relative to the available soil N. The second component, N-utilization efficiency, quantifies the amount of dry matter produced per unit of absorbed N. It results from a complex network of interlinked metabolic processes (for review see Hirel et al., 2007). For any fixed level of N supply, NUE simply is the dry matter yield at that N level. In the present chapter, the term NUE is specifically used for grain yield under low N supply, i.e. under N-deficiency stress.

Nitrogen-uptake efficiency depends on morphological and physiological root characters (Engels and Marschner, 1995) and on the activity of nitrate and ammonium transporters (Loqué and von Wirén, 2004). The latter also play a key rôle in the transportation, partitioning, and remobilization of N within the plant. These few considerations already demonstrate that NUE is a highly complex, polygenically controlled quantitative trait.

Since soil nitrate and ammonium can only be absorbed by the plant root if available in aqueous solution, N deficiency is frequently a consequence of drought. NUE then is confounded with water use efficiency (WUE), and several secondary characteristics such as asynchrony of male and female flowering (anthesis-silking interval), number of ears per plant, and kernels per ear are common symptoms of NUE and WUE (Lafitte and Edmeades, 1995; Bertin and Gallais, 2000; Ribaut et al., 2007).

2 Genetic Basis of NUE

Various classical and more recent studies revealed significant genetic variation for NUE and related traits in temperate and tropical materials. These studies include quantitative genetic analyses as well as structural and functional genomic approaches.

2.1 Quantitative Genetic Parameters

In most field experiments, similar heritability coefficients were estimated for grain yield under high and low N supply (HN and LN, respectively). Presterl et al. (2003)

reviewed 21 multi-location field experiments with representative samples of European breeding materials adapted to Germany and North France. Heritability estimates ranged among experiments from 36% to 96% under HN and from 41% to 88% under LN, averaging in both cases to 65%. However, the variance components due to genotype, genotype × location interaction, and error were about two-fold higher under LN. Deviating results were obtained by Bänziger et al. (1997) when analyzing 14 replicated trials with tropical materials at CIMMYT in Mexico. The heritability estimate for grain yield was 29% smaller under LN than under HN because of lower genotypic variance under LN but similar error variances. Both patterns of variation – and various intermediate situations – were also found by other authors underlining the importance of test conditions and genetic materials (Lafitte et al., 1997; Heuberger, 1998; Agrama et al., 1999; Bertin and Gallais, 2001; Ferro et al., 2007). Significant genetic variance was not only found in modern maize materials but also between (Lafitte et al., 1997) and within (Presterl et al., 2002a) tropical and temperate landraces, respectively.

Contrasting results were reported about the genetic variation for N-uptake and N-utilization efficiency. Presterl et al. (2002a) observed significant differences between N-deficiency tolerant and sensitive hybrids in N-uptake efficiency irrespective of the N-supply level but no differences in N-utilization efficiency. Gallais and Hirel (2004), on the other hand, found fewer QTL for N-uptake than for N-utilization efficiency under LN, whereas the reverse was true under HN. These results indicate that progress in adaptedness to low-N conditions may rest on different physiological mechanisms depending on the germplasm used.

In experiments of Presterl et al. (2002b), genetic correlations between S_2 line and testcross performance were significant for grain yield under both HN and LN but the association was stronger under LN. In accordance with this, the superiority of the testcrosses over the lines was smaller under LN than under HN. Early studies of Balko and Russell (1980) in Iowa Stiff Stalk (BSS) lines and their hybrids did not reveal a significant association between lines and hybrids at any of five N-supply levels. The discrepancy between the findings of these two studies may in part be due to the different selfing generations of the tested lines, S_2 in the former and $S_{>6}$ in the latter experiment. Different mutational loads of the two line samples might be another reason.

In genotype samples taken at random from a breeding population, generally moderate to strong genetic correlations exist between HN and LN testing conditions as long as the N-deficiency stress is not too severe. Correlation coefficients decline as the stress becomes stronger or if the tested sample is composed of genotypes divergently selected for adaptedness to HN and LN conditions, respectively (Bänziger et al., 1997; Presterl et al., 2003). As a consequence, the importance of genotype × N level interaction increases.

2.2 Quantitative Trait Loci (QTL)

The first QTL study targeting NUE and NUE-related traits was performed in a mapping population derived from a cross between a local inbred line from Egypt and the US-Corn-Belt line B73 (Agrama et al., 1999). Two hundred and fourteen

F_3 lines were evaluated for *per se* performance under HN and LN conditions over 2 years at one location in Egypt. The F_2 parent plants were genotyped with 185 RFLP probes. Five QTL for grain yield under HN were detected on chromosomes 1, 4, 5, 9, and 10 and 6 QTL under LN on chr. 1, 2, 7, 9, and 10. Four of the latter QTL were LN-specific, i.e. were not detected under HN (Table 1). Under LN, 1 QTL each for ear leaf area, plant height, kernels per ear, and kernel weight mapped to the 1-LOD confidence interval of 1 of the 4 NL-specific QTL for grain yield.

Bertin and Gallais (2001) investigated NUE in a mapping population comprising 99 inbred lines derived from a cross between the early-maturing French flint line F2 and a proprietary Iodent line. The inbred lines were crossed to an unrelated tester line (F252) and the testcrosses were evaluated over 2 years at Le Moulon south of Paris in replicated ($R = 2$) field experiments under HN and LN. Mapping was based on 152 RFLP markers. Five QTL located on chr. 1, 3, 4, and 5 were detected for grain yield under HN and two QTL on chr. 3 and 5 under LN. None of the latter was LN-specific. Genetic variation for N uptake at silking was only significant in the first year. Five QTL on chr. 1, 3, and 9 were found under HN but none under LN. Genetic variation for N-utilization efficiency at harvest was significant in both years. Two QTL map to chr. 3 and 9 under HN and five to chr. 1, 2, and 9 under LN. Four of the latter were LN-specific but none of them map in the confidence interval of a QTL for grain yield. For anthesis-silking interval, three QTL on chr. 5 and 10 were observed at both N levels. One of them colocalizes with a QTL for grain yield. Two QTL on chr. 3 and 8 were found for kernels per ear under both N levels and the one on chr. 3 colocalizes with a grain yield QTL. For grain N concentration two QTL occurred on chr. 1 and 3 under HN and four QTL on chr. 1, 3, 4, and 9 under LN. Two of the latter map close to the confidence intervals of QTL for grain yield (Table 1).

A very large mapping population was analyzed by Presterl et al. (2008). It consisted of 720 doubled haploid (DH) lines derived from a cross between two contrasting European dent lines. Testcrosses of the DH lines to a flint inbred line were evaluated in field experiments under HN and LN across seven environments in Germany and North France (see Sect. 2.1). The DH lines were genotyped with 188 SSR markers. Ten QTL for grain yield were detected under HN and eight under LN (Table 1). Four of the latter QTL on chr. 2, 4, 8, and 10 are LN-specific. Furthermore, eight QTL were detected for the reaction to N-deficiency stress measured as the difference in grain yield between HN and LN. All of them colocalize with the LN-specific QTL for grain yield.

Ribaut et al. (2007) published a QTL study in which 240 F_3 lines derived from a cross between a drought tolerant line from the CIMMYT population 'La Posta' and a drought susceptible line from 'Tuxpeño Caribe'. The F_2 parent plants were genotyped with 142 RFLP probes and evaluated for *per se* performance at a tropical CIMMYT research station (Poza Rica) in the 1996 dry and wet seasons under LN and in the 1996 wet season under HN. In this review only the wet-season data are compared. Three QTL for grain yield were detected on chr. 1, 3, and 10 under HN and eight on chr. 1, 2, 3, 4, 8, and 9 under LN. Six of the latter QTL are LN-specific.

For ears per plant, seven LN-specific QTL were observed, two of which colocalize with LN-specific QTL for grain yield on chr. 2 and 8. Three LN-specific QTL for kernels per ear were observed on chr. 2, 4, and 8 all of which colocalize with LN-specific grain yield QTL. Finally, four LN-specific QTL for anthesis-silking interval were detected two of which colocalize with N-specific QTL for grain yield on chr. 4 and 8.

Generally 60–70% of the genetic variance for grain yield was jointly explained by QTL in three of the aforementioned studies (Ribaut et al., 2007, do not report this statistic). The percentage was similar under LN and HN, indicating that marker-assisted selection is expected to work equally effective for both target conditions.

The chromosomal location of the LN-specific QTL for grain yield varies greatly between the four reviewed studies as might be expected in view of the differences in germplasm and test environments. Yet a certain QTL accumulation became apparent on chr. 2, 4, 8, and 9. LN-specific QTL for NUE-associated traits were mainly found on chr. 2, 4, 5, and 8. Colocalization between QTL for NUE-associated traits and NL-specific QTL for grain yield was restricted to chromosomes 2, 4, and 8. Altogether, chr. 2, 4, and 8 seem to be particularly important for improving NUE.

2.3 Candidate Genes

A large number of structural genes encoding enzymes of the N and C metabolism as well as nitrate and ammonium transporters have been mapped to the maize genome by various authors (http://maizegdb.org). Only a few of these genes colocalize with QTL for NUE or NUE-related traits. In a study of Bertin and Gallais (2001), genes for nitrate reductase (NR) colocalize with QTL for grain yield, grain weight and kernel number per ear on chr. 1, 4, and 5. Glutamine synthetase (GS) encoding genes colocalize with QTL for grain yield and kernel weight on chr. 1 and 5 and for anthesis-silking interval, leaf senescence, and N nutrition index on chr. 10. Genes for pyrophosphorylase (ADPG) on chr. 3, 4 and 8 colocalize with QTL for kernels per plant and vegetative development (mainly leaf area). A gene encoding sucrose phosphate synthase (SPS) on chr. 6 localizes with a QTL for grain yield and senescence and a second SPS gene on chr. 8 maps within overlapping QTL intervals for grain yield and kernels per plant. A sucrose synthetase (GUS) gene on chr. 9 colocalizes with overlapping QTL for grain yield and kernels per plant. Finally, four invertase (INV) encoding genes on chr. 2, 5, and 10 map to QTL intervals for kernel weight (chr. 2), grain yield (chr. 5), and anthesis-silking interval and N nutrition index (both on chr. 5 and 10). Only a few of the above cases of colocalization were specific to the N level under which the phenotypic traits were assessed, in particular no LN-specific cooccurence of candidate genes with QTL for grain yield was observed. Furthermore, no data on colocalization of candidate gene and QTL positions were reported in any of the three other QTL studies cited in the previous section.

Table 1 Chromosomal position [cM] of QTL for grain yield and various characters related to nitrogen-use efficiency under low and high-N (LN,HN) supply estimated in four mapping populations (% σ^2_g = percentage of genetic variance jointly explained by all QTL; bold figures refer to LN-specific QTL)

			N	Chromosome number										
Pop[a]	Reference	Trait[b]	level	1	2	3	4	5	6	7	8	9	10	% σ^2_g
A	Agrama *et al.* 1999	GY	HN	131			34	9				123	75	72
			LN	**47**	**91**					**111**		**60**	69	61
												121		
		K/E	HN	131								133		35
			LN	128					**148**	106				28
B	Berlin & Gallais 2001	GY	HN	160		78	178	186						59
				234										
			LN			86		162						46
		K/E	HN			120					96			54
			LN			90					**64**			–
		ASI	HN					90					110	29
								152						
			LN					96					112	42
C	Ribaut *et al.* 2001	GY	HN	95		39							63	32[c]
			LN	67	**18**	53	**128**				**136**	**64**		32
					101	**188**								
		E/P	HN				59		120	69	90	60	65	34
			LN	**67**	**12**	**53**	**125**	**148**	97	**115**	**149**		46	46
		K/E	HN			75							61	20
			LN		**103**	54	128				**153**			22
		ASI	HN	211					71	92			**44**	30
			LN	209		**65**	**117**		67	88	**70**		**46**	22
											134			

D	Presterl *et al*. 2008	GY	HN	116	79	138		33	14	7		24		72
					145			91	93					
			LN	114	**114**	134	**44**		12	6	**42**		**62**	70
			ΔN[d]	90	82	146	36				42		56	67
							94	16						
		PH	HN	216	62	12	18		26	4	32			77
						60	70		108	38	132			
						86	96							
			LN		62	60	24	**40**	96	0	40		**56**	82
					84	88	60	**96**		40	126			
							96							

[a] A: 214 F_3 lines derived from a cross between the US Corn-Belt line B73 and a local inbred line from Egypt.
B: 99 inbred lines derived from a cross between the French flint line F2 and an lodent line.
C: 240 F_3 lines derived from a cross between 2 tropical lines contrasting in drought tolerance.
D: 720 doubled haploid lines derived from two proprietary Central Europpan dent lines.
Phenotypic data refer to *per se* performance in population A and to testcross performance in populations B-D.

[b] GY= grain yield, E/P=ears per plant, K/E=kernals per ear, ASI=anthesis- silking interval.

[c] Percentage of phenotypic variance; estimates of % σ^2_g not presented.

[d] ΔN=performance difference between N levels (HN/LN)

An alternative approach of detecting candidate genes was used by Coque and Gallais (2006). The authors monitored the allele frequency change at marker loci under divergent recurrent selection for adaptedness to HN and LN conditions. 'Selective sweeps' of allele frequencies were expected to occur in the close neighbourhood of important candidate genes. Results indicate that the above approach is less powerful than a regular QTL mapping study but may be well suited for validation of 'strong' candidate genes. Interestingly, one of the QTL for grain yield was found to colocalize with a QTL for GS activity.

No results of association studies linking nuclear polymorphism of candidate genes with phenotypic NUE data have been published in maize so far. The same applies to expression studies in transgenic plants. Thus the relevance of hitherto studied putative candidate genes for the genetic improvement of NUE still needs critical validation.

3 Correlated Traits

Genetic correlation studies, particularly in tropical materials, revealed moderate to strong associations between grain yield and various NUE-related traits under LN conditions (Table 2). Both NUE components N-uptake and N-utilization efficiency were strongly correlated with NUE in materials adapted to the Northern Guinea Zone of West Africa (Heuberger, 1998; Kamara et al., 2003) whereas in US-Corn-Belt germplasm (Moll et al., 1982) no significant relationship was observed for N uptake and only a moderate one for N-utilization efficiency. Generally, significant associations occurred between grain yield under LN and ear-leaf area, leaf-chlorophyll concentration, leaf senescence rating, stay-green rating, anthesis-silking interval, kernels per ear, and kernel weight (Heuberger, 1998; Bertin and Gallais, 2000; Kamara et al., 2003, 2006; Gallais and Hirel, 2004; Paponov et al., 2005; Ribaut et al., 2007) Inconsistent results were obtained for ears per plant and plant height.

Under controlled greenhouse conditions, Subedi and Ma (2005) investigated N-uptake and partitioning patterns of three contrasting US-Corn-Belt hybrids: a conventional, a Stay-Green, and a Leafy variety. Restriction of N supply during the vegetative phase caused an irreparable reduction in ear size and grain yield (30%). Withholding N supply during and after flowering had a less severe effect on grain yield but dramatically decreased N uptake (53%). Interestingly, the Stay-Green trait was only expressed under adequate N supply and was not associated with greater N absorption or utilization compared to the conventional hybrid. The authors showed that N taken up during grain-filling is mainly accumulated in the stalk which may explain why neither the Stay-Green nor the Leafy trait were found to be indicators of enhanced N utilization. Gallais and Hirel (2004) reported significant genotypic associations of NUE with GS (glutamine synthetase) and GDH (glutamate dehydrogenase) activity but the correlation coefficients were low.

Coque and Gallais (2006) analyzed genome regions for colocalization of QTL for NUE with QTL for NUE-related traits. Under LN, they detected four such regions: one for grain yield and N uptake on chr. 1, one for grain yield and earliness

Table 2 Coefficients of phenotypic and genotypic correlations (r_p and r_g, respectively) between N-use efficiency (grain yield under N-deficiency stress) and various component and indicator traits reported in the maize literature

Correlated trait	Region[a]	r_p	r_g	Reference
N uptake	Corn-B.	0.10	–[c]	Moll *et al.,* 1982
	W. Afr.	0.91**; 0.97**[b]	–	Heuberger, 1998
	W. Afr.	0.88**	–	Kamara *et al.,* 2003
N utilization		0.90	–	Moll *et al.,* 1982
		0.53^{ns}; 0.64**	–	Heuberger, 1998
		0.67**	–	Kamara *et al.,* 2003
Ear-leaf area		0.55*	–	Heuberger, 1998
		0.72**	–	Kamara *et al.,* 2006
Leaf chlorophyll concentration		0.71**; 0.76**	–	Heuberger, 1998
		0.49**	0.40**	Ribaut *et al.,* 2007
Stay-green rating		–0.61*	–	Kamara *et al.,* 2006
Anthesis-silking interval	Mex.	–0.56*	–	Kamara *et al.,* 2003
		–	–0.81**	Gallais and Hirel, 2004
		–0.66**	–	Kamara *et al.,* 2006
		–0.41**	–0.39**	Ribaut *et al.,* 2007
Leaf senescence rating 2–4 week after anthesis		–0.38^{ns}; –0.74**	–	Schulte auf'm Erley *et al.,* 2007
Visual N-deficiency rating		–0.65**	–	Heuberger, 1998
Plant height		0.69**	–	Kamara *et al.,* 2003
		0.07	–	Kamara *et al.,* 2006
		0.51**	0.53**	Ribaut *et al.,* 2007
Ears per plant		0.61*	–	Kamara *et al.,* 2003
		0.07	–	Kamara *et al.,* 2006
		0.70**	0.80**	Ribaut *et al.,* 2007
Kernels per ear	W. Afr.	0.56*	–	Kamara *et al.,* 2006
		0.98**	0.98**	Ribaut *et al.,* 2007
Kernel weight		0.39**	0.13*	Ribaut *et al.,* 2007

*and **significant at $P = 0.05$ and 0.01, respectively.

[a] Geographic region in which the experiment was performed and to which the genetic material is adapted.

[b] Range of estimates among test environments.

[c] No estimate reported.

on chr. 5, and two on chr. 6, one for grain yield and grain filling and the other for grain yield and seed setting. In a follow-up experiment of the QTL study of Presterl et al. see Sect. 2.2), Guo, Tietze, and Westhoff (personal communication) observed a significantly stronger lateral root development in the N-use efficient than in the N-use inefficient parent line of the mapping population under controlled hydroponic LN conditions.

4 Genetic Improvement

4.1 Experimental Prerequisites

Developing breeding materials excelling in NUE requires field experiments under strong N-deficiency stress. In general, this can only be achieved by omitting N fertilizers and N-accumulating crops over 4–6 years. This preparatory soil N in period could be shortened by using fields with light sandy soils. However, depleting such fields mostly increases soil heterogeneity to an extent that no adequate precision can be achieved for differentiating among genotypes.

Since the response of maize to a stepwise increase of N supply is approximately linear (Brun and Dudley, 1989; Presterl et al., 2003), the greatest genotypic variance for tolerance to N deficiency is obtained under extreme stress condition resulting to yield reductions of 40–60%. In drought-prone regions, experiments need to be repeated in a wide range of test environments in order to minimize biases due to interactions between N and water supply.

4.2 Response to Selection

Pioneering research on selection for high NUE was performed at St. John and Manhattan, Kansas, by Muruli and Paulsen (1981). The authors used a genetically broad-based synthetic population composed of various tropical and temperate maize materials and divergently selected among half-sib families for adaptedness to low or high soil N supply. Best families from the two directions of selection were intercrossed to form an N-efficient and an N-inefficient synthetic, respectively, and these were field-tested under four N-supply conditions. Results clearly demonstrated that selection under LN leads to superior grain yields under LN and selection under HN to superiority under HN. One single cycle of divergent selection was enough to create a significant difference in NUE between the two selected fractions. Several other groups confirmed these early findings in other genetic materials and test environments (Geiger and Presterl, 1997; Chun et al., 2005; Coque and Gallais, 2006).

To increase breeding progress, Bänziger and Lafitte (1997) suggested to include correlated traits such as ears per plant, leaf senescence rating, and anthesis-silking interval (see Sect. 3.3) in the selection criterion. When selecting for an index combining grain yield and the foregoing traits, the authors were able to increase the genetic gain in NUE by 14%. Coque and Gallais (2006) proposed to additionally include major LN-specific QTL for grain yield and NUE-related traits. Geiger et al. (2006) analyzed a second-cycle DH-line population derived from crosses among selected genotypes of a large mapping population and observed a significant linear regression of grain yield under LN on the number of LN-specific yield QTL (Fig. 1). The yield increase per QTL amounted to about 1% on average.

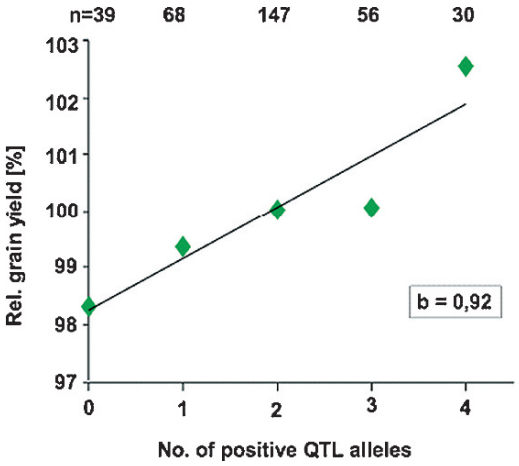

Fig. 1 Relative grain yield as a function of the number of positive QTL alleles under N-deficiency stress (means across three environments in Germany 2004; *n* = sample size, 340 entries in total; Geiger et al., 2006)

In practical breeding the question arises whether selection for LN and HN conditions should be approached in a single or two different, N-level specific programs. The former would be advisable if the genetic correlation between LN and HN was strong and the heritability lower under LN than under HN. Specific programs are more promising, if the correlation is only moderate or even weak and the heritability is not too much impaired by soil heterogeneity. Results obtained in the tropics under severe N-deficiency stress were in support of a dual approach (Bänziger and Lafitte, 1997) whereas those obtained in the temperate climate were more in favour of a unified approach (Presterl et al., 2003). Indeed, Landbeck (1995) demonstrated that selection based on an index composed of the grain yields under LN as well as HN may reveal genotypes being highly competitive under both conditions. Such genotypes generally display not only a superior yielding potential but also a high phenotypic stability (Presterl et al., 2003; W. Schmidt, personal communication).

Since NUE is the product of N-uptake and N-utilization efficiency we may ask how these two components react to selection for grain yield under LN versus HN. It was already mentioned that contrasting reports exist about the genetic variance for the two components at different levels of N supply. From this information, breeders may conclude that there is considerable potential for creating germplasm which combines significant variation for both NUE components, irrespective of the N-supply level of the respective target environment. Uribelarrea et al. (2007) analyzed N uptake and N utilization in Illinois High Protein (IHP) and Illinois Low Protein (ILP) germplasm. IHP genotypes were superior in N uptake and ILP genotypes in N-utilization efficiency. Thus crosses between parents contrasting in grain protein concentration may be a promising way to increase genetic variation for both NUE components.

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Seed Phosphate

Victor Raboy

1 Introduction

The management of phosphorus (P) in agricultural production represents an important issue for the twenty-first century and beyond (Sharpley et al., 1994; Brinch-Pedersen et al., 2002). Adequate soil available P is essential to optimal crop yields. After nitrogen, P is the second most common component of fertilizer, but both the production and use of P fertilizers, and application of P-rich manures, can have a negative impact on the environment and on water quality. A large fraction of the P taken up by plants during vegetative growth is translocated to developing seed. Therefore it is not surprising that the total amount of P annually stored in mature seeds of grain and legume crops is estimated to represent a sum equivalent to ~60% of the fertilizer P applied worldwide (Lott et al., 2000). Thus seed P represents an important bottleneck in the flux of P through the agricultural ecosystem. However, while fertilizer and manure P is applied to insure optimal yields, its ultimate fate as seed P, both in terms of seed P composition/chemistry and total amount, is not necessarily optimal for most grain end-uses. Engineering seed P composition/chemistry and total amount presents opportunities to enhance seed nutritional quality and end-use value, to enhance the efficiency of agricultural production, and to reduce its impact on the environment.

2 Practical Issues Concerning Maize Seed P

In 2007, ~55% of the total Unites States maize grain production was utilized in animal feeds, ~18% was used for biofuels production, and ~19% was exported (estimated from data collected by the USDA's Economic Research Service, www.ers.usda.gov/data/feedgrains). Of the ~18% used in biofuels production, a significant "coproduct", Distiller's Dry Grains (DGGs), is subsequently used in animal feeds (Rausch and Belyea, 2006). Also, the majority of exported maize is destined for use in animal feeds. Clearly use in feeds remains the major end-use of maize produced in the United States. Therefore feed end-use quality remains the most important end-use trait.

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Of the ~55% of maize used in feeds, about half is used in ruminant feeds and half used in non-ruminant, monogastric feeds (swine, poultry and fish). In the case of non-ruminant feeds, seed total P might present some opportunities for end-use improvement, but most concern and work has addressed the composition/chemistry of seed P (Ertl et al., 1998; Spencer et al., 2000a). The problem is that in most grain and legume crops from 65% to 85% of mature seed total P is found as a single, small molecular weight compound, *myo*-inositol hexa*kis*phosphate ($InsP_6$, mw 660), commonly referred to as "phytic acid" (Fig. 1; Raboy, 2007). It is interesting that each year nearly 50% of all P applied as fertilizer world-wide is synthesized into this one, small molecule (Lott et al., 2000). Non-ruminants do not efficiently digest and utilize seed-derived phytic acid. This simple fact has several undesirable outcomes. While crops like maize and soybean typically have enough total P to satisfy a chicken or pig's dietary needs, since most is in a form not readily digestible, feeds for these animals traditionally were supplemented with P. Supplementation with P satisfies the animal's nutritional requirement for P but feed phytic acid P is still excreted. This contributes to high waste P levels which represents an environmental hazard, potentially contributing to water pollution via eutrophication (Sharpley et al., 1994).

One alternative is to supplement feeds with an industrially-produced phytase enzyme (Brinch-Pedersen et al., 2002). Supplement phytase consumed by animals breaks down seed-derived dietary phytic acid, releasing its P for uptake and use by the animal. Thus more of the seed-derived P is used by the animal and less ends up in waste. Additional, alternative approaches include engineering crops to express active phytases in seeds, either to subsequently provide active phytases when seeds are consumed in feeds of foods, or to block phytic acid accumulation itself during seed development (Chen et al., 2007; Drakakaki et al., 2005). Various additional biotechnological and genetics/breeding approaches are aimed at producing "low-phytate/high available P" crops (Raboy, 2007; Shi et al., 2007). Since phytic acid P represents a large fraction of seed total P, another approach to producing low-phytate seed might be to develop genotypes that produce low-total-P seed, perhaps by blocking specific P transport functions such as a *Phosphate transporter 1* (*Pht1)* gene (see Sect. 5.1). Depending on the extent of seed total P reduction, and combination with other genotypes such as low-phytate, low-total-P seed may or may not

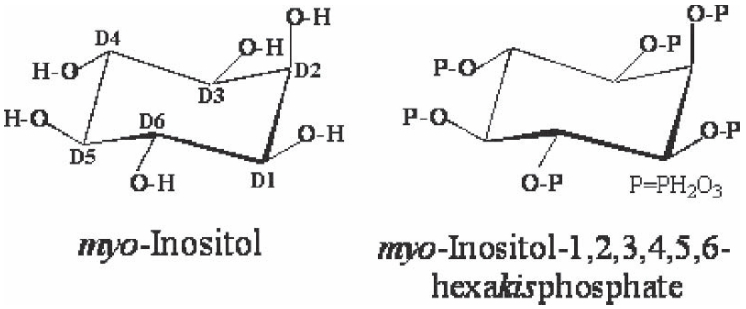

Fig. 1 The structures of phytic acid (*myo*-inositol hexaphosphate) and its backbone, *myo*-inositol

also have higher "available P". Seed phytic acid also presents a problem for human populations for which grains and legumes represent the bulk of foods consumed daily (reviewed in Raboy, 2007). Like other monogastric animals, humans do not efficiently digest seed-derived dietary phytic acid. Phytic acid binds tightly to nutritionally-important minerals such as iron, zinc, calcium and magnesium, and excretion of mixed phytate salts can contribute to deficiencies of these important minerals. Mineral deficiency such as iron-deficiency anaemia is an extremely important public health problem worldwide, impacting hundreds of millions of people. However potential health beneficial roles for dietary phytic acid must also be considered. It may function as an anti-oxidant, an anti-cancer agent, or in the prevention of renal stone formation (reviewed in Raboy, 2007). Thus when breeding or engineering maize for use in human foods, both positive and negative roles of phytic acid must be considered on a case-by-case basis.

The composition or chemistry of seed P is not considered a major issue when grain or legume seed products are used as part of ruminant (dairy and beef cow) feeds. Ruminants efficiently digest and absorb phytic acid. In this context it is not seed P composition but the amount of seed total P that may present an opportunity for crop end-use improvement. Grain and legume products might contain 25% more P than required by cattle, and this excess feed P contributes to elevated manure P, disposal of which is often subject to regulation based on P content (Wardyn and Russell, 2004; Environmental Protection Agency, EPA, 2002; Volk et al., 2000). This problem has been exacerbated by the increasing use of maize in ethanol production. DDGs have a higher concentration of P than does whole grain, and use of DDGs in feeds can lead to elevated manure P levels. Thus in the context of ruminant and biofuels production, reduced seed total P may be desirable (Maguire et al., 2007; Rausch and Belyea, 2006), but there is essentially no genetic understanding of seed total P, so few tools are currently available to breed or engineer "low seed total P" crops.

3 Genetics and Biochemistry of Seed P Composition

3.1 Genetics

In wild-type maize seed about 80% of seed total P is found as phytic acid P (Fig. 2). Typically <5% of seed total P is found as inositol phosphates with five or fewer phosphate esters (whereas phytic acid has six phosphate esters per molecule), and about 5% is found as inorganic P. The remaining seed P, such as nucleic acid, lipid and starch P ("Other P" in Fig. 2), represents about 10% of seed total P. The first two maize *low phytic acid* (*lpa*) mutants, maize *lpa*1-1 and *lpa*2-1 (Fig. 2), were isolated from a chemically-mutagenized population by testing individual seeds for reductions in phytic acid P, using a low-throughput ("brute force") chromatographic method, high-voltage paper electrophoresis (Raboy et al., 2000). Initial analyses of maize *lpa*1-1 and *lpa*2-1 revealed that reductions in phytic acid P (about 66% and

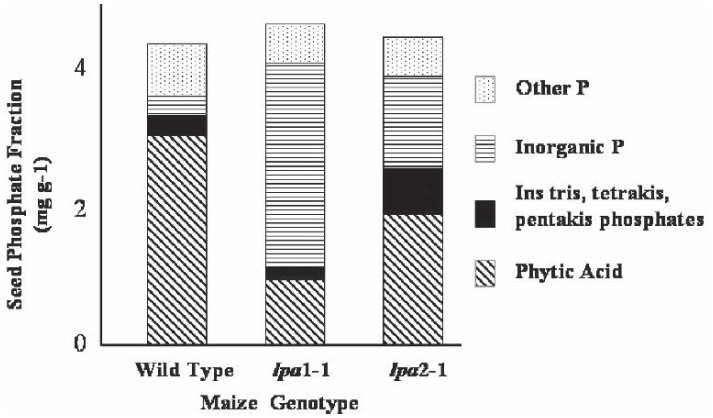

Fig. 2 Seed phosphate fractions in wild-type and two *low phytic acid* (*lpa*) genotypes of maize. The large increase in seed inorganic P in the *lpa* genotypes provides a high-throughput assay for homozygosity for an *lpa* allele, useful both in genetics and breeding. "Other P" includes all other cellular P-containing compounds other than inositol phosphates and inorganic P

40%, respectively), were mostly accompanied by molar-equivalent increases in inorganic P, so that seed total P was largely unchanged. Since wild-type seeds have relatively low levels of inorganic P, and low-phytic acid seeds have several-fold higher levels, and since testing for inorganic P is quick, easy, accurate and inexpensive, the "high inorganic P" (HIP) phenotype provides a high-throughput, single-seed assay for homozygosity for *lpa* alleles (Fig. 3).

This high-throughput assay was first used to identify additional *lpa* mutations in maize. A set of 26 heritable *lpa* mutations were isolated following screening of ~4,500 M_2s. Interestingly, while one of these was a second allele of *lpa*2, the remaining 25 were all additional alleles of *lpa*1 (Raboy, 1997; Raboy et al., 2001, unpublished data). This translates into a very high rate of recovery of mutations at a given locus following screening of a chemically-mutagenized population. In the case of maize *lpa*1-1 this rate was one "hit" per ~10^2 M_2s, an order of magnitude greater than that typical of most single genes, one hit per ~10^3 M_2s, such as that observed for *lpa*2. Subsequent studies also revealed high reversion rates and instability of selected alleles (unpublished data). Taken together, these results indicate an unusually high level of mutability for maize *lpa*1.

Variations of the high-throughput HIP assay were subsequently used to isolate what turned out to be an additional allele of maize *lpa*1 (Pilu et al., 2003), a third *lpa* gene in maize (*lpa*3; Shi et al., 2005), to identify *lpa* mutations and genes in a number of other species (reviewed in Raboy, 2007), and to facilitate genetics studies and breeding with the low-phytate trait in these various species. Maize *lpa*1, *lpa*2 and *lpa*3 all map to chromosome 1, to bins 1.02, 1.04/1.05 and 1.10, respectively (Raboy et al., 2000; Shi et al., 2005).

An alternative approach to a high-throughput assay useful in genetics and breeding of seed phytic acid is one that utilizes a simple, colorimetric assay for phytic

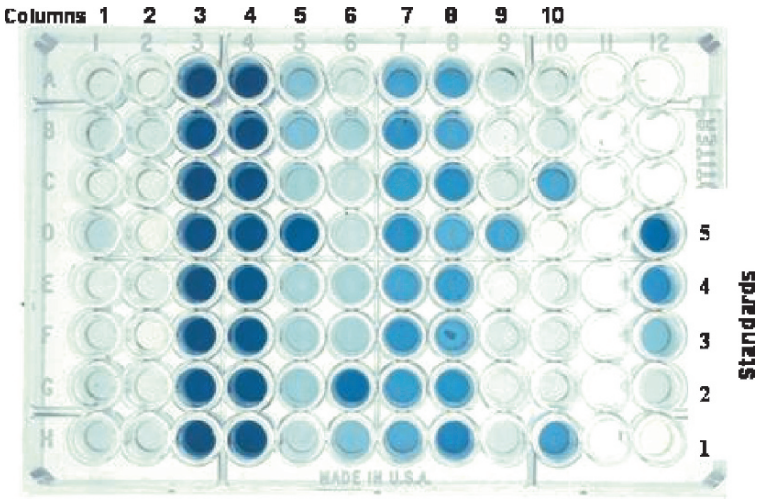

Fig. 3 Single-seed assay for the "high inorganic P" seed phenotype of *lpa* homozygotes. Individual seed are crushed, extracted overnight in 10 volumes per weight 0.4 M HCl, and then 10 μL aliquots are assayed for inorganic P using a simple colorimetric assay conducted in a microtitre plate. Columns: 1 and 2, 20 wild-type maize seed; 3 and 4, 20 homozygous *lpa*1-1 seed; 5 and 6, 20 seed segregating for *lpa*1-1; 7 and 8, 20 homozygous *lpa*2-1 seed; 9 and 10, 20 seed segregating for *lpa*2-1. Standards: colorimetric standards; No. 1 through 5 contains 0.0, 0.15, 0.46, 0.93, and 1.39 μg P

acid itself, rather than for the inorganic P (Gao et al., 2007). In this method single seeds are crushed and extracted as for the HIP test above, but, following treatment of the crude extracts with NaCl, colorimetric assays are conducted using the iron-containing "Wade's Reagent" (0.03% $FeCl_3$:0.3% sulfosalicylic acid). Phytic acid binds tightly to iron, reducing the absorbance of the solution. If conducted properly, there is a linear, inverse relationship between solution phytic acid and absorbance at 500 nm. Treatment of extracts with NaCl is required since inorganic P also interacts with iron in Wade's Reagent, interfering with phytic acid measurement, and there is high inorganic P in low-phytate seeds. There are advantages and disadvantages to both approaches, testing for HIP or testing directly for phytic acid. When testing for increases in seed inorganic P, one is testing for several-fold differences between wild-type and *lpa* genotypes. Consider that even a 10% reduction in seed phytic acid, probably a reduction too small to detect with the Wade's Reagent approach (probably less than the standard deviation), would result in a 100% increase in seed inorganic P, clearly distinguishable from the low background levels of a wild-type seed. However, direct assays for phytic acid may prove valuable in many applications. Labs interested in this area should try out both approaches and determine which method works best in their hands.

While these high-throughput assays have proven very valuable for genetics and breeding studies, chromatographic methods that fractionate inositol phosphates can also prove valuable for both routine screening purposes and more detailed analysis. Two relatively low-tech methods are paper electrophoresis (Raboy et al., 2000) and

thin layer chromatography (Rasmussen and Hatzack, 1998). These "intermediate"-throughput methods represent relatively low technologies that many labs can use without great difficulty. For more formal quantitative analysis of seed phytic acid in a relatively large number of samples, the "ferric-precipitation" method has proven useful (Raboy et al., 2000). This method involves extraction of phytic acid, precipitation as a ferric-salt, and measurement of the P content of the precipitate salt. However, essentially all inositol phosphates will be precipitated in the ferric salt. Perhaps the best method for both quantifying phytic acid and other inositol phosphates, albeit a relatively slow, time-consuming method that requires more equipment and expertise, utilizes some form of High Performance Liquid Chromatography (HPLC). Current methods of anion-exchange HPLC, where fractionated inositol phosphates are detected post-column using a version of the Wade's Reagent colorimetric assay, is sufficiently sensitive to quantitate phytic acid in single-seed assays.

Whichever method is used, care must be taken to recognize the most common types of false positives in testing for genetics of seed phytic acid: mutations which perturb embryo development, such as "germless" or "defective kernel" (DEK) mutations. These are common mutations in most mutagenized populations and one component of the DEK phenotype is reduced phytic acid accompanied by increased seed inorganic P (Raboy et al., 1990).

3.2 Biochemical Pathways to Seed Phytic Acid

The synthesis of phytic acid begins with the synthesis of its backbone, *myo*-inositol (Ins; Fig. 4) and with the supply of P to the developing seed (for more detailed review and references, see Raboy, 2003, 2007). P transport and supply will be discussed in Sect. 5. Most if not all of the Ins used in phytic acid synthesis is synthesized *de novo* in developing seeds via the action of *myo*-Ins(3)P_1 synthase (MIPS), which converts glucose-6-P to Ins(3)P_1 (Ins with a single phosphate ester at the "3" position). The maize genome contains up to seven loci containing MIPS-homologous sequences (Larson and Raboy, 1999), but these have not been studied in any great detail yet. The product of MIPS activity, Ins(3)P_1, may then be directly phosphorylated to yield phytic acid, but it is also possible that first it is broken down via Ins monophosphatase activity to yield Ins and P_1. In plants, Ins monophosphatases are often encoded by multi-gene families but this family of genes has not been studied in any detail in maize (Torabinejad and Gillaspy, 2006).

Studies of the biochemistry and molecular genetics of phytic acid synthesis in various eukaryotes indicate two basic pathways from Ins, or perhaps Ins(3)P_1, to phytic acid. One of these pathways, the "lipid-independent pathway", proceeds entirely via the sequential phosphorylation of Ins, beginning either with Ins or the Ins(3)P_1 product of MIPS activity (Stephens and Irvine, 1990). The second pathway, the "lipid-dependent" pathway, begins with the synthesis of phosphatidylinositol (PtdIns), a lipid-anchored form of Ins, and involves as initial intermediates PtdIns phosphates (York et al., 1999). Studies of maize *lpa* mutants have contributed

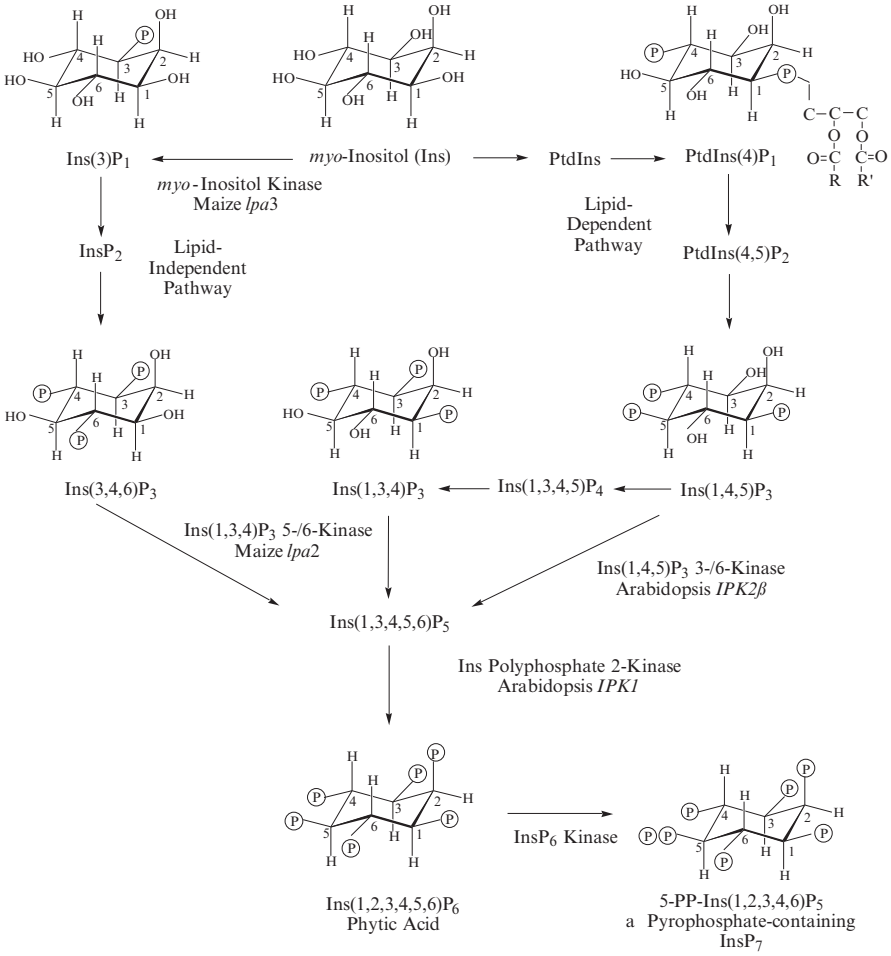

Fig. 4 Biochemical pathways leading from *myo*-inositol (Ins; top center) to Ins(1,2,3,4,5,6)P_6 or phytic acid (bottom centre) in developing seed. On the top left is the pathway which proceeds via "lipid-independent" precursors, while on the top right is the pathway that proceeds via "lipid-dependent" precursors that include phosphatidylinositol (PtdIns) and PtdIns phosphates. P = H_2PO4

significantly to understanding how these pathways work. Ground-breaking work with yeast indicated that the pathway to phytic acid might proceed solely via the lipid-dependent pathway (York et al., 1999). Forward genetics with maize mutants however, clearly indicates that the "lipid-independent" pathway involving the sequential phosphorylation of Ins might also play a role in seed phytic acid synthesis. Probably both pathways contribute to net phytic acid synthesis.

The lipid-independent pathway most likely begins with Ins kinase, which converts Ins to Ins(3)P_1, and possibly other Ins monophosphates. While known biochemically for many years, the first genetic proof of an Ins kinase in any organism, and of its

role in phytic acid synthesis, was the isolation of maize *lpa*3 and the demonstration that this gene encoded Ins kinase (Shi et al., 2005). Reduced seed phytic acid accumulation in plants homozygous for recessive alleles of maize *lpa*3 represents evidence that Ins kinase activity contributes to a lipid-independent pathway to phytic acid.

The "lipid-dependent" pathway from Ins to phytic acid was first documented genetically in yeast, but is also active in other eukaryotes including plants. It begins with the conversion of Ins and phosphatidic acid to PtdIns (Fig. 4). The action of two kinases then converts PtdIns to PtdIns(4,5)P_2, which is then cleaved to yield Ins(1,4,5)P_3. Up to this point this is the classic pathway to Ins(1,4,5)P_3 central to signal-transduction in many organisms. Ins(1,4,5)P_3 may then be converted to phytic acid by the action of two Ins polyphosphate kinases. First Ins(1,4,5)P_3 is converted to Ins(1,3,4,5,6)P_5 via an Ins(1,4,5)P_3 3-/6-kinase. Then Ins(1,3,4,5,6)P_5 is converted to phytic acid via the action of an Ins polyphosphate 2-kinase. Mutations in both a "3-6-kinase" and a "2-kinase" perturb phytic acid synthesis is *Arabidopsis* seed, so these enzymes clearly contribute to phytic acid synthesis in the plant seed (Stevenson-Paulik et al., 2005).

An alternative route from Ins(1,4,5)P_3 to phytic acid first involves its conversion to Ins(1,3,4)P_3, followed by conversion of Ins(1,3,4)P_3 to Ins(1,3,4,5,6)P_5 via the action of an Ins(1,3,4)P_3 5–6-kinase (Wilson and Majerus, 1996). Ins(1,3,4,5,6)P_5 is then converted to phytic acid via the previously mentioned Ins polyphosphate 2-kinase. This branch of the "lipid-dependent" pathway (Fig. 4 center) may be involved in maize seed phytic acid synthesis since maize *lpa*2 encodes this type of kinase and mutations in this gene partially block seed phytic acid synthesis (Shi et al., 2003). An alternative explanation is that while maize *lpa*2 encodes a "5-/6-kinase", it actually functions as part of the "lipid-independent" pathway, using substrates such as Ins(3,4,6)P_3 derived from the direct phosphorylation of Ins.

Studies in non-plant eukaryotic systems indicate that once synthesized, phytic acid and certain other Ins phosphates can be further phoshorylated to yield Ins phosphates with seven or eight moles of phosphate per mole of Ins, thus containing one or two pyrophosphates per mole of Ins (Fig. 4 bottom right; Stephens et al., 1993). Homologs of genes encoding enzymes that catalyze the phosphorylation of phytic acid are conserved throughout eukaryotes, including plants (Mulugu et al., 2007). Interestingly, the "Inositol pyrophosphate synthase" shown to have homologs in plants (*Arabidopsis*) has two domains; a kinase domain and a histidine phosphatase or "phytase" domain. Such a protein could function in the regulation of cellular P and Ins P levels via the synthesis and breakdown of phytic acid.

3.3 Compartmentalization of Phytic Acid Synthesis and Storage During Seed Development

Once synthesized, phytic acid accumulates primarily as a mixed potassium/magnesium "phytate" salt, found as a discrete inclusions referred to as "globoids". Globoids are in turn localized within one class of the storage microvacuoles referred to as Protein

Storage Vacuoles (PSVs; Fig. 5). Both the synthesis and deposition of phytic acid is localized within tissues of the maize seed. Approximately 80% of the mature maize seed's total phytic acid is found within the germ and scutellum, with the remainder in the aleurone layer (O'Dell et al., 1972). The central starchy endosperm contains little P or phytic acid. Until recently relatively little was known concerning the processes and functions involved in phytic acid transport/storage, and many questions remain. It is not clearly known whether components of the synthetic pathway are compartmentalized, or if the synthetic pathway is entirely cytoplasmic and only storage is compartmentalized. Also, it is possible that globoids have their own membranes and therefore represent a unique "vacuole-within-a vacuole" inside PSVs (Jiang et al., 2001).

Issues concerning phytic acid compartmentalization and transport are important across all eukaryotic cell biology, and again, a breakthrough recently occurred via forward genetics with maize. The maize *lpa*1 gene encodes a variant of the ATP-biding cassette (ABC) transporters. ABC transporters were first identified as "multidrug resistance-associated proteins" (MRPs) involved in drug-resistant human cancer cell lines, and since shown to play important roles in plant biology (Klein et al., 2006) including anthocyanin transport/storage in maize (Goodman et al., 2004). Maize *lpa*1 was shown to encode an MRP termed MRP4 (Shi et al., 2007). The specific role of maize *lpa*1/MRP4 in phytic acid synthesis or accumulation is not known. For example, its cellular location nor the metabolite it transports is known, but perhaps it is involved in phytic acid transport to its storage site.

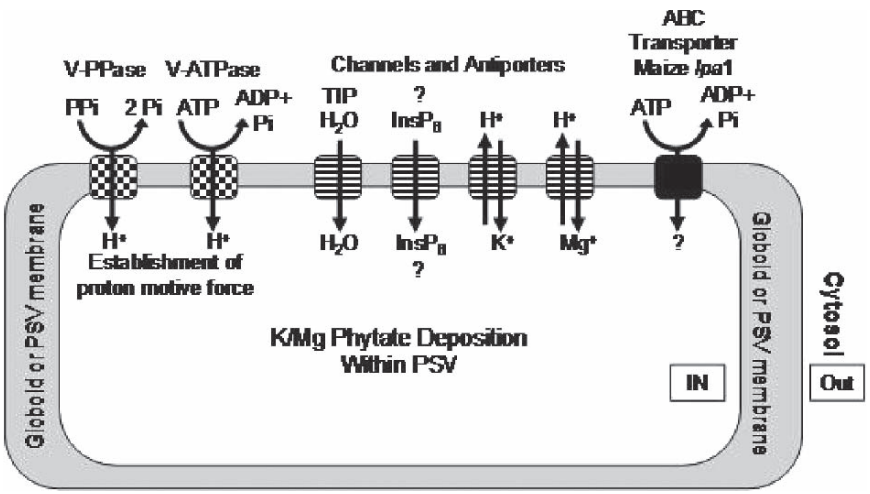

Fig. 5 The K/Mg phytate-accumulating membrane-bound organelle, either a Protein Storage Vacuole (PSV) or a membrane-bound globoid found within a compound PSV. *ABC Transporter* ATP-binding Cassete Transporter, *V-PPase* vacuolar inorganic pyrophosphatase, *V-ATPase* vacuolar ATPase, *TIP* tonoplast intrinsic protein, *PtdIns* phosphatidylinositol, *DAG* diacylglycerol. Question marks indicate purely speculative aspects of the diagram. (For references and detailed discussion, see Raboy, 2007)

4 Agronomic and Nutritional Quality Studies

4.1 Development of High-Yielding Low-Phytate Maize: Breeding Versus Genetic Engineering

Published reports of the agronomic impact of *lpa* alleles on maize hybrid performance are limited to the first such study which evaluated one allele of maize *lpa*1 (Ertl et al., 1998). For this study, the *lpa*1-1 allele was backcrossed from its original parent, the synthetic "Early ACR" population (Raboy et al., 2000), into a series of inbreds to produce backcross 3 (BC_3)-derived sib lines either homozygous wild-type or homozygous *lpa*1-1. These in turn were used to produce 14 hybrid pairs, again either wild-type or homozygous *lpa*1-1. These hybrid pairs were grown in two replications at three locations in Iowa and various agronomic traits were measured, such as grain yield, stand establishment, lodging, plant height, flowering date and "cold-test" germination. The only substantial effect observed was in grain yield. In eight of the 14 hybrid pairs, *lpa*1-1 grain yield was reduced as compared with wild-type. Overall, yield was reduced 5.5%. No effect on plant height, flowering date, germination, or other traits was observed. However, the wild-type and *lpa*1-1 hybrid pairs evaluated in this study cannot be considered near-isogenic, since they were derived only from the BC_3 obtained from very divergent parents (Early ACR and contemporary inbreds). Subsequent observations (Raboy, unpublished results) of more truly isogenic lines derived from the BC_5 has shown that homozygosity for *lpa1-1* or *lpa*2-1 also can reduce plant height, delay flowering and result in reduced germination rates. The lack of an observed effect on these traits in the initial Ertl et al. (1998) study probably was in part due to the insufficient "isogenicity".

Since the initial study of Ertl et al. (1998), a number of agronomic studies of *lpa* genotypes in other crops species, such as soybean and barley, have shown that there is often but not always an effect on yield, germination and stress tolerance. The net effect on yield typically ranges from 5% to 15%, but sometimes no effect is observed (reviewed in Raboy, 2007). Effects on seed and plant performance are both gene- and allele-specific, and vary dependent on genetic background. For example, the effect of homozygosity for various alleles of maize *lpa*1 range from little or no effect to lethality (Raboy et al., 2001). Where such effects are detectable, they may be more pronounced in relatively more stressful production environments (Bregitzer and Raboy, 2006).

In the case of maize, reduced grain yield of *lpa* genotypes can be attributed at least in part to reduced seed dry weight accumulation (Raboy et al., 2000). However, some component of the negative effect of essentially all recessive *lpa* alleles probably is on plant performance, since the genes in question probably have functions important to all vegetative and reproductive tissues. Most claims of seed-specific expression for an *lpa* gene probably won't stand up to close scrutiny. The impact of *lpa* mutations on plant performance is the rational behind the recent consensus in the plant biotechnology field that engineering seed-specific expression of the low-phytate trait would be one approach to achieving high-yielding,

low-phytate crops, demonstrated so far with the maize *lpa*1/MRP4 gene in both maize and soybean (Shi et al., 2007).

The problem with this consensus is that to date, no one working with maize has conducted recurrent selection for high yield, enhanced seed germination or seedling emergence within a population homozygous for an *lpa* allele. No one has reported on the results of selection or screening for modifiers of any given component of a maize *lpa* phenotype. Recent studies with a soybean low-phytate genotype have indicated that such selection can enhance yield or restore seedling emergence to levels observed with a conventional cultivar used as a control (Spear and Fehr, 2007; Oltmans et al., 2005). Thus it may be possible to select for high yielding low-phytate types without resorting to biotech approaches. Another approach to developing high-yielding low-phytate types would be to use recurrent selection within a conventional or "normal phytate" population for reduced phytate or elevated inorganic P (Lorenz et al., 2007).

4.2 Nutritional Quality Studies of Low-Phytate Genotypes

In terms of end-use quality for animal feeds, the primary interest in maize low-phytate genotypes concerns grain P availability for non-ruminants. The first animal nutrition studies of low-phytate genotypes used the first wild-type and *lpa*1-1 "isohybrids" described above (Ertl et al., 1998; Spencer et al., 2000a). These studies found that the nutritional availability of grain P for non-ruminants such as chicks (Ertl et al., 1998) or pigs (Spencer et al., 2000a) is increased in maize *lpa*1-1 grain, as compared with wild-type controls, in proportion to the increase in inorganic P (and decrease in phytic acid P) as illustrated in Fig. 2. Thus in the Ertl et al. (1998) study with chicks, various estimates of P availability in grain produced by the wild-type isohybrid ranged from 29.9% to 48.1% whereas estimates of P availability in the *lpa*1-1 isohybrid ranged from 69.7% to 91.2%. In the Spencer et al. (2000a) study with pigs, estimated P availability in wild-type and *lpa*1-1 grain was 9% and 62%, respectively. Depending on diet formulation, use of *lpa*1-1 grain in place of wild-type grain was shown to result in reductions in animal waste P of 10–40%, with no loss in animal growth or productivity. These basic results have been confirmed in a number of additional studies of low-phytate genotypes of maize, barley and soybean (reviewed in Raboy, 2007). Clearly genetic modification of grain P chemistry can provide one possible tool for managing P in animal production.

These studies have also shown that calcium, magnesium and zinc nutrition is enhanced in animals consuming diets prepared using low-phytate genotypes (reviewed in Raboy, 2007). This can result in enhanced animal health and productivity, and provides important evidence in support of the benefit of the low-phytate trait in grains used in human foods (see below). Also, since waste P can be reduced when *lpa* grains are used in feeds, the nitrogen/phosphorus (N/P) ratio in animal waste can be increased, resulting in manure that is better suited for soil application and more useful as a fertilizer (Spencer et al., 2000a).

The potential benefits, in terms of P and mineral nutrition, of *lpa* genotypes were largely predicted based on the grain P phenotypes illustrated in Fig. 2. An interesting and unanticipated finding of some of the initial nutritional evaluations of maize *lpa*1-1 is that animals consuming diets prepared with *lpa*1-1 maize as compared with normal maize were leaner, produced meat that was leaner, and produced meat and eggs with reduced cholesterol (Spencer et al., 2000b; Stilborn et al., 2002). How consumption of low-phytate versus normal phytate grains may result in less fat accumulation and reduced cholesterol is not known. The possible importance of such a finding to human nutrition and health in contemporary society is obvious.

The primary interest in phytic acid in terms of human nutrition and health historically has been its negative impact on mineral nutrition, primarily iron and zinc nutrition. The first study to evaluate a low-phytate genotype as a human food reported 49% greater iron absorption from tortillas prepared with *lpa*1-1 versus wild-type (Mendoza et al., 1998). A subsequent series of studies reported an inverse, linear relationship between the phytic acid:zinc molar ratio of a food and "fractional zinc absorption" (Hambidge et al., 2004). For example, fractional zinc absorption from test meals prepared with *lpa*1-1 maize versus wild-type maize was 0.285 as compared with 0.151, an improvement of 89%. A similarly designed study reported 43% greater fractional calcium absorption (Hambidge et al., 2005). Taken together, studies with human subjects or with animal models indicate that when whole grains or legumes represent a major component of a diet, use of low-phytate types can provide for enhanced P and mineral nutrition. However, some form of supplementation or fortification, either with P, minerals or phytase, can achieve similar benefits to those observed via reduction of dietary phytate. Also, any consideration of the "phytate" question in human nutrition and health must take into account its possible health-beneficial roles, and consider these issues on a case-by-case basis reviewed in Raboy (2007).

One interesting observation for geneticists is that if animal and human nutrition studies evaluate lines, cultivars or hybrids that differ only in the alleles at a single *lpa* gene and are otherwise near-isogenic, then any observed differences in nutrition, productivity, growth or health can be attributed to the different alleles of the *lpa* gene; they represent one component of the phenotype of the plant genotype.

5 Future Directions: Seed Total P Mutants

At present, there is much room for progress in using a genetic approach to investigate seed total P in maize or in any species. Seed total P is a function of P uptake by the parent plant and subsequent transport into the developing seed. Therefore mutations or allelic variants that impact plant P will probably impact seed P. This was demonstrated for the soybean *Np* locus (Raboy and Dickinson, 1984). With a few potential exceptions, there are essentially no mutations or alleles of specific genes known to greatly impact seed total P (by 25% or more) independent of plant P, thus clearly representing functions specific to seed P uptake. However, seed total

P is important to most end-uses of grains and legumes. Therefore developing genetics tools useful in engineering or breeding altered seed total P, such as a "low seed P", would be desirable.

Since high seed P has been shown to positively impact emergence and seedling growth, it might not be possible to develop "low seed P" genotypes without greatly impacting agronomic traits that ultimately contribute to yield (Thomson and Bolger, 1993; Zhu and Smith, 2001). The experimental model in these studies was to produce seed with "low" and "high" P by growing plants in nutrient culture with "low" and "high" nutrient P, and then to evaluate this seed in germination, emergence and seedling growth studies. A nice alternative experimental approach would be to evaluate high- or low-total P seed produced by plants grown with optimal nutrient P but that differ in their inheritance of single-gene allelic variants that have large impacts on seed total P. Thus developing a genetics approach to the question of seed total P in maize might be useful for breeding and engineering but also would be useful in studying seed biology. First, a few examples of candidate genes that may serve as targets for reverse genetics strategies to engineering altered seed total P will be considered. Second, screening approaches useful for forward genetics of seed total P will be described.

5.1 Targets for Reverse Genetics

There have been numerous studies addressing the genetics of P uptake by plants. Since high available soil P is so important to plant performance and yield, and since a large proportion of the world's soils have low available P, there is a large field of research devoted to the structural and regulatory biology of plant P uptake. Many mutations isolated in these studies no doubt impact seed P. The lack of data concerning seed P simply reflects the fact that the primary focus in these studies was on plant P. A good example is the *Pht1* gene family of P transporters. Most species contain multiple *Pht1* genes which can differ in tissue specificity and function. Studies have shown that some copies are important to P uptake by the root, while others are important to P transport within the plant. However it is not clear in any species which if any are specifically important to P mobilization to the developing seed. The maize genome contains six copies of the *Pht1* gene, but it is not clear which if any are specific to seed P uptake (Nagy et al., 2006).

A second example is the *Arabidopsis IPK*1 gene (Fig. 4). The Ins phosphate pathways to phytic acid in seeds are also central to signal transduction throughout plant and seed development, one aspect of which is P sensing. Mutations in *At IPK1* both blocks seed phytic acid synthesis, and perturbs the ability of plants to sense and regulate vegetative P level, resulting in luxury uptake of P and vegetative P toxicity (Stevenson-Paulik et al., 2005). While seed P chemistry (inositol phosphate and inorganic P) was analyzed in this study, seed total P was not reported. We (G. Hu and V. Raboy, unpublished results) are working on identifying the barley *lpa*1 gene. Mutations in the barley *lpa*1 gene results in an aleurone-specific block in seed

phytic acid synthesis (Ockenden et al., 2004). Embryo phytic acid is unaffected in mutations of this gene, indicating that it encodes an aleurone-specific function. Interestingly, in barley *lpa*1 mutations, the distribution of P in the seed is shifted (elevated in the embryo, reduced in the aleurone), and seed total P is reduced by about 15%, but plant P appears unchanged. Thus barley *lpa*1 represents a proof-of-principle that single-gene allelic variants can be identified that specifically impact seed total P. An *Arabidopsis* QTL has been identified for which allelic variants contribute to variation in seed total P (Bentsink et al., 2003). Contained within the chromosomal segment represented by this QTL is a H±ATPase of the type illustrated in Fig. 5, and it may function in P transport important to seed P.

The developing seed and its surrounding environment, proximal maternal tissue, represents in microcosm the developing plant and its surrounding environment, the soil solution. P uptake by the plant at the root-soil interface and P transport from maternal tissues into the developing seed probably are similar processes, involving in part P transport from an "apoplast" to the "symplast", utilizing similar molecular machinery.

Nutrient transport into the developing endosperm or aleurone layer probably occurs through cells in the "basal endosperm transfer layer" (BETL), or into the embryo through the "embryo-surrounding-region" (ESR), and there are genes whose expression is specific to these cells (Fig. 6). One that may be important to P

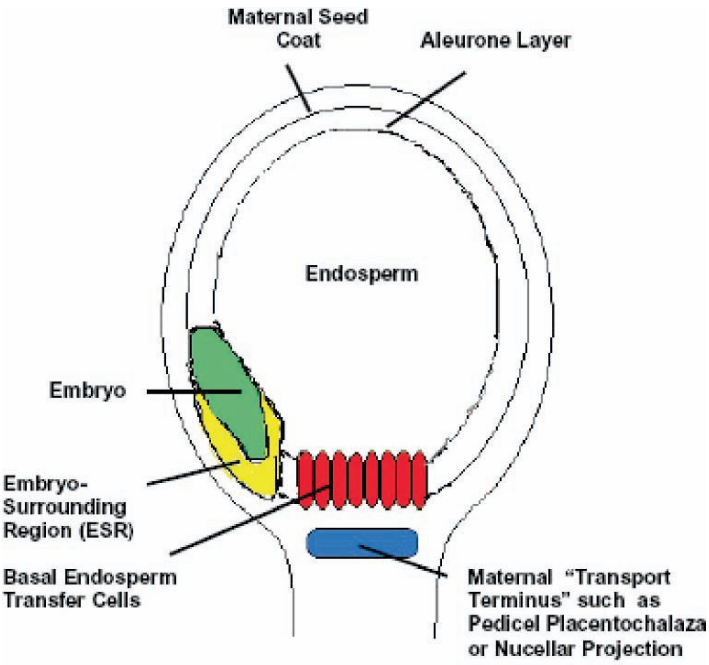

Fig. 6 Schematic of the developing cereal caryopsis and tissues important to transport processes (modified from Olsen, 2004)

transport via the BETL is maize *TCRR-1*, which encodes a "type-A" response regulator, a component of the "Histidine-to-Aspartate" (His-Asp) phosphorelay pathway (Muniz et al., 2006). This pathway represents the molecular mechanism for cytokinin signal transduction which in turn is important to P sensing and the regulation of P uptake (Martin et al., 2000). In fact, expression of a response regulator similar to *TCRR-1* is suppressed early in developing seeds homozygous for the barley *lpa*-M955 mutation (Bowen et al., 2007). In seeds homozygous for this mutation phytic acid synthesis is blocked by greater than 90%, resulting in very high levels of inorganic P. Perhaps seed cells of this genotype sense high inorganic P, and shut down the His-Asp phosphorelay pathway. This raises an important observation concerning the High Inorganic P phenotype. In nearly all *lpa* genotypes of several species, seed total P is largely unchanged as compared with wild-type. Even if the cells of barley M955 seed sense high inorganic P, and alter gene expression accordingly, P transport into the seed remains unchanged. This possibly indicates that there might be biological impediments to reducing seed total P. For example, it is possible that most seed total P results from movement down a concentration gradient from maternal to seed tissues, and across a poorly-selective poor into the seed (van Dongen et al., 2001). As a result, mutations that perturb high-affinity, energy-requiring P-transporters and the signalling machinery that regulates them might have relatively little effect on seed P levels.

5.2 Forward Genetic Screens

Forward genetic screens are important because of the large number of known functions for plant and seed P (Bucher, 2007), and an equally large number of unknown functions, many of which will be represented by multi-copy gene families. Thus it is probably impossible to successfully anticipate which small subset of genes might represent good targets for reverse genetics approaches. A primary goal might be to identify "low seed total P" genotypes, but "high seed total P" genotypes may also prove of practical value. Two types of high-throughput forward genetic screens designed to identify mutations that alter seed total P concentration are illustrated in Fig. 7; those that use assays for seed inorganic P (Fig. 7, Screen 1) and those that use assays for seed total P (Fig. 7, Screen 2).

For Screen 1, populations obtained following the chemical mutagenesis of *lpa* genotypes can be screened for mutations that alter the "High Inorganic P" (HIP) phenotype associated with a given *lpa* mutation. The rationale is that a substantial amount of the P in *lpa* seed has been converted to inorganic P, which allows for a high-throughput screen for mutations that alter that phenotype. One subset would be mutations that decrease HIP as a result of decreased seed total P (Fig. 7, 1A). A second subset would be those mutations that increase HIP by increasing seed total P (Fig. 7, 1B). A third subset of mutations (Fig. 7, 1C) would be those that have increased HIP but unaltered seed total P, due to second-site mutations that further impact phytic acid synthesis, such as an *lpa*2 or *lpa*3 mutation in an *lpa*1 genetic background.

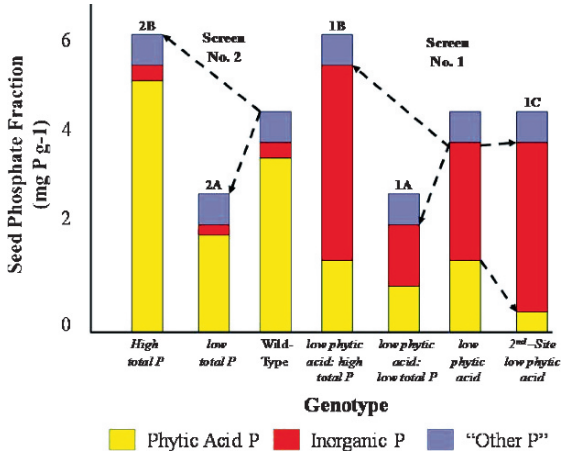

Fig. 7 Two types of screens for mutations that impact seed total phosphorus. In Screen No. 1, single seed of *low phytic acid* genotypes are screened for mutations that alter the "High Inorganic P" phenotype. In Screen No. 2, single seed of wild-type genotypes are screened directly for alterations in seed total P

Screen 2 can be used with any population of induced mutations, and would screen for mutations that alter seed total P by directly testing individual seed for total P. Individual seed would be weighed, crushed, placed in a digestion tube (resistant to acid and heat), wet-ashed and assayed for P, which would represent total P. I have set up a wet-ashing hot plate apparatus designed to provide sufficient through-put of single-seed total P assays. Again, one subset of mutations might result in reduced seed total P (Fig. 7, 2A) and a second subset would be mutations that increase seed total P (Fig. 7, 2B).

Putative "seed total P" mutations would be evaluated to determine which if any have little effect on plant P, and therefore are specific to seed total P. Since functions important to seed total P may in theory be active in either maternal or filial tissues, seed produced by both M_2 and M_3 plants must be screened.

6 Summary

Both the chemistry of seed P and the total amount of seed P represent traits important to maize end-use quality. There has been substantial progress in the genetics and biochemistry of seed P chemistry, and this progress continues. This work has contributed to enhanced knowledge of the biology of phytic acid ($InsP_6$) metabolism during seed development. One area of possible importance for which there has been little progress in maize or other plant systems concerns the biology of Ins pyrophosphates. Progress in the biology of seed P chemistry has provided a number of targets and resources for breeding or engineering the chemistry of seed P. In

contrast, there has been very little progress in the biology of seed total P. There are few genes or functions presently known to play a role specific to the transport of P from the parent plant to the developing seed. Therefore future work addressing seed P might focus on this later area.

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Seed Starch Synthesis

Martha James and Alan Myers

1 Introduction

Maize plants accumulate starch as a carbohydrate energy reserve in the seed endosperm. Starch is composed of two homopolymers of α-D-glucose, amylose (Am) and amylopectin (Ap), which are organized to form water insoluble semi-crystalline granules. Am comprises ~25% of starch, and is essentially a linear molecule in which glucosyl monomers are joined via α-(1→4) linkages to form long chains. Ap, the more abundant polymer that comprises ~75% of starch, contains a polymodal distribution of linear chains of various length that are joined via α-(1→6) linkages at a frequency of ~5% to form branches.

The molecular mechanisms responsible for the deposition of starch and formation of granules are complex and not well understood, and little is known about starch granule initiation, which takes place at the center of the granule in a relatively unorganized region termed the hilum. Starch granules are believed to grow by apposition, with successive layers of Ap transitioning from an unordered to an ordered state that results in crystallization of the polymer at the granule surface (Myers et al., 2000).

Much of the current understanding of the enzymatic steps required for starch synthesis in all plants has been acquired using maize as a model organism (Preiss, 1991; Nelson and Pan, 1995; Hannah, 1997, 2005). Synthesis begins with the formation of an activated glucosyl donor, ADP-glucose (ADPG), in a reaction catalyzed by ADPG pyrophosphorylase. The enzymatic reactions that utilize ADPG to build α-(1→4)-linked "linear" chains are catalyzed by the starch synthases (SSs). Branch linkages are introduced by branching enzymes (BEs) that catalyze cleavage of an internal α-(1→4) linkage and transfer the released reducing end to a C6 hydroxyl, creating a new α-(1→6) linkage. Presumably, SSs and BEs act non-randomly to produce the clustered arrangement of chains characteristic of Ap. Involvement of debranching enzymes (DBEs), which hydrolyze branch linkages, is indicated from the fact that DBE mutants produce a more highly branched, water-soluble polysaccharide at the expense of Ap (Morris and Morris, 1939). Multiple SS, BE, and DBE classes exist in all plant species, and their evolutionary conservation strongly suggests that each class functions uniquely in starch metabolism.

J.L. Bennetzen and S.C. Hake (eds.), *Handbook of Maize: Its Biology,*
DOI: 10.1007/978-0-387-79418-1_22,

2 Production of ADP-Glucose, the Activated Glucosyl Donor

Maize storage starch synthesis occurs following the transport of sucrose from the leaf to the developing seed. The pathway to endosperm starch synthesis is believed to begin with the conversion of sucrose to fructose and UDP-glucose, catalyzed by the enzyme sucrose synthase (SuSy) (Chourey and Nelson, 1976; Asano et al., 2002; Emes et al., 2003; Koch, 2004). The SuSy reaction products then are converted in the cytosol to hexose phosphates and to ADP-glucose (ADPG) for subsequent transport into the amyloplast, the sub-cellular compartment in which starch is produced. More complete descriptions of this complex process are available in a number of review articles (Winter and Huber, 2000; Koch, 2004; Spielbauer et al., 2006).

2.1 First Committed Step in Starch Biosynthesis

ADPG pyrophosphorylase (AGP) catalyzes the first committed step in starch biosynthesis, producing the activated glucosyl donor ADPG and pyrophosphate from glucose-1-phosphate and ATP. AGP comprises two large subunits and two small subunits, encoded by the maize genes *shrunken2* (*sh2*) and *brittle2* (*bt2*), respectively (Hannah and Nelson, 1976; Bae et al., 1990; Bhave et al., 1990). The enzyme is now known to be largely extra-plastidial (i.e., cytosolic) in cereal endosperm, but plastidial in other cereal tissues as well as in all tissues of non-cereal plants (Giroux and Hannah, 1994; Denyer et al., 1996; Thorbjornsen et al., 1996; Beckles et al., 2001). Thus, in the cereal endosperm the existence of an ADPG translocator function is predicted for the uptake of ADPG into the plastid. The identity of such a transporter remains under investigation.

In tissues where AGP is exclusively plastidial, the sucrose to starch pathway involves plastid import of hexose phosphates that potentially can also be used for a number of other metabolic pathways. In the cereals, however, starch biosynthesis has an advantage in that carbon (in the form of ADPG) is directly committed to the starch pathway in the cytosol prior to plastid import. This feature of AGP localization is likely to have functional significance for maize and other seed starch accumulators, allowing the partitioning of large amounts of carbon into starch when sucrose is plentiful.

2.2 Regulation of AGP

AGP is subject to allosteric regulation, with activity of the enzyme regulated positively by 3-phosphoglycerate (3-PGA) and negatively by inorganic phosphate (Pi) (Hannah, 1997). Interestingly, however, cereal endosperm AGP appears to be less sensitive to these effectors than AGP from other tissues (Gomez-Casati and

Iglesias, 2002). Heat lability of small and large subunit interactions within AGP also provides for indirect regulation of the endosperm enzyme (Greene and Hannah, 1998), and reduction/oxidation shifts have been proposed to regulate AGP activity, as well. From an agricultural standpoint, manipulation of AGP regulatory mechanisms could be important, particularly as applied to yield improvement. As one example, mutations in the maize AGP genes that both stabilize subunit interactions and reduce Pi inhibition were shown to result in the production of a more stable and enzymatically active AGP enzyme in transgenic wheat, correlating with increased biomass (Smidansky et al., 2002; Smidansky et al., 2003).

3 Starch Structural Organization

The amylopectin (Ap) component of starch is responsible for starch granule crystallinity. This is because the Ap polymer has a high degree of architectural organization, exemplified by the non-random distribution of linear chains and the clustered positioning of the branch points (Fig. 1). Within Ap, regions of high branch frequency alternate with regions devoid of branches, enabling intervening linear chains to align in parallel arrays of double helices. This conserved "cluster structure" is

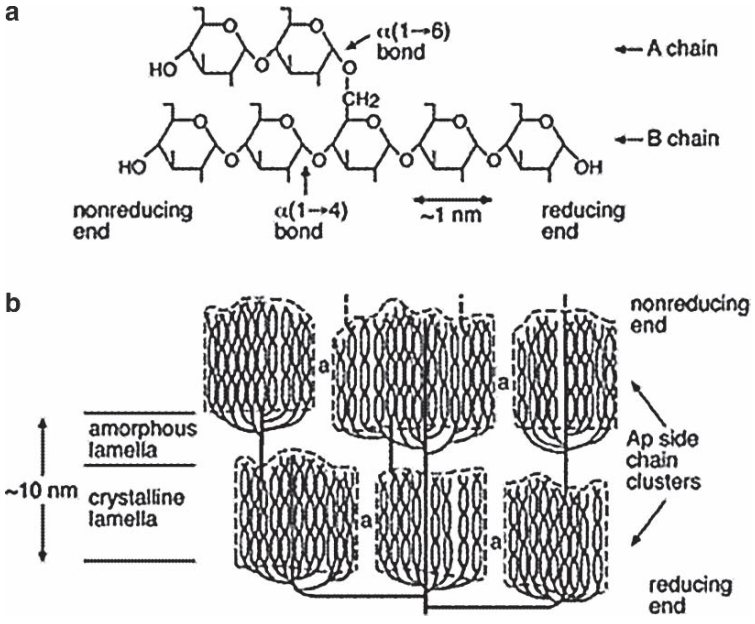

Fig. 1 Diagrammatic representations of Ap structure. **(a)** The connections of glucosyl units by a-1,4 and a-1,6 lingages. **(b)** Cluster model of Ap structure. Solid lines represent glucan chains and dashed lines represent the boundaries of Ap side-chain clusters, in which adjacent linear chains associate as double helices. Modified from Myers et al. (2000)

responsible for the semi-crystalline nature of starch granules and allows for the dense packaging of large amounts of glucose (Robin et al., 1974; French, 1984; Kainuma, 1988; Imberty et al., 1991). A higher-order organization within Ap gives rise to two types of crystalline structure, A-type and B-type, which differ with respect to the symmetry and packing of short Ap chains (Imberty et al., 1988; Imberty et al., 1991). A distinguishing feature of cereal starches is that they are 100% A-type, with double helices arranged so as to permit a minimal amount of bound water.

4 Synthesis of Amylose

Starch synthases (SS) utilize ADPG to elongate linear chains by catalyzing the formation of new α-(1→4) linkages. Since Leloir's pioneering work in the discovery of nucleotide sugar donors (Recondo and Leloir, 1961), the prevailing view in the field has been that glucose units are added at the non-reducing end of a linear chain. Recent studies using starch granules as a source of SS has challenged this view, suggesting instead that glucose units are added at the reducing end (Mukerjea et al., 2002; Mukerjea and Robyt, 2005). Further analysis using purified enzyme sources will be necessary to resolve this mechanistic aspect of SS function.

The maize endosperm contains multiple SS isoforms defined by conserved sequence relationships (Cao et al., 2000). Knowledge that one particular SS, granule-bound starch synthase I (GBSSI), is responsible for production of the long linear chains in the Am component of starch comes from analyses of mutations at the *waxy* (*wx*) locus in maize, which encodes GBSSI (Shure et al., 1983; Wessler and Varagona, 1985; Klösgen et al., 1986). The *wx* mutants of maize typically produce normal amounts of storage starch that lack Am and are composed solely of Ap. This phenotype occurs in GBSSI mutants in many other plant species, including rice, wheat, barley, pea, potato, and *Chlamydomonas* (Ball et al., 1998), indicating that GBSSI is universally required for synthesis of Am polymers. Furthermore, the fact that neither starch quantity nor granule morphology are compromised in *wx* mutants suggests that the inclusion of Am is not required for normal starch granule formation. Although the reason for the conservation of Am in starch remains unclear and a subject of investigation, some experimental evidence suggests that a function of Am may be to aid in the formation of the semicrystalline structure of Ap (Maddelein et al., 1994; Buleon et al., 1997; Fulton et al., 2002; Wattebled et al., 2002).

5 Synthesis of Amylopectin

5.1 Elongation of Linear Ap Chains by Starch Synthases

The starch synthase (SS) enzymes utilize ADPG as the glucosyl donor to elongate linear chains in starch, catalyzing the introduction of α-1,4 linkages at the non-reducing end of the chain. Five distinct classes of SS are known for all plants:

granule-bound SS (GBSS), SSI, SSII, SSIII, and SSIV. Genome and EST sequencing projects show that multiple isoforms (i.e., paralogs) can exist within a single SS class in cereal species, as indicated for GBSS, SSII, SSIII, and SSIV in maize and rice. In all plants, the SSs are highly similar in the C-terminal region, a span of ~450 amino acid residues comprising the regions necessary for catalysis. The SSs differ significantly, however, in their N-termini, which are unique within each class (Fig. 2). The SSIII polypeptide has the longest N-terminal arm by far, consisting of 1,228 of the total 1,764 amino acids.

The SSs other than GBSSI are believed to participate in elongation of the linear chains of the Ap component of starch. Genetic analyses have shown that at least three of the SS classes (SSI, SSII, and SSIII) provide unique functions in starch biosynthesis. Mutations in any of these genes cause specific alterations in Ap structure, indicating that the function of each of isoform is not fully compensated by any other SS. Examples from maize of SSII and SSIII function are provided by mutations at the *dull1* (*du1*) locus, which codes for SSIII (Gao et al., 1998; Cao et al., 1999), and the *sugary2* (*su2*) locus, which codes for SSIIa (Zhang et al., 2004). No forward mutation in maize has been identified in the gene encoding SSI; however, mutations in the rice and Arabidopsis orthologs are described (Delvalle et al., 2005; Fujita et al., 2006) and the effects can be extrapolated to maize. Further relevant information comes from analysis of double mutants in Arabidopsis lacking both SSII and SSIII, which shows there is significant overlap in the catalytic capacities of those two classes (Zhang et al., 2008). Little is known about the SSIV enzyme class in cereal plants, although recent mutant analysis of SSIV in Arabidopsis shows decreased numbers of starch granules, suggestive of a possible role for this isoform in priming starch granule formation (Roldan et al., 2007).

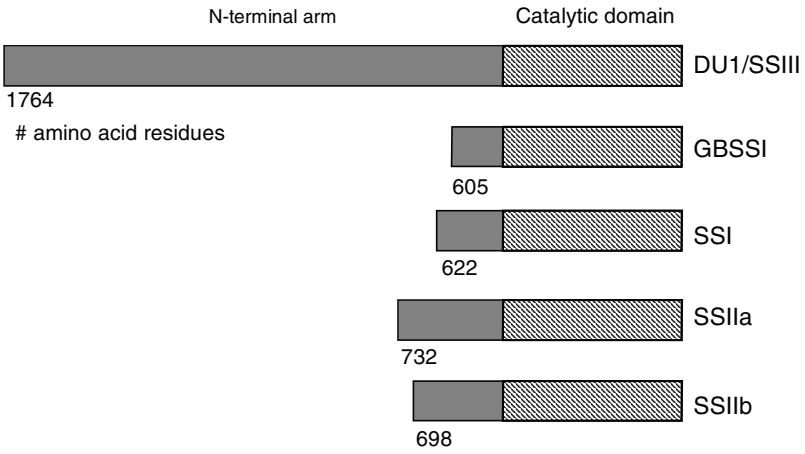

Fig. 2 Schematic diagram aligning the maize starch synthase isoforms from the C-terminal end. Catalytic and starch-binding domains are indicated by hatched lines, and N-terminal extensions unique to each isoform are in solid gray

A model to account for the roles of the different SSs in determining lengths of various Ap chains proposes that SSI is primarily responsible for synthesis of the shortest chains, SSII lengthens these to provide most of the chains that crystallize, and SSIII produces longer length chains extending between clusters (Fig. 3). A key feature of this model is the hypothesis that each SS is a distributive enzyme that dissociates from its substrate after each catalytic cycle. Thus, SSI, SSII, and SSIII could compete with each other for binding to any free end. Experimental evidence is consistent with this model. Loss of SSI in rice endosperm results in reduced numbers of short chains comprising 8–12 glucose units, suggesting chains of these lengths are produced by the action of SSI (Fujita et al., 2006). Furthermore, *in vitro* analysis of native and recombinant SSI shows the affinity of the enzyme is highest for short chain polymers (Commuri and Keeling, 2001). Intermediate length chains are affected by the loss of SSIIa, the endosperm specific form of the SSII class, which results in an increased proportion of the shortest chains in Ap and reduction of intermediate length Ap chains of ~12–25 glucose units that are likely to form the double helices within each Ap cluster (Zhang et al., 2004). The very longest length chains within Ap are most affected by the loss of SSIII catalytic function, which results in slightly elevated numbers of intermediate length chains and fewer chains longer than 30 glucose units (James and Myers, unpublished). Data from rice regarding the functions of SSII and SSIII (Umemoto et al., 2002; Hirose and Terao, 2004; Nakamura et al., 2005; Fujita et al., 2007) are supportive of the maize findings and the SS chain elongation model (Fig. 3), which predicts significant overlap in individual SS function. Accordingly, the model predicts that for any given chain length there is a probability of that particular chain serving as substrate for a particular SS, although access of each SS to the substrate at any given time or regulation of the activity of the SS is not known.

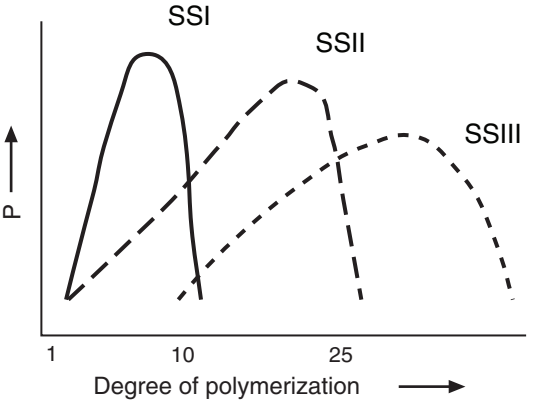

Fig. 3 Diagram of a model describing the probability (P) of SSI, SSII, or SSIII elongating an a-glucosyl chain of a given length in terms of degree of polymerization

5.2 Branch Linkage Introduction and Placement

Starch branching enzymes (BEs) introduce α-(1→6) branch linkages by cleaving an internal α-(1→4) bond within a linear chain and transferring the released reducing end to a C6 hydroxyl. Two classes of BE are known, BEI and BEII, with cereal species having two closely related BEII isoforms, BEIIa and BEIIb (Mizuno et al., 2001; Rahman et al., 2001). BEI and both BEII enzymes are active in maize endosperm, but BEIIb is unique to the reproductive tissues, including endosperm. *In vitro* analyses indicate the BEII class transfers shorter chains than BEI (Guan and Preiss, 1993; Takeda et al., 1993). Mutation of BEIIb increases the amount of Am in starch, hence designation of the maize mutation as "*amylose-extender*" (*ae*) (Vineyard and Bear, 1952). However, this phenotypic change is more likely attributable to greatly elongated Ap chains (i.e., "apparent Am") rather than increased Am *per se*, based on analyses of *ae wx* double mutants in rice that lack the Am starch component (Nishi et al., 2001). Altered Ap structure in *ae* mutants indicates that neither BEIIa nor BEI can compensate for the loss of BEIIb.

The roles of BEI and BEIIa in seed starch synthesis are less clear than that of BEIIb. In maize, wheat, and rice BEIIa expression occurs at early and mid-development of the grain; however, analysis of mutations in the maize gene encoding BEIIa indicates that loss of this isoform does not significantly affect starch composition or Ap fine structure (Blauth et al., 2001). Thus, it is possible that BEIIa does not play a critical role in endosperm Ap synthesis, or that BEIIb provides compensating activity. Analysis of BEI mutants reveals subtle deficiencies in intermediate and long length Ap chains, suggesting the possibility that this isoform has a role in the formation of starch chains of these lengths (Blauth et al., 2002).

6 Potential Functions of Starch Debranching Enzymes

Mutations in genes encoding starch debranching enzymes (DBEs) in maize and other plants point to the idea that selective removal of branch linkages in pre-Ap by DBEs is critical to normal starch synthesis. Two DBE classes exist in plants, termed isoamylase-type and pullulanase-type. Both enzymes cleave α-(1→6) linkages by hydrolysis but differ with respect to their substrate specificities. Mutations at the *sugary1* (*su1*) locus of maize, which encodes the isoamylase-type DBE ISA1, result in the accumulation of sugars and a water-soluble polysaccharide (WSP) termed phytoglycogen, and a major reduction in the amount of starch (Morris and Morris, 1939; James et al., 1995). Pleiotropically, *su1* mutants also condition reduced activity of the pullulanase-type DBE (Pan and Nelson, 1984). These *su1* mutant analyses, together with similar analyses in other plant species (Zeeman et al., 1998; Burton et al., 2002; Hussain et al., 2003; Delatte et al., 2005), indicate that isoamylase-type DBE activity is needed for the normal production of crystalline amylopectin.

The pullulanase-type enzyme in maize, PU1, is the product of the *zpu1* gene. The primary role of this DBE most likely is in starch degradation, but PU1 also provides an overlapping function with ISA1 during seed starch biosynthesis (Dinges et al., 2003). PU1 is an endo-acting enzyme that cleaves only very short branch chains, and is subject to activation by changes in redox status and inhibition in the presence of high sugar concentrations (Wu et al., 2002). Consequently, its role in starch synthesis is believed to differ somewhat from that of the isoamylase-type DBEs. Because sugar accumulation also is an effect of *su1* mutations, this may account for the secondary loss of pullulanase-type activity in *su1* mutants.

Several models could explain DBE function in starch synthesis. The most widely accepted is the glucan-trimming model, which proposes that DBEs directly participate in Ap synthesis, selectively removing branches that are inappropriately positioned for the formation of double helices (Ball et al., 1996; Myers et al., 2000). This implies that DBE activity is required to achieve and maintain the cluster structure of Ap that enables the dense packing of linear chains in water-insoluble granules (Fig. 4). Consistent with this idea is the observation of numerous short chains on the surface of premature starch granules that suggests an intermediary structure (Nielsen et al., 2002), and the fact that loss of ISA1 function in maize by mutation at the *su1* locus results in a structurally altered form of residual Ap with increased numbers of short glucan chains (Dinges et al., 2001). An alternative model proposes that DBEs function to eliminate soluble glucan from the plastid stroma, thereby removing a substrate that competes for BE and SS binding (Zeeman et al., 1998). This explains how DBE deficiency results in WSP accumulation at the expense of Ap, but does not address the question of how Ap structural differences occur. A third proposal for DBE function in seed starch synthesis stems from the observation that mutants lacking ISA1 have increased numbers of small sized granules (Jane et al., 1994; Burton et al., 2002). Consequently, this model suggests an involvement for ISAs in starch granule initiation, catalyzing the hydrolysis of bonds linking nascent glucosyl polymers to predicted initiator molecules. This idea has its basis in the knowledge that *Pseudomonas amyloderamosa* isoamylase releases a malto-oligosaccharide from the mammalian primer molecule glycogenin (Lomako et al., 1992). For plants, an

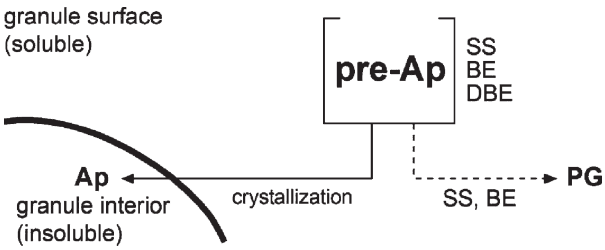

Fig. 4 Diagram of SS, BE, and DBE concerted activity at the surface of a nascent starch granule to achieve a crystallization competent organization of starch chains. Pre-Ap indicates a heterogeneous population of branched glucans that serve as substrates for the indicated enzymes. PG refers to the more highly branched glucan phytoglycogen that accumulates in the plastid stroma in the absence of DBE, according the "glucan trimming model"

analogous type of cleavage event is proposed but the identity of a glycogenin-like primer molecule remains under investigation (Chatterjee et al., 2005).

7 Regulation of Starch Biosynthetic Enzymes

Significant knowledge gaps limit the understanding of how regulation of starch synthesis occurs in seeds or any tissue. Post-translational mechanisms including protein complex formation, protein phosphorylation, and alteration of protein conformation via change in redox status all are implicated as providing regulatory mechanisms for specific starch biosynthetic enzymes. Transcriptional regulation of starch pathway genes also is indicated, particularly via sugar sensing and the resultant activities of transcription factors.

7.1 Protein Interaction and Enzyme Coordination

Regulation of the mechanisms of starch biosynthesis in maize endosperm is likely to involve physical interactions between enzymes. Genetic analyses repeatedly have revealed pleiotropic effects that indicate functional relationships among starch biosynthetic enzymes, and recent evidence from studies in maize and wheat reveal that many of these proteins are capable of forming stable physical interactions *in vitro*. Whether or not any of these specific interactions occur *in vivo*, and whether they have physiological relevance, remains to be determined. However, the genetic and biochemical data are suggestive that direct contacts between SSs, BEs, and DBEs are determinants of starch structure and may in part explain the evolution of this distinct physiological characteristic of plants.

Longstanding genetic studies in maize indicate that starch biosynthetic enzyme activities are coordinated, as opposed to acting independently through stochastic mechanisms. A classical example is the *du1* reference mutation, which was isolated as a second site modifier mutation of a weak *su1* allele, *su1-am* (Cameron, 1947; Mangelsdorf, 1947). The *su1-am* mutation causes a single amino acid substitution in ISA1, which has no phenotypic effect on the kernel or the starch (Dinges et al., 2001). The *du1-R* mutation causes loss of SSIII function and has only a minor effect on starch content and structure. The coupling of *du1-R* with *su1-am*, however, produces a strong synergistic phenotypic effect, suggesting the gene products SSIII and ISA1 function via a concerted mechanism in starch biosynthesis. Biochemical evidence supports this proposed interaction, specifically the observation that *du1* mutants have increased isoamylase activity (James and Myers, unpublished). SSIII has also been implicated in interactions with BEIIa and SSI. Mutation of *du1* affects the enzymatic activity of both BEIIa and SSI, in the former instance reducing activity and in the latter causing an increase in enzyme activity in cell extracts (Boyer and Preiss, 1981; Singletary et al., 1997; Cao et al., 2000).

The idea that these functional interactions have their basis in protein-protein interactions is supported by co-immunoprecipitation, co-affinity chromatography, and yeast two-hybrid data. Evidence that starch biosynthetic enzymes are capable of forming stable complexes was first obtained in studies of developing wheat endosperm, which showed BEIIb, BEI, and starch phosphorylase associate in a complex that bound antibodies against BEIIb (Tetlow et al., 2004). Assembly of this structure depends on phosphorylation at one or more serine residues in the complex. Recent work in both wheat and maize endosperm has extended these observations. In wheat, complexes have been detected that contain SSI, SSIIa, and either BEIIa or BEIIb (Tetlow et al., 2008). In maize, complexes are indicated that contain the following pairs of enzymes: SSI/SSII, SSI/SSIII, SSI/BEI, SSI/BEIIa, SSI/BEIIb, SSII/BEIIa, SSII/BEIIb, SSIII/BEIIa, SSIII/ISA1, and BEIIb/ISA1 (Hennen-Bierwagen et al., 2008). The evidence for SSIII involvement in so many assembly states with such a range of starch biosynthetic enzymes suggests the possibility that this particular polypeptide functions as a scaffold protein to provide coordination, substrate channeling, or regulation to the pathway in general (Fig. 5).

Binding of a trans-acting regulatory factor, a 14-3-3 protein, also is indicated for SSIII. 14-3-3 proteins constitute a family of regulatory factors present in all eukaryotic cells that modulate the activity of the target protein via phosphorylation-dependent, peptide-specific binding (Ferl, 2004). Arabidopsis plants with reduced levels of 14-3-3 proteins exhibit excess starch accumulation in leaves, which is postulated to result from decreased inhibition of SSIII activity in response to reduced 14-3-3 binding (Sehnke et al., 2001). Like Arabidopsis SSIII, maize SSIII has a consensus 14-3-3 binding sequence (RYGTIP) in the C terminus (Gao et al., 1998). Recent proteomic analysis of barley endosperm proteins indicates 14-3-3 binding to other starch biosynthetic enzymes as well, including BEII, SSI, and SSII (Alexander and Morris, 2006). Thus, 14-3-3 binding to starch pathway proteins is likely to provide at least some degree of regulation to the process of storage starch synthesis in seeds.

Regulation of the maize starch debranching enzymes (DBE) by protein-protein interaction also is likely. Maize and rice endosperm contain both a homomeric

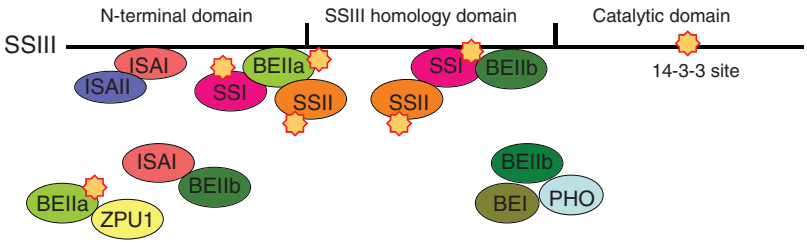

Fig. 5 Hypothetical model for protein complex assembly on an SSIII scaffold. Small octagonal shapes indicate putative 14-3-3 binding sites on specific proteins. Protein complex predictions are based on functional genetic interactions, yeast two-hybrid in vivo interaction data, affinity chromatography co-purification of the proteins, and/or co-immunoprecipitation of the proteins

enzyme comprising ISA1 and a heteromeric enzyme comprising ISA1 and ISA2 (Utsumi and Nakamura, 2006). In maize, three forms of ISA-type DBE purified from the endosperm all require *su1* gene expression and two also require *isa2* gene expression. These different assembly states have distinct enzymatic properties and are proposed to serve specific roles in starch biosynthesis as the endosperm tissue develops; however, the ISA1 homomeric complex is required for normal starch synthesis (as indicated by the severity of the effect of *su1* mutations) whereas the ISA1/ISA2 complex is not (as indicated by the lack of effect of *isa2* mutation). This is a different situation than that reported for dicot plants, in which only a heteromeric complex of ISA1 and ISA2 is known that is rendered completely non-functional by mutation at either the *isa1* or *isa2* locus (Hussain et al., 2003; Delatte et al., 2005).

Further evidence will be required in order to definitively test for *in vivo* function of starch biosynthetic enzyme complexes. Such studies will include *in vivo* crosslinking and/or *in vivo* BRET/FRET analyses to determine whether specific starch biosynthetic enzymes are located in cells within atomic distances from one other. Eventually, genetic analyses of site-specific mutations that prevent complex formation but have no other effects on the expression or activities of the enzymes involved will be necessary to investigate the molecular functions of the complexes that are proposed to exist *in vivo*. Suggested potential functions for the complexes include substrate channelling, and/or mediation of the crystallization of the linear chains of Ap to form crystalline laemellae.

7.2 Protein Modification

Regulation of the starch pathway in seeds by kinases and phosphatases that determine the phosphorylation state of individual proteins is likely, particularly in light of the isolation of a complex of phospho-proteins from wheat endosperm (Tetlow et al., 2004). This study showed that BEI and BEIIb bind each other and a starch phosphorylase at a particular mid-developmental stage in the endosperm, and that formation of this complex requires phosphorylation of one or more of the components. These observations expand the possibilities that must be considered for control of starch biosynthesis, and suggest that protein modification by phosphorylation of one or more residues can play an important role in regulating specific starch biosynthetic enzyme activities and protein interactions.

7.3 Redox Regulation of Enzyme Activity

Redox modulation is known to affect the activities of specific genes in the endosperm starch pathway, suggesting that change in redox status is a potential mechanism for regulating at least some aspects of seed starch synthesis. For example,

activity of the pullulanase-type DBE in maize and a number of other plant species is stimulated by thiol reducing agents. Over-expression of thioredoxin *h*, a small cytoplasmic protein with a redox-active disulfide bridge, results in a two- to four-fold activation of the PU1 enzyme in maize and barley grains (Cho et al., 1999; Wu et al., 2002). The relevance of this activation of PU1 with respect to starch synthesis, however, is not known. AGP activity also is increased by thiol reducing agents in response to sugars in both potato tubers and Arabidopsis leaves (Tiessen et al., 2002; Hendriks et al., 2003), although it is not clear whether this type or regulation affects maize seed AGP, given that the maize enzyme lacks the requisite cysteine residue that responds to redox signaling in the dicot plants (Hannah, 2005).

7.4 Transcriptional Regulation

Sugar availability and sugar signaling regulate the transcription of many of the starch biosynthesis genes. Sucrose in particular is well known to be a general inducer of storage starch biosynthesis (Wobus and Weber, 1999). For example, high levels of sugars enhance the expression of the maize genes *sh2* and *bt2* that encode the two AGP subunits (Giroux et al., 1994), and comparable regulation of AGP subunit genes is reported for potato, tomato, and rice (Muller-Rober et al., 1990; Li et al., 2002; Akihiro et al., 2005). In addition, the accumulation of sucrose as a result of *sh2* and *bt2* mutations has the effect of increasing the transcript abundance other sugar inducible genes, including *wx*, which codes for GBSSI (Giroux et al., 1994). As the starch branching enzyme gene expression patterns are temporally coordinated with *sh2*, *bt2*, and *wx* gene expression patterns in the maize endosperm, it is not surprising that the BE genes are up regulated by sucrose, as well. Notably, the maize *sbe1* and the barley *sbeIIb* gene promoters contain sucrose response elements (SURE), and transcription of both genes increases in response to increasing sugar levels in the endosperm (Kim and Guiltinan, 1999; Mutisya et al., 2006). SURE elements also are found in the barley ISA1 gene promoter and the gene is sugar inducible; consequently, the prediction is made that the maize ortholog would be regulated in the same manner given strong sequence similarities in these *isa1* promoters (Sun et al., 1999, 2003). Although the sugar responsiveness of other DBE genes and SS genes is not known for maize or other plants, investigations into of this mode of transcriptional regulation are likely to increase in the near future in attempts to elucidate the global nature of the regulation of seed starch synthesis by sugar availability.

Regulation of the storage starch synthesis genes by transcription factor (TF) binding is only beginning to be understood, largely through research into barley endosperm gene regulation. A novel WRKY transcription factor termed SUSIBA2 was identified in barley that binds W-box and SURE *cis* elements in the barley *isa1* gene promoter, inducing *isa1* transcription (Sun et al., 2003). Expression of the SUSIBA2 gene itself is induced by sucrose elevation, suggesting a role for this TF in the transduction of sugar signals in barley endosperm starch biosynthesis. SUSIBA2 orthologs have been identified in rice, wheat, potato, and barley, and most

likely exist in maize as well. Given that so little is known about transcriptional regulation of the starch pathway by TF binding, this field is predicted to be an exciting avenue for research efforts in the future.

8 Future Directions

Much remains to be understood about the molecular mechanisms that determine the architecture of starch and its ability to crystallize. In the future, a range of comprehensive genetic, biochemical, and genomic tools will be applied to investigate how SSs, BEs, DBEs, and possibly other enzymes function as biosynthetic system to produce starch.

From an applied perspective, starch produced in cereal seeds is an important agricultural commodity that provides the major carbohydrate component of most human and animal diets and serves a variety of industrial and manufacturing needs. Increasingly, starch is used as a renewable chemical feedstock for conversion products such as high fructose corn syrup, polymer-based fibers, and fuel ethanol. Most of these diverse applications are typically met using starch extracted from yellow dent corn that is subsequently modified by chemical and/or thermal treatment. For the future, breeding and engineering strategies are predicted that will be designed toward the goal of producing starches with properties tailored to specific fuel, food, or fiber applications. Manipulation of starch quality and starch quantity will be possible once key molecular regulators of the pathway are known.

Specific targets for starch modification are predicted to include: (1) improved yield; (2) specific alterations of Ap:Am ratios; (3) changes in Ap structure that translate into altered starch granule crystallinity; and (4) the incorporation of proteins or other molecules into starch granules. Some of the anticipated novel starch forms will be utilized for the production of functional foods that improve human or animal health and nutrition. Other starches may be applicable to the production of renewable biofuels that will enhance our energy security, and it is expected that some will be useful for the production of environmentally beneficial bio-based products that can be degraded over time. Ultimately, however, such applied research will require a clear understanding of the functional relationships among specific starch biosynthetic enzymes and how the starch pathway is regulated. For this reason, basic research efforts in the fields of starch metabolism and starch synthesis are likely to continue and to expand.

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Heterosis

Patrick S. Schnable and Ruth A. Swanson-Wagner

Abstract Although heterosis has been exploited commercially for close to a century, the molecular mechanisms underlying hybrid vigor are not well understood. Multiple models, including dominance and complementation, have been proposed. Historically, quantitative genetic approaches have been utilized to understand the regulation of heterosis as it affects traits such as yield. Recent technological advances have allowed for global studies of gene expression in inbreds and hybrids. These studies are consistent with multiple molecular mechanisms contributing to heterosis. An enhanced understanding of the regulation of cellular processes and their relationships to heterotic phenotypes are expected to enhance our ability to predict the performance of specific hybrids and hence to increase the rate of genetic gain.

1 History

Heterosis (also referred to as hybrid vigor) is the phenomenon in which the progeny of diverse inbred lines exhibit improved performance as compared to their inbred parents (Fig. 1). Although exploited by plant breeders and the seed industry for nearly a century, the molecular mechanisms underlying heterosis remain unexplained. Due to its biological and economic importance, several recent reviews have been published (Birchler et al., 2003, 2006; Troyer, 2006; Hochholdinger and Hoecker, 2007; Springer and Stupar, 2007b).

The effects of out-crossing versus selfing were first described by Darwin. For several traits, he observed that cross-pollinated plants of multiple species (including maize) were consistently superior to self-fertilized plants grown in the same environment (Darwin, 1876). At the turn of the last century, George Harrison Shull (Cold Spring Harbor Laboratory) and Edward M. East (Connecticut Experiment Station) were independently conducting experiments with self- and cross-fertilized maize plants. While studying quantitative inheritance of kernel row number, Shull noted that progeny of cross-fertilizations were more vigorous than the progeny of self-fertilized plants from the same source (Shull, 1908). Based on these and subsequent observations, Shull outlined a plan to use hybrids for agricultural production (Shull, 1909).

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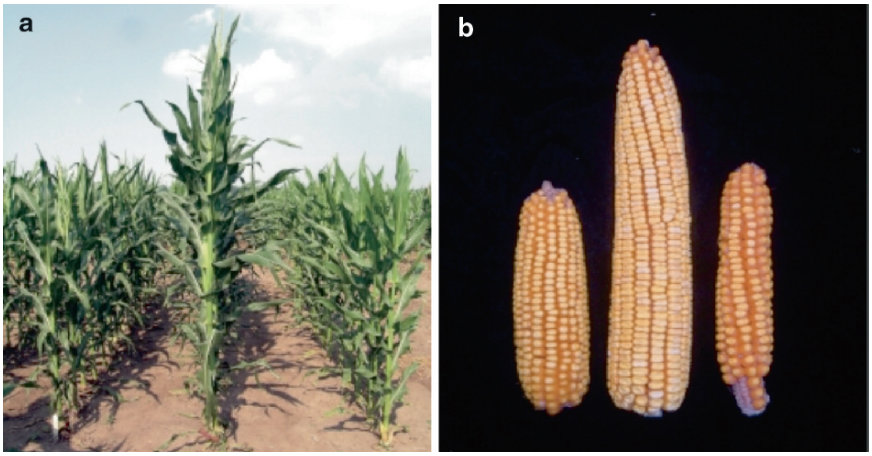

Fig. 1 **a** and **b** The hybrid (center) generated from inbred lines B73 (left) and Mo17 (right) exhibits heterosis. Photos by Jun Cao and Ruth Swanson-Wagner, Schnable Lab, Iowa State University

East also observed evidence of inbreeding depression. Based on Shull's reports, East applied Mendel's recently rediscovered report to the question of heterosis and hypothesized that homozygosity in the inbreds was enhancing deleterious effects (East, 1908, 1909). Shull and East are therefore often jointly credited for their contributions to the development of hybrid corn in plant breeding programs.

2 Inbred Lines, Hybrids, and Heterotic Groups

The first inbred lines were extracted from landraces such as Reid's Yellow Dent and Lancaster Sure Crop. These early inbreds were extremely weak. Consequently, their per se seed yields were too low to economically produce single cross hybrids. The utilization of double-cross hybrids overcame this challenge (Jones, 1918). Two pairs of inbred lines were crossed to generate a pair of vigorous hybrids (Inbred A × Inbred B and Inbred C × Inbred D). Because the AxB hybrid was vigorous, abundant quantities of [A × B] × [C × D] double-cross hybrid seed could be produced and marketed to farmers.

First generation inbreds were intercrossed to produce populations from which second generation inbreds were extracted. For example, the Iowa Stiff Stalk Synthetic (BSSS) population was developed by G.F. Sprague in 1933–1934 by intercrossing 16 inbred lines selected for resistance to stalk lodging, a major problem with landraces and early hybrids (Sprague and Jenkins, 1943). The resulting population had better than average performance for stalk quality and combining abilities relative to other populations (Hallauer and Malithano, 1976). It has since been subjected to more than 15 cycles of recurrent selection (Hinze et al., 2005) and has been the source of multiple important inbred lines, including B73 (Russell, 1972).

Because the molecular mechanisms responsible for heterosis are not understood, identifying specific pairs of inbreds whose progeny will exhibit high levels of heterosis remains a major challenge for plant breeders. It has been observed that hybrids generated from genetically divergent inbred parents typically exhibit more heterosis than do hybrids generated from genetically similar inbreds (Hallauer and Miranda Fo, 1981). Based on this observation, maize lines have been classified into heterotic groups. Progeny of crosses between heterotic groups typically exhibit more heterosis than do those within heterotic groups. Several heterotic groups have been described, viz., Reid Yellow Dent, Lancaster Sure Crop, European flints, and Minnesota 13 (Dubreuil and Charcosset, 1999; Troyer, 2006). Recently, another heterotic group has been defined among Chinese germplasm (Lu et al., 2002).

3 Gain from the Use of Hybrids

Less than one percent of corn acreage in the U.S. Corn Belt was planted to hybrid corn in the 1920s, when hybrids were first introduced. The first hybrid yields were ~15% higher than the open pollinated landraces they replaced (Duvick, 1999). As the per se performance of inbreds increased in the 1930s it was possible to economically produce single-cross hybrids. Compared to the double-cross hybrids, single-cross hybrid progeny exhibited more heterosis, in part because it is easier to identify two inbreds that interact well in hybrid combination than it is to identify four such inbreds. From the 1940s through the 1990s, maize yields increased to over 7 tons per hectare (Duvick, 1999) and since 1965, essentially all corn acreage in the Corn Belt has been planted to hybrids (Wallace and Brown, 1988). In the U.S. during 2004, nearly 12 billion bushels of seed-corn (with a value of $4.5 billion) were sold (Troyer, 2006). Results of several studies, compiled by Duvick, estimate that the genetic component for yield increase in the U.S. Corn Belt ranges from 33% to 89%, averaging 55% across all studies (Duvick, 1999). The remaining 45% of gain is due to improved management procedures such as fertilizer use and higher planting density (Duvick, 1999; Troyer, 2006). Since early testing for adaptedness to higher density planting (Mooers, 1910, 1920), typical planting densities have increased from ~15,000 plants/ha (in 1910) to nearly 70,000 plants/ha in 2005 (Troyer, 2006).

4 The Mechanisms Responsible for Heterosis

4.1 Proposed Models

Multiple models have been proposed to explain heterosis; the two most commonly accepted are discussed here. The complementation (dominance) model states that recessive, deleterious alleles present in the inbred parents are complemented in the progeny. According to this model, the additive effects of beneficial alleles result in

the superior phenotype of the hybrid. First presented in 1910, this model states that for heterosis to occur, the parent lines must differ in gene frequency, and dominance must be present (Bruce, 1910).

The other most widely known model for heterosis is overdominance, which states that heterozygosity per se is responsible for heterosis (Shull, 1908; East, 1936). According to this model, novel interactions between differing parental alleles result in phenotypes superior to either homozygous allele, as exemplified by the maize *pl* locus which encodes a transcriptional regulator of pigment biosynthesis. Particular alleles of this gene exhibit overdominance in the heterozygous state (Hollick and Chandler, 1998). Theoretically, if the complementation model were correct, an inbred line homozygous for beneficial alleles at all genomic loci could be generated that would perform as well as a hybrid. In contrast, according to the overdominance model heterozygosity per se is advantageous.

4.2 Quantitative and Molecular Approaches for Understanding Heterosis

4.2.1 Modes of Gene Action

Quantitatively, allele effects and gene action are described as: additive (each parental allele contributes a specific amount and the heterozygote value is equal to the sum of the individual effects from each allele), dominant (heterozygote value is equal to one of the two parental alleles), or over- or under-dominant (heterozygote value is more extreme (greater or lesser) than the sum of the individual allele effects). Gene or allele action estimates are used to identify particular alleles in a population that have significant or large effects on a trait and provide clues as to the mechanisms regulating the trait. The hybrid phenotype relative to the parental phenotype is quantitatively estimated using the d/a ratio. This ratio compares dominant (hybrid value-inbred average) and additive (each inbred value-average of inbreds) gene action. If the hybrid is equal to the average of the inbred parents, $d/a = 0$; if the hybrid is identical to one parent, $d/a = -1$ or 1.

4.2.2 Quantitative Trait Analyses

Measurable traits, such as kernel-row-number investigated by Shull, are considered quantitative traits and can be studied via Quantitative Trait Loci (QTL) mapping. With this approach, genomic regions regulating a measurable trait are identified in populations segregating for the trait. Traits subject to heterosis, including grain yield, plant height, biomass, and ear size, have all been investigated using QTL mapping (Stuber et al., 1992; Frascaroli et al., 2007). Historically, heterosis has been studied using quantitative genetic approaches, as evidence has suggested that heterosis is polygenic in nature (Kusterer et al., 2007). QTL mapping studies have been conducted to identify

regions of the genome that regulate heterotic phenotypes (Stuber et al., 1992; Lu et al., 2003; Zhang et al., 2006). Stuber et al. examined QTL effects for traits including yield, plant height, and leaf area. QTLs were identified, the majority of which were associated with overdominance (Stuber et al., 1992). The measured traits, however, are typically quite distant from the functional processes responsible for those phenotypes (Schadt et al., 2003), implying that intermediate steps between genotype and phenotype must be investigated to fully understand modes of gene action.

4.2.3 Complementation Model Does Not Explain Dosage Effects

A variety of observations suggest that heterosis cannot be due to simple complementation. Autotetraploid hybrid progeny derived from two parent lines exhibit heterosis, but the effect is increased in hybrid progeny derived from four parent lines, where four different alleles are present at a given locus. As the number of diverse alleles or genomes represented increases, so does hybrid vigor (Levings et al., 1967). In addition, selfing maize tetraploids results in inbreeding depression at rates as fast as in diploids (Randolph, 1942). Given the increased number of generations of selfing required to achieve homozygosity in tetraploids relative to diploids, this result is not compatible with complementation being the sole explanation for heterosis (Birchler et al., 2003). It has been hypothesized that regulatory genes and pathways underlying heterosis are dosage-dependent and that heterozygosity and ploidy level directly affect major regulatory pathways, resulting in heterosis (Birchler et al., 2003).

To further investigate this possibility, Auger et al. studied the accumulation of mRNA transcripts in eight maize genotypes; inbred lines B73 and Mo17, reciprocal hybrids, and triploid hybrids with unequal contribution of parental genomes (Auger et al., 2005). RNA samples were isolated from leaf tissue and the accumulation of transcripts from 30 genes was analyzed via RNA gel blots. Deviation of transcript accumulation from additivity (where additive is equal to the mid-parent level) was observed for 19 of the 30 genes investigated.

4.2.4 Global Analyses of Modes of Gene Action

Due to substantial public investments in maize genomics tools it has recently become possible to conduct global investigations of the molecular mechanisms underlying heterosis.

For example, several studies have used microarrays to identify genes with differential expression in hybrids relative to their inbred parents (Stupar and Springer, 2006; Swanson-Wagner et al., 2006). Each of these studies identified both additive and nonadditive patterns of transcript accumulation. In an experiment involving nine biological replications and a cDNA microarray, Swanson-Wagner et al. identified ~1,300 genes (9% of the ~14,400 genes on the array) that were differentially expressed in the inbreds B73 and Mo17, and their hybrid. Approximately 22% of the differentially expressed genes had patterns of expression that could be statistically

distinguished from additivity, including cases of over- and under-dominance (Swanson-Wagner et al., 2006). Stupar and Springer also investigated B73, Mo17, and their hybrids, but used three biological replications and an Affymetrix chip. This study also found ~20% of differentially expressed genes to exhibit nonadditive patterns of gene expression.

Stupar and Springer identified only a few genes with expression patterns outside the range of the parents (over- or under-dominant) and concluded that evidence was supportive of the complementation model. In contrast, Swanson-Wagner et al. concluded that multiple models, including over-dominance, could potentially contribute to heterosis. The differing results from these two studies could be due to a number of experimental differences, including the array platforms (cDNA versus Affymetrix), number of biological replications contributing to statistical power (nine versus three), and methods of statistical analysis. Even so, both studies agree that ~20% of maize genes exhibit modes of gene action that differ from simple additivity.

Several additional studies have reported analyses of gene expression across tissues and development in relation to heterosis. In an analysis of hybrid and inbred embryos, Meyer et al. found that additive gene expression patterns were predominant, but nonadditive patterns, including overdominance, were also observed (Meyer et al., 2007). Uzarowska et al. identified over 400 genes that exhibited significantly different expression patterns in apical meristem tissue across four inbred lines and their hybrids. In contrast to other studies, mostly nonadditive modes of gene action were detected. Over 50% of the significant genes in this study exhibited overdominance, 39% exhibited dominance, and only ~10% had expression levels in the hybrid that were within the range observed in the parent lines (Uzarowska et al., 2007).

Another mRNA profiling technology (GeneCalling) was used to examine transcript accumulation in immature ears (Guo et al., 2006). Sixteen hybrids and the 17 parental inbreds (all hybrids had same female inbred parent) were investigated. In agreement with Swanson-Wagner et al., Stupar and Springer, and Meyer et al., ~80% of the differentially accumulated transcripts exhibited patterns within the additive range. Interestingly, a correlation ($r = 0.89$) was found between the proportion of genes exhibiting additive gene action and heterosis for yield.

In another study, differential display was used to analyze twelve maize inbreds and over 30 hybrids. Genes expressed in both of the parent lines and the hybrid were identified, as well as novel patterns of transcript accumulation in the hybrid lines (Wang et al., 2007).

4.2.5 Global Analyses of Gene Regulation

Genetic variation contributes to differential expression of transcripts among inbreds, and combining diverged inbred genomes in a hybrid is likely to yield novel patterns of gene expression. In addition to allelic single nucleotide polymorphisms (SNPs) and insertions/deletion polymorphisms (IDPs), maize haplotypes exhibit noncolinearity ("Doonerization") including the presence/absence of genic fragments

(Fu and Dooner, 2002) and transposons (Fu and Dooner, 2002; Yao et al., 2002). Such variation in sequence content may explain the results of an analysis of leaf transcripts in ten inbreds and 38 hybrids via cDNA-AFLP analyses that revealed expression in only one parental inbred line, expression in both inbreds but not in the hybrid, and hybrid-specific expression (Tian and Dai, 2003).

Several studies have provided evidence supporting nonadditive gene expression and protein accumulation (reviewed by Springer and Stupar, 2007b), but only recently have studies focused on the mode of regulation producing such differences. Guo et al. used allele-specific RT-PCR to determine transcript accumulation from each parental allele in reciprocal hybrids for 15 maize genes (Guo et al., 2004). Of these, 11 exhibited differential accumulation of parental alleles. Springer and Stupar (2007a) examined 355 genes using allele-specific expression (ASE) assays. Genes with significantly different transcript accumulation were studied further regarding the regulation mechanisms. Gene expression levels can be regulated by *cis*- (regulating locus is the gene itself) or *trans*- (regulating locus is at alternative location to the gene) acting loci. Rather than using the genomic locations of the gene and regulating locus to define *cis*- and *trans*-relationships (as in QTL mapping), relative accumulation of transcripts contributed by each parental allele were used to draw conclusions regarding regulatory modes. Based on their interpretation of ASE assays, Springer and Stupar concluded that 70% of genes with significant allele expression differences are regulated by *cis*-effects. One limitation noted by the authors, however, is that ASE can only be used to investigate genes that contain SNPs (Springer and Stupar, 2007), which could bias the results in that a polymorphism may predispose a gene to exhibit *cis*-regulated differential transcript accumulation.

Expression QTL (eQTL) mapping is expected to yield a more representative analysis of *cis*- and *trans*-regulation of gene expression. An early eQTL study found that 80% of differentially expressed maize genes were *cis*-regulated (Schadt et al., 2003). Recent advances in the maize genetic map as well as the ongoing maize genome sequencing effort have provided an opportunity for more in-depth eQTL analyses. A study comparing homozygous versus heterozygous loci in a set of genotyped (Fu et al., 2006) Intermated B73 and Mo17 (IBM) Recombinant Inbred Lines (RILs) (Lee et al., 2002) and their crosses onto B73 and onto Mo17 revealed a high proportion (60–80%) of *trans*-regulating eQTLs (Swanson-Wagner et al., unpublished).

5 Investigations of Heterosis in Other Plants

Hybrid vigor is observed in many plant species, including rice, wheat, Arabidopsis, and tomato. Analyses from these species are expected to inform our understanding of heterosis in maize. Heterosis in rice was investigated using SAGE in a super hybrid variety and its inbred parents. Multiple tissues and time points were sampled. Gene expression was significantly different in at least one tissue type for ~1,700

genes of ~20,000 tags (mapped to ~20,000 genes). Expression patterns included both additive (25–45%) and nonadditive modes of gene action. In fact, over 500 gene expression patterns were classified as overdominant (Song et al., 2007a). Another rice study found that ~5% (438/8,422) of genes were differentially expressed in the hybrid and parental lines. Midparent heterosis of expression was calculated (F1-mid-parent/mid-parent) to identify significantly nonadditive genes. Nearly 25% (141/438) of the genes exhibited modes of gene action that were significantly different than additivity (Huang et al., 2006).

In wheat, differential display was utilized to examine gene expression in six inbred lines and their 18 hybrids. All combinations of presence/absence (in the inbred parent lines and the hybrid) of transcript accumulation were identified (Xie et al., 2003). Another wheat study identified nearly 50 proteins, involved in a wide variety of cellular processes, differentially accumulate in the roots of hybrid and parental wheat lines (Song et al., 2007b). The majority of differentially accumulated proteins (69%) were present in one parent and the hybrid, but absent in the other parent. The remaining significant proteins exhibited overdominant, underdominant, and high- or low-parent dominant patterns of accumulation. Identification of differentially accumulated proteins demonstrates that the differential transcript accumulation potentially influences protein accumulation differences in inbreds and their hybrids.

A QTL study using over 200 Arabidopsis recombinant inbred lines (RILs) and their crosses onto the parental lines and F_1 identified QTLs for traits including rosette diameter, growth rate, and biomass yield. The authors concluded that dominance and additive allele action, as well as epistatic interactions are important for biomass-related traits (Kusterer et al., 2007).

Introgression lines are able to control for epistatic interactions since the genome is uniform except the segment of interest. Crosses of over 75 tomato introgression lines to a reference inbred were used to detect QTL for 35 traits differentially affected by homozygous versus heterozygous genomic loci. The average number of QTLs detected per reproductive trait (~35/trait) was significantly higher than the average number (~18/trait) for nonreproductive traits (Semel et al., 2006). QTLs were classified based on the mode of regulation (dominance, additivity, and overdominance) and nearly all (21/26) overdominant QTL were associated with reproductive traits.

6 Future Directions

The complexity of heterosis demands multiple approaches for understanding the underlying mechanisms. Fortunately, new technologies are enabling global investigations of variation in genomic sequences, epigenetic states, and the accumulation and behavior of transcripts, proteins, and metabolites across inbreds and their hybrids. The resulting data are expected to stimulate the development of novel hypotheses.

Genetic approaches are also likely to prove fruitful. For example, eQTL mapping of segregating populations can identify chromosomal regions that contain regulatory

elements, regardless of the nature of those elements. Analyses of eQTL that co-segregate with phenotypic QTL associated with heterosis have particular promise. As technological advances make genotyping and profiling experiments easier it will be possible to combine high-resolution QTL experiments and chromosome walking to identify the specific regulatory mechanisms responsible for variation in the accumulation and behavior of transcripts, proteins, and metabolites in hybrids versus their inbred parents. Discoveries of novel regulatory processes via these or other approaches will provide insight into the mechanisms underlying heterosis. Understanding of these mechanisms and their relationships to plant phenotypes are expected to make it possible to predict which inbreds are likely to perform well in hybrid combination, reducing (but not eliminating) the necessity for empirical tests of thousands of hybrids, thereby speeding genetic gain.

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Increasing Yield

James B. Holland

Abstract Maize yields have improved dramatically in the past 80 years, largely due to genetic improvement through plant breeding. Maize yield is a quantitative trait affected by many genes, each with very small effects, and the environment. The inbred – hybrid breeding methods developed in the past 80 years exploit the genetic architecture of yield, including large additive and dominance variances as well as heterosis. This complex genetic architecture presents hindrances to the use of marker-assisted selection, although new approaches may overcome some of these difficulties. Future yield gains may also be achieved through the exploitation of the vast genetic diversity of maize. Unfortunately, improving yields for resource-poor farmers remains difficult.

1 Historical Trends in Maize Yield

Maize yield improvement in the twentieth century represents one of the great success stories of plant breeding and agronomy. Maize grain yield in the United States has increased on average by 0.122 metric tons per hectare per year since 1945 (Fig. 1). This is in sharp contrast to essentially zero gain in national yields from 1900 to 1944. What caused this dramatic increase after the Second World War? One cause of yield increases has been plant breeding. The period from 1937 to 1960 represents the period in which farmers shifted from planting open-pollinated cultivars to double-cross hybrids, and this coincided with the change from flat yield trends to annually increasing yields. Starting from 1960, single-crosses began to predominate, and this also coincides with an increase in the rate of annual gain for yield (Betran et al., 2004; Hallauer et al., 1988). As of 2006, the trend toward higher yield had not abated, testifying to the ability of plant breeders to continually improve maize hybrids.

Worldwide, maize yields increased by an average of 2.6% per year from 1950 to 1980, but the rate of gain slowed to 1.5% per year from 1980 to 1995 (Brown, 1998). Yield gains in some countries have been substantial, although they began much later than in the United States, for example China's maize yields began to take off about 1970 and Brazil's maize yields began increasing sharply around 1990 (Brown, 1998). In sub-Saharan Africa, however, yield gains were below 1% per

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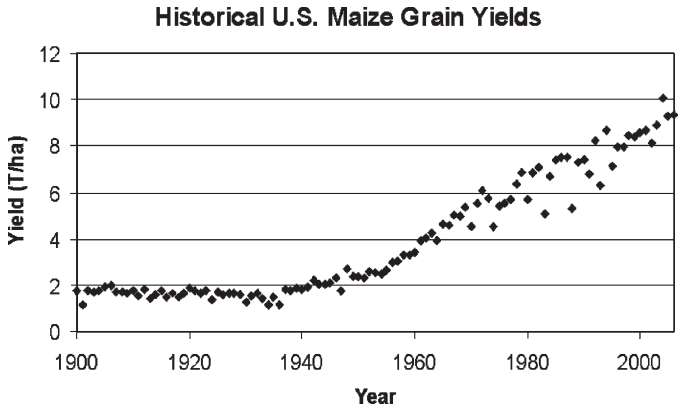

Fig. 1 Average grain yield of US maize (metric tons per hectare) for each year from 1900 to 2006. Data from United States Department of Agriculture–National Agricultural Statistics Service (2007)

year from 1956 to 1995 (Pingali and Heisey, 1999). In some countries, yield gains have been largely restricted to large-scale commercial farms and have occurred only to a limited extent on smallholder farms (Eicher and Kupfuma, 1997), where adoption of hybrids and improved agronomic practices are hindered by lack of resources and other factors (Pixley, 2006).

Genetic improvement is not the sole cause of yield gains, however. Coincident with the introduction of hybrid crops was a change in fertilizer practices, from organic to inorganic sources of fertilizer. In the major corn producing states of the Upper Mississippi River Basin, inorganic fertilizer inputs increased by more than an order of magnitude (0.5–25 kilogram per hectare) between 1949 and 1997 (Burkart and James, 2001). In addition, pesticide inputs for all of US agriculture, increased at a rate of 4.7% per year between 1948 and 1994 (Ahearn et al., 1998). In 1997, the corn crop represented the largest target of pesticide applications in the United States, mostly herbicides, with a smaller proportion of insecticides, and negligible amounts of fungicides (Padgitt et al., 2000).

Because hybrid adoption and genetic improvements due to breeding are confounded with changing production methods, inputs, and climate, experimentation is required to determine how much of the gains in maize yield are due to breeding. Comparison of older and newer varieties under common management practices and environments can be used to estimate rates of genetic gain for the time periods represented by those cultivars. Experiments have been performed using both public (Meghji et al., 1984; Russell, 1984) and private company (Castleberry et al., 1984; Duvick, 1984) Corn Belt cultivars representing time periods from the 1930s to the 1980s. Tollenaar (1989) also tested cultivars from Ontario released between 1959 and 1988. Based on these studies, from 56% to about 100% of the total yield gains in the respective regions could be attributed to genetic improvement. These authors all noted that part of the genetic improvement estimated from varietal comparisons under common growing conditions still cannot be separated from adaptation of more modern cultivars to improved cultural practices. Modern cultivars are

better able to exploit the advantages of modern agronomy. Duvick et al. (2004) extended the time series comparisons from 1930 to 2000 and observed that the trend of genetic improvement for yield continued unabated.

2 The Genetics of Yield

2.1 Quantitative Genetics of Yield

Yield is a quantitative trait, both in the sense that it is measured on a quantitative scale and that it is influenced by many genes and the environment. Maize yield is a model quantitative trait because its genetic basis appears to hew closely to the classical quantitative genetics assumptions of many genes, each with very small effects on the trait (Bulmer, 1985), giving rise to approximately normal distributions in randomly-mated populations (Falconer and Mackay, 1996). Further, the heritability of yield is low (Hallauer and Miranda, 1988), meaning that much of the phenotypic differences observed between individual observations on plants or lines are due more to environmental than genetic differences.

The overall effect of genes on yield can be partitioned into their additive effects, dominance interactions between alleles at a common locus, and epistatic effects due to interactions between non-allelic genes (Falconer and Mackay, 1996; Holland, 2001). Estimating the relative importance of these different genetic effects has been a major concern of quantitative geneticists. Since the genetic constitution of each maize plant or line in a population is unknown, the genetic effects of each locus cannot be estimated separately. Instead, quantitative geneticists devised mating designs that permit the estimation of genetic variances to provide insight to the overall combined effects of all genes affecting a trait and their gene action (Falconer and Mackay 1996; Hallauer and Miranda, 1988). Unfortunately, in most cases, there is no direct relationship between the magnitude of additive, dominant, and epistatic variances and the magnitude of their respective gene actions (Holland, 2001). Nevertheless, the magnitude of additive variance is a key parameter because it represents that proportion of total genetic variance that can be exploited for genetic gain by selection using nearly any breeding method. Thus, the ratio of dominance or epistatic variance to the additive variance indicates the level of difficulty that may be encountered in improving a trait by selection in random-mated populations. Hallauer and Miranda (1988) summarized the results of 99 studies of genetic variances for maize yield and found that, on average, the dominance variance was 61% of the additive variance. This is in contrast to almost all other traits, for which the dominance variance tended to be 25% or lower. Epistatic variance for maize yield appears to be much less important, although experimental and statistical difficulties hinder accurate estimation of epistasis (Hallauer and Miranda, 1988; Hinze and Lamkey, 2003; Holland, 2001; Mihaljevic et al., 2005).

Quantitative trait locus (QTL) mapping studies represent a refinement of classical quantitative genetics studies by permitting estimation of quantitative gene effects at the level of a genome region (typically 10–20 cM). Initially, QTL mapping appeared

to demonstrate genome regions with sizeable effects on maize yield (Austin and Lee, 1996; Stuber et al., 1992; Veldboom and Lee, 1994). In one case, fine-mapping revealed that at least two separable gene regions (with favorable alleles in repulsion phase linkage) underlay the initially identified QTL (Graham et al., 1997). However, simulation studies later suggested that QTL mapping in typical population sizes tends to lead to overestimated QTL effects (Beavis, 1994), therefore new studies were conducted with much larger population sizes to obtain more reliable estimates of maize yield QTL effects. These studies demonstrated the effects predicted by simulation: smaller population sizes led to underestimation of the number of QTL and overestimation of the effects of those QTL that were declared significant in a population sample (Melchinger et al., 1998; Schon et al., 2004). These experiments were conducted in populations developed from relatively elite parents created from many generations of selection that would be expected to eliminate any deleterious alleles of sizeable effect. Therefore, these results do not preclude the possibility of QTL with large effects on yield in maize, but they do suggest that such QTL should rarely segregate in crosses between highly selected lines. Instead, yield in most elite breeding populations is likely to be controlled by numerous genes, each with quite small effects, in accordance with classical quantitative genetics theory (Holland, 2007).

In summary, decades of quantitative genetics studies have demonstrated the following general points about the genetic architecture of maize yield. First, the low heritability of yield observed on individual plants or plots indicates that reliable evaluations of the yield of experimental lines can only be obtained from observations from numerous tests in independent environments. Selection for yield among individual plants is expected to have very little effect. Second, the predominance of additive variance suggests that most breeding strategies involving selection for improved yield should be effective if reliable evaluations can be performed. Third, the large amount of dominance variance suggests that breeding schemes that can select and fix specific allelic interactions will be most efficient. Fourth, many genes of small effect control yield, suggesting that long-term selection programs and recycling of superior lines should result in gradual, but steady increases in yield, as has been observed in most programs (Duvick et al., 2004; Hallauer and Pandey, 2006).

2.2 Heterosis

The two major theories competing to explain heterosis (or "hybrid vigor," Fig. 2) are that heterosis is caused primarily by overdominant gene action or primarily by dominance of favorable alleles (Crow, 1998; Hallauer and Miranda, 1988). The debate appeared largely settled in favor of the dominance hypothesis in the 1960s when independent experiments in maize populations demonstrated that the average level of dominant effects for yield were within the range of partial to complete dominance (but not overdominance) in populations that were sufficiently random-mated to approach linkage equilibrium for most pairs of genes (Crow, 1998). The use of populations near linkage equilibrium was important because in early generations of

Fig. 2 Inbreeding depression and heterosis expressed in ears of **(a)** an inbred line from the Stiff Stalk heterotic group, **(b)** F_1 of an intra-heterotic group cross (between two related non-Stiff Stalk lines), and **(c)** F_1 of an inter-heterotic group cross (between the Stiff Stalk inbred in part A and the non-Stiff Stalk F_1 in part B)

crosses, favorable dominant alleles at different loci that are in repulsion phase linkage can generate an effect similar to overdominance, called pseudo-overdominance (Hallauer and Miranda, 1988).

Recently, there has been renewed support for the overdominant hypothesis. The dominance hypothesis has been criticized because it supposedly implies that heterosis should decrease as genetic improvement for yield is achieved, and this has not happened (Birchler et al., 2003; Birchler et al., 2006). Changes in heterosis associated with genetic improvement were estimated by Meghji et al. (1984), and Duvick (1984, 1999), who both found that inbreds showed significant gain for yield over time, but at a lower rate than their respective hybrids. This indicates that heterosis has increased in response to selection for higher yield, which Birchler et al. (2003) has interpreted as evidence for overdominance. Nevertheless, these results are entirely consistent with the dominance theory, because heterosis is expected to increase if the frequency of favorable alleles changes from low to moderate, or if complementary sets of favorable alleles at different loci are selected for in different heterotic groups (Lamkey and Smith, 1987; Meghji et al., 1984). Finally, new germplasm sources have been accessed in modern hybrids, which may contribute to increased heterosis (Duvick et al., 2004).

Another argument made against the dominance theory is that, if true, it should be possible to produce inbreds as productive as hybrids (Hallauer and Miranda, 1988). Although this may be theoretically true, in practice it will be extremely difficult to accomplish if many genes control yield. Nevertheless, Duvick (1999) reported that at high plant density, modern inbred lines yield almost as much as hybrids from the 1930s!

2.3 *Breeding for Yield Improvement*

The inbred–hybrid breeding methods adopted by most maize breeding programs exploit the known aspects of the genetic architecture of yield (Fig. 3). The breeding materials are divided into heterotic groups based on their patterns of combining ability; pairs of lines that exhibit better than average heterosis should be assigned to different heterotic groups. If this is done appropriately, crosses between lines of different heterotic groups should produce more vigorous hybrids than crosses between lines of the same heterotic group (Fig. 2). Second, the additive genetic variance is exploited by inbreeding and selection within crosses between lines of the same heterotic group. Third, during line development, the best lines are crossed to a tester line from a different heterotic group to produce testcross (sometimes called "topcross") hybrids. Large quantities of seed can be produce for each topcross hybrid family, permitting their evaluation in multiple environments, which increases the heritability for selection on the basis of topcross mean yields. Favorable combinations of lines from different heterotic groups can be selected to produce hybrid

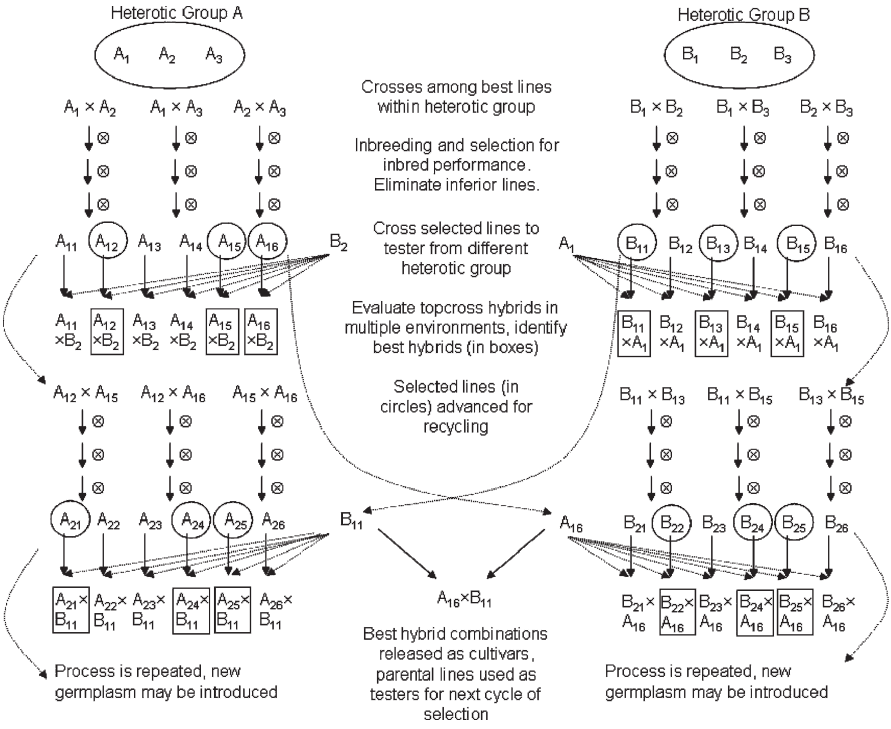

Fig. 3 Generalized and simplified inbred-hybrid breeding scheme for maize. In reality, many more breeding crosses are made, many more progenies evaluated, more testers are used, selection intensity is much higher, and breeders have materials representing each phase of the breeding program every year concurrently (Betran et al., 2004)

cultivars, thus fixing in heterozygous form the favorable allelic interactions and exploiting the dominance genetic variance. The best lines are then also recycled by crossing within heterotic groups to produce new breeding populations and they may also serve as testers for new lines created in a different heterotic group. New germplasm can be added to the breeding program after determining which heterotic group it belongs to.

In most breeding schemes, lines are first selected on the basis of inbred performance for traits that have moderate to high heritability, such as disease resistance, and for adequate agronomic performance. This is necessary to reduce the number of lines evaluated for topcross performance, which is more expensive. In addition, inbreds must have good seed yield to serve as commercially successful female parents of hybrids. A concern with selection for inbred performance, however, is that it is generally not highly correlated with topcross performance because of the importance of dominance interactions in hybrids (Betran et al., 2004). Therefore, most breeding programs attempt to compromise between selection for inbred performance and selection for hybrid performance to maximize genetic gain for both and to efficiently use resources.

3 Physiological Aspects of Yield Improvement

The time series experiments comparing newer versus older cultivars have provided an excellent opportunity to retrospectively assess what physiological and morphological changes have accompanied the observed yield gains. Summarizing numerous studies in Iowa and Ontario, the single most important change that occurred in the development of modern, high-yielding temperate maize hybrids was an increase in tolerance to high planting density stress (Duvick et al., 2004; Tollenaar and Wu, 1999). In fact, Duvick et al. (2004) noted that at very low planting density, there has been no observable change in yield per plant in Pioneer Hi-Bred's commercial cultivars after 70 years of breeding! However, yield per hectare has increased dramatically at high planting densities representative of modern agronomic practices. Yield of the oldest cultivars tended to decrease at the highest planting density, to which those cultivars were not adapted. Selection for adaptation to the stress of high planting density resulted in cultivars with more consistent ear production (less barrenness), minimal delay between anthesis and silk emergence, and more upright leaves, resulting in a canopy architecture with better light interceptance at high densities. Other trait changes observed over time included decreased tassel size, improved staygreen (and thus longer duration of photosynthesis during grain filling), and improved lodging resistance (Duvick et al., 2004; Tollenaar and Wu, 1999).

Perhaps most interesting are some of the traits that have not changed significantly during the development of modern maize hybrids. The most important physiological component of change in rice and wheat during the development of Green Revolution cultivars was the increase in harvest index (the ratio of grain yield to

total plant above-ground biomass), yet harvest index of maize has not changed over time (Evans and Fischer 1999; Meghji et al., 1984; Tollenaar and Wu, 1999). Similarly, leaf photosynthetic rate did not differ between old and new hybrids (Tollenaar and Wu, 1999).

These results suggest that much of the increase in maize yields due to breeding is from improved stress tolerance (Duvick et al., 2004; Tollenaar and Wu, 1999). Evans and Fischer (1999) suggested that breeding contributions to yield gains can be roughly partitioned into gains from improved stress tolerance and those from increased yield potential, "the yield of a cultivar when grown in an environment to which it is adapted; with nutrients and water non-limiting; … and other stresses effectively controlled." Changes in yield potential can be difficult to measure, however, because it can be difficult to provide an entirely stress-free testing environment, and because the environments to which older and newer cultivars are optimally adapted may be different. For example, high planting density is stressful on older cultivars, but newer cultivars are adapted to those conditions and require high densities to produce their maximum yield per area. Nevertheless, attempts have been made to compare genetic gains in environments with different levels of stresses to identify how much of the gain is due to stress tolerance versus improved yield potential. If greater gains are observed in more stressful environments, then stress resistance is the primary cause of yield improvement; conversely, if gains are greater in lower-stress environments, this would indicate that yield potential has improved more than stress resistance. Studies of cultivars representing different eras grown with lower and higher levels of nitrogen fertilizer indicated that genetic gains were observed both conditions, although the rate of gain was lower under low nitrogen stress (Castleberry et al., 1984). Duvick's (2004) studies over many years have demonstrated roughly similar rates of gain across environments with very different mean yields. Thus, it appears that both increased stress resistance and yield potential have contributed to the observed gains in yield, although Duvick and Cassman (1999) observed that maximum yields under irrigation in Nebraska exhibited no trend over almost 20 years, suggesting no increase in yield potential in modern hybrids.

4 The Future of Maize Yield Improvement

4.1 Yield Plateau?

A commonly expressed concern is that crop yields are approaching a yield plateau, which threatens world food security (Brown, 1998; Cassman, 1999). Identifying a yield plateau before it appears is likely impossible; all previous predictions of yield plateaus in cereal crops have been wrong. The United States national maize yield trends show no evidence of a plateau (Fig. 1), but, again, one cannot determine if a plateau has occurred until after the fact.

Specific concerns expressed about yield plateaus in the United States are that (1) yield potential under high input conditions has been trending flat in some regions, and (2) the amount of resources expended to achieve consistent yield gains are increasing (Cassman, 1999; Duvick and Cassman, 1999). While there is cause for concern, there are no proven methods for genetic improvement of yield beyond current breeding methods, so it is not clear what, if any, alternative methods should be adopted. Furthermore, even without gains in yield potential, substantial improvements to yield in farmer's fields can be made (Evans and Fischer, 1999; Tollenaar and Lee, 2002). Finally, much of the increased resources attributed to commercial corn breeding programs have been allocated to traits not associated with, or even detrimental to, yield (Duvick and Cassman, 1999). A sensible course of action is to maintain phenotypic selection for yield and agronomics as the cornerstone of breeding programs, adopt new technologies and methods once they are proven, and also promote improved crop and soil management.

4.2 Marker-Assisted Selection for Yield

While there is some evidence that marker-assisted selection (MAS) for maize yield can be effective (Stuber et al., 1999), there are also numerous reports where MAS has been cost-inefficient relative to phenotypic selection (Holland, 2004). A major hindrance to MAS for highly polygenic traits like yield is the difficulty in accurately estimating QTL effects (Beavis, 1994; Melchinger et al., 1998; Schon et al., 2004) and substantial genetic heterogeneity among populations (different sets of QTL segregating in each population, Holland, 2004; Holland, 2007).

A recently proposed method to circumvent the difficulties of QTL effect estimation bypasses the problem of determining which genome regions contain QTL and, instead, uses all available marker data to predict the genetic value of each line (Bernardo and Yu, 2007). To obtain better insight into the issue of genetic heterogeneity, simultaneous mapping of multiple populations that represent a major proportion of the genetic diversity of a breeding pool will be required (Holland, 2004; Holland, 2007). This will require massive investments in breeding program infrastructure, high throughput genotyping technologies, and large-scale data handling and analysis tools, but these investments are already being applied in large private companies (Crosbie et al., 2006; Niebur et al., 2004). The return on these investments may never be known, since there is no comparison to a similar large-scale investment in traditional breeding methods and infrastructure. The level of resources required to drive large-scale MAS programs are currently beyond what virtually all public breeding programs have available. Smaller-scale programs targeted at specific abiotic or biotic stress resistances or other characteristics where some single gene effects may be of larger size (at least in specific environments) may be a wiser target for investment where resources are more limited.

4.3 Untapped Genetic Resources

Another avenue toward maintaining and enhancing yield gains is to exploit more fully the genetic diversity of maize. Maize is one of the most genetically and phenotypically variable species known (Buckler et al., 2006). However, of the 300 or so races recognized in maize, only one, the Corn Belt Dent race, contributes substantially to the genetic base of modern United States maize hybrids (Goodman, 1990). Pedigree and DNA marker analyses demonstrate that pedigree duplication is common among commercial inbreds and hybrids (Darrah and Zuber, 1986; Mikel and Dudley, 2006; Smith, 1988; Smith et al., 1992). The best inbred lines are "recycled" as parents of new inbred lines (Mikel and Dudley, 2006), and commercial corn breeders introduce very little exotic germplasm into breeding populations. The result of this has been enhanced productivity, but the nearly exclusive reliance of more than 28 million hectares of US corn on the alleles of a small number of inbreds from a handful of cultivars of a single race of maize.

The greatest racial diversity in maize is represented by the tropical Latin American landraces (Tallury and Goodman, 2001). About 20,000 collections of maize landraces are available to select from, but very few have been used in breeding programs (Holland et al., 1996). A primary reason for the limited use of exotic germplasm in the development of modern commercial hybrids is its high level of photoperiod-sensitivity that delays flowering under long daylengths. Other factors include inbreeding depression, slow grain dry-down, poor lodging resistance, and susceptibility to smut disease. Nevertheless, some tropical germplasm sources possess alleles for increased heterosis when crossed with adapted temperate material (Betran et al., 2004; Goodman et al., 2000; Holland et al., 1996; Tarter et al., 2004; Uhr and Goodman, 1995). Long-term selection for adaptation of tropical germplasm to temperate production environments and for good hybrid performance in combination with temperate lines has resulted in the development of a new heterotic group that can be exploited by breeders in the US (Goodman et al., 2000; Tallury and Goodman, 1999). Prompted in part by these results, a large-scale public-private cooperative program to introduce exotic germplasm into the elite US maize breeding pool was initiated in the 1990s and continues today, resulting in the development of some exotic-derived inbred lines with excellent yielding ability and disease resistances (Balint-Kurti et al., 2006; Carson et al., 2006; Pollak and Salhuana, 2001).

4.4 Increasing Yield for Resource-Poor Farmers

As noted above, the yield gains observed in the United States for the past 80 years (Fig. 1) have not been repeated in all maize production areas worldwide. The less developed nations of sub-Saharan Africa have had among the lowest yield gains over time, including negative yield trends in some cases (Byerlee and Heisey, 1997; Pingali and Heisey, 1999). Many farmers in this region have limited resources and poor quality soils; as a result, among maize growing areas world-wide, the less developed nations

of sub-Saharan Africa have the lowest rates of adoption of improved maize varieties (Pixley, 2006) and fertilizer use (Byerlee and Heisey, 1997).

Given the very low input and management levels that occur on resource-poor farms, what can be done by researchers to help improve yields? One possibility is selection for tolerance to abiotic stresses such as drought and low soil nitrogen that are often encountered in low-input farming conditions (Banziger and Cooper, 2001). Another potential avenue of improvement is the development of improved open-pollinated varieties adapted to lower-input conditions. Hybrid seed must be purchased each growing season from seed dealers in order achieve maximum benefit from heterosis. Resource-poor farmers often cannot afford hybrid seed or the inputs required to make hybrids profitable, however. In contrast, seed saved from harvesting open-pollinated varieties can be sown in future seasons with little or no loss of vigor, reducing the input costs for farmers, while providing some of the benefits of genetic improvement (Pixley, 2006).

Farmer participatory breeding, wherein farmers actively participate in the selection process while evaluating experimental breeding materials on their own farms, has also been successful for improving maize yields in low input conditions on resource-poor farms (Smith et al., 2001). Farmer participation in the variety selection process should also have the advantage of increasing the rates of adoption of improved varieties (Edmeades et al., 2006).

Significant improvements in the rate of gain in yield improvement for resource-poor farmers may only be possible if breeding efforts are allied with intensification of agronomic practices to enhance soil productivity (Eicher and Byerlee, 1997; Evans, 1998). Nevertheless, plant breeding should be the cornerstone of agriculture improvement efforts, as improved varieties may be the easiest improved "technology" to be disseminated and adopted by resource-poor farmers (Evans, 1998). Many other factors, from the agronomic to the economic and societal levels, must also change for the benefits of plant breeding to be widely disseminated.

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The Illinois Long-Term Selection Experiment, Related Studies, and Perspectives

Torbert Rocheford

Abstract The Illinois Long-Term Selection Experiment was started in 1896 and represents the longest ongoing directional selection program in plants. The experiment was initiated to determine whether selection could alter levels of protein and oil in the maize kernel. The experiment readily demonstrated in the first several cycles that selection resulted in significant changes. Yet the experiment continues to this day, as gain from selection for high oil and for high protein is still progressing. The Illinois Long Term Selection Experiment (ILTSE) now addresses questions on the nature of gains from selection and limits to selection. This chapter focuses on insights developed through molecular marker studies on ILTSE. These studies involved the long term selection strains, mapping populations derived from the strains, and related mapping populations that underwent random mating. Studies on the oil and protein strains revealed trends in molecular markers consistent with response to selection, and that more heterozygosity remains in the strains than predicted earlier. The heterozygosity provides allelic variation for further selection, and also allows for intragenic recombination which may create new alleles. Studies involving populations derived from crosses of Illinois High Protein (IHP) × Illinois Low Protein (ILP), and Illinois High Oil (IHO) × Illinois Low Oil (ILO) detected QTL for kernel composition. Most of the markers associated with trends in response to selection were also associated with QTL. However, a relatively small number of QTL were detected in mapping populations, with six QTL explaining 65% of variation for protein, and six QTL explaining 52% of variation for oil, in the respective populations. These results were inconsistent with earlier predictions of 173 effective factors controlling protein concentration and 69 controlling oil. Subsequent studies involving random mating of related mapping populations, in order to break up large linkage blocks, revealed many more QTL, in a range more consistent with earlier predictions. For example, 50 QTL were associated with 50% of the variation for oil in a random mated population derived from IHO × ILO. Collectively, the large number of loci detected controlling oil and protein, and the much higher levels of heterozygosity maintained in the strains than predicted, help to explain some of the basis for long term gains in selection. Opportunities for use of the selection strains and derived genetic materials in the era of genomics are discussed.

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1 Introduction

The Illinois Long Term Selection Experiment (ILTSE) was started before the rediscovery of Mendel's laws, which highlights the selection work that plant breeders were performing at this time based simply on phenotypic data. In 1896 Cyril G. Hopkins started the first selection experiment on the chemical composition of maize kernels at the University of Illinois (Hopkins, 1899). The practical purpose was to determine if selection could alter maize chemical composition to improve grain for greater value for feeding animals. The chemical composition of feeds for the purpose of animal nutrition was at the forefront of agricultural science in the 1890s (Jones, 1944).

To begin the experiment, Hopkins analyzed 163 ears of the open pollinated variety Burr's White for percent protein and percent oil and small subsets of ears were selected to initiate four strains: Illinois High Protein (IHP), Illinois Low Protein (ILP), Illinois High Oil (IHO), and Illinois Low Oil (ILO). Considerable progress in response to cycles of selection has been observed in the strains. After 48 cycles of divergent selection, reverse selection was initiated in each of the four strains to test whether genetic variability for oil and protein concentration remained in IHO, ILO, IHP, and ILP. This created the Reverse High Protein (RHP), Reverse Low Protein (RLP), Reverse High Oil (RHO) and Reverse Low Oil (RLO) strains (Leng, 1962). Details of the selection procedures, chemical analyses and statistical evaluations have been published (Dudley et al., 1974; Dudley, 1977; Dudley and Lambert, 1992).

The average maize kernel is ~80% carbohydrate, 10% crude protein, 4.5% oil, 3.5% fiber, and 2% minerals (Jugenheimer, 1976). One hundred generations of selection for high protein concentration changed the level of protein from 11% for Burr's White open pollinated variety to 32% for IHP (Fig. 1). One hundred generations of selection for high oil concentration changed the level of oil from 4.7% for Burr's White to 22% for IHO. (Fig. 2). The experiment has now completed 108 cycles of selection. Results from 100 cycles of selection are discussed in this chapter since there is a recent publication that thoroughly summarizes 100 cycles of selection (Dudley and Lambert, 2004). The fundamental findings have not changed between cycles 100 and 108.

Selection changed percent protein from 11% to ~4% for ILP after 63 cycles. Protein remained at ~4% for another 32 cycles as no further progress was observed. Therefore selection for low protein was terminated after cycle 95. Selection for low oil changed percent oil from 4.7% to less than 1% by cycle 48. Oil essentially remained at this level until cycle 89 when selection for low oil was terminated (Dudley and Lambert, 1992). The low protein strain was terminated because it appeared to have reached a biological lower limit in protein at ~4%. The oil strains were at <1% and accurate measurement for oil concentration could not be obtained below 1%. Furthermore, for both the ILO and ILP strains, poor germination had become a problem. This created technical and genetic sampling challenges to the integrity of the selection process. The experiment had provided solid evidence on limits to selection for low protein and low oil.

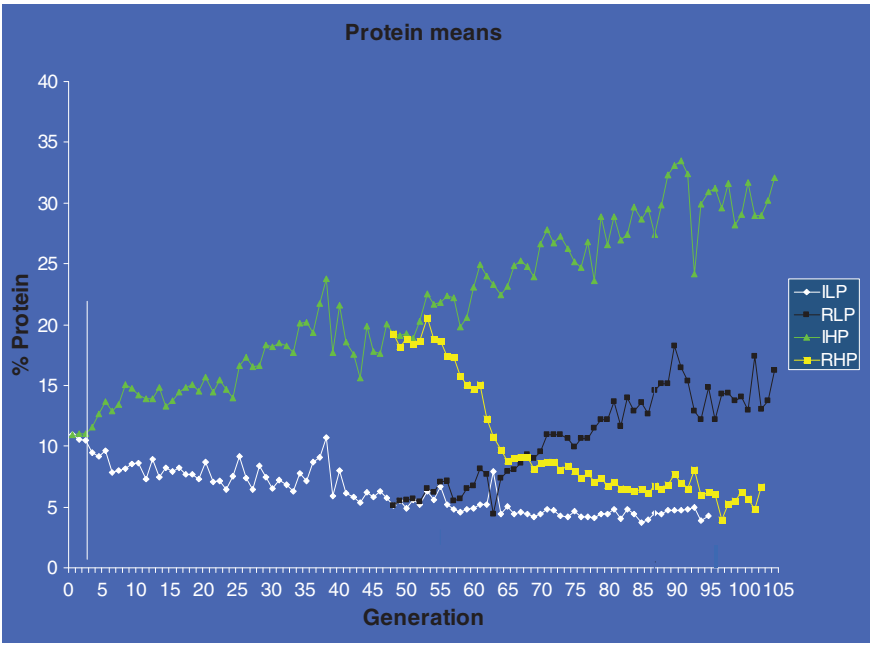

Fig. 1 Progress of Illinois long-term selection protein strains. Courtesy of J.W. Dudley

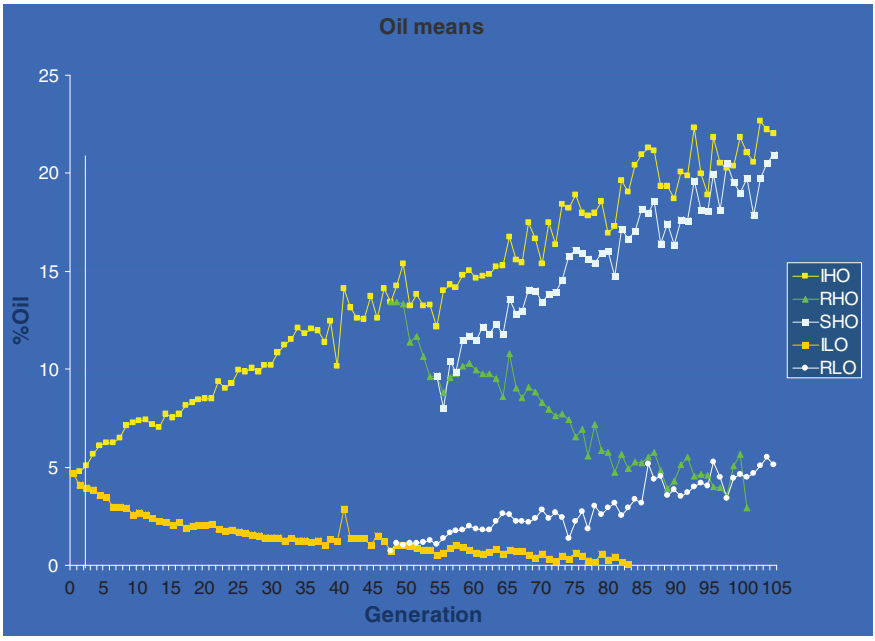

Fig. 2 Progress of Illinois long-term selection oil strains. Courtesy of J.W. Dudley

All of the reverse strains, RHO, RHP, RLO, and RLP, showed progress in the new direction of selection (Figs. 1 and 2). This indicated that despite 48 cycles of forward selection in these closed strains, enough variability remained to enable progress in the reverse direction. The slope of response to selection for RHO and RLO is somewhat similar to the slope of the IHO and ILO strains, respectively, just in the opposite direction. Similarly, The RLP strain has a slope similar to ILP but in the opposite direction. However, the RHP strain stands out in that is showed a dramatic reduction in protein levels, with a rapid rate of response to selection for ~20 cycles of selection, followed by a more gradual response to selection.

All of the strains are routinely measured for oil, protein, and starch concentration. Increasing or decreasing the percentage of one of these major chemical components of the maize kernel altered percentage of one or both of the other chemical components. Increasing protein concentration in IHP was associated with both a decrease in starch and a lesser decrease in oil concentration. Decreasing protein concentration in ILP was associated with higher starch concentration, whereas oil did not change much for this strain. Selection for higher oil in IHO was associated with reduced starch concentration but minimal changes in protein (Dudley and Lambert, 1992).

Many classical genetic studies have been performed on the Illinois long-term selection chemical strains (Hopkins et al., 1913; Smith, 1918; Winter, 1929; Woodworth et al., 1952; Dudley and Lambert, 1992). Some studies used population and quantitative genetic models to develop estimates of the number of genes involved in controlling oil and protein concentration. Dudley estimated the number of loci separating the different high and low strains after 90 cycles of selection. He calculated 173 effective factors associated with protein concentration in IHP versus ILP and 69 associated with oil in IHO versus ILO (Dudley and Lambert, 2004).

2 Molecular Marker Studies

Brown (1971) was the first to study the long-term selection strains at the molecular level, estimating the allozyme frequencies for six enzyme loci at generation 68. He concluded that levels of fixation could be explained by neutral drift, and were not in response to directional selection. Brown's study was noteworthy, but interpretations were limited in that only six enzyme loci were assayed. The subsequent availability of DNA based molecular marker systems enabled examination of a much greater portion of the maize genome than was possible with isozymes (Stuber, 1992).

The first DNA marker work on the Illinois long-term strains determined RFLP variant and genotypic frequency differences among cycle 90 of IHO, ILO, RHO, RLO (Sughroue and Rocheford, 1994). They found a high degree of variant polymorphism within and among the four oil strains. Notably, 37–57% of the 49 RFLP loci assayed were still segregating within the different oil strains even after 90 generations of selection as closed strains. Comparison of RFLP variant frequencies between IHO versus RHO and ILO versus RLO, and then among all four strains,

identified markers that showed trends consistent with response to directional selection for levels of oil. A parallel study performed on cycle 91 of the Illinois Protein strains IHP, RHP, ILP, RLP revealed a high degree of variant polymorphism within and among the strains. Thirty four loci were still segregating in more than one strain (Mikkilineni and Rocheford, 2004). The protein strains showed variant frequency trends consistent with response to directional selection.

The study on the protein strains also included earlier cycles of selection so that inbreeding coefficients (change in level of heterozygosity) could be calculated. Samples from cycle 65 or 69 were assayed in the protein strains, which were the earliest cycles with remnant seed available. Before John Dudley took over direction of the Illinois long-term selection experiment at cycle 65, the standard practice had been to save remnant seed of only the two previous cycles of selection. Dudley had the good wisdom to start saving seed from all cycles. Availability of seed from earlier cycles enables experiments such as this one. Inbreeding coefficients ranged from 36% for IHP to 3% for RHP (The higher the number, the more inbreeding or fixation of loci). There was less inbreeding observed from cycles 65 to 91 for all four strains than the 42% predicted for each strain by classical models (Dudley et al., 1974).

The finding of lower inbreeding coefficients (higher levels of heterozygosity) than predicted from comparison of cycles 65–91 in the protein strains is noteworthy. There are differing opinions on estimates of effective population size, number of alleles that could be maintained in the strains, early bottlenecks, and how this may impact estimates of expected inbreeding coefficients (Walsh, 2004). The pollination scheme for the strains, which is designed to prevent self-pollinations, appears relevant to the finding of higher levels of heterozygosity. The 12 ears with the highest or lowest values for oil or protein are selected from a set of 60 well-pollinated ears from within each strain. The 12 selected ears are used to create the next cycle, and are split into two groups of six, with two balanced bulks of seed made. The two bulks are planted in blocks next to each other. Pollen from many tassels from one block is bulked and used to pollinate many plants from the adjacent block, and vice versa. Thus many alleles could be present in the bulk of pollen used, as it comes from many different plants.

However, even with estimates of larger effective population size that come with this pollination scheme, the estimated inbreeding coefficients are higher than those calculated from RFLP marker data (Walsh, 2004). Furthermore, early in the history of the protein and oil strains there appears to have been bottlenecks, such as the protein strains tracing to one ear and the oil strains tracing to three ears. This would have limited the effective population size of gametes in the protein strains to four at this point (plants were cross pollinated and two different gametes could be contributed if each plant was heterozygous at a locus) and 12 in the oil strains. Notably most of the loci still segregating in the oil and protein strains show two RFLP alleles, and a very small number show three. This observation is not inconsistent with the protein and oil strains tracing to just one and three ears, respectively.

A fundamental aspect of the biology of the maize plant may impact levels of inbreeding in the selection strains. Maize exhibits inbreeding depression, resulting in reduced vigor and height. For the pollination process within each selection strain,

a conscious effort is made to use a random set of plants from each adjacent block growing in the field. However, through the usual steps of human administered tassel-bagging and pollination, some of the more vigorous, healthier plants are likely to be chosen more often than expected by random chance. Plants that do not produce ears, or have other morphological limitations that make them less suitable for pollinations, tend not to be selected. Furthermore seeds that do not germinate, due to lethal homozygous conditions, obviously do not produce plants that can be selected. Occasional unintentional selection of more vigorous plants may contribute to maintenance of heterozygosity, given that maize shows inbreeding depression and the more heterozygous plants tend to be more vigorous.

The molecular marker studies provided some fundamentally new insights to the question: What is the basis of the long-term continual gain from directional selection? Classical theories and models suggested that most of the natural allelic variation in the base population likely would have been used up after perhaps 40–50 cycles of selection (Hill and Robertson, 1966; Hill, 1982; Hill and Keightley, 1988). Further gain from selection after this point was considered to be increasingly due to exploiting newly created mutational variance (Clayton and Robertson, 1955; Clayton et al., 1957). The molecular marker studies showed that there is more heterozygosity in the strains than predicted. Although RFLPs only assay variation at that marker locus, they are indicators of the likely status of linked loci. The levels of heterozygosity in cycle 90–91 of the oil and protein strains, and lower inbreeding coefficients in the protein strains, suggest higher levels of heterozygosity and thus variation in genes in the selection strains than predicted. The classical estimates suggest that there would be considerable fixation of loci by cycle 90, ~98% (Dudley et al., 1974), and thus little variation remaining for further selection. More allelic variation appears available to exploit for directional selection, and likely contributes to the continual gain from selection.

The maintenance of higher levels of heterozygosity in the strains also provides greater opportunity for intragenic recombination of different haplotypes of genes that may be under selection, which can create new allelic variation. As indicated, within the individual strains two different RFLP alleles were maintained at most heterozygous loci. Different RFLP variants ranged from two to four among the oil strains and two to three among the protein strains. Many of the predominant variants present in the different protein or oil strains were likely in response in part to directional selection, likely linked to alleles of genes under selection. By maintenance of heterozygosity, these more favorable alleles can recombine intragenically and potentially create a new even more favorable alleles that can respond to further selection. Furthermore, any new mutation in an allele at a heterozygous locus can potentially undergo intragenic recombination to create other novel haplotypes.

RFLP variants were compared across the four protein strains to look for novel variants detected in only one strain. A novel variant can be an indicator of mutation since the initial point of separation and isolation of the different protein strains. Only one marker had an RFLP variant that was detected in just one strain. All of the other markers had variants that were present in more than one strain, which suggests these variants were likely present in the base population. This single

marker with a variant detected in only one strain could have been a rare variant in the base population, created due to mutation during cycles of selection, or due to recombination that created a new variant. The number of loci assayed was limited, only 35, but the search for novel variants was over four strains and two cycles of these strains, surveying 50 gametes in each. This indicates there may not be a lot of novel RFLPs within the strains, perhaps suggesting mutational variance may not be a major factor. However, RFLP markers have considerable limitations, and do not reveal many linked point mutations.

Despite the limitations of RFLPs, the molecular marker studies provided genome-wide coverage at the DNA level for the first time. This enabled findings from a different perspective than the phenotypic evaluations and the population and quantitative genetic model estimates available for approximately the first 90 cycles of the experiment. There may be slight bias that influenced the results of these molecular marker studies. The RFLP markers used were selected based on showing easily scored polymorphism between bulk DNA samples IHO versus ILO and IHP versus ILP High Oil. Approximately 60% of 155 RFLP markers screened between IHO and ILO cycle 90 were polymorphic and ~30% of 200 RFLP markers were polymorphic between ILP and IHP cycle 91. The choice of polymorphic markers does increase the likelihood of finding different variants among the strains.

The use of polymorphic markers distributed throughout the genome facilitated assessment for effects of reverse selection on RFLP variant frequencies. Variant differences between the high and low strains could be due to directional selection, or could be simply due to sampling and neutral genetic drift effects. Thus evaluation of the forward and reverse strains and assessing for an associated response to reverse selection enables a powerful analysis. In the ILTSE, the forward strains were selected for 48 generations before the reverse strains were created, so considerable selection had occurred by this point. Thus a variant that is in high frequency in a forward high strain, but in low frequency in the respective reverse high strain, is likely responding to directional selection. The variants present in the low and reverse low strains can also be compared in a similar manner. Comparison across all four strains adds to the power of assessment. For example, selection for high oil is imparted to both the high oil and reverse low oil strains. So comparisons can be made among all four oil strains, and assessment for consistent variant frequency trends in more than just two strains.

Evaluation of cycle 90 of the IHO, RHO, ILO, RLO strains reveal trends consistent with selection for three loci in RHO, and eleven loci in RLO, and one locus significant in both. Evaluation of cycle 91 of the IHP, RHP, ILP, and RLP strains revealed trends for eight loci in RHP and twelve loci in RLP, and seven in both. The evaluation of two cycles of selection of the protein strains also enabled assessment for changes of variant frequencies within a strain. Seventeen to 29% of a total of 35 markers showed significantly different frequencies in protein strains between cycles. Some markers were only at intermediate frequencies at cycle 65, but had increased considerably to high frequency by cycle 91. This suggests that there may not have been strong selection on some of these allele variants through the first 65 cycles of selection, and then there was much stronger/effective selection in the

subsequent 25 cycles. What may be the basis for this observation? There may have been fixation or near fixation of other loci with larger effects on protein levels by cycle 65. Then selection may function more effectively on other loci with smaller effects. Or the new higher protein or oil phenotypic and genotypic background developed over 65 generations of selection may have created a new context, such that selection would now act on other loci. Mutation may have occurred in a linked gene that provides a superior selective advantage. The key point is that rapid frequency changes can occur in later cycles of selection, and that frequency changes did not appear to have a similar rate of change over time for all loci.

3 Quantitative Trait Loci Studies

The long-term selection strains provided materials well suited to investigate mapping of QTL, particularly during the early period of QTL studies. Initially, some questioned whether QTL could be detected and whether initial reports of QTL were statistical artifacts. Ideal sets of material to search for QTL were mapping populations derived from crosses of high by low selection strains. A population derived from such a cross would be an excellent place to search for QTL. It did not involve a wide cross involving a domesticated cultivar by wild species (Paterson et al., 1988). Rather, it was created from two natural breeding populations created by human imposed divergent selection from a common genetic background. Furthermore, markers associated with trends from selection in the strains would be polymorphic and could be assayed in IHO × ILO and IHP × ILP mapping populations to determine if QTL were also associated with these same markers. Detection of QTL in the same region that a trend was detected provides supportive evidence for presence of a QTL.

One concept in population and quantitative genetics is the infinitesimal model which proposes the basis of genetic control of the quantitatively inherited traits is due to very large number of loci (Lynch and Walsh, 1998). This could be up to hundreds of loci with very small and equal effects. The long term divergent selection of high and low oil and high and low protein from a common base population provided materials well suited to examine whether this model is relevant. The continual, gradual gain from long term selection over many cycles would suggest that large numbers of loci were pyramided in each strain, with differing, contrasting alleles in the high versus low strain.

The first QTL study using the Illinois Long Term Selection Strains involved a cross of IHP × ILP cycle 76 and evaluation of 100 derived F2 S1 progeny in replicated trials (Goldman et al., 1993, 1994). This was a population cross of several IHP plants by several ILP plants. Single factor analysis of variance and multiple regression analyses were performed for QTL analysis. QTL for protein, starch and oil concentration and kernel weight were readily detected. There was a considerable range in the magnitude of effect of different QTL. Notably, the multiple regression model had only six loci which explained 65% of the variation

for protein concentration. This was in sharp contrast to estimates of ~173 factors responsible for control of variation in protein in IHP versus ILP cycle 90 (Dudley, 1977; Dudley and Lambert, 1992), not to mention perhaps hundreds that would be predicted from the infinitesimal model. However only 65% of the total variation was explained by the regression model in the IHP × ILP mapping population (Goldman et al., 1993) and it is unknown how many loci with smaller effects, and thus not detectable by QTL analysis, were involved in control of the other 35% of variation for protein.

Starch was inversely correlated with protein and showed considerable variation in the IHP × ILP mapping population. The detection of QTL for starch was similar to that for protein, with a range in magnitude of effect of QTL, and seven loci explaining 66% of the variation. A couple large QTL explained a considerable portion of the variation for starch concentration. One of these mapped to the chromosome region with the *Shrunken2* (*Sh2*) locus (Goldman et al., 1993). *Shrunken2* encodes the large subunit of ADP-glucose pyrophosphorylase, considered a key or rate limiting enzyme in the starch biosynthetic pathway (Stark et al., 1992). This provided one of the very first logical candidate genes that may be the underlying the basis of a QTL.

A QTL study was performed on material derived by a cross of a single plant of IHO cycle 90 by a single plant of ILO cycle 90, so that only two alleles would segregate in the derived 200 F2 S1 progeny. This made generating a molecular marker map much easier than for a population cross. A number of QTL for oil, starch and protein concentration and kernel weight were detected with analysis of variance of mean performance of marker classes (Berke and Rocheford, 1995). Again a relatively small number of loci (six, seven) explained a large portion (52%, 44.5%) of the variation for oil and starch, respectively. A QTL on chromosome 6 had a very large additive effect. The finding of small number of loci explaining a large proportion of the variation, and detecting a single major QTL region that explained a large amount of variation, was also inconsistent with earlier predictions of 69 effective factors responsible for control of oil (Dudley, 1977; Dudley and Lambert, 1992) and certainly the infinitesimal model.

The results of these two QTL studies were consistent with a model presented by Beavis (1994). He suggested that the experimental design of QTL studies limits significant detection of only a subset of QTL controlling the trait, and that there is usually one very large QTL detected. The structure of a simple F2 derived population with 100–200 families limits the number of QTL that can be detected in a sample of progeny (Lynch and Walsh, 1997). Thus some of this inconsistency between prior predictions on number of genes controlling kernel traits in the long-term strains and the QTL results may be related to limits of QTL mapping in general, and particularly in population sizes of 100 to 200 progeny. The mapping results did show that QTL with different magnitude of effects were controlling oil and protein.

An important question in the early period of QTL mapping was whether QTL detected in academic experimental crosses could be effectively transferred to more elite breeding material. Detection of QTL for oil concentration in an IHO × ILO cross was one thing, but the long term selection strains are not elite

material and not used commercially. They essentially have the agronomic performance of an 1890s open pollinated variety. Furthermore, due to the nature of QTL analysis, a QTL is only detected if there are contrasting alleles in the parents of the mapping population. Thus if an allele from ILO has a large negative effect it can cause a significant QTL association, even if the IHO allele has little positive effect on oil. Thus a timely question was "Could some of the oil QTL from IHO be expressed and detected in more elite germplasm?" A single plant of IHO cycle 90 was crossed with B73, a historically elite and important line, backcrossed to B73, and selfed to generate 150 BC1S1 progeny. Evaluation of these materials readily detected QTL for oil, starch and protein concentration, demonstrating that QTL could be transferred across genetic backgrounds (Rocheford, 1994).

The initial results of the (IHO/B73)B73 population held up in replicated environments over multiple years (Wassom et al., 2008). Again a relatively small number of QTL explained a large portion of the variation (47%) for oil concentration. The QTL on chromosome six again showed very large effects on oil concentration. The transfer of the effect of chromosome 6 segment detected initially in IHO × ILO to a B73 backcross background greatly lessened the likelihood that detection of this large QTL in IHO × ILO was due to chance or experimental design limitations. Near-isogenic lines developed for the IHO chromosome 6 segment confirmed the QTL and importantly the large magnitude of effect (Johnson, 2003). This demonstrated that a single QTL region with very large effects on oil concentration was present in IHO.

During selection for extreme high and low protein levels, IHP and ILP were indirectly selected for extreme low and high levels of starch in IHP and ILP, respectively. A logical question then was whether starch concentration can be improved in more elite germplasm using ILTSE strains. The maize inbred B73 and a single plant from ILP cycle 90 were crossed, F1 backcrossed to B73, and BC1 plants self-pollinated to generate 150 BC1S1 progeny. Evaluation of these materials revealed QTL for starch, protein and oil (da Silva, 2006). A relatively small number of QTL, eight, explained a large amount of variation (35.2%) for starch concentration. The IHP × ILP materials showed more marker loci associated with both starch and protein concentration (16/90) than the (ILP × B73)B73 background (5/142). The detection of more QTL for both protein and starch in the IHP × ILP background was likely related to the parents and how the populations were created. The IHP versus ILP parents likely had a greater allelic contrast than ILP versus B73, and the IHP × ILP F2 derived materials had three marker classes and not just two for (ILP/B73)B73 backcross derived materials, which reduces power in detection. Three QTL in (ILP/B73)B73 study showed larger effects on starch concentration than the others, demonstrating QTL with differing magnitudes of effect. Six of the eight QTL for high starch came from ILP, indicating that this germplasm could be used to improve starch in B73 type materials. However, not all QTL for higher starch came from ILP. Thus although 90 generations of selection for low protein and high starch had occurred, there are still alleles in other germplasm that are superior for increasing starch concentration.

4 Random Mated QTL Mapping Population Studies

Collectively the QTL mapping results indicated that a relatively small number of loci were responsible for a large amount of variation segregating in mapping populations derived from the long term selection strains. The long-term selection process likely puts together coupling phase linkages of favorable alleles over the many cycles of selection. Creation of F2 or Backcross derived mapping populations involves only one highly heterozygous meiosis, resulting in relatively large linkage blocks remaining intact. Thus the QTL detected in the studies reported here may actually represent a number of closely linked small effect QTL, with collective larger effects that remain intact in the large linkage blocks. To address this question, mapping populations were random mated for a number of cycles in the F2, and then lines extracted. Random mating in an F2 allows more cycles of meiosis in a highly heterozygous state, and enables the breaking up of linkage blocks. Therefore the selection strains, which underwent many cycles of selection that created coupling phase linkage blocks, were very well suited to examine effects of random mating on QTL detection.

The first study on random mating involved a cross of IHP × ILP cycle 70 in which the F2 was random mated (RM) for four cycles and then S1 lines extracted. The same molecular markers were used in the F2 and RM4 generations. This study involved a multi-parent cross, a linkage map was not developed, and interval analysis not performed. Random mating was associated with reduction in the number of markers showing significant effects for protein, starch and oil concentration (Dijkhuizen et al., 1998; Dudley et al., 2004). This would be expected if random mating resulted in more crossovers, reducing size of linkage blocks, and thus creating more recombinants between markers and QTL. Random mating would effectively increase the genetic distance between markers, and between markers and QTL, and thus reduce ability of a constant set of markers to detect significant associations after random mating. Furthermore, if a QTL association was due to multiple linked genes with small effects, then random mating may dissipate the coupling phase linkages and make the individual effects effectively non-detectable. Notably, this study demonstrated that most of the QTL significant for both protein and starch (with opposite effects) in the F2 remained significant for both traits in the RM4 generation. This suggests that these QTL had pleiotropic effects on protein and starch, and there were not two closely linked QTL, one for protein and one for starch.

The results from this random mating study are noteworthy as it was one of the first studies to show reduced number of QTL associations due to random mating. Similar results were obtained for an IHO × ILO RM population (Willmot et al., 2006). However these experimental materials did not allow addressing the question related to inconsistency in estimates of effective factors controlling protein or oil based on the strains versus QTL studies on populations derived from crosses of strains. The above studies simply showed that random mating resulted in fewer associations using the same number of markers. If linked QTL that cosegregate in an F2 population as a single QTL were being broken up, then more markers would be needed in the RM population to detect them. This question was better pursued

by developing a larger mapping population with more individuals (499), more cycles of random mating (10), and higher marker density (488). The larger population size and more dense marker coverage better enables detection of independent closely linked QTL that may have been detected as a single QTL in an F2 derived population. Evaluation of these materials revealed a much larger number of QTL, with ~50 detected for oil concentration, explaining ~50% of the variation (Laurie et al., 2004; Clark et al., 2006). This result is notable as it showed that there were considerably more QTL associated with oil than previously estimated based on simple F2 derived populations.

The finding of 50 QTL for oil concentration is noteworthy in that Dudley had predicted 69 loci controlling oil concentration in IHO versus ILO cycle 90, and these numbers are not considerably different. Furthermore it is not known how many loci control the other 50% of variation not explained by the 50 detectable QTL. Furthermore, there may be limits to what can be detected even with this larger RM population. It may take both 2,000 or more progeny and markers to get a more complete and accurate estimate on number of QTL controlling oil or protein concentration. The findings from the IHO × ILO RM study are relevant to the long term selection strains and to QTL studies in general. With a larger random mated population with greater probe density, the number of genes detected is somewhat comparable to classical estimates (69) for oil concentration on the strains themselves. The study also suggests that 75–100 genes (or more) controlling a kernel composition trait may be reasonable numbers. However, this RM study suggests that the infinitesimal model, with predictions of perhaps hundreds of loci involved, may not be very realistic.

The availability of cloned genes, for example in the starch biosynthetic pathway, enabled gaining further insight into some of the long standing questions related to the selection strains. As noted, analysis of the IHP × ILP population detected a QTL for starch concentration that mapped to the chromosome region with *Sh2*. Sequence analysis revealed two different *Sh2* alleles were present in IHP and ILP, including an amino acid difference, two small insertion sequences in introns, and detection of a new transposable element-like insertion sequence, ILS-1 in ILP (Alrefai et al., 1994). Diverse germplasm from the United States and the world was assayed with three *Sh2* PCR markers, which detected the insertions in combinations of one, two, or all three (Shaw, 1997). There results suggested a distinct order in which the insertion events occurred that was completely consistent across the diverse germplasm. Insertion one was found alone, insertion two was only found along with insertion one, and insertion three was only found along with insertion one and two. Only two inbreds had the rare haplotype with all three insertions, and one of the inbreds was derived from an old Illinois open pollinated variety. Thus the rare *Sh2* haplotype with three indels could have easily been in the Burr's White open pollinated variety base population.

Survey of plants from the protein strains for *Sh2* insertion sequences revealed some genotypes in ILP that were different than what could have resulted from homozygotes and heterozygotes of haplotypes revealed from the germplasm survey. The simplest explanation for the novel variation would be one intragenic recombination event in the strains. Without full length sequencing, an excision of

insertion sequences can not be excluded, however these elements are essentially nonactive and this is very unlikely. Although this data does not prove intragenic recombination, it revealed *Sh2* alleles in the strains that were not present in a broad survey of diverse germplasm. Regardless of mechanism, 90 cycles of selection for low protein (high starch) was associated with a presence of novel *Sh2* genotypes based on these PCR markers (Shaw, 1997).

Association analysis for starch biosynthetic genes performed on the maize genetic diversity panel of inbreds revealed that *Sh2* had some association with starch concentration but it was more significantly associated with genotype x environment interactions (Wilson et al., 2004). These marginal results for *Sh2* are consistent with a study in which a *Sh2* marker and umc63 showed similar effects on starch in an F2 derived mapping population but in the random mated RM4 generation the effect of Sh2 had diminished more than for the *umc63* marker (Dijkhuizen et al., 1998; Dudley et al., 2004). This provides information consistent with a hypothesized model of linked QTL in this region that influence starch concentration. One is the structural gene for ADP-GPP and the other is of unknown function. Selection for high starch (low protein) may have combined linked loci of different function that both contribute to starch concentration, with the unknown locus linked to *umc63* having larger effects.

Relevant to the QTL of unknown gene identity linked to *Sh2*, QTL studies on the strains and related association analysis studies on starch biosynthetic pathway genes have revealed very few QTL associated with starch biosynthetic pathway structural genes (Wilson et al., 2004). Similarly, for oil concentration there are limited QTL and significant association analysis results for oil biosynthetic pathway genes. This suggests that there may be other types of genes, perhaps transcription factors, which may quantitatively influence the biosynthesis of starch or oil. The availability of the full genome sequence of maize and resources such as the very large nested association mapping population will be helpful in identifying the genes underlying QTL that are not associated with known biosynthetic genes.

5 Variation for Other Traits in the Strains

The strains were selected only for levels of protein or oil, yet other phenotypic traits show considerable variation among the strains. These include days to flower, plant height, and ear and tassel inflorescence architecture. Analysis of these traits in the same mapping populations studied for kernel composition detected QTL for plant height, days to flower, ear and tassel inflorescence architecture. A QTL detected for tassel branch number in IHO × ILO population provided the first candidate gene for a QTL for inflorescence architecture, mapping to *ramosa1* (Berke and Rocheford, 1999; Vollbrecht et al., 2005). The question has been asked whether the variation for days to flower or inflorescence architecture is directly related to selection for levels of protein or oil. In some cases QTL for inflorescence architecture and days to flower have been found in the same general or nearby

chromosome regions as QTL for kernel composition. Thus selection for oil levels may have selected a high oil QTL and indirectly selected a linked locus for days to flower or inflorescence architecture.

An example related to changes in different traits in ILTSE was detection of a QTL for oil on chromosome bin 7.02 region that is linked to *ramosa1* locus (Wassom et al., 2008). These two loci may have reinforced each other, for example, selection for oil may have also selected the linked allele for higher branch number, and thus plants with larger tassels and more pollen that is passed on to the next generation. In contrast ILO has a small tassel, which might be related to chance linkages of ILO alleles with fewer branches in early generations of selection, or just chance selection of unlinked loci in very early cycles that resulted in a smaller tassel. Days to maturity also varies among the strains, for example, IHO is much earlier than ILO. However selection for early maturity in ILO, starting with cycle 70 was able to change late maturity to earlier maturity without changing oil levels (Dudley and Lambert, 1992). This indicates that late maturity was not necessary for low oil concentration. These results and correlation analyses in mapping populations provide evidence that traits such as days to flower or tassel inflorescence architecture were not strongly associated with levels of kernel composition traits. One trait that may be worth further investigation is perhaps the samller ears of IHP than ILP, which perhaps is related to how the plant produces high levels of protein.

In contrast to the above traits, evidence suggests that variation in nitrogen (N) use components among the protein strains may be in response to selection for protein concentration in the grain (Uribelarrea et al., 2004, 2007). Selection for protein may have altered how the plant responds to nitrogen availability, in order to increase levels of protein in the kernel. Testcrosses of inbreds derived from protein strains were field evaluated at different N rates. Results showed that all the protein strains had similar nitrogen use efficiency, but they clearly differed in their N use components. IHP and RLP (lower starch) exhibited a higher uptake efficiency, and ILP and RHP (higher starch) exhibited high utilization efficiency. The strains have therefore created a useful set of germplasm with physiological differences that can be used to study nitrogen efficiency and uptake. With increasing concerns about groundwater contamination, understanding nitrogen uptake, utilization, and overall efficiency is becoming increasingly important (see chapter by Geiger, this volume).

6 Current and Future Directions

Studies enabled by availability of molecular markers have been useful for generating new information and insights on the basis of progress in response to long term selection, and genetic control of kernel composition. The strains are also very well suited for use with contemporary genomic resources and tools (Moose et al., 2004). Expression profiling was performed with RNA from developing kernels of IHO and ILO, identifying cDNAs that were differentially expressed. SNP markers were generated for many of these genes and many used in the IHO × ILO RM study. Some

of the markers showed strong association with oil QTL and provide logical candidate genes (Laurie et al., 2004). Expression analysis has been performed using tails of the phenotypic distribution for starch concentration of the (ILP × B73)B73 population and revealed genes that are differentially expressed. A related expression analysis study comparing inbreds derived from ILP and IHP, and B73 revealed genes that are differentially expressed (daSilva, Rocheford and Moose, unpublished results). These genes are now being placed on mapping populations to assess for linkage with QTL as well as being assayed on the maize diversity panel to search for associations with kernel composition. The strains have been used in the private sector to search for candidate genes associated with kernel composition. Some of these candidate genes are used in transgenic approaches to attempt to alter a kernel composition trait significantly with a single transgenic event. The rationale is to make modification of kernel composition more rapid and simpler for breeding purposes.

With changing times and technologies, different experimental approaches have been, and will be, applied to the long term selection strains to seek answers. A comprehensive series of genomic experiments could be performed on the strains. SNP marker analysis could be performed on hundreds and thousands of genes to look at allelic frequency changes over cycles of selection. This would enable more comprehensive assessment of selection of genes that potentially may have favorable effects. The assay of large numbers of genes may enable some assessment of genes that may have epistatic interactions. This is supported by the recent study on the IHO × ILO RM population suggesting the potential importance of QTL involved in epistatic interactions in control of oil levels and long-term gain from selection (Dudley, 2008). Further expression analysis could be performed not only between high and low strains, or high and reverse high strains, but also between different cycles of selections within a strain, to examine for changes in expression over cycles. With the advent of cheaper DNA sequencing costs, sequencing large genomic regions from many samples from the strains, or whole genome sequencing of some samples from the strains would be useful. The results may provide information on possible organization of favorable coupling phase alleles, and to provide information on intragenic recombination. Assessing the possible role of epigenetics on gain from long term selection is logical future study. The molecular marker analyses summarized here provided a set of new insights into the long term selection strains. A comprehensive set of large scale genomic analyses will likely provide another, potentially more revealing set of insights relevant to understanding allelic variation and response to selection in the long term strains.

7 Summary

The long-term selection strains have and continue to provide a resource that is useful in diverse ways, whether it is marker assisted selection or transgenics. There has been interest in high oil concentration maize for advantages in feeding monogastric animals. More recently there is considerable interest in high starch concentration in

maize grain for ethanol production efficiencies. Enhancing nitrogen efficiency for reducing groundwater contamination and cost savings is receiving increasing attention. Some of these studies would not be possible without the selection and maintenance of the strains over a long period of time. The strains provide a resource for academic inquiry and debate on the basis of gain from selection and limits to selection. These are questions relevant to all of plant breeding. The use of molecular markers provided new insights on levels of heterozygosity, allelic variation and frequency trends, mapping QTL controlling kernel composition, and an initial suggestion on possible role of intragenic recombination in the strains. The arsenal of current and future genomic technologies will enable further investigations of important questions and will take our understanding of the long term selection strains to the next level.

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QTL for Agronomic Traits in Maize Production

Roberto Tuberosa and Silvio Salvi

Abstract The utilization of molecular markers and genomics platforms offers unprecedented opportunities to discover, select, and clone the quantitative trait loci (QTL) that govern grain yield and other agronomic traits in maize. The dissection of the genetic basis of quantitative traits into their single components provides direct access to valuable genetic diversity for the morpho-physiological features that in maize regulate yield potential per se and the adaptive response to abiotic and biotic constraints. This in turn, enables us to utilize marker-assisted selection for enhancing maize performance and/or producing isogenic lines at target QTL to be cloned. This chapter summarizes the main results on the large number of studies that have described QTL for grain yield and other agronomic traits in maize. The availability of the sequence of the entire genome coupled with other genomics platforms will accelerate QTL discovery and cloning while facilitating a more effective exploitation of the allelic richness present at key QTL.

1 Introduction

Maize is presently the third most important source of calories for humankind after rice and wheat, and will most likely become the most important crop by 2020 (Rosegrant et al. 2001). The impressive surge in the utilization of maize grain for biofuel production further underlines the urgency to improve the agronomic performance of maize to ensure that a suitable and sustainable domestic supply will not be outpaced by the rapidly expanding global demand for maize-derived food, feed, and fuel.

Notwithstanding the pivotal contribution of conventional breeding to the spectacular increase in maize yield during the past century (Russell, 1991; Troyer, 1996; Duvick, 2005), maintaining and, hopefully, enhancing the yearly gain in maize production will require an unprecedented, multidisciplinary effort for the identification of agronomically superior alleles and their introgression into elite germplasm. These challenges are even more daunting in view of the increasing unpredictability and vagaries of rainfall and temperature patterns consequent to global climate change, the dwindling availability of irrigation water and phosphate fertilizer, the escalating

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cost of nitrogen fertilizer, and the necessity to improve the long-term sustainability of maize production. Because agronomic traits are quantitatively inherited, quantitative trait loci (QTL) discovery represents an unavoidable crossroad for enhancing yield and yield stability of maize production while maximizing its sustainability. Clearly, genomics approaches allowing for a more efficient discovery and manipulation of QTL will be instrumental and increasingly important for coping with the challenges faced by maize production (Lee, 2007; Ragot and Lee, 2007).

2 Mapping QTL in Maize: An Historical and Methodological Perspective

Although the concept of mapping loci with major effects on quantitative traits was first introduced by Sax in 1923, the earliest maize maps contained only a few linked loci for morphological traits and, as such, were ineffective for a meaningful scanning of the genome. After the pioneering work that laid the foundations and provided the statistical framework to enable QTL discovery (Soller et al., 1976, 1979), the first results for a genome-wide QTL search in maize were described two decades ago (Edwards et al., 1987; Stuber et al., 1987) based on isozyme markers. At about the same time, restriction fragment length polymorphisms (RFLPs) became a standard marker system (Helentjaris et al., 1985, 1986; Burr et al., 1988) to elaborate comprehensive genetic maps and engage more effectively in genome-wide QTL discovery. In the 1990s, the concerted efforts of the maize community laid the foundation for the assembly of a reference map (Beavis and Grant, 1991; Coe, 1993, 1998; Gardiner et al., 1993; Lee, 1995; Davis et al., 1999). Additionally, new marker types such as simple sequence repeats (SSRs; Taramino and Tingey, 1996), amplified fragment length polymorphisms (AFLPs; Vuylsteke et al., 1999), and single-nucleotide polymorphism (SNPs) accelerated the systematic dissection of quantitative traits and enabled a high-throughput marker-assisted selection (Salvi et al., 2001; Rafalski, 2002; Morgante and Salamini, 2003; Crosbie et al., 2006; Ragot and Lee, 2007).

Functional maps reporting the position of expressed sequence tags (ESTs) added a new dimension to QTL studies and ushered in the candidate gene approach for discovering the sequence underpinning a QTL (Causse et al., 1996; Okagaki et al., 2001; Tuberosa et al., 2002a; Falque et al., 2005). The first functional map in maize incorporated data on 275 loci obtained from four segregating populations (Causse et al., 1996). To further refine the mapping of ESTs, Falque et al. (2005) used two populations of intermated recombinant inbred lines (RILs), which allowed for a higher map resolution than non-intermated RILs. The first panel derived from B73 × Mo17 (IBM: Intermated B73 × Mo17), is publicly available from the Maize Genetics Cooperation Stock Center. The second panel (LHRF) was developed from F-2 × F252 to map loci monomorphic on IBM. In total, 1,454 loci that corresponded to 954 previously unmapped cDNA probes were mapped (Falque et al., 2005).

A number of articles and reviews have surveyed the statistical approaches that have made possible the emergence of the QTL archipelago in maize and other crops (Churchill and Doerge, 1994; Beavis, 1998; Kao et al., 1999; Zeng et al., 1999; Korol et al., 2001; Doerge, 2002, 2007; Jansen et al., 2003; Flint-Garcia et al., 2005; Ersoz et al., 2007; Gallais et al., 2007; Gao and Zhu, 2007). One factor that was quickly recognized for its importance for the accurate outcome of QTL studies was the size of the progeny under analysis. In fact, a major shortcoming of any QTL study is the low accuracy in detecting the real number and effects of QTL (Beavis et al., 1994; Charcosset and Gallais, 1996; Melchinger et al., 1998), particularly with mapping populations with fewer than 150–200 RILs. Beavis et al. (1994) conducted a study to verify if QTL for 24 agronomic traits could be identified by a small but representative sample of topcrossed and $F_{2:4}$ progeny derived from B73 × Mo17. The estimated number of QTL identified per trait varied from three to five. Nonetheless, the yield QTL identified by Beavis et al. (1994) differed from those previously reported for B73 × Mo17 by Stuber et al. (1987). Among the factors listed as the cause of these conflicting results, the different representativity of the samples of progeny was suggested as the most likely explanation.

Simulation studies and large-scale experiments in maize have been instrumental for testing new QTL models and for evaluating the factors influencing the false positive (Type-I error) and false negative (Type-II error) frequency. The study of Beavis (1998) showed that it is very difficult to detect QTL of small effect in populations with fewer than 500 progenies. These predictions were confirmed in experiments carried out with mapping populations sufficiently large (>400) to allow for meaningful sub-sampling (Openshaw and Frascaroli, 1997; Melchinger et al., 1998). In terms of scale, the most notable example is provided by the study of Openshaw and Frascaroli (1997) with 976 F_5 testcross progenies that were evaluated in 19 environments. Subsequently, a simulation study carried out to reanalyze the same set of data has demonstrated that the allocation of experimental resources (sample size, number of test environments, and significance threshold) greatly affects the power of QTL detection as well as the accuracy and precision of QTL estimates (Schon et al., 2004). The number of detected QTL and the proportion of genotypic variance explained by QTL generally increased more with increasing sample size than with a higher number of environments (Schon et al., 2004). These findings provide a useful framework for optimizing QTL mapping and resource allocation, a crucial factor particularly with agronomic traits of moderate and low heritability. Notably, the simulation study by Bernardo (2004) showed that preventing false-positive QTL will require the utilization of highly stringent significance level.

2.1 Segregating Populations, Congenic Progenies, and Panels

An important advancement for a more accurate mapping of genes and QTL was introduced by Darvasi and Soller (1995) with the concept of advanced intercrossed lines (AILs), an experimental population providing more accurate estimates of QTL

map location than conventional mapping populations. AILs are produced by randomly and sequentially intercrossing a population that initially originated from a cross between two inbred lines or some variant thereof. This increases recombination frequency between any two linked loci. Consequently, the genetic length of the entire genome greatly increases, providing a parallel improvement in mapping resolution. In this way, for example, with the same population size and QTL effect, a 95% confidence interval of QTL map location of 20 cM in the F_2 is expected to show an average five-fold reduction after eight additional random mating generations (F_{10}).

In maize, Lee et al. (2002) applied the AIL approach to assemble the IBM population, which has been widely used to map QTL for agronomic traits. Recently, an improved version of the map (ISU-IBM Map4) that integrates 2,029 existing markers with 1,329 new indel polymorphism markers has been released (http://magi.plantgenomics.iastate.edu; Fu et al. 2006). The advantage provided by the utilization of the IBM population was evident in the mapping of QTL for flowering time and resistance to southern leaf blight (Balint-Kurti et al., 2007). The results of this study showed that the IBM, when compared to a conventional RIL population, provided between five- and, in one case, 50-times greater mapping resolution.

Among the different categories of congenic strains, introgression library lines (ILLs) provide us with a valuable platform for a genome-wide search of QTL, and particularly, for rapidly advancing their fine mapping and positional cloning (Eshed and Zamir, 1995; Paran and Zamir, 2003). Surprisingly, despite the well-documented effectiveness of ILLs for the mapping and cloning of QTL in tomato (Paran and Zamir, 2003; Gur et al., 2004), this approach was only recently exploited in maize. Szalma et al. (2007) have assembled a set of 89 near-isogenic lines (NILs) representing 19 regions of the genome of a donor line (T × 303) introgressed into B73. MAS accelerated the recovery of the recurrent parental line genome during three backcrosses. The size of the genomic regions introgressed from T × 303 ranged from 10 cM to 150 cM, with an average length of ca. 60 cM. The NILs and their testcrosses with an unrelated line (Mo17) were evaluated for flowering time traits in two environments that differed greatly for water availability. Different sets of QTL were detected across environments and between category of accessions (inbreds vs. hybrids), thus highlighting the genetic complexity of flowering time in maize.

A similar approach was also undertaken by Salvi et al. (2007a) using Gaspé Flint as a donor and B73 as recurrent parent. In this case, 70 partially overlapping genomic regions (from ca. 10 cM to ca. 50 cM) representing all chromosomes were introgressed through five marker-assisted backcrosses. In view of the extreme earliness of Gaspé Flint, the B73 × Gaspé Flint ILLs are ideal for identifying QTL for flowering time. A preliminary evaluation has singled out five QTL whose additive effect varied from 2 days to 6 days (Salvi et al., 2007a). Additionally, the B73 × Gaspé Flint ILLs have also been screened for root traits and a number of relevant QTLs have been mapped (S. Salvi, personal communication).

An important breakthrough in QTL mapping and cloning came about with the introduction of panels of unrelated accessions suitable for association mapping and characterized by low linkage disequilibrium (Flint-Garcia et al., 2005; Buckler et al., 2006; Yu and Buckler, 2006; Ersoz et al., 2007; Yu et al., 2008). Despite the

distinct advantages of association mapping on biparental linkage mapping (e.g. multilallelism, higher genetic variability and genetic resolution, shorter time to obtain the results, etc.), a major constraint to its utilization is represented by the high rate of Type-I error, hence spurious association, that can be generated by the presence of population structure. To reduce the risk of detecting spurious associations, Yu et al. (2006) have developed a unified mixed-model approach for QTL dissection able to account for multiple levels of relatedness simultaneously as detected by random genetic markers. This approach was applied to a sample of 277 diverse maize inbred lines with complex familial relationships and population structure. As compared to other methods, this new one allowed for an improved control of both Type-I and Type-II error rates (Yu et al., 2006; Ersoz et al., 2007).

A panel of 302 lines was made available by Buckler and coworkers as a valuable research tool that captures a large proportion of the alleles present in cultivated maize (Flint-Garcia et al., 2005). On average, Flint-Garcia et al. (2005) estimated that population structure accounted for 9.3% of phenotypic variation among lines with a high of 35%. Therefore, inclusion of population structure in association models is critical to meaningful analyses. This new association population has the potential to unveil QTL with small effects, a valuable feature for the dissection of agronomic traits.

A clear example of the resolution power of association mapping was recently provided by the work of Salvi et al. (2007) for the validation of the role of *Vgt1*, a ca. 2.0 kb-long, non-coding sequence that controls a major QTL for flowering time in maize. In this case, a study conducted to investigate the association between flowering time of 100 unrelated lines and the polymorphisms present at 192 sequences within the 700 kb flanking the *Vgt1* region, pointed out that functional natural allelic variation was present at *Vgt1* and absent at the nearby locus *ZmRap2.7*, a structural gene that encodes for a transcription factor known to regulate flowering time in *Arabidopsis*.

An additional alternative to QTL detection is offered by in silico mapping based on existing phenotypic and genomic databases. The validity of in silico mapping was tested by Parisseaux and Bernardo (2004) with multilocation data collected during 7 years on 22,774 single crosses belonging to nine heterotic groups and derived from 1,266 inbreds that were profiled with 96 SSRs. Using a mixed-model approach, the SSR loci with significant general combining ability effects for plant height, smut (*Ustilago maydis* [DC.] Cda.) resistance, and for grain moisture were consistent with previous results obtained with biparental populations. Each trait had many loci with small effects and few loci with large effects. Based on these results, Parisseaux and Bernardo (2004) concluded that in silico mapping (a) can detect associations that are repeatable across different populations and (b) will be more useful for gene discovery than for selection.

2.2 QTL Galore: Consensus Maps and Meta-Analyses

In any crop, a more effective exploitation of the plethora of published QTL data greatly benefits from the availability of a consensus map and a comprehensive database

able to provide a unified picture of the QTL landscape. Consensus QTL maps coupled with haplotype profiling are a useful resource to more effectively capture valuable allelic variation at target regions while avoiding adverse effects due to linked loci. A more comprehensive understanding of the QTL landscape of target traits would enable the breeders to take more informed decisions for a more effective MAS. Clearly, major QTL that might be highlighted in different genetic backgrounds are preferable targets for MAS as compared to QTL of small effect and/or that are detected exclusively in one genetic background (Schaeffer et al., 2006).

The comparative analysis between QTL data of two or more mapping populations is made possible by directly using the information provided by markers common to the maps being compared and/or indirectly by referring each mapping population to a reference map. In maize, a widely-used reference is the UMC map (Davis et al., 1999; Coe et al., 2002; available at MaizeGDB: http:/www.maizegdb.org). To facilitate the cross-referring among QTL studies, the UMC map has been subdivided into 103 sectors (bins), each spanning ca. 10–20 cM, i.e. an interval roughly equivalent to the level of resolution of most QTL. The boundaries of each bin are defined by flanking markers included in a public set of Core Markers (Gardiner et al., 1993; Davis et al., 1999). The UMC map reports over 15,000 loci and include genes, probed sites, cytological breakpoints, and QTL (Schaeffer et al., 2006). Because the bin framework integrates over 130 independent map sets and includes all mapped loci stored in MaizeGDB (http://www.maizegdb.org), it allows for the comparison of QTL positions across genetic backgrounds (Lin et al., 1995; Khavkin and Coe, 1997; Tuberosa et al., 2002b, 2003, 2005; Chardon et al., 2004; Sawkins et al., 2004; Schaeffer et al., 2006; Wang et al., 2006). Gramene (http://www.gramene.org) is another database that reports information on maize QTL and allows for comparative searches of maize genomics data with other grasses (Ware et al., 2002). Additionally, the UMC map allows us to compare the map position of mutants (Neuffer et al., 1997) with that of QTL, thus contributing relevant information for the identification of possible candidate genes for a particular trait.

In maize, following a few empirical attempts to identify key regions controlling developmental features (Khavkin and Coe, 1997) and morpho-physiological traits (Tuberosa et al., 2002b, 2003), a more systematic approach towards consensus QTL maps has been pursued with the adoption of computational platforms that allow for the integration of information from multiple sources (Schaeffer, 2006). Goffinet and Gerber (2000) proposed a meta-analysis approach that provides clues on how many QTL are actually represented by the QTL detected in different experiments and allows for a consistent reduction of the QTL confidence interval when there are only very few actual QTL locations. The validity of this approach was tested using an extensive set of data from the MaizeGDB. Sawkins et al. (2004) have developed a Comparative Map and Trait Viewer (CMTV http://cropwiki.irri.org/icis/images/0/0c/AG06_CMTV.pdf) tool that can be used to construct dynamic aggregations of a variety of types of genomic datasets and to display syntenic regions across taxa, combine maps from separate experiments into a consensus map, or project data from different maps into a common coordinate framework. The utility of the CMTV software was tested with a large maize dataset collected at CIMMYT.

Once a genomic region of interest is identified, the CMTV can search and display additional QTL reaching a particular threshold for that region or other functional data such as sets of differentially expressed genes located in the region. An example of the use of the CMTV to align and visualize QTL data from different traits and across experiments is reported in Sawkins et al. (2004). Recently, Veyrieras et al. (2007) have presented a new computational and statistical package, called MetaQTL (http://bioinformatics.org/mqtl), for carrying out whole-genome meta-analysis of QTL mapping experiments. Differently from the previous methods, MetaQTL establishes a consensus model for both the marker and the QTL positions on the entire genome. In particular, MetaQTL deploys a new clustering approach to estimate how many QTL underline the distribution of the observed QTL. Simulations showed that MetaQTL leads to a reduction in the length of the confidence interval of QTL location provided that across studies there are enough observed QTL for each underlying true QTL location. The usefulness of the MetaQTL approach was illustrated on the basis of published QTL detection results for flowering time in maize (Veyrieras et al., 2007).

2.3 Searching for Valuable QTL Alleles in Unadapted and Wild Germplasm

The domestication process has drastically reduced the genetic variability present in crops compared to their wild counterparts. This limitation can be partly overcome through advanced backcross-QTL (AB-QTL) analysis, an approach that offers the opportunity to quickly discover and exploit beneficial QTL alleles present in wild germplasm (Tanksley and Nelson, 1996). The AB-QTL approach relies on the evaluation of backcross (BC) families between an elite variety used as recurrent parent and a donor accession, usually a wild species sexually compatible with the crop. In maize, three studies have adopted the AB-QTL strategy to identify QTL for yield and other agronomic traits (Ho et al., 2002; Birolleau-Touchard et al., 2007; Li et al., 2007). Nonetheless, these three AB-QTL studies have utilized elite parental lines and not unadapted germplasm.

Wild relatives can provide breeders with important clues to identify novel alleles for agronomically relevant traits by focusing on loci targeted by selection during both domestication and modern breeding (Vigouroux et al., 2002). To this end, the comparative analysis of a screening of the allelic diversity present in elite accessions, landraces, and the undomesticated wild relative of a target crop allows for the identification of loci devoid of genetic variation within the elite germplasm as a result of domestication and subsequent man-made selection (Yamasaki et al., 2005). In this case, the assumption is that the loss of genetic diversity observed from the wild parent to the cultivated crop underpins the strong selection at loci controlling traits of agronomic importance. Therefore, this diversity screen approach provides the distinct advantage of identifying agronomically valuable loci that would otherwise go undetected owing to a lack of allelic diversity in the

genetic pool presently cultivated. Additionally, the diversity screen approach allows for the identification of candidate genes of potential agronomic importance even without prior knowledge of gene function and the phenotype of interest. Nonetheless, Yamasaki et al. (2005) have recognized that the applicability of the diversity screen approach is limited by a number of factors, most notably that some of the identified genes may only be hitchhiking with selected genes present in their vicinity. The validity of diversity screening has been tested in maize (Yamasaki et al., 2005), a species particularly suited for this approach due to the existence of a known wild progenitor (teosinte), its extensive allelic richness, and at the same time, the existence of many landraces still cultivated (Doebley et al., 1990, 1995; Doebley, 1992; Bennetzen et al., 2001; Westerbergh and Doebley, 2002).

3 QTL for Traits of Agronomic Interest

The agronomic performance of maize is influenced by many morpho-physiological features whose variability has been empirically exploited by breeders to steadily improve grain yield (GY) and its stability. The landmark review of Duvick (2005) presents a detailed, critical assessment of the traits that have contributed to improve the agronomic performance of maize since the introduction of the first hybrids. Several decades of breeding have brought noticeable changes for architectural features of maize both at the single plant level and crop level. A better understanding of the QTL that underlines these traits would provide new impetus and opportunities for more targeted selection programs based on MAS of the relevant QTL. Additionally, QTL studies allow us to investigate cause-effect relationships between traits (Lebreton et al., 1995; Prioul et al., 1997, 1999; Sanguineti et al., 1999; Tuberosa et al., 1998, 2002b). Hereafter, the results of some of these studies have been summarized for a number of morpho-physiological features known to affect field performance of maize.

3.1 Plant Architecture

Plant architecture plays a predominant role for maximizing the capture of the resources (water, nutrients, and radiation) required by each crop to produce a harvestable product. The remarkable importance of plant architecture in maize is well underlined by the retrospective analysis of the hybrids released during the past 50 years (Russell, 1974, 1984, 1985, 1991; Duvick, 1977, 1992, 2005; Tollenaar, 1989, 1991; Duvick and Cassman, 1999; Sanguineti et al., 2006). Among the agronomic traits that have changed more markedly as a result of selection, leaf angle (Crosbie, 1982) and root lodging (Duvick, 1977, 1992; Russell, 1974, 1985) have played a predominant role in driving the historical increase (ca. four-fold) that has occurred in plant density from 1950 to the present, the single most important factor

responsible for the improvement observed in biomass and grain production per unit of cultivated surface. High plant density increases the adverse effects of both abiotic and biotic stresses and thus requires an improved stress tolerance (Troyer, 1996). Interestingly, this improvement is not evident under spaced conditions, a finding that underlines the importance of the management component to maximize at the crop level the yield potential of each genotype (Duvick, 2005).

3.1.1 Root

The difficulty in measuring root characteristics in the field has hindered the identification in such conditions of the relevant QTL. Additionally, studies on roots of field-grown plants often require destructive approaches and are not very informative owing to the low heritability of root features consequent to high heterogeneity in soil structure and composition. As an alternative to root surveys in field-grown plants, studies implemented in controlled conditions (e.g. hydroponics, aeroponics, pots, etc.) facilitate the measurement of root characteristics in a large number of plants. Nonetheless, the unnatural environment in which the root grows and the early growth stage that is usually considered in such studies are major shortcomings that should be cautiously considered before extrapolating the results to field-grown plants.

In maize, a significant, albeit weak, positive association has been reported between seminal root traits in hydroponics and root pulling resistance in the field (Landi et al., 2001). Seminal roots play a prominent role in nutrient acquisition by plant seedlings and thus influence early vigour, a feature particularly relevant under conditions of zero or minimum tillage characterized by low agronomic input and a limited use of herbicides. The length and number of seminal roots may be particularly important in the acquisition of immobile nutrients such as phosphorus (Kaeppler et al., 2000; Zhu et al., 2005, 2006; Lynch, 2007). In maize, as the plant reaches flowering, the importance of the seminal roots declines as compared to shoot-born roots, commonly named adventitious nodal roots (Kiesselbach, 1949; Hochholdinger et al., 2004), which in water-limited conditions can play a rather beneficial role on GY (Navara et al., 1994; Jesko, 2001). A comprehensive survey on QTL for root traits in maize was presented by Tuberosa et al. (2003).

Following the early report of Lebreton et al. (1995) on QTL controlling root pulling force (RPF) and abscisic acid (ABA) concentration in the root of Polj17 × F-2, Tuberosa et al. (2002b) described several QTL affecting root architectural features in 171 F_3 families derived from Lo964 × Lo1016 and grown in hydroponics. The rather high LOD values (>5.0) of 10 of the 37 QTL and their sizeable R^2 values (from 14.7% to 32.6%) suggested that a number of QTL with major effects on root traits segregate in this population. In order to verify to what extent the QTL regions influencing variation in root traits in hydroponics may also modulate root traits in the field, a random sample of 118 Lo964 × Lo1016 F_3 families were investigated near flowering for RPF in field trials (Landi et al., 2002). Among the 30 bins with QTL for number of brace roots and/or RPF, 15 (representing 19% of the 80 bins explored by the Lo964 × Lo1016 map) also harboured a QTL for one or more of

the root traits measured in hydroponics. A comparative analysis of the QTL studies on root architecture highlighted the important role of bin 1.06. This region has been shown to harbour QTL for root traits in Polj17 × F-2 (Lebreton et al., 1995), F288 × F271 (Barriere et al., 2001), Ac7729 × Ac7643/TZSRW (Tuberosa et al., 2003), B73 × Mo17 (Kaeppler et al., 2000; Zhu et al., 2005), and Lo964 × Lo1016 (Tuberosa et al., 2002b). Additionally, bin 1.06 also harbours QTL for GY at different water regimes in Lo964 × Lo1016 (Tuberosa et al., 2002b) and at two N levels in F-2 × Io (Hirel et al., 2001). These results highlight the importance of bin 1.06 for determining GY under conditions characterized by limited water availability and/or N supply. Near isogenic lines (NILs) differing for the parental segment at this QTL region on bin 1.06 have now been produced to more accurately evaluate its direct effect on root traits and the associated effects on GY as well as other agronomic traits (S. Salvi, personal communication).

Strong effects on root architecture have also been reported for bin 1.03 in DTP79 × B73 (Steve Quarrie, personal communication) and in Lo964 × Lo1016 (Tuberosa et al., 2002c). Interestingly, the *rtcs* (*rootless for crown and lateral seminal root*) locus recently cloned by Taramino et al. (2007) has been mapped ca. 15–20 cM away from *umc11* in bin 1.03 (Hochholdinger and Feix, 1998).

Another interesting QTL for root architecture has been assigned to bin 2.04 (Giuliani et al., 2005). The effects of this QTL on root traits and ABA concentration were first reported by Lebreton et al. (1995) in Polj17 × F-2. Subsequently, this region was shown to affect root architecture, root lodging, leaf ABA concentration (L-ABA), and GY in the cross Os420 × IABO78 (Tuberosa et al., 1998; Landi et al., 2001; Giuliani et al., 2005). Based upon such results, a tentative model accounting for the effects of this QTL (*root-ABA1*) was suggested by Giuliani et al. (2005). In order to gain further insights on the effects of *root-ABA1*, NILs at this QTL have been developed from each parental inbred following a marker-assisted backcross procedure (Landi et al., 2005). These sets of backcross-derived lines were evaluated in two watering regimes (well-watered and water-stressed), both *per se* (Landi et al., 2005) and in hybrid combination (Giuliani et al., 2005; Landi et al., 2007). These evaluations confirmed the effect of *root-ABA1* on L-ABA, root traits, and GY. The fine mapping of *root-ABA1* is underway (S. Salvi, personal communication).

In maize, QTL have been identified for root hair length and plasticity in response to phosphorus stress. By enhancing soil exploration, root hairs play an important role in the uptake of immobile nutrients such as phosphorus. A paper-roll culture system was used to investigate root hair length (RHL), tap root length, root thickness, and root biomass in B73 × Mo17 (Zhu et al., 2005). One QTL was associated with RHL plasticity, three QTL with RHL under high fertility, and one QTL with RHL under low phosphorus. Six QTL accounted for 53.1% of the total variation for seed phosphorus among RILs. Root biomass plasticity was significantly correlated with RHL induced by low phosphorus, taproot length plasticity, and seed phosphorus reserves.

Root architecture plays an important role also in tolerance to soil flooding or waterlogging. Root and shoot traits were investigated in two experiments to identify QTL associated with waterlogging tolerance in HZ32 × K12. Several major

QTL for shoot dry weight. root dry weight, total dry weight, plant height, and their waterlogging tolerance coefficient each mapped on chrs. 4 and 9. These QTL were detected consistently in both experiments. Secondary and more trait- or environment-specific QTL influencing tolerance were also identified on chrs. 1, 2, 3, 6, 7, and 10.

Adventitious root formation (ARF) at the soil surface is one of the most important adaptations to soil flooding or waterlogging. QTL for ARF were evaluated by Mano et al. (2005b) under flooding conditions in 110 F_2 plants derived from a cross between the dent line B64 with the tropical Caribbean flint line Na4. The QTL for ARF were located on bins 3.07-8, 7.04-5, and 8.05. At all QTL, the alleles of line Na4 increased ARF. The comparison of ARF QTL in the B64 × Na4 population with those in a B64 × teosinte (*Zea mays* ssp. *huehuetenangensis*) population showed the consistency of the chr. 8 QTL (Mano et al., 2005a). *Zea mays* ssp. *huehuetenangensis* contributed all of the favorable QTL alleles for ARF, suggesting its potential to transfer waterlogging tolerance to maize and thus supporting the conclusions of Campos et al. (2004) concerning the value of exploring genetic variation from outside cultivated maize to improve its root architecture and functions. On a similar line, QTL for aerenchyma formation in roots, another important feature for adaptation to water logging, were identified by Mano et al. (2007) using an F_2 population generated from the B64 × teosinte (*Zea mays* ssp. *nicaraguensis*) cross. Seedlings of *Zea nicaraguensis* clearly formed aerenchyma in the cortex of adventitious roots in non-flooding conditions, whereas the maize inbred line B64 did not. Four QTL for aerenchyma formation under non-flooding conditions were located to two regions of chr. 1 (*Qaer1.02-3* and *Qaer1.07*), chr. 5 (*Qaer5.09*), and chr. 8 (*Qaer8.06-7*); collectively, these regions accounted for 46.5% of the total phenotypic variance for aerenchyma formation (Mano et al., 2007). SSR markers linked to QTL for aerenchyma formation in drained soil conditions could be deployed for the development of maize hybrids with an increased tolerance to flooding and a greater yield stability under such conditions.

3.1.2 Leaf and Inflorescence

Light penetration into the canopy is an important factor for photoassimilate production in any crop. Agricultural practices (e.g. planting density) and plant morphological features such as leaf angle, leaf size, and tassel size are the main factors that affect light penetration in maize. The importance of these factors in modern maize production is witnessed by the substantial change that has occurred during the past 50 years of breeding in the canopy architecture of maize (e.g. more upright leaves) and the sizeable reduction in tassel size (Duvick, 2005). While canopy architecture has a prevailing impact on the capture of incoming radiation, tassel size is also relevant for its high metabolic cost, an important factor in view of the strong competition for assimilates with the ear at flowering, when a depletion in photoassimilate flow to the ear has been shown to reduce reproductive fertility (Boyle et al., 1991; Schussler and Westgate, 1995; Zinselmeier et al., 1995;

McLaughlin and Boyer, 2007). Additionally, a worthy aspect is that tassel inflorescence architecture is also important for an efficient commercial production of hybrid seed.

The genetic basis of leaf growth in maize has been investigated with a novel approach that has allowed Tardieu and coworkers (Reymond et al., 2003, 2004; Tardieu et al., 2005) to identify QTL affecting organ growth in response to environmental factors (e.g. temperature, soil moisture, etc.). Their work provides an example on how ecophysiological modelling and QTL analysis can be integrated to investigate the genetic basis of the plasticity of organ growth based on QTL for the response to environmental constrains (e.g. drought, high temperatures, soil salinity, etc.) and how this approach might help us to more appropriately resolve genotype × environment (G × E) interactions (Yin et al., 2003, 2004; Hammer et al., 2005, 2006). Several factors have been postulated to affect leaf growth: leaf carbon balance, turgor pressure, cell wall plasticity, and cell division rate, with contributions from growth regulators such as ABA or ethylene. Because the precise role of each of these mechanisms is still controversial and involves several gene families, it has been argued that gene regulatory networks are not feasible for modelling such complex systems (Tardieu et al., 2005). Conversely, leaf growth could be better modelled via response curves to environmental conditions that are considered as "meta-mechanisms" acting at a higher degree of organization through the integration of various metabolic factors and external cues.

QTL for leaf growth in relation to water vapour pressure difference, soil water status, and meristem temperature were first described in maize by Reymond et al. (2003, 2004). The responses of leaf elongation rate to evaporative demand and to soil water status in 100 RILs derived from Io × F-2 were common to several experiments for each genotype, implying that the ranking of genotypes across experiments was consistent. QTL of ABA concentration in the xylem sap colocalized with QTL of response to soil water deficit and conferred a low response. Interestingly, a strong and stable QTL for leaf width was unrelated to QTL for leaf length, hence suggesting that leaf length and width are governed by different genetic factors. Remarkably, a model based on the combined QTL effects predicted 74% of the variability for maize leaf elongation rate measured among a random sample of RILs of the population used for QTL detection (Reymond et al. 2003). Of the several QTL identified, most were specific for their response to only one variable.

Leaf growth and anthesis-silking interval (ASI, i.e. the interval between pollen shed and silk extrusion) are major determinants of source-sink relationships in maize and are both influenced by the expanding ability of the relevant organs (leaves and silks). ASI is negatively correlated with GY, particularly under water-stressed conditions (Bolanos and Edmeades, 1993, 1996; Campos et al., 2004, 2006). Welcker et al. (2007) investigated to what extent leaf growth and ASI may share a common genetic basis in Ac7643 × Ac7729/TZSRW, F-2 × F252, and F-2 × Io. Maximum leaf elongation rate per unit thermal time and the slopes of its responses to evaporative demand and soil water status were measured in greenhouse and growth chamber experiments, while ASI was measured under both well-watered and water-stressed field conditions. Maximum elongation rate per unit

thermal time in well-watered plants was accounted for by five QTL, three of which co-localized with QTL of ASI of well-watered plants. The additive effects for leaf elongation rate were positively associated with those for high rate of silk elongation (i.e. low ASI). The responses of leaf elongation rate to evaporative demand and to soil moisture were linear, a finding that allowed for the characterization of each RIL with the slope of the response curves (Welcker et al., 2007). Three QTL for the variability in such slopes overlapped with those for ASI of water-stressed plants. Also in this case, the allele for leaf growth maintenance was positively associated to maintenance of silk elongation rate. Conversely, a number of QTL for ASI did not influence leaf growth. Based on these findings, Welcker et al. (2007) postulated that their results may have profound consequences for modelling G × E interaction and for tailoring drought-tolerant plants. In a companion study, a larger set of RILs (318 in total) derived from Ac7643 × Ac7729/TZSRW were evaluated for daily pattern of leaf growth under naturally fluctuating temperature and evaporative demand (Sadok et al., 2007). The RILs carrying QTL alleles conferring a high response to temperature had markedly higher night-time plateau of leaf growth than those with low responses. Groups of RILs with high response to evaporative demand had rapid decreases in leaf growth at the transition between night and day, while this decrease was slower in groups of RILs with low response. Similarly to the study of Welcker et al. (2007), these results provide important insights for a better understanding and manipulation of the genetic basis of the kinetics of the responses of maize to environmental cues.

QTL studies can provide useful insights on the evolutionary mechanisms that have shaped the architecture of modern maize (Doebley, 1990, 2006). The evaluation conducted by Bomblies and Doebley (2006) with different genetic backgrounds showed significant associations between several QTL and copy number of the duplicate FLORICAULA/LEAFY orthologs *zfl1* and *zfl2*, two important regulatory genes. In particular, *zfl1* was more strongly associated with flowering time, while *zfl2* showed a stronger association with branching and inflorescence structure traits, suggesting some divergence of function. Since *zfl2* associates with quantitative variation for ear rank and also maps near a QTL on chr. 2 controlling ear rank differences between maize and teosinte, Bomblies and Doebley (2006) tested whether *zfl2* might have been involved in the evolution of this trait using a QTL complementation test. The results indicated the importance of *zfl2* activity for the QTL effect, and supported *zfl2* as a candidate gene for a role in morphological evolution, hence domestication, of maize.

Westerbergh and Doebley (2002) analyzed the genetic basis of tassel architecture using a large (425 plants) F_2 population derived from the cross of two wild species of teosinte (*Zea diploperennis* and *Zea mays* ssp. *parviglumis*). For each one of the six traits that were investigated, 2 to 8 QTL were detected, for a total of 33 QTL. The location of the QTL suggested pleiotropy and/or linkage of QTL for several traits. Additionally, the small to moderate magnitude of QTL effects suggested the absence of major QTL of large effect on tassel morphology.

QTL for leaf angle and tassel morphology were described in a B73 × Mo17 RIL population that was tested in multiple environments (Mickelson et al., 2002).

Three QTL for tassel branch angle accounted for 36% of the phenotypic variation. For tassel branch number, three of the six relevant QTL were mapped on chr. 2. For leaf angle, nine QTL were detected in one or more environments. The significant phenotypic correlations detected between tassel branch angle versus tassel branch number and between tassel branch number versus leaf angle were partially supported by a corresponding QTL overlap on chr. 2 near *umc53a*, and also on chr. 5 near *bnl6.10*, a finding that suggests a common genetic basis for these traits.

Upadyayula et al. (2006a, 2006b) searched for QTL affecting a number of measured and calculated features of tassel architecture using a backcross population derived from two lines (IHO and B73) diverging for tassel architecture. A Principal Component (PC) analysis approach was used to examine relationships among traits and identify the main genetic factors responsible for such associations. Traits with high loadings for PC1 were branch number and branch number density, for PC2 were spikelet density on central spike and primary branch, and for PC3 were lengths of tassel and central spike. In total, 45 QTL for individual architecture traits and eight QTL for the three PCs were detected. Some of these calculated traits QTL were detected in regions that did not show QTL for the measured traits, suggesting that calculation of ratios may reveal developmentally relevant patterns of tassel architecture (Upadyayula et al., 2006a). For control of inflorescence architecture, important QTL were identified in bins 7.02 and 9.02. The interval *phi034-ramosa1* (*ral*) in bin 7.02 was associated with six individual architecture trait QTL and accounted for the largest amount of phenotypic variation (17%) for PC1. The interval *bnlg344-phi027* in bin 9.02 explained the largest amount of phenotypic variation (15%) for PC2. Although a number of inflorescence architecture QTL were detected in regions corresponding to candidate genes (e.g. *thick tassel dwarf1* and *ral*), the vast majority of QTL mapped to regions on chrs. 6, 8, and 9 without known candidate genes, thus providing useful information towards the discovery of new genes for control of inflorescence architecture.

3.1.3 Plant and Ear Height

In temperate maize, breeding activities conducted during the past decades appear to have slightly reduced ear height (EH) but not plant height (PH; Duvick, 2005). The slight decrease in EH likely relates to the selection for reducing the incidence of lodging. Among the agronomic traits considered in this chapter, PH is the one for which the largest number of mutants have been mapped, a feature that provides an interesting opportunity for comparing their map position with the peaks of the profiles of QTL for PH.

The early work of Beavis et al. (1991) showed that QTL for PH identified in a given small population were not consistent with QTL identified in other small populations. Additionally, it was noted that most of these QTL mapped in close proximity to known qualitative genetic loci, thus supporting Robertson's hypothesis (Robertson, 1985), and providing indirect evidence that real QTL can be

identified even with RIL population of relatively modest size. The validity of Robertson's hypothesis was investigated by Touzet et al. (1995) with a segregating population derived from the cross of two NILs differing for a QTL that controls PH on chr. 9 near the centromere. Both map position and physiological criteria suggested the gibberellin biosynthesis gene *dwarf3* as the most suitable candidate gene for this QTL. More recently, Tang et al. (2007) have reported six QTL for PH which were consistent with previously reported ones. Additionally, they reported eight QTL for leaf number, seven for internode number, and six for average internode length. Four of six QTL detected for average internode length were located on the same chromosomal region as the QTL affecting PH and shared common molecular markers. Based on this latter result, Tang et al. (2007) suggested that average internode length was the main contributor to PH in maize.

As compared to temperate germplasm, maize tropical germplasm has been less extensively explored for QTL discovery (Bohn et al., 1997; Groh et al., 1998; Khairallah et al., 1998). Because tropical germplasm has a broader genetic base than temperate synthetic materials, the evaluation of tropical material may uncover novel QTL that are devoid of significant variability in temperate maize. Based on this consideration, 400 $F_{2:3}$ lines derived from L-08-05F × L-14-4B were evaluated in five environments for PH, EH, GY, and grain moisture (Sibov et al., 2003). Based on the mean values in the five environments, four QTL were mapped for PH on chrs. 1, 2, and 7, explaining 24.8% of phenotypic variation. For EH, five QTL were detected on chrs. 1, 7, and 9 explaining 20.9% of the phenotypic variation. QTL profiles for PH and EH overlapped on chr. 1 in the *bnlg0615-phi0037* interval and on chr. 7 in the *dupssr13-umc1154* interval where a GY QTL was also mapped. The comparative analysis of these results with those reported for temperate maize indicated that the QTL described in L-08-05F × L-14-4B had already been identified in maize (Beavis et al., 1994; Berke and Rocheford, 1995; Austin and Lee, 1996a, b; Ribaut et al., 1997; Melchinger et al., 1998).

Recently, a meta-analysis on QTL for PH and other agronomic traits was carried out by Wang et al. (2006) based on 1,201 published QTL. The integrated map showed QTL clustering for various traits in all chromosomes. As to PH, a total of 127 QTL were refined to 40 consensus QTL. A comparative analysis suggested colinearity of 22 PH QTL of maize with 64 PH QTL of rice, and 43 GY QTL of maize with 7 GY QTL of rice.

3.2 Lodging Resistance

The evaluation of the historical series of maize hybrids indicates that although the overall trend is toward improved resistance to lodging, this improvement seemingly stopped from time to time (Duvick, 2005). Nevertheless, modern hybrids are considerably more resistant to lodging than older hybrids, particularly at high planting density, a condition that clearly accentuates this difference.

3.2.1 Root Lodging

Although a rather large genotypic variability in root lodging has been reported in maize (Melchinger et al., 1986), the low heritability and unpredictability of root lodging in the field coupled with the high cost required to carry out a large-scale evaluation using artificial devices (Guingo and Hebert, 1997) hinder the improvement of this trait. The identification of QTL for root lodging would allow breeders to select irrespectively of the local environmental conditions and seasonal constraints. It has been shown that root architecture is a major factor influencing root lodging (Ennos et al., 1993). Guingo et al. (1998) measured for two seasons a number of root traits in 100 field-grown RILs from the cross between F-2 (root-lodging susceptible) and Io (root-lodging resistant). The only QTL that concomitantly influenced a number of root traits (adventitious root number at internodes 7 and 8, and root angle at internode 7) mapped in the *SC343B-C403* interval on bin 5.05. Epistasis was invoked by Guingo et al. (1998) to account for the small number of QTL detected in their study. A larger number of QTL for root features was identified by Barriere et al. (2001) in a population of 131 RILs derived from F288 × F271. Interestingly, among the nine QTL that were shown to influence root diameter, root angle, and/or the number of adventitious roots at internodes 6, 7, and 8, the most sizeable one was on bin 1.06, where QTL for root architecture were reported in four other populations (Tuberosa et al., 2003).

A QTL with a marked effect on root lodging and root architecture has been described on bin 2.04 in the Os420 × IABO78 background (Giuliani et al., 2005; Landi et al., 2005, 2007; for details see Sect. 3.1.1).

3.2.2 Stalk Lodging

Stalk lodging occurs when the stalk breaks at or below the ear, which may in turn cause loss of the ear at harvest. The main determinants of stalk lodging are the mechanical strength of the stalk and the stalk-tunnelling action of the second-generation of the European corn borer (2-ECB) (*Ostrinia nubilalis* Hubner). Worldwide, it has been estimated that stalk lodging in maize causes yield losses estimated to range from 5% to 20%. Because the intensity of stalk lodging can vary greatly from season to season and because accurate phenotyping for stalk lodging is rather laborious, breeders have used rind penetrometer resistance (RPR) to measure stalk strength and improve stalk lodging resistance. Accordingly, selection for RPR has proven useful in enhancing germplasm for stalk strength, and therefore improving stalk lodging resistance. QTL have been described for both RPR and 2-ECB damage (Cardinal et al., 2001, 2006).

Flint-Garcia et al. (2003a) searched for RPR QTL in four $F_{2:3}$ populations obtained by means of combinations of MoSCSSS-High (selection for high RPR), MoSCSSS-Low (selection for low RPR), and MoSQB-Low (selection for low stalk-crushing strength), B73, and Mo17. In total, 8, 10, 8, and 9 single-effect QTL, and 4, 2, 0, and 5 epistatic interactions were detected for RPR in each one of the four populations. Multilocus models, including the single-effect QTL and epistatic interactions, accounted

for 33%, 45%, 48%, and 59% of the total phenotypic variation. Only one QTL was common to all four populations, while two QTL were detected in three of the four populations and five QTL were in common between two populations. These data highlighted the complex nature of stalk strength in maize. Based on the information available in the database, candidate genes that map within the QTL confidence intervals were identified; these genes are involved in lignin synthesis, the phenylpropanoid pathway, and the timing of vegetative phase change (Flint-Garcia et al., 2003b). The same three populations were used by Flint-Garcia et al. (2003a) to compare the efficiency of phenotypic selection (PS) versus MAS for both RPR and 2-ECB. Although MAS was effective in all three populations, in two of the populations PS for RPR was more effective than MAS. Interestingly, in the third population, MAS for high RPR based on QTL effects from the same population was more effective than PS, and using QTL effects from a separate population was just as effective as PS. MAS for resistance and susceptibility to 2-ECB using QTL effects from the same population increased susceptibility, but not resistance. MAS using QTL effects from a separate population was effective in both directions of selection. Thus, MAS was effective in selecting for both resistance and susceptibility to 2-ECB. These results validated the locations and effects of QTL for RPR and 2-ECB resistance identified in earlier studies and led Flint-Garcia et al. (2003b) to conclude that MAS can be applied effectively to improve both RPR and 2-ECB resistance.

Because a number of studies had indicated a significant negative correlation between RPR and EH, the correlation between RPR and EH is of interest in attempting to understand to what extent selection for high RPR has resulted primarily in increased stalk strength *per se* and coincidentally lower EH, or whether selection for high RPR resulted in lower EH and a subsequent increase in stalk strength. To unravel the cause-effect relationship between RPR and EH, Flint-Garcia et al. (2003c) measured EH in the same three $F_{2:3}$ populations used for their companion study aimed to identify QTL for RPR and ECB (Flint-Garcia et al., 2003b). Adjusting RPR for EH across populations caused loss of significance for 11 of the 26 original RPR QTL. Because EH clearly had an effect on RPR, adjusting RPR for EH likely resulted in more accurate descriptions of QTL for stalk strength *per se*. These results demonstrate that QTL analysis can be used to separate the effects of correlated traits from the genetic effects of the target trait, and underline the importance of appropriately considering which correlated traits may influence measurement of the target main trait. Similar considerations are particularly important when dealing with genotypes (e.g. mapping population or panel of accessions suitable for association mapping) where GY correlates to the degree of a particular disease or, commonly in the case of terminal drought, after flowering.

3.3 Flowering Time and Maturity

Variability in flowering time is the single most important factor that has allowed the cultivation of maize from the tropics to both Canada and Northern Europe.

After domestication from the Central America native teosinte, maize was gradually adapted to temperate climates, through selection of flowering time and maturity to match the local climatic features. Under water-limited conditions, the importance of flowering time is substantiated by the higher correlation of GY with kernel number/plant, a feature mostly determined at flowering and shortly after, rather than kernel weight (Duvick, 2005).

Maize flowering time and related traits such as PH and total node number are largely determined by the timing of the transition from vegetative to reproductive development at the shoot apical meristem. Because only few flowering time mutants have been mapped in maize, our knowledge of the genetic control of this important agronomic trait remains scanty (for a detailed analysis, see Colasanti and Muszynski, this volume) and much more limited if compared to Arabidopsis, rice, barley, and wheat. The identification and cloning of flowering time QTL would thus be valuable to advance our understanding of the molecular mechanisms responsible for the switch from vegetative to reproductive phase in maize and to manipulate maize phenology via MAS and/or genetic engineering.

Due to the ease at which flowering time is recorded, a large number of relevant QTL have been reported. Chardon et al. (2004) used the meta-analysis approach of Goffinet and Gerber (2000) to synthesize the information provided by 313 QTL identified in 22 studies where flowering time was recorded. The meta-analysis doubled the precision in QTL position estimation and highlighted the presence of 62 consensus QTL, with 6 playing a major effect. This study thus provides the best summary for QTL influencing flowering in maize. The 62 consensus QTL were then compared with the positions of the few flowering-time candidate genes that have been mapped in maize and also with rice candidates using a synteny conservation approach based on comparative mapping. This approach highlighted 19 associations between maize QTL and genes that have been shown to influence flowering time in rice and in *Arabidopsis*. The work of Chardon et al. (2004) indicates that the combination of meta-analysis within a target species and synteny-based projections from related model plant provides an efficient way for identifying novel candidate genes for trait variation. However, it should be pointed out that the collinearity between such distantly related species like maize and *Arabidopsis* is very poor (van Buuren et al., 2002) similarly to what was shown also between rice and *Arabidopsis* (Devos and Gale, 2000).

Following the early work of Phillips et al. (1992), six consensus QTL were singled out in the meta-analysis of Chardon et al. (2004). The region by far with the strongest effects on flowering time corresponds to bin 8.05. In the same region, two major QTL for the transition from vegetative to generative phase (*Vgt1* and *Vgt2*) were described by Vladutu et al. (1999) based on the evaluation of a population derived from a cross between N28 and an early isogenic version (N28E) carrying an introgressed chr. 8L segment from Gaspé Flint, a Northern Flint open-pollinated population characterized by extreme earliness. Homozygous recombinant lines with a variable length of the introgressed segment confirmed the presence of the two linked QTL. In the N28 background, Gaspé Flint QTL alleles at both loci induce a reduction in node number (ca. 1), height (ca. 20–30 cm), and days to

pollen shed (from 5 days to 15 days, according to the average temperature). Given the determinate growth pattern of maize, the phenotypic effects indicated that the two QTL are involved in the transition of the apical meristem from vegetative to generative structures. The effects of the two QTL in the background of N28 suggested the presence of two general developmental factors affecting the timing of pollen shed. The primary factor is the timing of the transition of the apical meristem. The second, derivative factor is the global extent of internode elongation. By separating the two linked QTL, Vladutu et al. (1999) laid the foundation for the positional cloning of *Vgt1*, the QTL with a larger effect (Salvi et al., 2007b). Interestingly, the *Vgt1* locus corresponds to a 2.7 kb, non-coding region positioned 70 kb upstream of an *Ap2*-like transcription factor whose up- or down-regulation via genetic engineering was shown to radically alter flowering time. The effect of the two *Vgt1* alleles on the expression levels of the downstream gene indicated that *Vgt1* functions as a *cis*-acting regulatory element. Additionally, within *Vgt1* Salvi et al. (2007) identified evolutionarily conserved non-coding sequences across the maize–sorghum–rice lineages, a result supporting the notion that changes in distant *cis*-acting regulatory regions are a key component of plant genetic adaptation throughout breeding and evolution.

The characterization and mapping of a spontaneous early flowering mutation, *Vgt-f7p*, in the same position of *Vgt1* suggests a correspondence between these two loci (Chardon et al., 2005) and further supports Robertson's hypothesis. Similarly to *Vgt1*, this mutation shortens the time from sowing to pollen shed by about 100 growing degree days, and reduces the number of nodes and PH. *Vgt-f7p* was mapped to a ca. 6 cM confidence interval on chr. 8 that overlaps with the position of *Vgt1* (Vladutu et al., 1999; Salvi et al., 2002, 2007). QTL analysis of a mapping population derived from the cross between the mutant F7 line and the Gaspé Flint population showed that *vgt-f7p* is probably allelic to *Vgt1*. In keeping with Robertson's hypothesis, *vgt-f7p* affects earliness more strongly than the Gaspé Flint allele at *Vgt1*. In summary, the work of Chardon et al. (2005) confirmed the findings reported by Vladutu et al. (1999) on the presence of two consensus QTL, *Vgt1* and *Vgt2*, and suggests contrasting allelic effects at these loci: rare alleles conferring extreme earliness at *Vgt1* versus greater diversity and milder effects at *Vgt2*. Our work based on an introgression library of Gaspé Flint × B73 identified major QTL for flowering time on chrs. 1, 3, 9, and 10, and confirmed the major QTL (*Vgt1* and *Vgt2*) on chr. 8 (Salvi et al. unpublished). All these QTL appear to influence flowering time primarily by changing the time of transition of the apical meristem and hence the number of nodes/leaves, similarly to *Vgt1* and *Vgt2* loci.

The cultivation of corn in cool climates benefits from a rapid grain moisture (GM) loss rate, a valuable feature that also reduces post-harvest production costs relative to artificial grain drying. Therefore, hybrids with low GM at harvest are desirable in mid- to short-season environments. The wide range reported in the literature for GM has precluded its use to estimate physiological maturity (Sala et al., 2007). The reason for this variability remains unclear. One of the most important factors determining GM is field grain drying rate (FDR). Genetic variation for FDR has been documented with precipitation being the single most important

environmental factor that contributed to G × E interaction (Magari et al., 1997). Sala et al. (2006) tested 181 $F_{2:3}$ families to locate QTL for GM at harvest and FDR as a starting point for MAS. Among the 16 QTL that were identified, only 2 affected both GM and FDR. Significant QTL×E interactions were evidenced for both traits, which led Sala et al. (2006) to conclude that MAS per se will not be an efficient method for reducing GM at harvest and/or increasing FDR. A selection index including both molecular marker information and phenotypic values, each appropriately weighted, was advocated as the best selection strategy. QTL for ear moisture were investigated by Sibov et al. (2003). In this case, the target trait showed a low heritability (0.23) and no QTL was identified.

3.4 Growth Rate and Grain Yield

Among all agronomic traits, GY is the most valuable and complex in terms of genetic basis (Duvick, 1997). Maize breeders have developed new inbred parents of hybrids through topcrossed and *per se* evaluation of agronomic traits in segregating progeny derived from the crosses planned at each selection cycle. Yield improvement through conventional approaches has been achieved empirically through a direct selection for yield, with limited or no knowledge of the underlying morpho-physiological determinants (Edmeades et al., 1997; Duvick, 2005). Further complexity is added by the occurrence of abiotic and biotic constrains that curtail yield potential and can influence greatly the identification of "real" QTL for GY. Within this complex scenario, the QTL approach allows us to identify chromosome regions able to affect GY and other agronomic traits on either a prevailingly adaptive or constitutive (i.e. *per se*) basis, provided that the genetic materials are tested under the relevant experimental conditions (e.g. well-watered vs. water-stressed, high N vs. low N, etc.). Table 1 summarizes the main features of a number of studies that have reported QTL for GY in maize.

3.4.1 Testing for Heterotic QTL

The spectacular increase in GY during the past decades of maize breeding has heavily relied on the exploitation of heterosis (Duvick, 2005). Several QTL studies have investigated the genetic basis of heterosis in maize (Beavis et al., 1994; Ajmone Marsan et al., 1995; Cockerham and Zeng, 1996; Graham et al., 1997; Austin et al., 2000; Lu et al., 2003; Frascaroli et al., 2007; Ma et al., 2007). This notwithstanding and despite the new insights provided by transcriptome analysis (Guo et al., 2006), the genetic basis of heterosis has not yet been elucidated (Springer and Stupar, 2007).

From a methodological standpoint, searching QTL in a highly heterotic species like maize adds further complexity to the approaches adopted to discover and manipulate the relevant QTL alleles. In view of the low correlations between trait

Table 1 Summary of the main features of studies in maize reporting results on QTL for grain yield and its components. Studies have been listed alphabetically according to the genetic background.

Background	Reference	Type of genetic material	Trait	Number of QTL[1]	Total R^2 (%)	Marker or marker interval for the major QTL (bin)	Action[2]	R^2 (%) of major QTL[3]	Notes
Ac7643S5 ×Ac7729/ TZSRWS5	Ribaut et al. (1997)	234 F_3 lines evaluated in three water regimes (well watered: WW; internediate stress: IS; severe stress: SS)	Yield	4–5	16–26	umc33a (1.07) WW	PD	9	
						umc119 (1.06) IS	PD	12	
						umc64 (10.04) SS	A	10	
			Ear number	1–7	6–40	umc105 (9.02) WW	A	9	
						csu60 (6.05) IS	OD	8	
						umc11 (1.03) SS	OD	6	
			Kernel number	2–3	9–21	umc33a (1.07) WW	A	10	
						bnl8.01 (3.06) IS	OD	13	
						umc114 (9.04) SS	OD	7	
	Ribaut et al. (2007)	240 F_3 lines evaluated under low and high N conditions	Grain yield at low N	8	27–32	bnl3.06 - umc114 (9.02-9.03)	OD	2–9	
			Ear number at low N	9	22–46	umc150a - umc65c (8.06)	PD	1–12	
			Kernel weight at low N	1	0–8	npi114b - umc10 (3.04)	D	0–8	
			Yield at high N	3	32	umc64 - csu86 (10.04)	A	18	
			Ear number at high N	1	8	umc64 - csu86 (10.04)	PD	8	
			Kernel weight at high N	5	39	npi114a - umc91b (8.01 - 8.02)	OD	19	
B73 × A7	Ajmone-Marsan et al. (1995)	232 F_3 lines testcrossed with two testers (A1 and Mo17)	Grain yield	2–3	35	umc59a - umc21 (6.02-6.05)	NA	25	QTL identified in both tester experiments
B73 × H99	Frova et al. (1999)	142 RILs evaluated in two water regimes (WW and WS)	Kernel weight	4 (WW)	49–58	bnl5.40 (5.06) WW	NA	11	
				4 (WS)		bnl5.40 (5.06) WS	NA	12	
			Kernel number per ear	4 (WW)	47–49	php20523 (5.08) WW	NA	11	
				4 (WS)		phi093 (4.08)	NA	11	
	Frascaroli et al. (2007)	142 RILs and three testcrosses with B73, H99, and B73 × H99	Grain yield	21	20–49	phi121 - bnlg666 (8.05)	PD	7–20	QTL identified also in Beavis et al. (1994) and Melchinger et al. (1998)
			Kernel weight	13	10–33	phi008a - bnlg557 (5.03)	A	3–13	

(continued)

Table 1 (continued)

Background	Reference	Type of genetic material	Trait	Number of QTL[1]	Total R^2 (%)	Marker or marker interval for the major QTL (bin)	Action[2]	R^2 (%) of major QTL[3]	Notes
B73 × Mo17	Stuber et al. (1992)	264 F_3BC1 (B73 and Mo17 as testers)	Grain yield	6–8	60	amp3 (5.04)	NA	30-35	No overlaps with grain yield QTLs of the same study
			Ears per plant	2–2	NA	npi296 (3.05)	NA	NA	
	Beavis et al. (1994)	112 $F_{2:4}$ lines and 112 $F_{2:3}$ topcross lines (tester:V78)	Grain yield	5	57	php10005 - bz1 (9.01) bnl12.30a - bnl2.369 (8.05)	A	22	
				2	22		NA	13	
			Kernels per row	0 ($F_{2:4}$ lines)	-	-	-	-	
			Kernel rows per ear	4 ($F_{2:4}$ lines)	37	php10005 (9.01)	PD	10	Overlaps with major yield QTL of the same study
B73 × X178	Xiao et al. (2005)	234 $F_{2:3}$	Yield WW	2	21	umc1657 (9.05)	D	14	QTLs for grain yield on bin 9.07 were also found by Agrama and Moussa (1996), Ajmone-Marsan et al., (1995), Veldboom and Lee (1996), Ribaut et al., (1997).
			Yield WS	1	14	bnlg1525 (9.07)	PD	14	
			Kernel number WW	4	24	umc1657 (9.05)	D	8	
			Kernel number WS	2	16	umc1657 (9.05)	D	10	
			Kernel weight WW	4	24	phi109642 (2.03-2.04)	PD	6	
			Kernel weight WS	5	28	umc1003 (2.05)	PD	7	
			Ear number WW	3	3	bnlg1525 (9.07)	D	21	
			Ear number WS	2	2	bnlg1525 (9.07)	PD	31	
Dan232 × N04	Li et al. (2007)	220 $BC2F_2$ in a AB-QTL mapping strategy	Grain yield (Grain weight per plant)	4	29	phi115 - bnlg1863 (8.03)	NA	19	
			Kernel weight	3	32	bnlg1863 - umc2147 (8.04)	NA	15	
			Kernel row number per ear	2	15	umc2017 - umc1877 (10.03-10.07)	NA	9	
			Kernel number per row	1	8	phi115 - bnlg1863 (8.03)	NA	8	

H99 × Mo17	Austin and Lee (1996)	194 $F_{2:3}$ and 185 $F_{6:7}$	Grain yield	8–5	28–48	npi280 (6.05-6.06)	NA	13–31	Ten QTLs in common with Austin et al. (2000). Major QTL not detected in Austin et al. (2000).
			Ear number	8–4	48–66	bnl10.06 - bnl7.71 (5.03-5.04)	NA	12–23	
			Kernel weight	10–13	27–50	umc165a - bnl3.18 (3.06-3.07)	NA	17–27	
	Austin et al. (2000)	194 $F_{2:3}$ and 186 $F_{6:8}$, crossed with A632, B73, and B91 as testers	Grain yield	24	24–44	npi212 - isu1 - sh2 (3.07-3.09)	NA	NA	Major QTL detected as small QTL in Austin and Lee (1998).
	Veldboom and Lee (1996)	150 $F_{2:3}$ lines	Grain yield	1	47	bnl5.47 - npi280 (6.05-6.06)	D	39	
			Kernel weight	7	90	umc121 (3.01)	PD	24	
			Ear number	2	65	umc165a (3.06)	PD	24	
			Kernel rows per ear	2	51	umc78 (2.02)	PD	35	
KW1265 × D146	Schon et al. (1994)	380 F_3 lines crossed with two testers (KW4115 and KW5361)	Kernel weight	8–6	55-58	umc32b (8.03)	NA	18–20	
	Melchinger et al. (1998)	Two experiments with two samples of 344 and 107 F_3 lines crossed with two testers (KW4115 and KW5361)	Grain yield	2–7 (exp. 1) 1–4 (exp. 2)	15–31 (exp. 1) 4–32 (exp. 2)	bnl14.07 - umc151 (7.04) (exp. 1) umc59a - umc65 (6.03) (exp. 2)	NA	0–10 (exp. 1) 0–17 (exp. 2)	Limited correspondence of QTLs was found between experiments and testers
			Kernel weight	11–12 (exp. 1) 4–5 (exp. 2)	53–64 (exp. 1) 42–44 (exp. 2)	umc32b - bnl9.44 (8.03) (exp. 1) bnl5.71[a] - umc126a (5.04-5) (exp. 2)	NA	17–26 (exp. 1) 7–19 (exp. 2)	

(continued)

Table 1 (continued)

Background	Reference	Type of genetic material	Trait	Number of QTL[1]	Total R^2 (%)	Marker or marker interval for the major QTL (bin)	Action[2]	R^2 (%) of major QTL[3]	Notes
LH200 × LH216	Lu et al. (2003)	351 backcross families over the two parents from a F_2Syn_3 population	Grain yield	15–18	85–90	A1792-A1808 (chr. 7)	OD	4-17	Three generation of selfing after the F_2 cross to break up repulsion linkage phase
Lo964 × Lo1016	Tuberosa et al. (2002c)	118 F_3 families evaluated at two water regimes (WW and WS)	Grain yield in WW regime	7	59	PGAMCTA205 (1.03)	PD	47[4]	
			Grain yield in WS regime	4	34	PGCMCTA260 (5.05 approx)	PD	27[4]	
SD34 × SD35	Agrama and Moussa (1996)	230 F_3 lines grown in drought conditions	Grain yield	5	50	umc76 (1.03)	PD	22	
			Ear Number	3	35	umc132 (6.07)	A	19	
Z3 ×871	Ma et al. (2007)	294 F_8 RILs	Grain yield	7	14	bnlg339 - umc1865	NA	3	
			Kernel row number	13	42	umc1460 - umc1562	NA	8	
			Kernel weight	2	5	umc1911 - umc2043	NA	3	

[1] Number of QTL always refers to a single experiment. When two numbers are given they report results obtained with two different tests.

[2] Type of gene action given in publications or assigned when possible based on the criteria given in Stuber et al (1987), after the computation of *d/a*: Additive (A) = 0 to 0.20; partially dominant (PD) = 0.21 to 0.80; dominant (D) = 0.81 to 1.20; overdominant (OD) = > 1.21.

[3] Computed after fitting a multiple regression model

[4] Computed after fitting a multiple regression model,

values observed in inbred and hybrid progeny, a comparative assessment of QTL controlling inbred *per se* and hybrid performance is required to understand the underlying genetic factors, and to gauge the value of QTL detected in progeny types differing in heterozygosity. The difficulty in properly assessing the genetic make-up of QTL for GY was properly addressed by the work of Graham et al. (1997) aimed at the dissection of a major GY QTL previously identified on chr. 5 in B73 × Mo17 and also in other backgrounds. The evaluation of testcrosses of two sets of reciprocal BC_2S_1 lines, each containing a fragment at the target region of one parental line introgressed in the other line, indicated that the QTL on chr. 5 included at least two smaller QTL that appeared to act in a dominant manner in repulsion phase linkage, thus lending support to the dominance theory of heterosis. Additionally, these results underline the drawbacks to undertake a MAS for this type of QTL based on data from an early generation (e.g. backcross, F_2, or F_3) that does not allow to resolve the true nature of a heterotic region. Eventually, cloning of heterotic QTL will provide valuable information on the underlying genetic cause (dominance and overdominance) of heterosis. Further details on the genetic and molecular basis of heterosis in maize are reported elsewhere in this volume (chapter by Schnable and Swanson-Wagner).

3.4.2 Physiology of Biomass Accumulation and GY

From a physiological standpoint, heterosis for GY in maize can be mainly attributed to (a) heterosis for dry matter accumulation before silking, which results mainly from greater light interception due to increased leaf size; (b) heterosis for dry matter accumulation during the grain-filling period as a result of greater light interception due to greater maximum leaf area index and increased stay green, and (c) heterosis for harvest index (Tollenaar et al., 2004). Therefore, the identification of QTL for early vigour, photosynthetic efficiency, and reproductive fertility would prove beneficial for increasing yield potential of maize.

The work carried out by Boyer and coworkers (Boyle et al., 1991; Boyer, 1996; Boyer and Westgate, 2004; McLaughlin and Boyer, 2004a, b, 2007), Schussler and Westgate (1991a, b, 1994, 1995), and Zinselmeier and coworkers (Zinselmeier et al., 1995, 1999, 2002) has shown the key role of sucrose level on reproductive fertility. Hexoses, as well as sucrose, have been recognized as important signal molecules in source-sink regulation. Increasing evidence indicates the presence of crosstalk, modulation, and integration between signalling pathways responding to phytohormones, nutrients, light, sugars, as well as biotic and abiotic stress-related stimuli (Roitsch, 1999). Thus, the QTL approach provides an opportunity to dissect the genetic basis of these complex interactions that are likely to play a central role in the regulation of source-sink relationships and reproductive failure, particularly under unfavourable conditions.

A number of studies have searched for QTL underlying the physiological basis of GY by measuring the level of activity of some of the key enzymes influencing photosynthetic capacity (Prioul et al., 1997, 1999). Causse et al. (1995) contributed

the first results to link QTL for the activity of sucrose phosphate synthase, ADP-glucose pyrophosphorylase, invertases, and sucrose synthase with QTL for growth. Causal relationships that had been suggested by previous physiological studies were reinforced by common locations of QTL for different traits. In particular, the strong correlation between growth rate and invertase activity, which may reflect sink organ strength, was to a large extent explained by a single region of chr. 8. Interestingly, some of the structural genes of the enzymes mapped to regions with QTL affecting the activity of the encoded enzyme and/or concentration of its product, and sometimes growth traits.

In order to investigate the role of ADPglucose pyrophosphorylase, sucrose-phosphate-synthase, and invertases, the concentration of their substrates or products were subjected to QTL analysis (Prioul et al., 1999). This approach was applied at three- or four-leaf stage, under well-watered and water-stressed conditions and on grain, at maturity. Several QTL were detected for each trait, particularly for two enzyme activities measured in mature leaves. Apparent co-locations between QTL for activity and structural locus were observed for sucrose-phosphate-synthase (chr. 8) and acid-soluble invertase (chrs. 2 and 5). A QTL for leaf acid-soluble (vacuolar) invertase on chr. 5 explaining 17% of variability co-located with the *Ivr2* gene encoding a vacuolar invertase protein which was strongly water-stress inducible. A similar finding was reported in the grain for an amylose QTL co-located with the *Sh2* gene of ADPglucose-pyrophosphorylase. The role of this candidate was further tested through the examination of the *Sh2* DNA polymorphism in 46 unrelated lines. This analysis supported the correlation between this polymorphism and kernel starch content, further validating *Sh2* as a feasible candidate.

To better understand the relationship between early grain-filling traits and carbohydrate composition in mature grain, QTL linked to soluble invertase, sucrose synthase, and ADPglucose pyrophosphorylase activities and to starch, sucrose, fructose, and glucose concentrations were investigated at 15, 25, and 35 days after pollination (Thevenot et al., 2005). Although several QTL were revealed for all traits, only one was consistently observed at the three sampling periods. Numerous possible candidate genes of the starch synthetic pathway co-located with QTL; in particular, four QTL were found close to the locus *Sh1* (bin 9.01) coding for the sucrose synthase.

Biochemical factors other than sucrose supply appear to be involved in kernel abortion during the early stages of kernel growth (Boyle et al., 1991; Setter et al., 2001; Zinselmeier et al., 2002; McLaughlin and Boyer, 2004a, b, 2007). As an example, ABA has been implicated as one of such factor which, either *per se* or through complex interactions, may allow the plant to gauge the availability of environmental factors (e.g. soil moisture) with a key role in determining reproductive fertility. Interestingly, the evaluation of an historical series of maize hybrids has indicated a significant decrease in the capacity to accumulate ABA of modern maize hybrids when exposed to a given level of water stress (Sanguineti et al., 2006). QTL for ABA concentration in the maize leaf and in the xylem sap, but not in reproductive organs, have been described (Quarrie et al., 1994; Lebreton et al.,

1995; Tuberosa et al., 1998, 2002a; Sanguineti et al., 1999; Reymond et al., 2003; Pelleschi et al., 2006).

An important aspect of maize productivity relates to the capacity of the plant to efficiently absorb soil nitrogen (N), store it in the leaves and stalk, and relocate it during kernel growth (Hirel et al., 2007). The chapter by Geiger (this volume) provides the details on the genetic and molecular basis of N-use efficiency (NUE). Here, we report the essential results of the work of Hirel and coworkers on the QTL for NUE. The findings of Hirel et al. (2001) suggested that increased productivity was positively associated with the ability to accumulate nitrate in the leaves during vegetative growth and to efficiently remobilize this N during grain filling. Coincidences of QTL for GY and its components with genes encoding cytosolic glutamine synthase (GS) and the corresponding enzyme activity were detected, particularly at the GS locus on chr. 5, whose metabolic activity was positively associated with kernel weight and yield. Because at this locus coincidences of QTL for GY, GS, nitrate reductase (NR) activity, and nitrate content were also observed, Hirel et al. (2001) postulated that leaf nitrate accumulation and the reactions catalyzed by NR and GS are co-regulated and represent key elements controlling NUE in maize. Additional work further confirmed the putative role of the chr. 5 locus in NUE and leaf senescence (Gallais and Hirel, 2004).

3.4.3 Testing for QTL × Environment Interaction

A number of studies have addressed the issue of QTL × Environment interaction, often with conflicting results (Crossa et al., 1999; Moreau et al., 2004; LeDeaux et al., 2006; Lima et al., 2006). The environmental factor that most frequently influences GY and QTL effects is the availability of water (Epinat-Le Signor et al., 2001; Moreau et al., 2004). Regrettably, future projections indicate that maize production will also face a reduction in irrigation volumes, even in regions where supplemental water is essential for securing a profitable harvest (Rosegrant and Cai, 2002; Rosegrant et al., 2002). Therefore, significant yield losses in maize caused by drought are expected to increase with global climate change as temperatures rise and rainfall distribution changes (Campos et al., 2004). With only a few exception (Frova et al., 1999; LeDeaux et al., 2006), most QTL for GY and related traits detected under drought conditions are stress-adaptive, hence detected prevailingly under such conditions (Ribaut et al., 1997; Tuberosa et al., 2002b), a result that is in keeping with the sizeable G × E interaction usually evidenced when traits are measured across different water regimes. As an example, only 14% of the 84 QTL evidenced in the Io × F-2 population were common to well-watered and water-stressed conditions (Pelleschi et al., 2006). These findings are somehow counterintuitive when compared to the widespread notion that modern breeding has improved maize yield across a broad range of water regimes (Duvick, 2005). This apparent contradiction can be in part reconciled considering that mapping populations have often been derived from publicly available lines which as compared to elite materials, likely harbour greater allelic diversity at drought-adaptive QTL.

Conversely, elite lines used for the production of commercial hybrids have undergone a very strong selection for the best-performing alleles at such loci, particularly in terms of yield stability under unfavorable environmental conditions. A detailed haplotype analysis of the drought-adaptive QTL regions in the historical series of maize hybrids (Duvick, 2005) would provide useful insight in this direction. The chapter by Ribaut et al. (this volume) reports more extensively on the QTL that regulate maize yield under water-limited conditions.

Linking QTL information to crop modelling has shown that QTL analysis removes part of the random errors of measured model input parameters, thus indicating that this information can successfully be coupled with crop models to replace measured parameters (Tardieu et al., 2003). The QTL-based modelling relies on the estimation of parameters of response curves to environmental variables and may allow for a more effective selection based on QTL information. Crop modelling has also the potential to help resolving G × Environment interactions as well as the genetic basis of traits' plasticity (Chapman et al., 2003; Reymond et al., 2004; Cooper et al., 2005, 2007). For this approach to be effective, crop models that are capable of predicting yield differences among genotypes in a population under various environmental conditions are needed (Tardieu et al., 2003; Cooper et al., 2005; Hammer et al., 2005, 2006; van Eeuwijk et al., 2005). The ultimate goal of the modelling approach is the possibility to implement an in silico selection able to identify the combinations of the desirable alleles at the target QTL. Nonetheless, further work is required to test the validity of models when more of such environmental variables vary simultaneously, the condition typically encountered by crops in the field.

Recent experiments have highlighted the importance of epigenetic mechanisms (e.g. cytosine methylation) and RNA interference (RNAi) for the regulation of the expression of genes and QTL (Lukens and Zhan, 2007). It is becoming increasingly evident that the plant genome's response to environmental stress generates both novel genetic and epigenetic methylation polymorphisms that may increase phenotypic diversity and plasticity to abiotic stress.

3.4.4 Testing for QTL Epistasis

From a breeding standpoint, the studies so far conducted in maize to determine the genetic basis of the historical increase in performance of both elite inbreds and hybrids suggest that epistasis did not play a prevalent role (Duvick, 2005). Nonetheless, as the agronomically superior alleles become fixed at non-epistatic major loci controlling yield performance in the elite populations, further gains in yield potential may well require a more extensive exploitation of additional epistatic QTL effects.

For untangling the complex web of "cross-talk" among QTL, an issue that still remains largely unsolved is the detection of epistatic interactions (Gao and Zhu, 2007; Jannink, 2007; Ma et al., 2007), particularly those of trigenic and higher order. Stich et al. (2007) adopted a new mapping strategy in order to investigate

higher-order epistatic interactions in a study whose main objectives were to (a) compare three- versus four-step genome scans to identify three-way epistatic interactions among QTL belonging to a metabolic pathway in maize, (b) investigate by computer simulations the power and proportion of false positives (PFP) for detecting three-way interactions among QTL in RIL populations derived from a nested mating design, and (c) compare these estimates to those obtained for detecting three-way interactions among QTL in RIL populations derived from diallel and different partial diallel mating designs. Compared to the three-step genome scan, the power to detect three-way interactions was higher with the four-step genome scan. The power and PFP to detect three-way interactions using a nested design with 5,000 RILs were for both the 4-QTL and the 12-QTL scenario of a magnitude that appeared promising for their identification. Although the technical advance described by Stich et al. (2007) facilitates the detection of higher-order epistasis, the high number of lines required to detect such interactions remains a major obstacle toward a more complete detection of higher-order epistatic interactions.

Mihaljevic et al. (2005) searched for epistatic QTL affecting *per se* and testcross performance for GY and grain moisture in four crosses among four elite European flint maize lines. Significant epistatic interactions were found with only a few marker pairs that did not improve the fit of the model after including main-effect QTL previously detected by composite interval mapping. Based on these results, Mihaljevic et al. (2005) concluded that epistasis is of minor importance for GY and grain moisture in maize.

4 Concluding Remarks

Almost two decades separate the first report on genome-wide search of QTL in maize (Edwards et al., 1987; Stuber et al., 1987) from the first positional cloning of a maize QTL (Wang et al., 2005). Remarkably, it is now possible to Mendelize and clone major QTL for agronomic traits also in genomes of high complexity such as that of maize (Salvi and Tuberosa, 2005; Bortiri et al., 2006; Salvi et al., 2007b). The identification of major QTL suitable for MAS (Stuber, 1997; Bernardo and Charcosset, 2006) and cloning will be facilitated by meta-analysis and association mapping (Buckler et al., 2006; Tuberosa and Salvi, 2006). MAS is already an important component of maize breeding programs, particularly in the private sector (Morandini and Salamini, 2003; Crosbie et al., 2006; Lee, 2007; Ragot and Lee, 2007). A number of major QTL for agronomic traits are presently in the cloning pipeline, the major limiting factor often being the collection of adequately accurate phenotypic data. An example is provided by QTL that control root architecture, one of the traits whose direct manipulation would more greatly benefit from the isolation of the relevant genes (de Dorlodot et al., 2007).

As the data on the breeding value of QTL haplotypes for agronomic traits accumulate, SNP profiling or direct sequencing will allow for a more informed and effective choice of the parental lines to cross for developing new segregating

populations (Bernardo and Yu, 2007). The information on the sequence and mode of expression of thousands of genes in maize and related species will be soon superseded by the availability of the entire maize genome sequence (Wessler, 2006; Sofi et al., 2007). Additionally, the decreasing cost of sequencing coupled with the evaluation of suitable panels of unrelated accessions will allow us to more systematically apply genome-wide association mapping to identify feasible candidates for QTL of agronomic interest (Yu et al., 2008). The new QTL discovery paradigm ushered in by association mapping further highlights the necessity to develop accurate, high-throughput platforms able to generate phenotypic data in quantity and quality sufficient to match properly the level of genetic resolution offered by the genomics platforms (Campos et al., 2004; Montes et al., 2007). In turn, the availability of highly accurate, relevant phenotypic data would speed up the path from a QTL to a candidate sequence. Clearly, the urgency to fill in the genotype-phenotype gap has never before been more evident. Nevertheless, the limitations imposed by quantitative traits suggest that only a fraction of the available genotypic variability will be amenable to a direct manipulation via MAS. Most likely, the plethora of minor QTL that control agronomic traits will largely remain undetected even with the most accurate phenotyping platforms and sophisticated statistical approaches. In this case, conventional breeding will undoubtedly continue to play a key role to improve maize productivity.

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The Mexican Landraces: Description, Classification and Diversity

Jean-Philippe Vielle-Calzada and Jaime Padilla

Abstract The domestication of maize gave rise to a group of ancestral landraces that eventually diversified and adapted to a wide range of climatic and geographic conditions. Although biologists do not always agree in the total number of landraces currently existing in Mexico, there are at least 59 that can be clearly and consistently distinguished on the basis of biochemical and morphological characteristics. Following a historical perspective, this chapter reviews our current knowledge of the phenotypic and geographical distinctions among Mexican landraces, and illustrates their most recent classification. It also discusses some of the opportunities that the genomic characterization of landrace germplasm could offer for the study of maize functional diversity and molecular evolution.

1 Introduction

Mexico as a nation is not conceivable without the existence of maize. For most Mexicans, more than being domesticated, maize was handcrafted through thousands of years of ancestral work. The plant has been at the heart of cultural creativity for all generations and remains an essential element of popular Mexico. As described by Guillermo Bonfil (1982), the establishment of maize as the fundamental Mexican crop required the design and implementation of the technical procedures necessary for its cultivation and consumption, including the specific organization of time and space in response to a physiological rhythm imposed by the plant. It also guided the emergence of immensely diverse religious practices, allowed the development of a unique culinary art, and became the reference necessary to understand the popular organization of the Mexican life style. In contrast to developed countries where it is fundamentally used for agro-industrial or animal production, maize in Mexico is mainly cultivated for human nutrition, in all states, and under a wide range of climatic conditions. Its consumption represents the main source of protein and energy in rural regions, particularly in the poorest communities. Corn is at the table of millions of Mexicans daily, either in the form of a wide diversity of regional meals, or simply as its most important derivative, the tortilla.

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Maize was domesticated from its wild progenitor teosinte (derived from "*teocintli*" in nahuatl language: "*teotl*" = sacred and "*cintli*" = dried ear of corn), a common name given to a group of annual and perennial species of the genus *Zea* native to Mexico and Central America (see Chap. 20; reviewed in Doebley, 2004; Matsuoka, 2005). An extensive phylogenetic analysis based on molecular comparisons of landraces and wild taxa indicates that maize arose from a single domestication event occurred in southern Mexico about 9,000 years ago (Matzuoka et al., 2002). Domestication resulted in a group of ancestral landrace varieties that subsequently spread throughout the continent and eventually to the rest of the World, adapting to human practices in different ecological and geographical regions where the crop has been established. As the main center of origin and domestication, Mexico has the largest diversity of maize genetic resources. Through continuous divergent selection, the plant has diversified into a multitude of populations with distinct adaptations to climate, latitude, and length of growing season, and has specialized to fulfill a wide range of specific human purposes (Hemández, 1985; Ortega Paczka, 2003).

In this chapter we review our current knowledge of the genetic variability found in Mexican landraces, and discuss their most recent classification on the basis of morphology, geographical distribution, and some biochemical characteristics. We also anticipate some of the future implications that landrace genome characterization will have for the understanding of the molecular and functional diversity of maize.

2 Conservation of Mexican Maize Germplasm

There is a substantial confusion in the terminology used to refer to maize germplasm. As indicated by Ortega Paczka (2003), the term "*variedad criolla*" or "*criollo*" is commonly used by many Mexican agronomists or breeders to name native local populations for which a pedigree is absent. The term "*criollo*" was used to name people from Spanish descent that were born in the Americas, and therefore has little in common to maize native populations maintained by Mexican farmers.

The term "*raza*" (race or landrace) should be in principle less confusing, as it is generally accepted that a landrace is a population of individuals that share a large number of genetically inherited traits that allow a clear and consistent phenotypic distinction from other maize populations. The name of a landrace can sometimes refer to an obvious phenotypical trait such as "*Cónico*" for a conical ear shape, or "*Negrito*" for prevalent seed color. In other cases it refers to a location where the germplasm was initially collected or is predominant (*Tuxpeño* or *Mixteco*), or it can also reflect indigenous names given by local populations in their native language (*Nal Tel* or *Dzit Bacal*).

In practice, the identification and description of a maize landrace is not obvious. In contrast to modern breeding schemes used by seed companies, traditional maize improvement is mostly empirical and relies on complex factors that include

elements of tradition, intuition, affection, and improvisation. Since cross-pollination prevails as a reproductive habit, maize heterozygosity is usually high and advantageous in common selection procedures followed by rural communities. Owing to these practices, what is generally observed in Mexican local populations is a continuous variation in quantitative traits such as plant height, ear size, kernel row number, or flowering time. Additional traits such as seed color are also highly variable among individuals of a same landrace. The majority of native maize planted to date is the result of hybridization between several landraces, and often between native germplasm and modern varieties recently introduced through neighboring practices or human migration (Bellón et al., 2003; Bellón and Berthaud, 2004; Pressoir and Berthaud, 2004a, b).

While the effort of creating and preserving Mexican maize diversity is intimately related to social and economic activities of indigenous communities, peasants, and farmers, it is the enthusiasm and dedication of a relatively small group of scientists that has enable the systematic identification, description, and conservation of present collections of maize germplasm. Although a general interest for maize diversity dates back to pre-Colombian times, the systematic study and collection of maize germplasm initiated in the twentieth century, with the work of Chávez (1913), Vavilov (1931), Anderson and Cutler (1942), and Anderson (1946). The work of Kuslehov (1930) was essential to demonstrate for the first time that the variability of seed morphology is exceptionally high in Mexico. Based on these pioneering initiatives, from 1938 to 1951 the Mexican Ministry of Agriculture supported important efforts to identify the best native materials that could serve as parental lines for a national program of maize improvement. Although the majority of the results were never published, these efforts indirectly derived in a successful partnership between the Ministry of Agriculture and the Rockefeller Foundation, giving rise to the most valuable *ex situ* collections of Mexican maize germplasm existing to date: the collection kept at the Centro Internacional de Mejoramiento de Maíz y Trigo (CIMMYT), and the Germplasm Bank of the Instituto Nacional de Investigaciones Forestales y Agropecuarias (INIFAP). With more than 11,000 samples, the latter represents the most important collection of Mexican maize germplasm; a substantial portion has been duplicated and transferred both to CIMMYT and the National Seed Storage Laboratory (USDA-NSSL) in Forth Collins, Colorado. Unfortunately, the INIFAP infrastructure is from 1950 and a limited budget impedes renovation. Additional collections include the Native Species Germplasm Bank at the Autonomous University of Chapingo (Texcoco, México) with 2,500 samples from native local populations, and the collection of Colegio de Postgraduados (Montecillos, México) with 4,000 samples. CIMMYT international accessions total more than 22,000 and according to official authorities new introductions are constantly being added from cooperative regeneration projects around the world. Passport data on CIMMYT maize germplasm bank accessions have been compiled and is available on CD-ROM (for details see www.cimmyt.cgiar.org). To safeguard CIMMYT collections, duplicate samples of about four fifths are kept at the USDA-NSSL.

3 Identification and Classification of Mexican Landraces

Maize biologists do not always agree in the total number of maize landraces that exist in Mexico (Wellhausen et al., 1951; Sánchez et al., 2000; Ortega Paczka, 2003; Ron-Parra et al., 2006). The classical monograph published by Wellhausen et al. (1951) has been an essential reference for all subsequent reports. Based on general architecture, kernel cytological traits, and physiological characteristics (time of flowering, yield, and disease resistance), they classified 25 landraces into 5 major groups. The first one included four Ancient Indigenous Races believed to have arisen from a primitive pod corn (*Palomero Toluqueño*, *Arrocillo Amarillo*, *Chapalote*, and *Nal Tel*). Other groups included landraces believed to be introduced from other regions of Central or South America (Pre-Colombian Exotic Races), or to have arisen after hybridization of ancient native and introduced landraces with teosinte (Prehistoric Mestizos); the fourth and fifth groups were considered to be composed of Modern Incipient Races or simply Poorly Defined Races.

A renewed interest for developing comprehensive systems of classification of Mexican landraces was initiated by Goodman (1972), Goodman and Bird (1977), and Cervantes et al. (1978) with the use of numerical techniques to classify morphological traits and geographical distribution. These studies resulted in a first set of well-defined racial groups that generally followed the relationships proposed by Wellhausen et al. (1951). Subsequent studies emphasized maize cytogenetic characteristics to report an extensive analysis of the chromosome constitution and geographic distribution of knob complexes (Kato, 1976, 1984; McClintock et al., 1981). These authors showed that specific knobs in chromosome 1, a small knob in the short arm of chromosome 3, and a large knob in the short arm of chromosome 7 are only distributed in landraces of the Pacific Coast, showing for the fist time a genetic link between primitive landraces such as *Chapalote*, *Nal Tel*, *Harinoso del Ocho, Reventador* and *Zapalote Chico*, and their relatives *Blandito de Sonora*, *Cristalino de Chihuahua*, *Dulcillo de Sonora*, *Palomero Toluqueño*, and *Onaveño.*

One of the first systematic attempts to biochemically characterize the genetic diversity of Mexican landraces was conducted by Doebley et al. (1985), who analyzed isozyme variation corresponding to 23 loci in 34 landraces. Their results indicated that the levels of variation in maize landraces are as high as in the teosintes, demonstrating that maize is among the most genetically variable crops. Although they could not identify well-defined racial complexes, their study allowed the recognition of three groups of morphologically similar landraces: (1) The high elevation Mexican Pyramidal group, represented by *Palomero Toluqueño*, *Cónico*, *Chalqueño*, and *Cacahuacintle*, (2) The Northern and Northwestern group, represented by *Apachito*, *Azul*, and *Gordo*, and (3) The remaining races, including the Southern and Western low-elevation dent and floury landraces.

Additional identification of Mexican landraces has been reported by Ortega Paczka (1979), Benz (1986), and Sánchez (1989). After establishing the existence of two racial complexes (the “Mexican Pyramidal Ear Complex” and the “Mexican Narrow Ear Complex”), Benz (1986) proposed five more types (*Chatino Maizón, Choapaneco, Mixeño, Mixteco, and Serrano Mixe*); however, a published illustration

and description of these landraces has not been available, and their distinction from previously established accessions has been sometimes questioned (Ortega Paczka, 2003). Using morphological traits in combination to numerical taxonomy, Sánchez (1989) was able to describe four of the "poorly defined races" mentioned by Wellhausen and co-workers (*Blandito de Sonora, Dulcillo del Noroeste, Mushito, and Zamorano Amarillo*), and confirmed the existence of three types identified by Ortega Paczka (1979): *Coscomatepec*, *Motozinteco*, and *Elotero de Sinaloa*.

Following these previous reports, and to assess the overall genetic diversity of Mexican germplasm, Sánchez et al. (2000) collected accessions from 50 landraces and analyzed their isozymatic and morphological characteristics under diverse growing conditions and locations. Although a very high level of variability was found among and within landraces, more than 65% of the alleles were present at frequencies below 1%, with some populations having low levels of genetic diversity, particularly those corresponding to rare variants planted in small fields in which seed for the next cycle comes from a small number of ears. Although Ortega Paczka et al. (1991) recognized the existence of at least 41 landraces, the study of Sánchez et al. (2000) reports the largest number of classified landraces on the basis of both morphological and biochemical evidence.

4 The Classification of Sánchez et al. (2000)

The most complete classification was proposed by Sánchez et al. (2000) and includes 50 out of 59 landraces divided in 5 major subdivisions (Fig. 1 and Table 1) on the basis of biochemical and morphological markers (Ron-Parra et al., 2006):

- The Central and Northern Highlands Group is composed of 15 landraces. With one exception (*Dulce de Jalisco*), all landraces of this group grow at elevations higher than 2,000 m. All group members are characterized by a low frequency of tassel branches, a weakly developed root system, and strongly pubescent leaf sheaths often pigmented by anthocyanins. *Apachito*, *Azul*, *Gordo* and *Cristalino de Chihuahua* are restricted to Northwest highlands at 2,000–2,600 m. These landraces have short plants (140–190 cm), early flowering (53–68 days), and rounded kernels. The group also includes the large *Cónico* subgroup (*Arrocillo Amarillo*, *Cacahuacintle*, *Chalqueño*, *Cónico*, *Cónico Norteño*, *Elotes Cónicos*, *Dulce de Jalisco*, *Mixteco*, *Mountain Yellow*, *Mushito*, *Negrito*, *Palomero de Chihuahua, Palomero de Jalisco, Palomero Toluqueño*) showing conically shaped ears and high kernel row number (14–18).
- The Eight-Rowed Group includes 12 landraces distributed at elevations comprised between 1,000 m and 1,800 m, with some along the Pacific Coast growing at elevations comprised between 100 m and 500 m (*Ancho*, *Blandito de Sonora*, *Bofo*, *Bolita*, *Elotes Occidentales*, *Harinero de Ocho*, *Jala*, *Onaveño*, *Tablilla de Ocho*, *Tabloncillo*, *Tabloncillo Perla*, and *Zamorano Amarillo*). These landraces are 200–250 cm in height, and characterized by early-to-medium maturity cycles in which flowering is reached 70–80 days following germination.

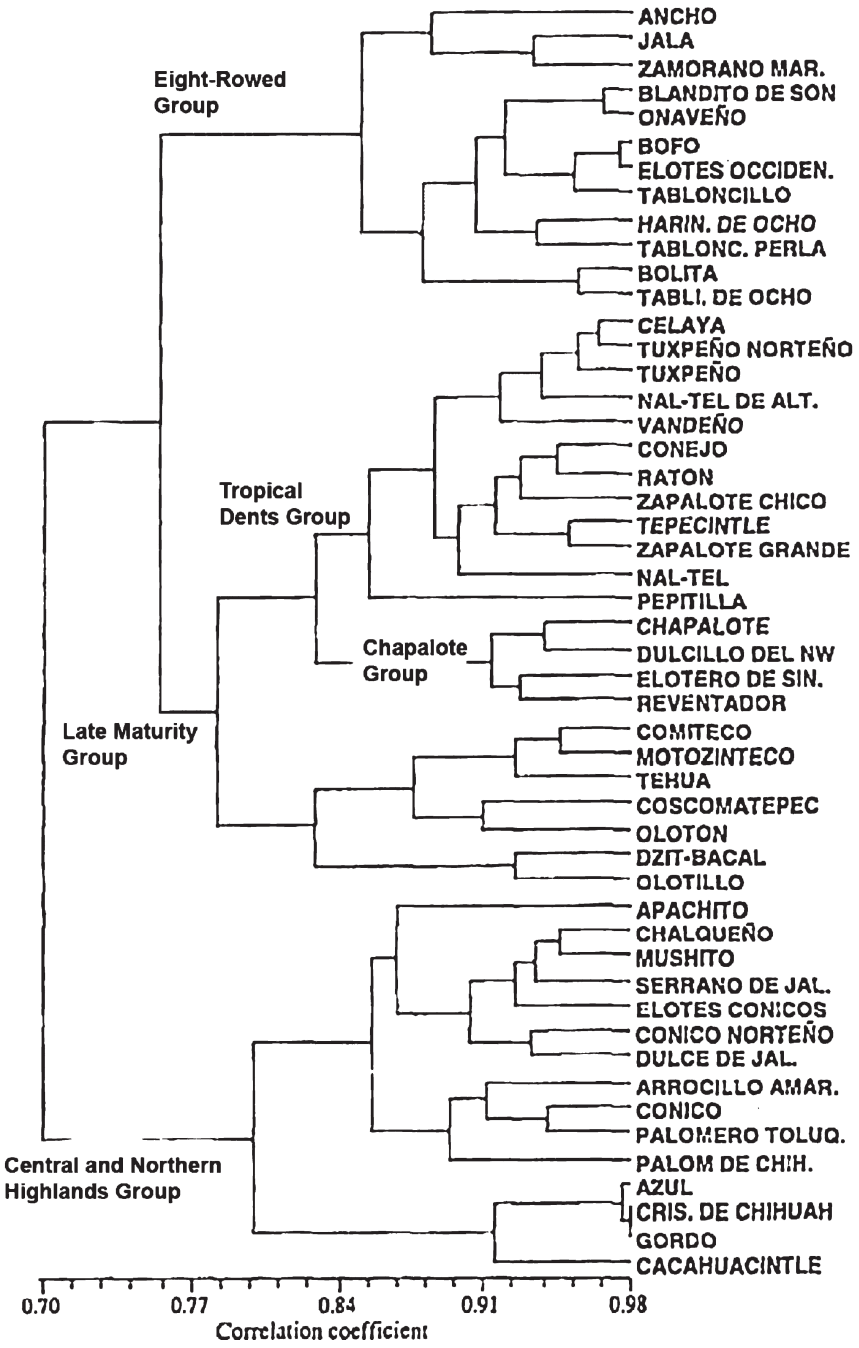

Fig. 1 Dendogram of 50 Mexican landraces based on morphological variables and isozyme frequencies (adapted from Sánchez et al., 2000)

Table 1 General characteristics of 57 Mexican landraces

Name	Distribution	Kernel type	Identification	Description
Group I: Highlands				
(A) *Apachito*	Chihuahua (high Babícora region); rare	Semi-crystalline; white or pinkish	Hernández and Alanís (1970)	Hernández and Alanís (1970)
(B) *Chalqueño*	Edo. de México, DF, Puebla, Hidalgo, Tlaxcala, Oaxaca, Zacatecas	Semi-crystalline to floury; white, ivory, red, or rarely purple	Wellhausen et al. (1951)	Wellhausen et al. (1951)
(C) *Mushito*	Michoacán, Oaxaca	Dent; white, bronze, purple or black	Ortega Paczka (1979)	Sánchez (1989)
(D) *Serrano de Jalisco*	Highlands of Southern Jalisco	Crystalline, dent; white, yellow, or purple	Wellhausen et al. (1951)	Wellhausen et al. (1951)
(E) *Elotes Cónicos*	Edo. de México, DF, Puebla, Hidalgo, Tlaxcala	Floury; purple or red	Wellhausen et al. (1951)	Sánchez (1989)
(F) *Cónico Norteño*	From Querétaro to Southern Chihuahua, 1400–1800 m	Dent; white	Wellhausen et al. (1951)	Wellhausen et al. (1951)
(G) *Dulce*	Southern Jalisco, Nayarit, Michoacán, Guanajuato, and Durango	Sweet endosperm, wrinkled; white, yellow, red, or purple	Wellhausen et al. (1951)	Wellhausen et al. (1951)
(H) *Arrocillo*	Northern Highlands of Puebla and Veracruz	Dent; white, yellow, or purple	Wellhausen et al. (1951)	Wellhausen et al. (1951)
(I) *Cónico*	From Oaxaca to Southern Querétaro, above 1,700 m	Dent; white, yellow, or purple	Wellhausen et al. (1951)	Wellhausen et al. (1951)
(J) *Palomero Toluqueño*	Edo. México (Valley of Toluca); rare	Popcorn; white	Wellhausen et al. (1951)	Wellhausen et al. (1951)
(K) *Palomero de Chihuahua*	Chihuahua (high Babícora region); rare	Popcorn; white	Ortega Paczka (1979)	Ortega Paczka (1985)
(L) *Palomero de Jalisco*	Jalisco; rare	Popcorn; white	A complex group of populations that has not been clearly identified or described (see Ron-Parra et al., 2006)	No published description

(continued)

Table 1 (continued)

Name	Distribution	Kernel type	Identification	Description
(M) *Mixteco*	Highlands of Oaxaca (Apizaco, Chalcatongo); rare	Dent; black, red, or purple	Benz (1986)	Benz (1986)
(N) *Negrito*	Highlands of Oaxaca	Floury; black	Sánchez et al. (2000)	No published description
(O) *Azul*	Chihuahua (high Babícora region)	Crystalline, dent: blue or slightly purple	Hernández and Alanís (1970)	Hernández and Alanís (1970)
(P) *Cristalino de Chihuahua*	Chihuahua (high Babícora region)	Crystalline; white or yellow	Wellhausen et al. (1951)	Wellhausen et al. (1951)
(Q) *Gordo*	Chihuahua (high Babícora region); rare	Crystalline to floury, dent; white	Hernández and Alanís (1970)	Hernández and Alanís (1970)
(R) *Cacahuacintle*	Edo. de México (Valley of Toluca)	Floury; white, ivory	Wellhausen et al. (1951)	Wellhausen et al. (1951)
Group II: Eight rowed				
(A) *Ancho*	Guerrero and Morelos	Large and wide; dent, floury; white	Ortega Paczka (1979)	Ortega Paczka (1979)
(B) *Jala*	Nayarit (Valley of Jala); nearly extinguished	Dent; white or yellow	Wellhausen et al. (1951)	Wellhausen et al. (1951)
(C) *Zamorano Amarillo*	Michoacán (Zamora region) and Jalisco; rare	Semi-crystalline, dent; yellow	Wellhausen et al. (1951)	Sánchez (1989)
(D) *Blandito de Sonora*	Sonora, Sinaloa and Nayarit; rare	Floury; white	Wellhausen et al. (1951)	Sánchez (1989)
(E) *Onaveño*	Sonora, Sinaloa y Baja California	Crystalline; white	Wellhausen et al. (1951)	Ortega Paczka (1979)
(F) *Bofo*	Nayarit (highlands region), Durango and Jalisco	Floury; white, red or purple	Hernández and Alanís (1970)	Hernández and Alanís (1970)
(G) *Elotes Occidentales*	Guanajuato (Bajío region) and Jalisco	Floury; purple or red	Wellhausen et al. (1951)	No published description
(H) *Tabloncillo*	Jalisco (mid-altitude regions); rare	Dent; white or ivory	Wellhausen et al. (1951)	Wellhausen et al. (1951)
(I) *Harinoso de Ocho*	Lowlands of Nayarit and Sinaloa	Floury; white or yellow	Wellhausen et al. (1951)	Wellhausen et al. (1951)
(J) *Tabloncillo Perla*	Nayarit to Sonora and Baja California	Crystalline; white or yellow	Wellhausen et al. (1951)	Wellhausen et al. (1951)
(K) *Bolita*	Oaxaca (mainly in central valleys); rare	Dent, floury; white, yellow, purple or red	Wellhausen et al. (1951)	Wellhausen et al. (1951)
(L) *Tablilla de Ocho*	Jalisco, Nayarit, Zacatecas	Dent, floury; white, yellow, red or pinkish	Hernández and Alanís (1970)	Hernández and Alanís (1970)

Group III: Tropical dents				
(A) *Celaya*	Guanajuato (Bajío region), Jalisco	Dent; white, transparent aleurone and pericarp	Wellhausen et al. (1951)	Wellhausen et al. (1951)
(B) *Tuxpeño Norteño*	Oaxaca, Puebla	Dent; white	Ortega Paczka (1979)	Ortega Paczka (1979)
(C) *Tuxpeño*	From Tamaulipas to Nayarit	Dent; white or yellow, rarely purple	Wellhausen et al. (1951)	Wellhausen et al. (1951)
(D) *Nal-Tel de Altura*	Northern Oaxaca (highlands region)	Semi-crystalline, dent, sweet; yellow or white	Ortega Paczka (1979)	Benz (1986)
(E) *Comiteco*	Chiapas (Comitán region) and Oaxaca	Dent; white or yellow, rarely purple	Wellhausen et al. (1951)	Wellhausen et al. (1951)
(F) *Vandeño*	Pacific coast, Michoacán to Chiapas; rare	Dent, generally white	Wellhausen et al. (1951)	Wellhausen et al. (1951)
(G) *Choapaneco*	Oaxaca (lowlands North of Sierra Juárez); rare	Dent; white	Benz (1986)	Benz (1986)
(H) *Conejo*	From Michoacán to Oaxaca (Tierra Caliente region)	Semi-crystalline, dent; white	Wellhausen et al. (1951)	Wellhausen et al. (1951)
(I) *Ratón*	Tamaulipas and Coahuila	Dent; white	Ortega Paczka (1979)	Ortega Paczka (1979)
(J) *Motozinteco*	Chiapas Midlands	Dent; white	Ortega Paczka (1979)	Sánchez (1989)
(K) *Zapalote chico*	Tabasco and Oaxaca (Tehuantepec region)	Dent; white	Wellhausen et al. (1951)	Wellhausen et al. (1951)
(L) *Tepecintle*	Oaxaca and Chiapas; rare	Dent; white or yellow	Wellhausen et al. (1951)	Wellhausen et al. (1951)
(M) *Zapalote Grande*	Oaxaca and Chiapas; rare	Dent; white or yellow	Wellhausen et al. (1951)	Wellhausen et al. (1951)
(N) *Nal-Tel*	Yucatán Peninsula; rare	Semi-crystalline; yellow, rarely white or purple	Wellhausen et al. (1951)	Wellhausen et al. (1951)
(O) *Tehua*	Chiapas (Central Lowlands region); very rare	Dent; white or orange	Wellhausen et al. (1951)	Wellhausen et al. (1951)
(P) *Pepitilla*	Guerrero (Mid-Highlands); rare	Thin, ending in a sharpen point; floury endosperm, aleurone and pericarp often transparent; white, ivory	Wellhausen et al. (1951)	Wellhausen et al. (1951)

(continued)

Table 1 (continued)

Name	Distribution	Kernel type	Identification	Description
(Q) *Serrano Mixe*	Oaxaca (Mixe Choapaneca region; midlands North of Sierra Juárez); rare	Dent; white, yellow	Benz (1986)	Benz (1986)
(R) *Negro de Chimaltenango*	Chiapas (Southern region).	Dent; dark, purple	Ortega Paczka (1979)	No published description
(S) *Chatino Maizón*	Oaxaca (West of Tehuantepec region)	Dent; white	Benz (1986)	Benz (1986)
(T) *Coscomatepec*	Veracruz; rare	Dent; white	Ortega Paczka (1979)	Sánchez (1989)
(U) *Olotón*	Oaxaca and Chiapas (Midland regions)	Semi-crystalline, dent; white, yellow, purple, or red	Wellhausen et al. (1951)	Wellhausen et al. (1951)
(V) *Dzit Bacal*	Yucatán Penninsula	Dent; white or yellow, rarely purple	Wellhausen et al. (1951)	Benz (1986)
(X) *Olotillo*	Chiapas (Central Lowlands region); lower region of the Balsas River Valley	Dent; white or yellow, rarely purple	Wellhausen et al. (1951)	Wellhausen et al. (1951)
Group IV: Chapalotes				
(A) *Chapalote*	Sinaloa and Sonora; rare	Crystalline to floury; red, bronze, purple or black	Wellhausen et al. (1951)	Wellhausen et al. (1951)
(B) *Dulcillo del Noroeste*	Nayarit, Sinaloa and Baja California	Sweet endosperm; yellow or orange	Wellhausen et al. (1951)	Sánchez (1989)
(C) *Elotero de Sinaloa*	Nayarit, Sonora and Baja California; rare	Crystalline; purple or dark	Ortega Paczka (1979)	Sánchez (1989)
(D) *Reventador*	Nayarit, Sonora and Baja California	Crystalline or popcorn; white or yellow	Anderson (1946)	Anderson (1946)

Letters indicate the index depicted in Fig. 2

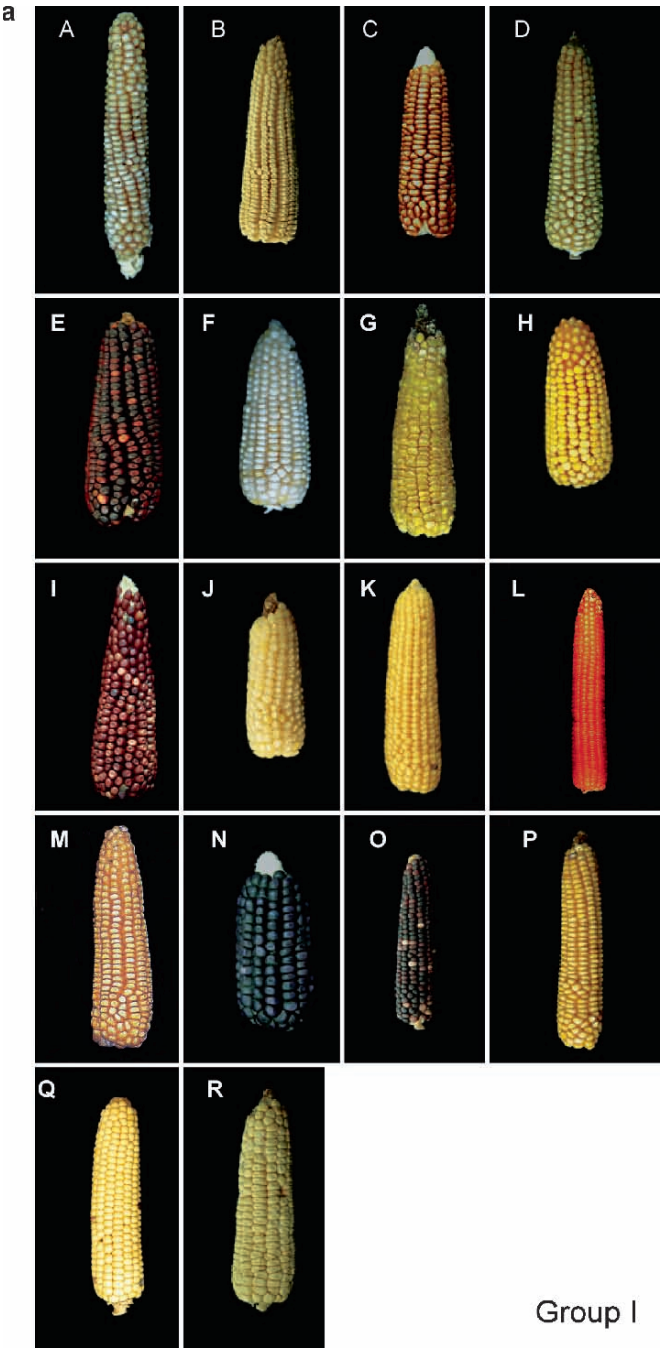

Fig. 2 Examples of ears from 57 Mexican landraces. Groups correspond to the classification of Sánchez et al. (2001). A detailed description of these landraces and their distribution is presented in Table 1

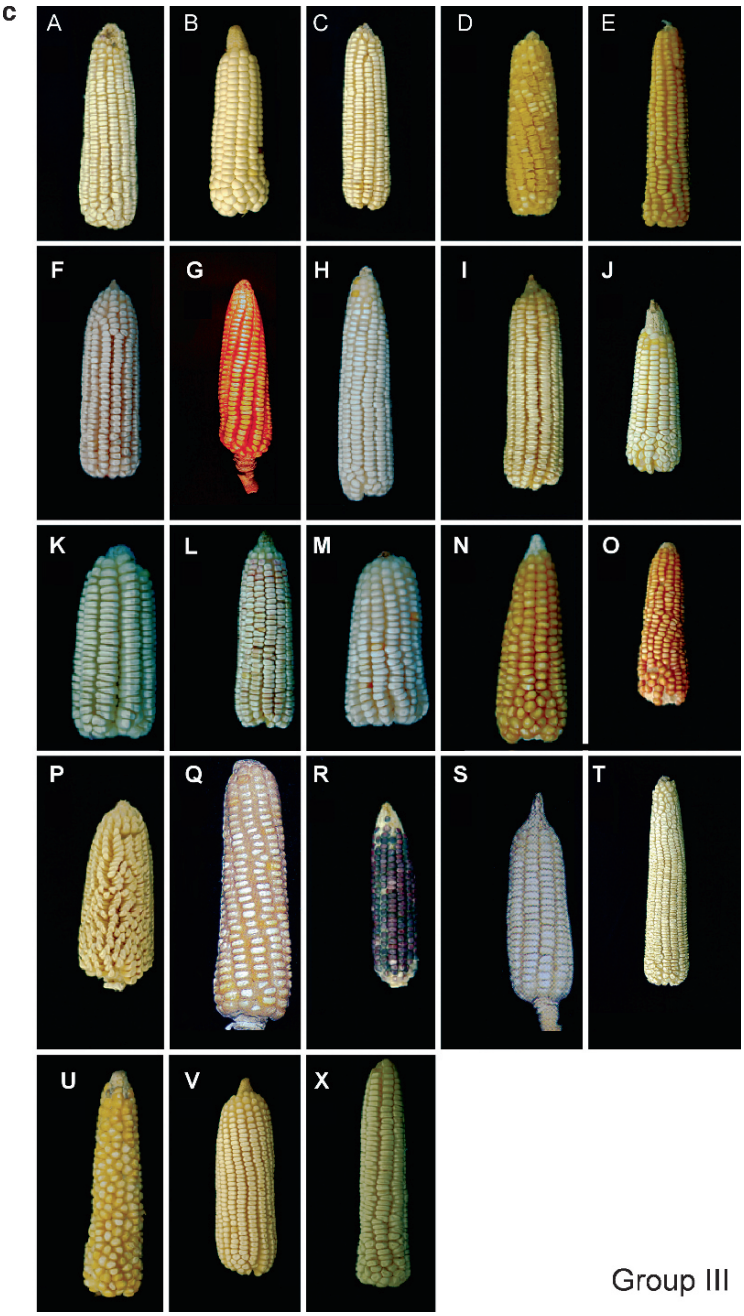

Fig. 2 (continued)

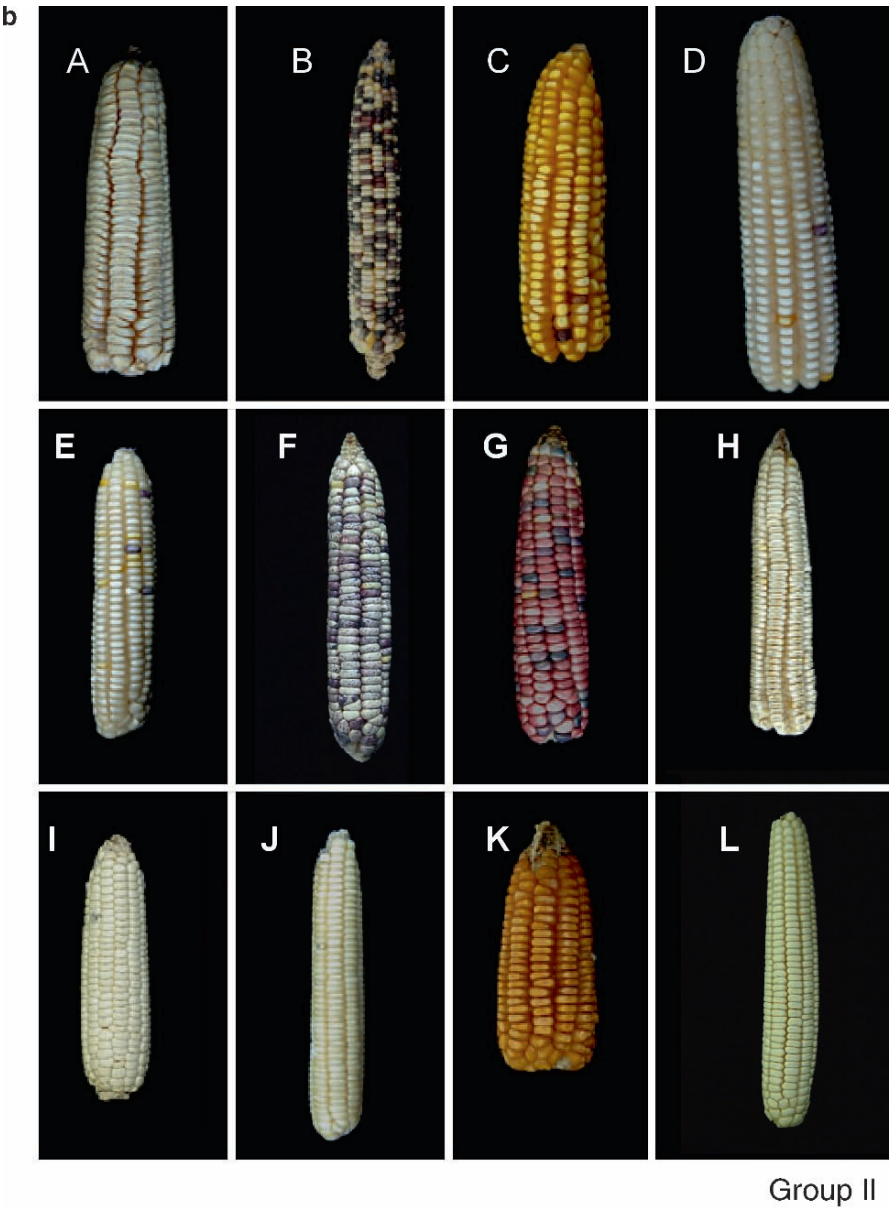

Fig. 2 (continued)

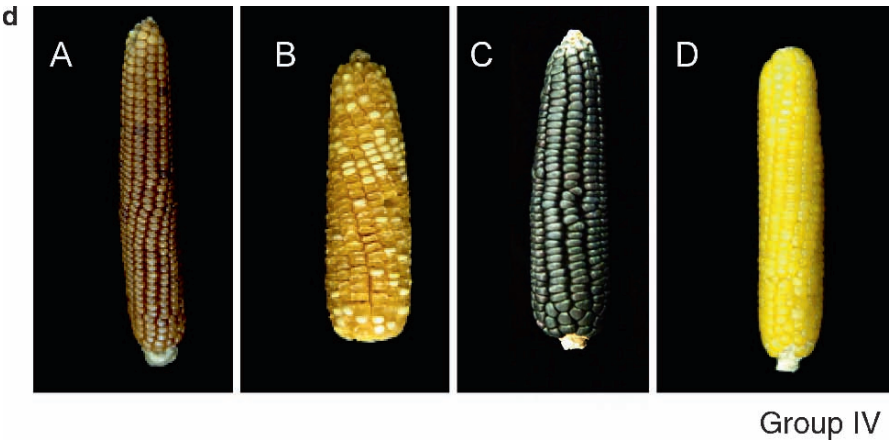

Fig. 2 (continued)

– The Late Maturity Group includes seven landraces. Whereas *Comiteco*, *Coscomatepec*, *Motozinteco, Olotón* and *Tehua* are all from Southern Mexico and distributed at elevations comprised between 900 m and 2,200 m, *Dzit Bacal* and *Olotillo* are from Southern States but cultivated at elevations between 500 m and 700 m. All are characterized by tall plants (290–380 cm), late-maturity cycles (88–110 days to flowering); many tassel branches, and high sensitivity to photoperiod and temperature fluctuations. Ears from *Olotillo* and *Dzit Bacal* have 8 rows; all others have 10–16 rows.
– The Tropical Dents are 12 landraces subdivided in two subgroups. The first one includes *Conejo*, *Nal Tel*, *Ratón*, *Tepecintle*, *Zapalote Chico* and *Zapalote Grande*. All are characterized by short fast maturing plants adapted to low elevations. The second subgroup consists of *Celaya*, *Nal Tel de Altura*, *Pepitilla, Tuxpeño*, *Tuxpeño Norteño*, and *Vandeño*; all races are adapted to low to medium elevations (0–1,700 m) and characterized by tassel branched plants with long cylindrical ears (12–16 rows) and deeply dented kernels.
– The Chapalote Group includes only four landraces (*Chapalote*, *Dulcillo del Noroeste*, *Elotero de Sinaloa*, and *Reventador*). Although the isozymatic data had a tendency to suggest similarities with Tropical Dents and the Late Maturity groups (Fig. 1), members of the Chapalote group were classified in a separate class mainly because of their distinctive morphological characteristics. *Chapalote* is one of the most distinctive and ancient landraces of Mexico (Wellhausen et al., 1951). These races have slender cigar-shaped ears, weak tapering at both ends, and are commonly found at low elevations (100–500 m in the Pacific Coast).

Although no morphological data was available for some of the additional landraces, passport data was used to place *Palomero de Jalisco*, *Mountain Yellow*, *Mixteco* and *Negrito* in the Highlands Group, with the Cónico complex. With the same procedure, *Mixeño* and *Negro de Chimaltenango* were assigned to the Late Maturity Group, and *Choapaneco* to the Tropical Dents (Sánchez et al., 2000).

5 Genetic Erosion of Mexican Maize Diversity

Genetic erosion refers to germplasm and gene loss by the elimination of native local populations caused by exogenous factors such as the adoption of hybrid commercial varieties or drastic changes in land use (Plucknett et al., 1992). Several factors have eroded the endemic diversity of Mexican landraces. Over the last 70 years, the adoption of hybrid varieties has been particularly strong in regions like Central Mexico, the States of Jalisco and Nayarit, or Northeastern regions such as the State of Tamaulipas and the Laguna basin in Coahuila (Ron-Parra et al., 2006). The global economic integration of Mexico, in particular through the North American Free Trade Agreement (NAFTA), has increased pressure on agro-biodiversity, promoting monoculture specialization on farmers that now focus on high-yield varieties. At the same time, the Mexican government has constantly discouraged the production of colored maize by subsidizing the production of white corn, a policy that almost caused the extinction of pure landraces such as *Tehua* or *Jala*. According to Ortega Paczka (2003), there is at least one nearly extinguished landrace (*Tehua*), 6 that are endangered (*Jala, Zamorano Amarillo, Vandeño, Zapalote Grande, Pepitilla, and Motozinteco*), and 13 that are rare or difficult to find under cultivated conditions (*Apachito, Palomero Toluqueño, Palomero de Chihuahua, Mountain Yellow, Gordo, Blandito de Sonora, Tabloncillo, Choapaneco, Tepecintle, Nal Tel, Mixeño, Serrano Mixe, and Coscomatepec*).

6 Landrace Genome Sequencing and Functional Maize Diversity

The phenotypic and molecular diversity of maize has been essential to harness important traits for crop improvement. On the basis of landrace germplasm, the activity of modern plant breeders gave rise to inbred lines currently used in hybrid production, causing significant improvements in yield, grain quality, resistance to biotic or abiotic stress, and maturity (Walden, 1979). For example, it is well known that members of the Tropical Dents such as *Tuxpeño* and *Tuxpeño Norteño* were fundamental for several breeding programs in Mexico and around the world. Interestingly, and despite the importance of selection-dependent bottleneck effects that drastically reduced genetic diversity, most maize genes have retained high levels of nucleotide diversity as compared to other cereals (Tenaillon et al., 2001; Wright et al., 2005; Yamasaki et al., 2005). Wright et al (2005) compared SNP diversity between maize inbred lines and teosintes and concluded that the number of genes that show signs of human selection was close to 1,200. This estimation is in agreement with subsequent studies indicating that less than 3% of maize genes have been the targets of intentional or unintentional modification of individuals in a population through human action (artificial selection; Yamasaki et al., 2007). All other genes remain unselected but show evidence of a population bottleneck associated with domestication and crop improvement. It is currently estimated that

selected alleles at loci regulating plant architecture and seed nutritional quality were genetically fixed at least 4,400 years ago (Jaenicke-Depres et al., 2003).

A genome wide survey of gene content in B73 and Mo17 revealed that more than 20% of gene fragments examined in allelic contigs were not shared between these two inbred lines (Morgante et al., 2005), a divergence that can be largely due to the activity of transposable elements such as *helitrons* (Fu and Dooner, 2002; Lai et al., 2005). Considering that single nucleotide polymorphisms that distinguish two maize inbred lines are on average as significant as those distinguishing humans form chimpanzees (Tenaillon et al., 2001), reasonable predictions anticipate that the genomic divergence between two landraces is far more important. The activity of specific families of transposable elements diverge in maize landraces. For example, transcriptionally active *MuDR* elements, the regulatory element of the *Mutator* transposon family that had been found only in the two specific maize lines, is also present in specific accessions of *Zapalote chico* (Gutiérrez-Nava et al., 1998). It is likely that the large non-homologies that characterize maize genomes will substantially contribute to landrace structural diversity (Fu and Dooner, 2002). As a consequence, large-scale sequencing efforts concentrated in B73 will not be sufficient to fully understand maize genome organization and identify all genes. To complement the large-scale B73 sequencing initiative and explore landrace genomic diversity, we recently undertook the structural and functional characterization of the *Palomero Toluqueño* genome after estimating its size at ~1,950 Mb (Mexican Maize Genome Team, unpublished results). A total of 1.2 million Sanger reads (10% HCot; 90% enzyme-based methyl-filtration) and 213 pyrosequencing runs (50% methyl-filtered, 50% whole genome sequencing) were sequenced at the National Laboratory of Genomics for Biodiversity (Langebio). The total sequence generated represents coverage of more than 3X the full genome; it has been complemented by in-depth pyrosequence-based global transcriptional analysis of the same landrace. As expected, at least 15% of codifying transcripts are not reported in publically available databases (Julio Vega-Arenguín et al., unpublished results), suggesting that a large portion of the molecular and functional diversity contained in Mexican landraces remains unexplored; the process of assembly and annotation is well underway.

Access to large-scale structural genomic information is rapidly transforming QTL mapping, positional cloning, and association approaches by allowing dissection of complex traits down to the gene or nucleotide level (Buckler et al., 2006). Since the resolution of association mapping is dependent upon the structure of linkage disequilibrium (LD or the non-random association of alleles between loci; Yu and Buckler, 2006) and does not require the generation of a mapping population, the diversity of Mexican landraces might represent the most suitable maize germplasm for these approaches. Whereas LD can be over 100 Kb for commercial elite inbred lines, it extends less than 1,000 bp for maize landraces (Buckler and Gore, 2007; Remington et al., 2001). Although the local prevalence of genomic non-homologies might result in potential difficulties by reducing recombination and preserving LD, the access to large-scale landrace sequence information will represent

an invaluable source of polymorphic information for exploring maize natural variation and exploiting allele diversity and recombination. We expect that a renewed interest in landrace germplasm will emerge with the development of new international initiatives to explore the functional diversity of maize.

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Production, Breeding and Process of Maize in China

Jiansheng Li

Abstract Maize has become one of the most important crops in China recently. The total output of maize grain increased 12-fold during past six decades. The average grain yield of maize ranks the second place among cereal crops. Maize is mainly used for animal feed, accounting for 67.45% of total production in 2006. Much of maize production is concentrated in the slope long narrow regions from northeast to southwest, which is called the Chinese Corn Belt. Agronomically, maize in China is classified into two major types, spring and summer maize. Maize disease prevalence in China varies with areas, planting seasons and cropping patterns. Asian maize borer is a predominant maize pest. Hybrid maize. Today, hybrids cover 97% of total national planting areas of maize, which contributes 40% of grain yield increase in China. Maize germplasm introduced from the US Corn Belt has played an important role in developing elite hybrids but local germplasm is also important in China. During the past decade, biotechnology as a powerful tool has gradually been adapted into maize breeding programs in China. For the processing industry of maize in China, wet milling is a majority processing technology, which accounts for 60–70% of total processing maize. In the future, to meet the demand of a large domestic market, the increase of total output will depend more on average grain yields so that genetic improvement could play a more important role in raising average yield than before.

1 Introduction

1.1 History of Maize in China

Historically, maize is an exotic crop in China. The earliest literature on maize appeared in a local historical book, "Yingzhou Records," in 1511, indicating that maize arrived in China from the outside world before 1511, during the Ming Dynasty. Thus, China has been growing maize for nearly 500 years. But it is still a mystery when and how maize was introduced into China. According to some agronomic historical studies, there may have been two possible ways in which maize

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was introduced in China. One is called the land-way spread, in which maize was first brought to Tibet from India, then to Sichuan Province in southwest China. The other way says that maize came from the oceans, first shipped to the coastal areas of southeast China by boats, from where it spread to other parts of Mainland China. By the 1700s, maize was widely grown in southern China. During the eighteenth century, a rapid population expansion forced the Chinese to turn to new crops that could grow in the hills and mountains outside the rice-growing plains, to meet dietary needs. At this time, maize became widely established in northern China.

The early-introduced maize was the flint type while dent maize (e.g., Gold Queen, and While Italy) was first introduced in the second decade of the twentieth century from the USA and Italy. After human and natural selection for 500 years, the local maize germplasm in China generated four major races: Northern Dent, Northern Eight-Row Flint, Semi Dent, and Southern Waxy. Most varieties of the Northern Dent originated from the USA's dent maize. The Northern Eight-Row Flint may have come from New England Flint and Long Fellow also in the USA. A cross between flint and dent maize produced the Semi Dent maize. The Southern Waxy maize is distributed across the mountain areas of southwest China. An American missionary, Collins, first described waxy maize using a Chinese waxy maize collection, which was brought to the USA in 1908. Except for four races mentioned above, it is likely that Chinese maize was first introduced to southwest China through the "land-way," a race of Southwest Flint could also exist because there are a large number of local maize varieties of which the flint type is dominant, based on the germplasm survey in southwest China.

1.2 Significance of Maize in Chinese Economy

Maize is one of the most important crops in China. In order to feed the large population, China has become the second largest maize producer in the world, just behind the USA, and its annual production has made up 18–20% of the world total. During the past 46 years, the area planted to maize in China more than doubled from 11.07 million hectares in 1949 to 26.35 million hectares in 2005. The national average yields of maize rose from 1.06 tons per hectare in 1949 to 5.29 tons per hectare in 2005, representing a fivefold increase. The total output of maize grain reached 139.4 million tons in 2005, 12-fold increase from the 11.75 million tons produced in 1949. The increase in maize production in China is mainly attributed to the introduction of hybrid maize and fertilizer use except an increase in planting areas.

In recent years, the significant change in Chinese agriculture has been the increased importance of maize among the three major cereals, rice, wheat and maize. It used to rank behind rice and wheat on planting areas, average yields and total outputs. With the increase in living standard, Chinese people are consuming more meat-based foods, thus more maize is needed as animal feeds. Since 1996, both the total outputs and average grain yield of maize have exceeded those of wheat, and maize now ranks second among cereals, after rice. In the past decade,

maize contributed 76.1% of the increased yield of cereals in China while rice and wheat only contributed 12.1% and 11.1% respectively. Maize plays a more important role in food security in China.

1.3 Utilization and Marketing of Maize

In China, maize is used for human food, animal feed and industrial material. During the past two decades, with economic and technological development, some changes in the proportions of maize used for different purposes have taken place. Since 1978 when China started its economic reform, the proportion of maize used for feed has increased by 2–3% per year. In contrast, the proportion used as human food gradually declined. In 2005–2006, 94 million tons of maize was consumed as animal feed, which accounted for 67.45% of total maize production in China and only 9.2 million tons of maize was used for human food, which is 6.6% of total production.

In recent years, the industrial demand for maize use has increased sharply. From 1991–1992 to 2003–2004, maize consumption for industries had a gradual increase from 5 million tons to 14.5 million tons, which accounted for 5% and 12.5% of maize harvest respectively. However, in 2005–2006, about 17.2% of maize was used for industrial proposes, reaching to 24 million tons. More recently, a small proportion of maize has been used to produce fuel ethanol and a number of plants have been set up, which consumed 1.1 million tons of maize in 2006. In order to insure food security, the Chinese government issued a strict regulation to limit the establishment of plants for ethanol production with maize in 2006.

The rapidly emerging changes in the national economy have dramatically altered the way farmers market their maize. Maize produced by farmers used to be fed to livestock and poultry directly in farmer households. Now, much of it is sold to local cereal dealers. Even if farmers want to raise livestock and poultry in their household, they would like to sell their harvested maize first, and then purchase formulated feed from markets. With the transition from a centrally planned to a market economy, especially with China's entry to the WTO in 2001, agro business has become more open to the outside world. Therefore, international markets directly influence domestic maize prices in China.

2 Maize Production in China

2.1 Ecological Characterization of Primary Maize-Growing Zone in China

As a widely adaptive crop, maize grows across all of China, including Tibet, which has the highest altitude in the world, where 3.3 thousand hectares of maize were grown in 2005. Ecologically, the maize producing region in China can be

divided into six primary zones, the north zone, the Yellow and Huai River zone, the southwest zone, the south zone, the northwest zone and the QinHai-Tibet altiplano (Table 1).

The north zone includes Heilongjiang, Jilin, and Liaoning provinces, Inner Mongolia and Ningxia municipalities, and parts of Hebei and Shanxi provinces. It is the largest maize production zone in China, where 10.55 million hectares of maize are grown annually. In this zone, the average rainfall varies from 400 mm to 800 mm per year and 60% of the rainfall occurs between July and September. In the early stage of maize growth, there is often a low temperature. The annual cumulative total temperatures above 10°C in a day range from 2,000°C to 3,600°C (Table 1). The maize growing area of Shandong and Hebei provinces, as well as the parts of Hebei and Shanxi provinces, and the north of Anhui and Jiangsu provinces is located along the middle-lower reaches of the Yellow and Hui Rivers. Therefore it is called the Yellow and Huai River maize zone, which is the second largest maize production zone in China, with a maize-growing area of 9.39 million hectares. In this zone, the annual rainfall ranges from 500 mm to 800 mm and the cumulative total temperatures above 10°C in a day varies from 3,600°C to 4,700°C (Table 1).

The southwest maize zone includes Sichuan, Yunnan and Guizhou provinces, Guangxi municipality, and the parts of Hubei, Hunan and Shanxi provinces. In the zone, most of maize is planted on the hill and mountain areas from 250 m to 2,500 m above see level and the annual average rainfall varies from 800 mm to 1,200 mm. The some of areas in the southwest maize zone belong to tropical or sub-tropical regions, such as Yunnan and Guizhou provinces and Guangxi municipality.

Table 1 Characterization of primary maize-growing zone in China

	North China	Yellow and Huai River	Southwest China	Northwest China	South China	QinHai–Tibet
Areas (millions hectare)	10.55	9.39	5.01	1.01	0.54	0.0033
Percentage of total areas	40.0	35.6	19.0	3.8	2.0	0.01
Average yield (tons per hectare)	5.7	5.2	4.4	6.2	4.5	7.9
CT temp > 10°C	2,000–3,600	3,600–4,700	4,500–5,500	2,500–2,600	4,500–9,000	2,400–3,200
Rainfall (mm)	400–800	500–800	800–1,200	200–400	1,000–2,500	370–450

Note: CT Temp: cumulative total temperatures above 10°C

The south maize zone includes much of southern China, where there is much more rainfall than other zones during the maize growing season, varying from 1,000 mm to 2,500 mm per year. Across the zone, maize is grown sporadically because rice is the major crop. The northwest maize zone including Xinjiang municipality and Guansu province only has 300–400 mm of annual rainfall and 2,500–3,600°C of cumulative total temperatures above 10°C. Maize is only cultivated in irrigated areas. In the QinHai-Tibet maize zone, maize was first introduced as a new crop in the 1950s. Since then, it has been grown in valleys along rivers below 4,000 m of sea level.

Although maize can be planted in almost all of China's agricultural lands, much of its production is concentrated in the slope long narrow regions from northeast to southwest between latitudes 45° and 20° N, which is called the Chinese Corn Belt (Fig. 1). Two areas in the Chinese Corn Belt, the Northeast China and the Yellow and Huai River, are predominant for maize production. In Northeast China, about 60–70% of farmlands are used to cultivate maize with total planting area of 8.59 million hectares, and yield of 50.45 million tons for an average of 5.9 tons per hectare in 2005, accounting for 32.6% of total national maize-growing areas and 36.2% of total national output respectively. Similarly, in the area of the Yellow and Huai River, 9.39 million hectares of maize were planted, and 48.75 million tons of maize were produced with an average yield of 5.2 tons per hectare in 2005. They contribute 35.6% of national maize-growing areas and 35.0% of national total output respectively.

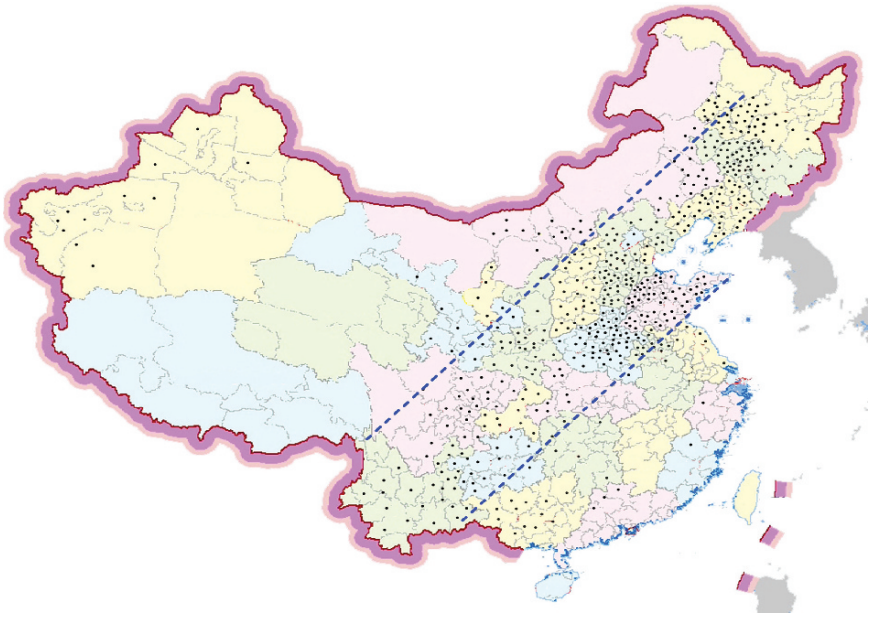

Fig. 1 The Chinese corn belt (one dot = 50,000 hectares)

2.2 Cropping Systems and Cultural Practices

On the basis of growing seasons, maize in China is classified into four types, spring, summer, autumn and winter maize. Probably, China could be the only country in the world, where maize is planted during the four seasons in a year. Both spring and summer maize are the most important in the cropping system of China. Nearly all maize planted in the north zone is spring maize. It is sown in April, planted one season per year as a mono-crop, and accounts for 70–80% of total farming lands. Some of the spring maize is rotated with soybean, sorghum or millet. But with the recent sharp decline in planting areas of soybean in the northeast China, the maize rotation system has become less practiced than before. Summer maize is a predominant type in the Yellow and Huai River maize zone, where it is almost always double-cropped with winter wheat. Maize is sown after wheat is harvested. The time for sowing summer maize varies between the early and the end of June, from the southern part of the Yellow and Huai River maize zone to the northern part around the Beijing-Tianjing areas. There is a small amount of autumn maize grown in the southern maize zone while only a little of winter maize is grown in tropical regions of the southwest maize zone, such as the south of Guangxi and Yunnan.

Since Chinese maize is planted in widely different areas with varying environments, there is diversity of cropping patterns. The considerably small farm sizes, from 0.1 hectares to 2 hectares per farm family on national average, makes the cropping patterns more complicated. In the Chinese Corn Belt, most maize is grown as a solid stand in a sequential cropping pattern because there is limited farmland relative to the populations. In the Yellow and Huai River maize zone, maize is also frequently intercropped with soybean, peanut, and different vegetable crops while its intercropping with sweet potato is a more popular pattern in the southwest maize zone, as in Sichuan province. In some parts of China, various relay cropping patterns exist for different purposes. In some parts of north China, e.g., Inner Mongolia and Ningxia municipalities, spring maize is planted as a relay crop between rows of spring wheat so that farmers can choose late maturing varieties for high-yield. There is a similar relay-cropping pattern in Southwest China. Farmers can plant maize much early in order to avoid drought damage, which often occurs from mid-July to mid-August.

In China, most of the cultural practices from sowing seeds to harvesting are done manually because farm sizes are considerably small. Generally, farmers sow two or three hybrid seeds per hill and plant populations at harvest are 37,500 to 60,000 per hectare. Weed control used to be manual, but now herbicides are widely used to control weeds in maize fields. More recently, some machines for sowing maize and harvesting ears have been adopted in some parts of the Chinese Corn Belt, such as Heilongjiang, Jilin, Shandong and Hebei provinces, where farmers have considerably larger farm sizes and better income than other regions.

2.3 Disease and Pest Control in Maize

Maize disease prevalence varies with areas, planting seasons and cropping patterns since maize is distributed in diverse environments across the Chinese Corn Belt. In the Northeast maize area, the northern leaf bright, leaf spot, stalk rot, and dwarf virus can cause yield losses, but heat smut frequently occurs because there is a low temperature during planting and maize seeds stay in the cold soil for quite a long time. Yield damage caused by heat smut is often most severe. In maize regions of the Yellow and Huai River, sugarcane mosaic virus, southern and northern leaf bright, leaf spot, dwarf virus, kernel and stalk rot are frequently prevalent, but sugarcane mosaic virus causes the most damage especially when maize is planted relaying with un-harvested wheat. In the Southwest China maize zone, southern and northern leaf bright, kernel and stalk rot, sugarcane mosaic and dwarf virus often cause yield losses. However, banded leaf and sheath blight seems to have become more important diseases because a few cases of host resistance have been found. Southern rust often occurs in tropical and sub-tropical maize areas, like Yunnan and Guangxi province. Using resistant hybrids is the most efficient measure to control maize diseases in China.

Asian maize borer is a predominant maize pest, which may cause 5–7% of grain loss and chemical control is widely used against it. So far, there is no GMO (genetically modified organism) maize that can be used against maize borer in China.

3 Maize Breeding in China

3.1 History and Current Status of Hybrid Maize

The earliest breeding program for maize hybrids started in 1926 at Nanjing Jinling University, China. Then, several scientists in the different parts of China selected inbred lines from local OPVs. In that time, although a few of improved maize varieties were obtained, they did not have any chance to come into use because of the Sino-Japanese War and the Civil War. The development of hybrid maize as a modern breeding system was initiated in China, in 1950. Two pioneers of modern maize breeding made a great contribution to promote the development of hybrid maize and train maize breeders. One was Dr. Shaokui WU, who received his Ph.D. degree at Minnesota University. Another was Dr. Jiangxiong LI, who obtained his Ph.D. at Cornell University. Both returned to China from the USA in the 1940s.

The history of modern maize breeding in China can be divided into three periods (Fig. 2), OPV (1954–1959), double hybrids (1960–1970), and single hybrids (1971–now). The change trends for average grain yield in different historical

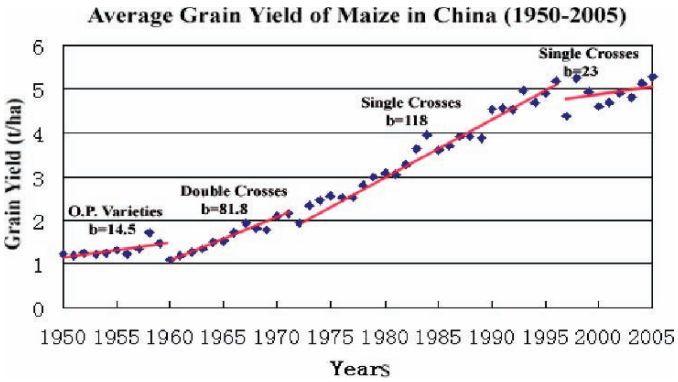

Fig. 2 National average grain yield of maize from 1950 to 2005 in China

Table 2 Percentage of areas growing hybrids with yield increase during different years

No.	Year	Areas of hybrids[a] (%)	Grain yield (tons per hectare)
1	1975	55	2.5
2	1980	70	3.1
3	1987	80	3.9
4	1996	90	5.2

[a]Percentage of areas growing hybrids over total maize planting areas

periods of modern maize breeding are similar to the USA. The only difference is the time delay. A number of first generation double hybrids were released around 1960. The first single hybrid, Xinduan No. 1, was released in 1968. Superior single hybrids significantly out-yielded the best OPVs and double hybrids so that they were rapidly extended across the Chinese Corn Belt. The wide extension of single hybrids resulted in average yield increasing from 2.5 tons per hectare in 1975 to 5.2 tons per hectare in 1996 in China (Table 2). During 1971–1998, *b* values (regressions) of average yield per year reached to 118 (Fig. 2). Except for fertilizer uses, soil improvements, and other cultivating measures, heterotic utilization contributes 40% of grain yield increase in China. Today, hybrids cover 97% of total national planting areas of maize. Taking 2005 for example, 372 hybrids were grown in China, among which, each of the top ten hybrids was planted over 0.4 million hectares of harvested areas. However, after 1998, average yield increase trends began to slow down considerably (Fig. 2), suggesting new breeding technologies and elite germplasm will be needed for further improving maize hybrids.

In recent years, special maize is needed in China to meet market demand. One significant change is that fresh maize including super sweet, normal sweet and

waxy maize as a vegetable and a process crop has become more popular especially in South China. Planting areas of fresh maize increased from 3,000 hectare in 1996 to 325,000 hectare in 2006. With a dramatic increase of dairy and beef cattle numbers, there is increasing interests in silage maize. A total annual area planting silage maize hybrids is 100,000 hectare approximately. Since 1984, Professor Song's team at China Agricultural University (CAU) has developed nine high-oil maize populations. Their oil concentration in grain reaches to 12.25–18.85% after long-term selections (Song and Chen, 2004). A number of high-oil single hybrids with more than 6% of oil concentration have been released. The areas planting high-oil hybrids were 62,600 hectare in 2004. A few numbers of QPM (quality protein maize) hybrids that contain partial CIMMYT germplasm have been extended in Yunnan Province in Southwest China.

3.2 Germplasm Resources and Heterotic Groups

Maize germplasm introduced from the US Corn Belt has played an important role in developing elite hybrids in China. Historically, some public elite inbred lines from the USA, such as C103, Oh43, Mo17, B73, were widely used in breeding program for hybrids. For example, Mo17 used to be crossed with a local inbred line, Zi330, as an elite single hybrid (Zhongduan No. 2), which was widely grown in China with two million hectares of harvest areas in 1986 alone. Local germplasm is also important for maize breeding because it has been broadly adapted to local environments after an ~500 year evolution. A larger number of local varieties from the different parts of China have been collected and stored in the national germplasm bank at China Agricultural Academy of Science (CAAS). Only in the southwest maize zone, 3,913 local maize varieties were collected during 1950–1980. Among the local germplasm, a group of inbred lines containing pedigree of Tang Sipingtou was more outstanding. They combine a number of elite hybrids that are widely extended either in the northeast China maize zone or the Yellow and Huai River maize zone. In the tropical and subtropical regions of China, CIMMYT germplasm is also used to develop inbred lines for hybrids.

Recently, two research groups at CAU and at CAAS have employed molecular markers to classify heterotic groups of Chinese germplasm using different lines (Yuan et al., 2001; Teng et al., 2004). Their results are similar and supported by pedigree data. Generally, Chinese maize germplasm can be classified into five major heterotic groups, Lancaster, Reid, Tang Sipingtou, Zi330, and Tem-tropic I. According to results from CAU (Teng et al., 2004), Lancaster group includes Mo17 while Raid group contains B73. Based on pedigree data, the Zi330 group may be related to Oh43. These data indicate that germplasm originating from the US Corn Belt plays an important role in hybrid maize breeding in China. The elite inbred lines having pedigree of local germlasm are clustered together, among which, lines derived from Tang Sipingtou are predominant. Tem-tropic I group may be related to tropic or subtropical germplasm, like Suwan synthetics from Thailand.

3.3 Production of Hybrid Seeds

In the past decade, the economic reform and technological progress in China have dramatically altered the way to produce hybrid seeds of maize. The production of hybrid maize seeds used to be operated only by public seed companies. After the Seed Law of the People's Republic of China was issued in 2000, a large number of private seed companies were set up. The leading international seed companies, such as Pioneer, Monsanto, Syngenta, also entered the Chinese seed market. As a result, the quality of commercial hybrid seed has significantly improved recently.

Today, the single cross is the predominant type of maize hybrid in the China Corn Belt, which accounts for 98% of total hybrids; although a small number of modified single crosses and three-way crosses are used in poor regions. Annually, there is about 0.3 million hectares of harvest areas for hybrid seed production and around 1.12 million tons of hybrid seeds are produced. Northwest and North China are the most important zones for hybrid seed production, where about 80% of total hybrid seeds are produced and they only have 60–70% of harvest areas for seed production. Especially, in Guansu province, annual planting areas for seed production reach 0.08 million hectares, which accounts for about 25% of national total areas, because there is adequate irrigation system and a few maize diseases occurs.

In the hybrid production field, the ratio of female to male depends principally on the amounts of pollen of the male parent, ranging from 4:1 to 6:1. All tassels from female rows are manually removed in China. In order to increase seed yield, farmers sometime conduct artificial pollinations. After harvesting ears, most commercial hybrid seeds are dried in the sun. Recently, a few relatively large seed companies in the major seed production areas have set up some seed drying systems through heating. During seed processing, harvesting, husking and sorting are done manually while shelling, cleaning and grading are done with different machines. Isozyme markers are widely used through starch gel electrophoresis to test genetic purity of hybrid seeds in relatively large companies. Today, most hybrid seeds sold in China are treated with fungicides and insecticides.

In order to protect the intelligent property right of the new varieties, regulations of the People's Republic of China on the protection of new varieties of plants (PVP) was issued by the States Department of China in 1997. The application numbers for maize PVP accepted by the PVP office increase dramatically from 56 in1999 to 327 in 2006. Until 2006, the rights of 391 maize varieties have been granted.

3.4 Application for Bio-Technology in Maize Breeding

During the past decade, biotechnology as a powerful tool has gradually been adapted into maize breeding programs in China. The modified *Bt* gene that is resistant to the maize borer was constructed in CAAS and was successful transferred

into maize inbred lines at CAU, although the transgenic maize is still waiting to be approved for commercialization in China. Molecular markers, such as RFLP, SSR, were first used to classify the heterotic groups, and then to map some important quantitative trait loci (QTL). At CAU, the high-oil line from BHO (Beijing high-oil) population was employed with SSR marker to map QTL for oil concentration (Song et al., 2004) and a major QTL accounted for over 15% of variation was detected on chromosome 1. A number of QTL and epistatic loci associated with grain yield and yield components were identify using populations derived from an elite hybrid (Yan et al., 2006; Ma et al., 2007). In order to isolate diseases resistant genes, a total of 197 unique RGAs (resistance gene analog) were identified, and at least 185 of them were derived from the putative expressed resistance genes in maize (Xiao et al., 2006). At Huazhong Agricultural University, Zheng' group identified 20 QTL related to drought tolerance under water-stressed condition, and 25 and 34 QTL associated with water-logging tolerance respectively over 2 years (Xiao et al., 2005; Qiu et al., 2007). At Sichuan Agricultural University, Zhao et al. (2006) mapped 11 QTL for resistance to banded leaf and sheath blight in maize. Based on the sequence information of O_2 gene, a research group at CAAS developed dominant markers based on PCR to identify the O_2/o_2 genotype for QPM breeding. These results provide the potential molecular markers for marker assistant selection during future maize breeding.

4 Maize Process and Products

4.1 Traditional and Current Maize Food

There is a considerable range for maize uses as human foods in China, depending on the differences of economy and people customs. Generally, maize foods can be divided into three major parts, mixture of flour with grit, grit only and whole kernels according to raw stuff types. The maize food is mainly processed at home with traditional methods. There are some differences in details of recipes for each class of maize food among different households, communities, and regions.

Porridges are the most common and simplest foods prepared with maize flour, which are divided into two types. Thick porridges are "solid" and can be eaten with the fingers or utensil, while thin porridges are "fluid" and may be drunk or eaten by a utensil. The basic procedure for the preparation of porridge includes adding flour to boiling water and stirring continuously until the flour forms a thick, homogeneous and well-gelatinized mass. The flint maize with hard endosperm is preferred to make porridges because it has the special flavor.

Maize flours are often used to make different kinds of breads. The maize flatbread is the most popular type of unfermented dry pancakes. Sometimes maize flour is mixed with wheat, legume flour or milk, eggs in various proportions to suit

taste and palatability. Consumers usually prefer yellow flatbreads made with yellow maize flour, which are called golden flatbreads. Maize flours are also used to make Chinese noodle based on the procedure of wheat noodle. The steam-cooked product made from maize is more popular in China, which called wowotuo. Generally, maize grits are preferred to prepared rice-like products. In Southwest China, yellow maize grits are often mixed with rice at a ratio of 1:1 to cook for a meal, which is named as the golden and silvery rice.

There are numerous variations in snack foods prepared from maize in China. Popped maize grains mixing with syrup is a famous snack food. After heating for popping, popping grains are mixed with cereal syrup to make ball-shape products. They may be consumed directly or ground and mixed with other ingredients in various snacks. Recently, new food produced by maize is fast being developed with advanced food processing technology. The maize fried chip is a kind of new food in China, which is used for snack. It has becoming more popular in the urban areas of China. Usually, the chips are made with coarse maize grits. Maize cereal is another new food in China. Some people use it as breakfast in urban areas. In order to make maize cereal, steeped maize grains are mixed with fat, sugar, and salt to improve the taste, and then are cooked in the autoclave to allow gelatinization of maize grains.

4.2 Processing Industry of Maize and its Products

For the processing industry of maize in China, wet milling is a majority processing technology compared with dry milling, which accounts for 60–70% of total consumed maize. Usually, plants for processing grain and fuel ethanol plants employ dry milling. Some farmer households have their own small size device of dry milling to produce maize flours. The plant size of wet milling is considerably large. Recently, plant numbers and plant size have rapidly increased. In 1999, there were eight wet milling plants in China and only two plants with a capacity of processing 0.1 million tons of maize per year. In 2006, the wet milling plant number rose to 37 and four plants were able to process one million tons of maize annually. The top ten plants consumed 73.74% of processed maize and produces 3/4 of maize starch.

The main products from dry milling are maize grits, meals and flours. These products are used as raw materials to produce beer, alcohol, chips, and cereal in food industries. Maize starch is a major product of wet milling, being about 11.3 million tons in 2006. Starch is further processed into HFCS (High Fructose Corn Syrups), and various chemicals, such as sorbitol, lysine, citric acid, and lactic acid. Only 10% of maize starch is modified by chemical or physical methods. The major products of modified maize starch are pre-gelatinized starch and cationic starch.

In China, there is a long history to use maize as a resource to produce syrup for sugar food applications. Now, the traditional method for maize syrup has been

replaced by modern methods. Maize syrup is produced through concentrating sugar solution with heating. For the high qualitative maize syrup, its color should be pale yellow with slightly transparent and reducing sugar reaches to 36% by dry weight. The production of alcoholic drinks is one of most important food industries in China, and also one of the major revenue resources in China. The Chinese alcoholic drinks made from fermented mixed cereals, such as sorghum, maize and rice, have complicated classifications and distinct aroma and flavor features, because of different processing methods adopted and different raw materials used in the production of spirits (Qin and Zhang, 1998).

5 Future Prospects

In the future, with a further raise of people's living standard and the development of maize process industry, maize will increase in importance as an animal feed and industrial material in China. If areas planted in maize keep increasing as the last 5 years, maize could become the number one crop over rice. However, the increase of planting areas will be limited in China. In order to meet the demand of a large domestic market, the increase of total output will depend more on average grain yields so that genetic improvement could play a more important role in raising average yield than before. Therefore new breeding technology, like biotechnology, and new elite germplasm will be needed to enhance maize heterosis. Drought will be a most important factor to constrain maize production. Thus, drought tolerance should be a priority target trait for maize breeding. On the other hand, the appropriate increased density of planting population could be an efficient cultivating measure to raise average yield of maize. If maize total output could not keep the continue increase in the next decade, China could turn from self-supported country for maize into importing country.

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Index