Chittaranjan Kole (Ed.)

Genome Mapping and Molecular Breeding in Plants

Fruits and Nuts

Springer

Genome Mapping and Molecular Breeding in Plants
Volume 4

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Chittaranjan Kole (Ed.)

Fruits and Nuts

With 50 Illustrations, 2 in Color

Springer

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Preface to the Series

Genome science has emerged unequivocally as the leading discipline of this new millennium. Progress in molecular biology during the last century has provided critical inputs for building a solid foundation for this discipline. However, it has gained fast momentum particularly in the last two decades with the advent of genetic linkage mapping with RFLP markers in humans in 1980. Since then it has been flourishing at a stupendous pace with the development of newly emerging tools and techniques. All these events are due to the concerted global efforts directed at the delineation of genomes and their improvement.

Genetic linkage maps based on molecular markers are now available for almost all plants of significant academic and economic interest, and the list of plants is growing regularly. A large number of economic genes have been mapped, tagged, cloned, sequenced, or characterized for expression and are being used for genetic tailoring of plants through molecular breeding. An array of markers in the arsenal from RFLP to SNP; tools such as BAC, YAC, ESTs, and microarrays; local physical maps of target genomic regions; and the employment of bioinformatics contributing to all the "-omics" disciplines are making the journey more and more enriching. Most naturally, the plants we commonly grow on our farms, forests, orchards, plantations, and labs have attracted emphatic attention, and deservedly so. The two-way shuttling from phenotype to genotype (or gene) and genotypte (gene) to phenotype has made the canvas much vaster. One could have easily compiled the vital information on genome mapping in economic plants within some 50 pages in the 1980s or within 500 pages in the 1990s. In the middle of the first decade of this century, even 5,000 pages would not suffice! Clearly genome mapping is no longer a mere "promising" branch of the life science; it has emerged as a full-fledged subject in its own right with promising branches of its own. Sequencing of the *Arabidopsis* genome was complete in 2000. The early 21st century witnessed the complete genome sequence of rice. Many more plant genomes are waiting in the wings of the national and international genome initiatives on individual plants or families.

The huge volume of information generated on genome analysis and improvement is dispersed mainly throughout the pages of periodicals in the form of review papers or scientific articles. There is a need for a ready reference for students and scientists alike that could provide more than just a glimpse of the present status of genome analysis and its use for genetic improvement. I personally felt the gap sorely when I failed to suggest any reference works to students and colleagues interested in the subject. This is the primary reason I conceived of a series on genome mapping and molecular breeding in plants.

There is not a single organism on earth that has no economic worth or concern for humanity. Information on genomes of lower organisms is abundant and highly useful from academic and applied points of view. Information on higher animals including humans is vast and useful. However, we first thought to concentrate only on the plants relevant to our daily lives, the agronomic, horticultural and technical crops, and forest trees, in the present series. We will come up soon with commentaries on food and fiber animals, wildlife and companion animals, laboratory animals, fishes and aquatic animals, beneficial and harmful insects,

plant- and animal-associated microbes, and primates including humans in our next "genome series" dedicated to animals and microbes. In this series, 82 chapters devoted to plants or their groups have been included. We tried to include most of the plants in which significant progress has been made. We have also included preliminary works on some so-called minor and orphan crops in this series. We would be happy to include reviews on more such crops that deserve immediate national and international attention and support. The extent of coverage in terms of the number of pages, however, has nothing to do with the relative importance of a plant or plant group. Nor does the sequence of the chapters have any correlation to the importance of the plants discussed in the volumes. A simple rule of convenience has been followed.

I feel myself fortunate to have received highly positive responses from nearly 300 scientists of some 30-plus countries who contributed the chapters for this series. Scientists actively involved in analyzing and improving particular genomes contributed each and every chapter. I thank them all profoundly. I made a conscientious effort to assemble the best possible team of authors for certain chapters devoted to the important plants. In general, the lead authors of most chapters organized their teams. I extend my gratitude to them all.

The number of plants of economic relevance is enormous. They are classified from various angles. I have presented them using the most conventional approach. The volumes thus include cereals and millets (Volume I), oilseeds (Volume II), pulse, sugar and tuber crops (Volume III), fruits and nuts (Volume IV), vegetables (Volume V), technical crops including fiber and forage crops, ornamentals, plantation crops, and medicinal and aromatic plants (Volume VI), and forest trees (Volume VII).

A significant amount of information might be duplicated across the closely related species or genera, particularly where results of comparative mapping have been discussed. However, some readers would have liked to have had a chapter on a particular plant or plant group complete in itself. I ask all the readers to bear with me for such redundancy.

Obviously the contents and coverage of different chapters will vary depending on the effort expended and progress achieved. Some plants have received more attention for advanced works. We have included only introductory reviews on fundamental aspects on them since reviews in these areas are available elsewhere. On other plants, including the "orphan" crop plants, a substantial amount of information has been included on the basic aspects. This approach will be reflected in the illustrations as well.

It is mainly my research students and professional colleagues who sparked my interest in conceptualizing and pursuing this series. If this series serves its purpose, then the major credit goes to them. I would never have ventured to take up this huge task of editing without their constant support. Working and interacting with many people, particularly at the Laboratory of Molecular Biology and Biotechnology of the Orissa University of Agriculture and Technology, Bhubaneswar, India as its founder principal investigator; the Indo-Russian Center for Biotechnology, Allahabad, India as its first project coordinator; the then-USSR Academy of Sciences in Moscow; the University of Wisconsin at Madison; and The Pennsylvania State University, among institutions, and at EMBO, EUCARPIA, and Plant and Animal Genome meetings among the scientific gatherings have also inspired me and instilled confidence in my ability to accomplish this job.

I feel very fortunate for the inspiration and encouragement I have received from many dignified scientists from around the world, particularly Prof. Arthur

Kornberg, Prof. Franklin W. Stahl, Dr. Norman E. Borlaug, Dr. David V. Goeddel, Prof. Phillip A. Sharp, Prof. Gunter Blobel, and Prof. Lee Hartwell, who kindly opined on the utility of the series for students, academicians, and industry scientists of this and later generations. I express my deep regards and gratitude to them all for providing inspiration and extending generous comments.

I have been especially blessed by God with an affectionate student community and very cordial research students throughout my teaching career. I am thankful to all of them for their regards and feelings for me. I am grateful to all my teachers and colleagues for the blessings, assistance, and affection they showered on me throughout my career at various levels and places. I am equally indebted to the few critics who helped me to become professionally sounder and morally stronger.

My wife Phullara and our two children Sourav and Devleena have been of great help to me, as always, while I was engaged in editing this series. Phullara has taken pains ("pleasure" she would say) all along to assume most of my domestic responsibilities and to allow me to devote maximum possible time to my professional activities, including editing this series. Sourav and Devleena have always shown maturity and patience in allowing me to remain glued to my PC or "printed papers" ("P3" as they would say). For this series, they assisted me with Internet searches, maintenance of all hard and soft copies, and various timely inputs.

Some figures included by the authors in their chapters were published elsewhere previously. The authors have obtained permission from the concerned publishers or authors to use them again for their chapters and expressed due acknowledgement. However, as an editor I record my acknowledgements to all such publishers and authors for their generosity and good will.

I look forward to your valuable criticisms and feedback for further improvement of the series.

Publishing a book series like this requires diligence, patience, and understanding on the part of the publisher, and I am grateful to the people at Springer for having all these qualities in abundance and for their dedication to seeing this series through to completion. Their professionalism and attention to detail throughout the entire process of bringing this series to the reader made them a genuine pleasure to work with. Any enjoyment the reader may derive from this books is due in no small measure to their efforts.

Pennsylvania,
10 January 2006

Chittaranjan Kole

Preface to the Volume

Fruit and nut crops make perhaps the largest group of species of economic importance and they by far outnumber any other major groups of domesticated plants. However, progress of genetic or genomic researches on fruit and nut crops is indeed much slower than the pace they deserve. Relatively more importance attached to the agronomic crops might be one of the reasons. The most important reason, to our mind, however, is the constraints inherent to the long life cycle, heterozygosity, space required to raise large populations often required, and difficulty in recording phenotypic trait data for most of the fruit and nut crops. The common constraints in most of these crops include too long juvenile period, problems of sterility and incompatibility, large plant size, the randomness of artificial mutations, limitations of the sexual system to incorporate small changes, the dependence upon natural origin of variation and the exorbitant costs needed to select, detect, and evaluate desirable recombinants those lead to the difficulties for genetic analysis and breeding. Most of these crops invoke for formulating strategies specific to the above problems and limitations, employment of pseudo-testcross method and use of SDRF markers, for examples.

Appreciable progress has been made in some fruit crops, mostly temperate, including say apple, grapes, stone fruits, cherries, citrus fruits. Still many others remain neglected, particularly the tropical and subtropical fruit and nut crops grown in the developing countries, litchi, custard apple, guava to name a few. These 'orphan' fruit and nut crops are too many and deserve global attention for concerted efforts. The presentation of the chapters in this volume, therefore, has nothing to do with the production statistics and relative economic importance of the fruit and nut crops at world level, but has been done primarily envisaging the quantum of works accomplished. We have included 20 chapters in this volume including seven chapters perhaps with the first time comprehensive review such as on mango, banana, olive, pineapple, pistachio, persimmon and papaya. How we wish to have independent volumes on temperate, and tropical and subtropical fruits and nuts in near future.

Due to some unavoidable circumstances there was delay for this volume to go to press and obviously the authors had to take pain to rework on the manuscripts for updating. I remain grateful to them for their co-operations and perseverance. I am also thankful to them for presenting the most current commentary on genomic researches on fruit and nut crops.

The former three volumes of this series have earned appreciation from all levels of readers and we hope this volume also will be liked by them. In that case the credit must go to the authors and the publishers for their contributions and care. I take the sole responsibility of all the shortcomings, and look forward to the readers for their suggestions for improvement in contents and format of this volume in its future edition(s).

Pennsylvania,
15 April 2006

Chittaranjan Kole

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Abbreviations

ACC	1-Amino-Cyclopropane-1-Carboxilate
ACC	Asian Citrus Canker
AFLP	Amplified Fragment Length Polymorphism
AP	Andhra Pradesh
AP-PCR	Arbitrary Primer-PCR
ARO	Agricultural Research Organization
BA	Benzyladenosine
BAC	Bacterial Artificial Chromosome
BIBAC	Binary Bacterial Artificial Chromosome
bp	Base pair
BSA	Bulked Segregant Analysis
BSV	Banana Streak Virus
CAPS	Cleaved Amplified Polymorphism Sequence
CARBAP	Centre Africain de Recherches sur Bananiers et Plantains
cDNA	Complementary DNA
CENARGEN	Centro Nacional de Recursos Genéticos e Biotecnologia
CIRAD	Centre de Coopération Internationale en Recherche Agronomique pour le Développement
CITA	Centro de Investigación y Tecnología Agroalimentaria de Aragón
CLM	Citrus Leaf Miner
cM	centi-Morgan
CMA	Chromomycin A3
CNPMF	Centro Nacional de Pesquisa de Mandioca e Fruticultura Tropical
CP	Coat Protein
cpDNA	Chloroplast DNA
CPMR	Coat Protein Mediated Resistance
CpTi	Cowpea protease Trypsin inhibitor
CSIC	Consejo Superior de Investigaciones Científicas
CTV	Citrus Tristeza Virus
C-value	Amount of DNA contained within a haploid nucleus
CVC	Citrus Variegated Chlorosis
DAPI	4′-6-Diamidino-2-Phenylindole
DARE	Durable Apple Resistance in Europe
DNA	Deoxyribose Nucleic Acid
EAGMPP	European Apple Genome Mapping Project
ELISA	Enzyme-Linked Immunosorbent Assay
EMBRAPA	Empresa Brasileira de Pesquisa Agropecuária
EMS	Ethyl Methane Sulfonate
EST	Expressed Sequence Tag
FAO	Food and Agriculture Organisation
FHIA	Fundacion Hondurenea de Investigacion Agricola
FISH	Fluorescent In Situ Hybridization
FPC	Fingerprinted contigs
GDR	Genome Database for Rosaceae
GFP	Green Fluorescent Protein

GMC	Grape Microsatellite Collection
GMO	Genetically Modified Organism
GRIN	Germplasm Resources Information Network
GSI	Gametophytic Self-Incompatibility
GUS	β-Glucuronidase
HiDRAS	High-Quality Disease Resistant Apples for a Sustainable Agriculture
HMW-DNA	High Molecular Weight DNA
HRI	Horticulture Research International
IARI	Indian Agricultural Research Institute
ICGC	International Citrus Genome Consortium
ICM	Integrated Crop Management
IGGP	International Grape Genome Program
IITA	International Institute of Tropical Agriculture
INRA	Institut National de la Recherche Agronomique
IPB	Institute for Plant Biotechnology
IPM	Integrated Pest Management
IRGC	International Rosaceae Genome Consortium
IRTA	Institut de Recerca i Tecnologia Agroalimentàries
ISSR	Inter-Simple Sequence Repeat
ITS	Internal Transcribed Spacer
IVIA	Instituto Valenciano de Investigaciones Agrarias
IWBT	Institute for Wine Biotechnology
kb	Kilobase
KUL	Katholieke Universiteit Leuven
LD	Linkage Disequilibrium
LecRK	Lectine/Kinase Receptor
LG	Linkage Group
LINE	Long Interspersed Element
LOD	Logarithm Of Odds
LRR	Leucin-Rich Repeat
LTP	Lipid Transfer Proteins
MAB	Marker-Assisted Breeding
MAS	Marker-Assisted Selection
Mb	Megabase
MBC	Map-Based Cloning
Mbp	Mega base pairs
MFLP	Microsatellite Fragment Length Polymorphisms
MS	Murashige and Skoog
MSY	Male Specific Y
mtDNA	Mitochondrial DNA
NAA	Napthalene Acetic Acid
NAD	Nicotinamide Adenine Dinucleotide
NBS	Nucleotide Binding Site
NCBI	National Center for Biotechnology Information
NIL	Near Isogenic Line
NPTII	Neomycin phosphotransferase
ORF	Open Reading Frame
OVERGO	Overlapping Oligonucleotide
PaLCuV	Papaya Leaf Curl Virus
PCA	Pollination-Constant and Astringent
PCNA	Pollination-Constant and Non-Astringent

PCR	Polymerase Chain Reaction
PD	Pierce's Disease
PDO	Protected Designation of Origin
PGI	Protected Geographical Indication
PGI	Phosphoglucoisomerase
PGM	Phosphoglucomutase
PGRI	Plant Genome Research Initiative
PPO	Polyphenol Oxidase
PPV	Plum Pox Virus
PRI	Purdue-Rutgers-Illinois
PRSV	Papaya Ring Spot Virus
PSDM	Papaya Sex Determination Marker
PTGS	Post-Transcriptional Gene Silencing
PTSL	Peach Tree Short Life syndrome
PVA	Pollination-Variant and Astringent
PVNA	Pollination-Variant and Non-Astringent
PVP	Polyvinylpyrrolidone
QTL	Quantitative Trait Loci
QUT	Queensland University of Technology
R gene	Resistance Gene
RACE	Rapid Amplification of Complementary DNA Ends
RAG	Resistance Associated Gene
RAPD	Random Amplified Polymorphic DNA
rbcL	Large subunit of riblose-1,5-bisphosphate carboxylase/oxygenase
rDNA	Ribosomal DNA
Rep	Replicase
RFLP	Restriction Fragment Length Polymorphism
RFRS	Regional Fruit Research Station
RGA	Resistance Gene Analog
RGC	Resistance Gene Candidate
RKN	Root-Knot Nematode
SAM	Sexually Ambivalent Male
SCA	Specific Combining Ability
SCAR	Sequence Characterized Amplified Region
SCRI	Scottish Crop Research Institute
SDH	Shikimate Dehydrogenase
SDRF	Single-Dose Restriction Fragment
SFB	S haplotype-Specific F-Box protein
SINE	Short Interspersed Element
SNP	Single Nucleotide Ppolymorphism
SSCP	Single-Strand Conformation Polymorphism
SSR	Simple Sequence Repeats
STMS	Sequence-Tagged Microsatellite Site
STS	Sequence Tagged Sites
TAC	Transformation-Competent Artificial Chromosome
TBLASTX	National Center for Biotechnology Information Blast Analysis
Ti	Tumor inducing
TIGR	The Institute for Genomics Research
TILLING	Targeting Local Lesions in Genomes
TIR	Toll/Interleukin-1 Receptor
TNAU	Tamil Nadu Agricultural University

TNL	TIR-NBS-LRR
TSG	Traditional Specialty Guaranteed
TSS	Total Soluble Solids
UP	Uttar Pradesh
URGV	Unité de Recherche en Génomique Végétale
USDA	United States Department of Agriculture
VMC	Vitis Microsatellite Consortium
VNTR	Variable Number Tandem Repeats
VPg	Viral Protein genome-linked
WGS	Whole-Genome Shotgun
YAC	Yeast Artificial Chromosome

1 Apple

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1.1 Introduction

1.1.1 Origin of the Domesticated Apple

The genus *Malus* belongs to the Rosaceae family and forms with its closely related fruit (*Pyrus* and *Cydonia*) and ornamental (*Amelanchier, Aronia, Chaenomeles, Cotoneaster, Crateagus, Pyracantha, Sorbus*) genera, the subfamily Maloideae (Challice 1974). This subfamily is believed to be an allopolyploid, that evolved from a hybridization between a Spiraeoidae ($x = 9$) and a Prunoidae ($x = 8$) ancestor resulting in the basic haploid number of $x = 17$ for the Pomoidae (Lespinasse et al. 1999). Most *Malus* species are diploids ($2n = 34$), but a few are triploid (e.g., *M. hupehensis* and *M. coronaria*), or tetraploid (e.g., *M. sargentii*), while some species show variable levels of ploidy (Way et al. 1989). Little information is available on the karyotype of apple. The lengths of the chromosomes in haploid *M. domestica* range from 1.5 to 3.5 μm, with 11 of them being submetacentric, and six being metacentric with respect to the position of the centromere (Bouvier et al. 2000). The longest, and possibly a second chromosome carry a satellite.

With the number ranging from eight to about 122 (Robinson et al. 2001; Harris et al. 2002), there is no agreement among taxonomists as to how many species this genus comprises. The higher estimates may also include many interspecifics, as the species are widely compatible and readily interbreed (Korban 1986). This characteristic has been deployed in apple breeding for the introgression of pest and disease resistance genes. For this reason as well as the assumed interspecific origin of the eating apple in general (Korban 1986; Korban and Chen 1992; Robinson et al. 2001), it seems appropriate to identify the domesticated apple as *M. x domestica* Borkh. However, more recently it has been argued that the correct nomenclature is *M. pumila* Mill. (Korban and Shirvin 1984), and that this species should include the wild apple identified as *M. sieversii* (Lebed.) Roem. (Mabberley et al. 2001). Vavilov (1951) also referred to the wild apple as *M. pumila* when describing the centers of origin of cultivated plant species, which is in complete opposition to the view of another well-known Russian botanist, Ponomarenko, who denied the existence of this species (Way et al. 1989). However, the relatedness of the domesticated and wild apples is strongly supported by the small degree of morphological, biochemical and molecular variation between the two species (Harris et al. 2002). The same could be said of the European wild crabapple *M. sylvestris*. This also belongs, together with *M. sieversii*, to *M. pumila* Mill. (Westwood, in Way et al. 1989), and may have been the result of a separate introduction of the wild apple into Europe. However, the UK research team has not adhered to its own recommendation in later papers and refers to the domesticated apple as *M. domestica* Borkh., while recognizing *M. sieversii* from Central Asia as a separate species (Robinson et al. 2001; Harris et al. 2002). As it suits a purpose of these reviewers, we adhere to the nomenclature according to Way et al. (1989), who identify *M. domestica* and *M. sieversii* as separate species.

The domestication of the apple went hand in hand with the civilization of mankind and has been described extensively by Morgan and Richards (1993). There is evidence of fruit gathering having started as early as the Neolithic times (Juniper et al. 1999). Cultivation increased with propagation through cuttings and also with the discovery of grafting techniques (Morgan and Richards 1993). The fixing of genotypes had a long-lasting effect on apple production, enabling varieties to be grown in orchards and pro-

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viding horticulturalists with the possibility of selecting the best varieties from the many that would have only suited processing because of their bitterness and astringency. Even today, apple production is dominated by cultivars, such as McIntosh (1800s), Jonathan (1820s), Cox's Orange Pippin (1830s), Granny Smith (1860s), Delicious (1870s), Golden Delicious (1890s) and Braeburn (1940s), which were mostly selected from chance seedlings over 100 years ago. By this period, apple had reached all the corners of the world, as emigrants from the Old World introduced them into their new home countries. In Asia, these varieties often replaced the local varieties selected from the native species *M. prunifolia* and its cultivated species *M. asiatica* (Morgan and Richards 1993). It is only recently that bred cultivars developed in the 1930/40s and introduced in the 1960/70s, such as Royal Gala (Kidd's Orange Red × Golden Delicious), Jonagold (Delicious × Jonathan), Fuji (Ralls Janet × Delicious), and Elstar (Ingrid Marie × Golden Delicious), have made major inroads in some countries, even completely replacing existing cultivars. For example, China's enormous growth in apple production is entirely due to the introduction of Fuji.

1.1.2 Apple Production and Exports

With the advent of the new bred cultivars, apple production started to increase rapidly, with several Southern Hemisphere countries, into which apple was introduced, starting to develop major apple industries as they took advantage of the seasons being opposed to those in the Northern Hemisphere (Morgan and Richards 1993). Table 1 shows that in 2004, the world production of apples was an estimated 59 million metric tonnes (MT) produced on 5,280,638 ha of trees (http://faostat.fao.org). After bananas (71 million MT), grapes (65 million MT) and oranges (63 million MT), apples are the fourth biggest fruit crop in the world and production is more than three times that of pears (18 million MT). At 20.5 million MT, China produced over one third of the world production, with the USA being a distant second at 4.3 million MT (Table 1). However, many of the large producers do not export much of their crops, as they have large internal markets, with most of the fruit probably being processed. At 6.2 million MT, about 10% of the world production of apples is exported.

1.1.3 Breeding Strategy

The traditional method of apple improvement by selecting the best phenotypes from seedlings grown from open-pollinated seeds was replaced by deliberate hybridization about 200 years ago. The science of breeding started with the first controlled cross-pollinisation carried out by Thomas Knight early in the nineteenth century (Brown 1975). However, initially little progress was made in improving apple cultivars through controlled crossing, which has been attributed to poor selection of parents (Janick et al. 1996). The success of the relatively recent introductions must be attributed to the selection of parents with good fruit quality. Royal Gala, Fuji, and Jonagold were selected in the first generation from the best commercial cultivars, notably Golden Delicious and Delicious, available at the time of crossing.

Table 1. Estimated apple production (for 2004) and exports (for 2003) (× 1000 metric tonnes) by country (FAOSTAT data)

Country	Production	Export
China	20,503	609
USA	4,290	546
Poland	2,500	349
France	2,400	804
Iran	2,350	109
Turkey	2,300	19
Italy	2,012	708
Russian Federation	1,900	1
Germany	1,600	70
India	1,470	9
Argentina	1,262	200
Chile	1,100	601
Brazil	978	76
Japan	881	17
Ukraine	850	10
Romania	810	0
South Africa	701	326
Hungary	680	8
North Korea	660	0
Spain	614	73
New Zealand	550	323
Mexico	503	0
Uzbekistan	500	4
Egypt	485	0

Apple is self-incompatible and highly heterozygous, which results in very diverse progeny with only a few of them being a major improvement on the parents. As most characters are under polygenic control, low efficiency in genetic improvement of breeding lines together with a long juvenile period make breeding in this crop a slow and expensive process. Hence most apple breeders cannot afford long-term breeding strategies based on recurrent selection achieving incremental gains for a range of characters in each generation (Bringhurst 1983; Oraguzie et al. 2004). Instead, the most common breeding strategy in apple is a limited version of recurrent selection, which is applied to fewer but larger progenies derived from a limited number of parents, selected for a few characters to be improved in a new cultivar (Janick et al. 1996). As breeders cannot afford the time to develop test-crosses to assess the ability of crossing combinations to achieve the breeding goals (Bringhurst 1983), there will be an aspect of chance in the parent selection for a high specific combining ability (SCA) with regard to quantitatively inherited traits. The effect of parents with poor fruit quality is illustrated by the breeding of scab-resistant cultivars carrying the *Vf* gene from *M.* x *floribunda* 821, a crabapple with small fruit of low quality. The first cultivar, Prima (Dayton et al. 1970), is an F_4 descendant of *M.* x *floribunda* and was introduced about 30 years after the Purdue-Rutgers-Illinois (PRI) breeding program started with the specific objective of developing pest and disease resistant cultivars (Crosby et al. 1992). In spite of an "unceasing, single-minded emphasis on moving the *Vf* gene into an adapted type" (Janick et al. 1996), 35 years later there still are no cultivars that have had a considerable impact on pipfruit production by replacing major susceptible cultivars. Breeders have not been able to make the scab-resistant cultivars "catch up" with the eating quality expected of new cultivars today. Nevertheless, the program might have made still less progress if the breeders had been aiming to achieve too many breeding objectives at the same time, which creates inefficiencies as large numbers of seedlings are required to improve the chances of meeting all selection criteria (Brown 1975; Oraguzie et al. 2004).

1.1.4 Breeding Objectives

The principal breeding objective for apple is to increase the marketability of the fruit (Janick et al. 1996). As most breeding programs aim to develop new cultivars for the fresh market, the emphasis is on appearance and eating quality meeting the consumers' expectation of pleasurable fruit consumption, linked with storability to extend the market window. Selection criteria for external quality mostly pertain to skin color, the pattern and amount of fruit covered with color, and the size and shape of the fruit, while internal quality is predominantly determined by flesh texture and flavor (Janick et al. 1996). However, selection criteria may differ in accent, as different breeders aim to develop new cultivars specific to the particular market they target (Laurens 1999) and long-term breeding goals are being increasingly determined by consumer preference research. For example, in reply to an increased consumer interest in the nutritional value of fruit and vegetables, apple is currently being investigated particularly as a source of antioxidants (Davey and Keulemans 2004; Thielen et al. 2004; Lichtenthaler and Marx 2005), which may help prevent diseases and ageing (Raskin and Ripoll 2004; Graziani et al. 2005). On the other hand, a health concern is that apple is a well-known source of allergens. Genetic markers have been identified for genes controlling development of allergens in apple (Gao et al. 2005a, b) (see also Sect. 1.3.2.5.6) and ways are being sought to reduce their negative effect (Hoffman-Sommergruber and the SAFE consortium 2005).

Breeding for pest and disease resistance comes a close second as a major objective (Laurens 1999). Apple is host to a wide range of pests and diseases (Way et al. 1989), many of which need to be controlled in order for commercial production to be profitable. The use of plant resistance is widely regarded as the preferred means of controlling pests and diseases. There are major socio-economic advantages in using resistant cultivars, because they help reduce production costs and diminish the effects on the users and environment because of the reduced requirements for equipment, labour, and fossil fuels (Way et al. 1989; Hogenboom 1993). However, while the potential benefits of resistance breeding are large with regard to the wider impact of pesticide use, the savings to the grower in the direct costs of disease protection are only about 4% of the value of the annual crop (Merwin et al. 1994). The savings also may easily be offset by

market fluctuations and may be reduced by the emergence of other diseases requiring additional control (Merwin et al. 1994). Consumer objection to the use of pesticides was a significant driver for apple breeders to include resistance breeding as a major objective in the development of new cultivars (Laurens 1999), but this to date has not translated into consumers showing a preference for resistant varieties. Although new resistant selections with improved fruit quality are available (Crosby et al. 1992; Fischer et al. 1999), their success in the market place is determined foremost by their ability to differentiate themselves based on appearance and texture in direct competition with the current susceptible cultivars (Murphy and Schertz Willet 1991; Merwin et al. 1994). Therefore, the value of disease resistance to the marketers may prove to be only incremental, until resistant varieties provide an opportunity to rapidly reap the financial benefits of increased demand for fruit produced with reduced chemical inputs, e.g. in organic production systems. These gains will be realised in the long-term only if resistances are durable.

Climatic adaptation is a general breeding objective that ensures trees are productive, bear regularly, and produce fruit with minimal defects, and is achieved by selecting for tree habit, vigor, duration of the juvenile period, and flowering season (Janick et al. 1996). A few breeding programs have more specific objectives to meet the needs of their industries, e.g. adaptation to cold hardiness for climates with severe winters, or low chilling requirements for some subtropical climates. New cultivars often are selected to replace cultivars occupying certain market windows, but in some cases the aim is to extend the marketing period by selecting for very early, or very late maturing cultivars (Laurens 1999).

1.1.5 Molecular Markers and Genetic Maps

Most of the molecular research to date has focused on identifying genetic markers for pest and disease resistance genes, as apple has proved to be a rich source of simply inherited resistance genes with major effects (Table 2). Initially, isoenzymes were used, but they were rapidly superseded by DNA-based markers (see Sect. 1.2). Many different types of markers are available to breeders now, but it has become clear that highly informative markers, such as microsatellite (SSR) and single nucleotide polymorphism (SNP) markers are required to identify resistance genes that are linked or residing in clusters (e.g. Bus et al. 2005b). To date, the primary use of genetic markers in resistance breeding has been in the application of marker-assisted selection (MAS) for pyramided resistance genes in seedling progenies, but they also are an important tool for germplasm screening for sources of resistance (see Sect. 1.5), in host-pathogen interaction research, and map-based cloning of resistance genes (see Sect. 1.6). The mapping of resistance gene loci increasingly shows that they are often linked (Hemmat et al. 2003; Bus et al. 2005a, b), or form part of a gene cluster (Vinatzer et al. 2001; Xu and Korban 2002b). Recent research has also shown that quantitative trait loci (QTL), e.g. for scab resistance, map to the same chromosomal regions as major genes (Durel et al. 2003; Calenge et al. 2004), which suggests that these QTLs probably include residual resistance of "defeated" major effect genes (Pedersen and Leath 1988). The same research has shown that some QTLs are isolate-specific, which suggests that they conform to a gene-for-gene relationship and therefore are subjected to the same risk of resistance "breakdown" as major effect genes (see Sect. 1.4). In apple, gene-for-gene relationships have been demonstrated for *Venturia inaequalis* (Boone and Keitt 1957; Williams and Shay 1957; Bagga and Boone 1968a, b); and apple-cedar rust *Gymnosporangium juniperi-virginianae* (McNew 1938; Niederhauser and Whetzell 1940; Aldwinckle 1975b). The presence of biotypes overcoming major resistance genes suggests that gene-for-gene interactions exist for woolly apple aphid (*Eriosoma lanigerum* Hausm.) (Giliomee et al. 1968; Sandanayaka et al. 2003) and the rosy leaf curling aphid (*Dysaphis devecta* Wlk.) (Alston and Briggs 1968, 1977). Major gene resistances against powdery mildew are also common, while resistance to diseases, such as fire blight and crown rot are predominantly under polygenic control. The same applies to polyphagous insect species, such as leafrollers, although it recently was shown that the resistance to the New Zealand native leafroller species *Ctenopseustis obliquana* Walk. in Prima is controlled by a major gene (Wearing et al. 2003).

QTL mapping is becoming more important in apple breeding as more QTLs are detected not only for pest and disease resistance characters, but increasingly for fruit and tree characters as well (King et al. 2000, 2001; Durel et al. 2003; Liebhard et al. 2003a, c; Calenge et al. 2004; Stankiewicz-Kosyl et al. 2005). Successful mapping of QTL for use by breeders re-

Table 2. Major genes for resistance or susceptibility[z] in apple

Gene	Species	*Malus* source	Reference
Apple scab			
Va	*Venturia inaequalis*	Antonovka PI172623	(Hough et al. 1970)
Vb	*Venturia inaequalis*	Hansen's baccata #2	(Dayton and Williams 1968)
Vc	*Venturia inaequalis*	Cathay	(Korban and Chen 1992)
Vbj	*Venturia inaequalis*	*Malus baccata jackii*	(Dayton and Williams 1968)
Vd	*Venturia inaequalis*	Durello di Forlí	(Tartarini et al. 2004)
Vf	*Venturia inaequalis*	*M. floribunda* 821	(Hough et al. 1953)
Vfh	*Venturia inaequalis*	*M. floribunda* 821	(Bénaouf and Parisi 2000)
Vg	*Venturia inaequalis*	Golden Delicious	(Bénaouf et al. 1997)
Vh8	*Venturia inaequalis*	*M. sieversii* W193B	(Bus et al. 2005a)
Vj	*Venturia inaequalis*	Jonsib	(Korban and Chen 1992)
Vm	*Venturia inaequalis*	*M. micromalus* 245-38	(Dayton et al. 1970a)
Vh2	*Venturia inaequalis*	Russian apple R12740-7A	(Bus et al. 2005b)
Vr2	*Venturia inaequalis*	Russian apple R12740-7A	(Patocchi et al. 2003)
Vh4	*Venturia inaequalis*	Russian apple R12740-7A	(Bus et al. 2005b)
Powdery mildew			
Pl-1	*Podosphaera leucotricha*	*M.* x *robusta* OP 3762	(Knight and Alston 1968)
Pl-2	*Podosphaera leucotricha*	*M.* x *zumi* OP 3752	(Knight and Alston 1968)
Pl-8	*Podosphaera leucotricha*	*M. sargenti* 843	(Korban and Dayton 1983)
Pl-d	*Podosphaera leucotricha*	D12	(Visser and Verhaegh 1980)
Pl-m	*Podosphaera leucotricha*	Mildew Immune Selection	(Dayton 1977)
Pl-w	*Podosphaera leucotricha*	White Angel	(Batlle and Alston 1996)
Aphids			
Er-1	*Eriosoma lanigerum*	Northern Spy	(Knight et al. 1962)
Er-2	*Eriosoma lanigerum*	*M.* x *robusta*	(King et al. 1991)
Er-3	*Eriosoma lanigerum*	Aotea	(Bus et al. 2000)
Sd-1	*Dysaphis devecta*	Cox's Orange Pippin	(Alston and Briggs 1968)
Sd-2	*Dysaphis devecta*	Northern Spy	(Alston and Briggs 1977)
Sd-3	*Dysaphis devecta*	*M.* x *robusta* OP MAL59/9	(Alston and Briggs 1977)
Sm-h	*Dysaphis plantaginea*	*M.* x *robusta* OP MAL59/9	(Alston and Briggs 1970)
Miscellaneous pests and diseases			
Cob-1	*Ctenopseustis obliquana*	Prima	(Wearing et al. 2003)
Gb[z]	*Glomerella cingulata*	Golden Delicious	(Thompson and Taylor 1971)
Gy-a	*Gymnosporangium juniperi-virginianae*	Spartan	(Aldwinckle et al. 1977)
Gy-b	*Gymnosporangium juniperi-virginianae*	Spartan	(Aldwinckle et al. 1977)
Pc	*Phytophthora cactorum*	Northern Spy	(Alston 1970)
Ps-1[z]	*Phyllosticta solitaria*	Jonathan	(Mowry and Dayton 1964)
Ps-2[z]	*Phyllosticta solitaria*	Idared	(Mowry and Dayton 1964)

quires appropriate and rigorous phenotyping techniques, as well as maps saturated with markers that are transportable across genetic backgrounds. The development of the genetic marker maps, e.g. Liebhard et al. (2002, 2003b), perhaps is the easier task, as the meaningful measurement of some quantitatively inherited characters, such as fruit texture (King et al. 2001), is difficult and further complicated by environmental factors (Kearsey and Luo 2003).

In this chapter we describe the advances made in the development and application of molecular techniques in apple breeding to date. We cover the areas of genetic map construction, gene mapping, identification of QTLs, the application of MAS and map-based cloning, following the gene annotation of Alston et al. (2000). Finally, we will discuss the most advanced technologies that are being developed, and future directions of cultivar improvement.

1.2 Construction of Genetic Maps

1.2.1 Brief History of Genetic Mapping in Apple

The earliest genetic maps of apple were developed in the USA and took advantage of the ready availability of Random Amplified Polymorphic DNA (RAPD) markers during the nineties. They also included a small number of isoenzyme markers (Hemmat et al. 1994; Conner et al. 1997). These maps were specific to the genetic background of the mapping parents because of the poor transferability of RAPD markers. For that reason, an international initiative based in Europe developed a genetic map with a number of codominant transportable markers. These were mostly Restriction Fragment Length Polymorphisms (RFLPs) plus a few microsatellite markers (Maliepaard et al. 1998). The most complete map to date is constructed with 129 microsatellites, as well as larger numbers of dominant Amplified Fragment Length Polymorphisms (AFLPs) and RAPDs to assist in filling in gaps (Liebhard et al. 2003b). Such robust polymerase chain reaction (PCR)-based saturated reference maps are essential for whole genome scanning and for understanding complex traits controlled by several Quantitative Trait Loci (QTLs). Several groups worldwide are currently developing transportable genetic maps for apple and a fully saturated consensus map of apple is still required.

1.2.2 First-Generation Maps

Progress in construction of apple genetic maps is summarized in Table 3. The first map (Hemmat et al. 1994) exhibits isoenzyme, RFLP and RAPD markers distributed over 21 and 24 linkage groups, for the cultivars Rome Beauty and White Angel, respectively. Neither of these cultivars was being used in the Cornell University breeding program at the time. However, the second set of maps, for accessions Wijcik McIntosh, NY 75441-67 and NY 75441-58, that were being used in that breeding program, also relied heavily on the contribution of RAPD markers, limiting their usefulness in other progenies. The number of linkage groups (19, 16 and 18 respectively) had been reduced to a number closer to that of the chromosome number of *Malus* ($n = 17$), indicating that these maps were more saturated than previous ones (Conner et al. 1997).

Because of the low transferability of RAPD markers between different cultivars and laboratories, several groups have developed more specific microsatellite markers (also called SSRs or Simple Sequence Repeats). These highly polymorphic and transferable markers proved to be the marker of choice. The first microsatellite markers mapped in apple included some of those identified by Guilford et al. (1997) and Hemmat et al. (1997), as well as four developed by Horticulture Research International (HRI), Wellesbourne, UK. The use of these markers, plus a number of codominantly segregating isoenzymes and RFLPs in a Prima × Fiesta population of 152 seedlings, permitted alignment of the 17 linkage groups and construction of the first integrated apple map (Maliepaard et al. 1998). This initial apple reference map utilized a small number of AFLP markers as well as RAPDs to assist in filling the longer intervals. The cultivars Prima and Fiesta are used in European breeding programs and as such are central to the succession of research programs on genetic mapping in apple situated there: European Apple Genome Mapping Project (EAGMP), Durable Apple Resistance in Europe (DARE) (Lespinasse and Durel 1999) and High-Quality Disease Resistant Apples for a Sustainable Agriculture (HiDRAS). Information from this collaboration, plus that from the mapping of 41 microsatellite markers in the White Angel × Rome Beauty population (Hemmat et al. 2003) enabled cross-referencing of US linkage group numbering with that adopted in Europe. This Prima × Fiesta population has been used to map QTL for apple scab (Durel et al. 2003) and fireblight (Calenge et al. 2005b) (see Sect. 1.4.2).

The genetic map constructed in a Fiesta × Discovery population of 267 individuals (Liebhard et al. 2003b) contains the largest core of robust PCR based markers to date, namely 129 microsatellites, including loci identified by Gianfranceschi et al. (1998) and Liebhard et al. (2002). These markers are supplemented by 710 dominant RAPDs and AFLPs, enabling a good coverage of the 17 linkage groups. The construction of this map was aided by the use of a robotic workstation to set up the large number of PCR reactions required. This reference map has already been used as the framework for mapping QTL (Liebhard et al. 2003a, c; Calenge et al. 2005a, b) – (see Sect. 1.4 below) and Resistance gene analogs (RGAs) that are homologues of nucleotide binding-site (NBS)/leucine-rich repeat resistance genes (LRRs)

Table 3. Genetic maps of apple

Cross	Pop size	Number of markers		Marker Type						Length of map	Reference	Traits
		Female parent	Male parent	Isoen-zyme	RFLPs	RAPD	AFLP	Micro-satellite	Others	cM (female, male)		
Rome Beauty × White Angel	56	156	253	34	8	367	–	–	–	–, 950	Hemmat et al. 1994	*Pl-w*
Wijcik McIntosh × NY 75441-67	114	238	110	6	–	138	–	–	–	1206 (integrated WM), 692	Connor et al. 1997	Skin color, *Vf*, columnar habit, juice pH
Wijcik McIntosh × NY 75441-58	172	181	183	6	–	266	–	–	–	1206, 898	–	
Prima × Fiesta	152	194	163	17	124	133	9	10	SCAR = 1 *Rf*, BC226	842, 984	Maliepaard et al. 1998	*Vf*, *Sd-1*, *Ma*, *SI*
Fiesta × Discovery	112	202	227	–	–	217		118		914, 1015	Liebhard et al. 2002	–
Fiesta × Discovery.	267	439	499	–	–	235	475	129	SCAR = 1 *Rf*, BC226	1144, 1455 (F × D integrated 1371)	Liebhard et al. 2003b	–
	–	–	–	–	–	–	–	–				–
Fiesta × Discovery	44 (subset of 112)	–	–	–	–	–	–	–	18 RGAs (NBS LRR)	Partial map, based on Liebhard et al. 2003b	Baldi et al. 2004	RGAs
Discovery × TN10-8	149	–	–	13	–	–	102	62	22 RGAs (43 bands generated by NBS profiling mapped)	1,219 (integrated map)	Calenge et al. 2004, 2005	*Vg*, scab QTL, RGAS
Telamon × Braeburn	257	259	264	–	–	–	463	20		1039, 1245	Kenis and Keulemans 2005	For QTL analysis growth habit and fruit quality

(Baldi et al. 2004). These RGAs were isolated using a PCR-based strategy based on degenerate primers for conserved sequence motifs in the NBS region and include members of the two major groups of NBS-LRRs described in plants – those possessing a toll-interleukin repeat region, and those lacking it. Eighteen NBS-LRR analogues were mapped as either cleaved amplified polymorphic sequences (CAPS) or single-strand conformation polymorphism (SSCP) markers. These RGAs were distributed widely over the apple genome, covering 12 linkage groups. Potential clustering and association with loci conferring pathogen resistance was noted.

Employment of at least two microsatellite markers per linkage group from this map enabled Calenge et al. (2004, 2005b) to orientate an integrated map constructed in a related Fiesta × Discovery progeny with regard to the two earlier reference maps (Maliepaard et al. 1998; Liebhard et al. 2003b). They then used NBS profiling (Van der Linden et al. 2004) to simultaneously amplify and map 23 polymorphic markers with sequence homology to *Malus* NBS-LRRs, as well as 20 markers with unrelated homologies (Calenge et al. 2005a). Most of the NBS markers were organized in more or less extended clusters, as found in other plant species (Michelmore and Meyers 1998; Young 2000). An extended cluster comprising 13 markers on Linkage Group 2 in a region around *Vr2* is particularly noteworthy (Calenge et al. 2005a). Tight clusters displaying no recombination events were observed on LG 2, LG 10 and LG 17. NBS markers mapped close to major scab and powdery mildew resistances on LG 2, LG 8, LG 10 and LG 12 (*Vr2, Pl-w, Vd* and *Vg* respectively) and to QTLs for resistance to scab and powdery mildew identified previously in this progeny (Calenge et al. 2003, 2004, Calenge and Durel 2006).

The recent construction of a genetic map in a progeny from Telamon × Braeburn has added a further resource for QTL analysis of columnar growth habit and of fruit quality (Kenis and Keulemans 2005) Interestingly, this is the first map in Braeburn, a key cultivar in the New Zealand breeding program, where a population of at least 600 plants of Royal Gala × Braeburn is being developed for mapping of fruit quality attributes (R. Volz, unpublished). The application of the planned QTL analyses from the Telamon × Braeburn progeny to studies of fruit quality in other progenies would be improved by adding more microsatellite markers, as these are low (20) in comparison to the Fiesta × Discovery and Discovery × TN-8 maps (129 and 62) respectively. It has been reported that in Europe a molecular marker map is already under construction for Fuji × Mondial Gala, and that one for Fuji × Braeburn was initiated in 2005 (Costa et al. 2005). These maps in aggregate will provide a valuable resource that will enable rapid progress to be made in establishing the genetics of apple fruit quality.

The recent development of large numbers of Expressed Sequence Tag (EST) sequences for apple (Crowhurst et al. 2005; Korban et al. 2005; Newcomb et al. 2006) has given apple researchers access to a new source of a vast number of potential markers (there are currently nearly 260,000 *Malus* sequences in the public database, GenBank). Polymorphic microsatellite sequences have been identified in great numbers in the EST databases. These EST-microsatellites are being added to those previously developed from both genomic DNA and from the untranslated regions of apple cDNAs. The first apple genetic map utilizing EST-microsatellites is under construction in a cross between Royal Gala and A689-24 (E. Rikkerink et al., work in progress). Single Nucleotide Polymorphism (SNP) markers are also being developed using ESTs. These markers are present throughout the genome and can be used directly to map genes that are hypothesized to be involved in the trait of interest (i.e. are candidate genes). SNPs are already widely used for genetic mapping and association studies in human, animals and plants and represent the markers that will be used in second generation maps in apple.

1.2.3 Genome Organization and Homeology

There are several hypotheses concerning the allotetraploid (or amphidiploid) origin of domestic apple (see Sect. 1.1 and Maliepaard et al. 1998), each of which imply a certain level of duplication within the genome. Maliepaard et al. (1998) were the first to identify the duplication of an entire linkage group (LG 5 and LG 10) by examining the positions of multilocus EST-RFLP markers on a molecular marker linkage map of the cross Prima × Fiesta. An update of this map (Van de Weg et al., in preparation) identified additional duplications (Fig. 1). The amount of homology differed across linkage groups. Some linkage groups seem to be entirely homoeologous to a single other linkage group, such as LG 5 and LG 10, LG 13 and LG 16, and LG 9 and LG 17. Other linkage groups are composite, with several segments, each of which is homoeologous to a segment of a differ-

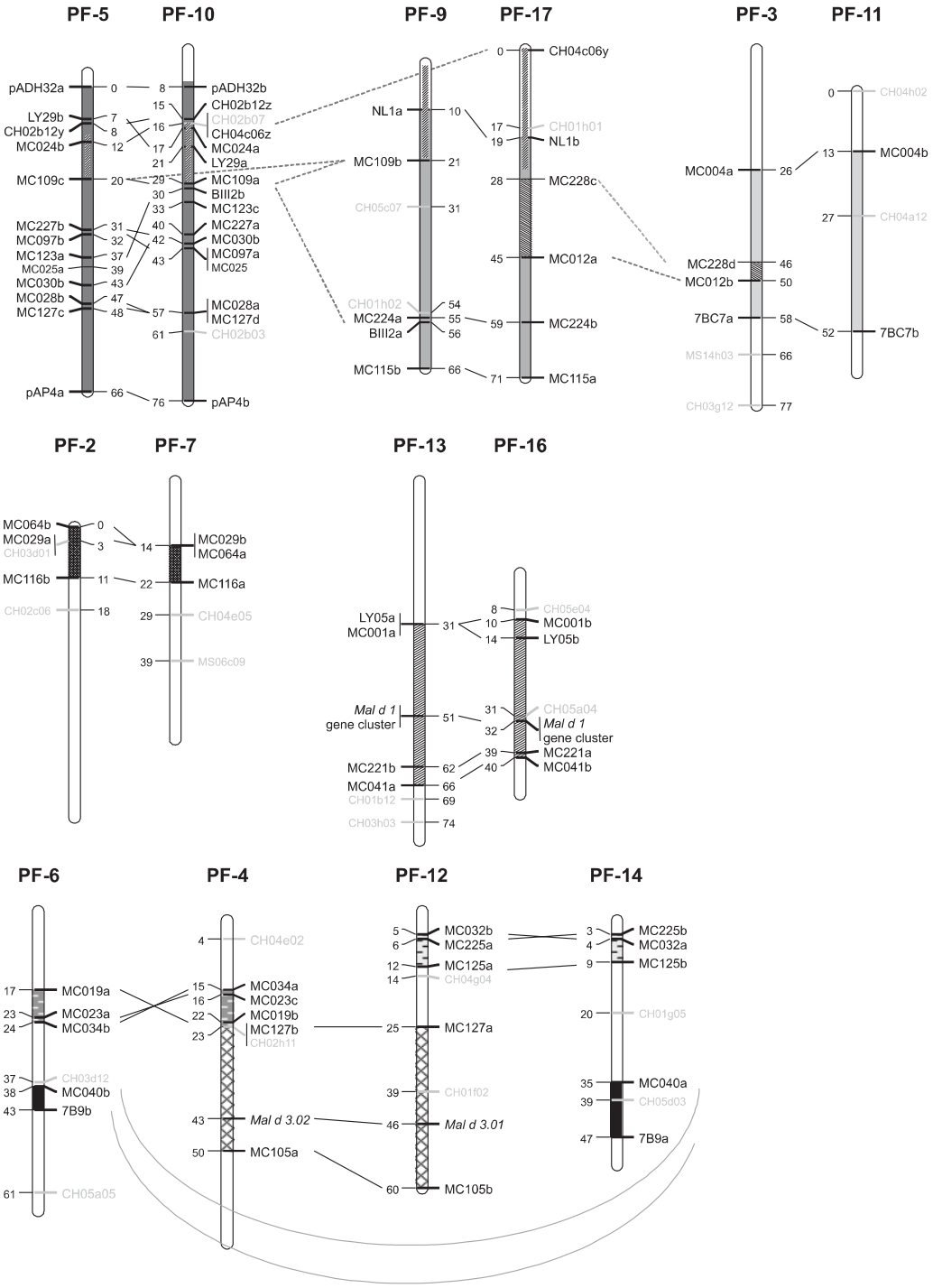

Fig. 1. Map positions of multi-locus markers reveal duplication patterns within the apple genome. Map positions are according to an update of the Prima × Fiesta map of Maliepaard et al. (1998) (van de Weg et al. in prep). The different duplicated (mostly homoeologous) chromosome segments are indicated by different filling patterns of bar segments. Duplicated markers are printed in *black* and are connected by *lines*. Single locus microsatellite-markers (in *grey*) were added as points of reference. Linkage groups are named and orientated according to Maliepaard et al. (1998), except for the orientation of the LG 2, LG 5 and LG 13 that were inverted according to the orientation of their homoeologous linkage groups. Map positions of the *Mal d 1* and *Mal d 3* genes are according to Gao et al. (2005a, b). This figure was kindly supplied by Eric van de Weg and arose in part within the framework of the project EU-DARE (Durable Apple Resistance in Europe, FAIR5, CT97-3898)

ent linkage group. For instance, the proximal part of LG 4 is homoeologous to the proximal part of LG 6, while the more distal part of LG 4 is homoeologous to the distal part of LG 12 (Fig 1). While LG 5 and LG 10 are clearly homoeologous to each other, they also have some markers in common with LG 9 and LG 17 (MC109, MC224, CH04c06). Similarly, LG 3 and LG 17 have two EST-RFLP markers in common, and thus share partial sequences. These shared markers may be due to different genes of a gene family that are dispersed over pairs of homoeologous linkage groups, e.g. *Mal d 1* (Gao et al. 2005b; and Sect. 1.3.2.5.6) where *Mal d 1* represents a gene cluster of seven genes on LG 13 and nine on LG 16.

The order in map position of markers is sometimes slightly different between homoeologous segments. It is not clear whether these differences are real, and arose from genomic rearrangements, or are artefacts due to tension among marker scores during the integration of unbalanced maternal and paternal data sets. For the composite LG 4, LG 6, LG 12 and LG 14, an inversion may have occurred during translocation events, either for the proximal parts of LG 4 and LG 6, or the distal parts of LG 6 and LG 14. Based on these results, Van de Weg et al. (in preparation) proposed to change the Maliepaard (1998) orientation of LG 2, LG 5 and LG 13 to make them consistent with that of their homoeologous linkage groups, thus facilitating comparative analysis within the apple genome.

1.2.4 Comparative Mapping Across Genera

In comparison to other plant systems, for which comparative genome mapping has proven to be a valuable approach both to study genome evolution and to transfer mapping information between genera, only preliminary studies have been carried out to compare the genome maps of apple with others.

1.2.4.1 *Malus* and *Pyrus*

The first example of comparative genome mapping between apple and other members of the Maloideae has been the alignment with the linkage maps of pear. In the course of developing maps for Japanese (Hosui) and European (Bartlett) pears, Yamamoto et al. (2004) located 36 apple microsatellite loci (Liebhard et al. 2002) on the pear map. All pear linkage groups were aligned to the apple consensus map, suggesting conservation of genome organization between apple and pear. This was confirmed in the conservation of the order of loci and the distances between them, which is in agreement with the conserved karyotype between the two genera. An extension of this study to include 69 apple microsatellites and the pear cultivar La France confirmed this finding (Yamamoto et al. 2005). In the same way, apple microsatellite markers from LG 10, 12 and 14 have been mapped in detail on three pear linkage groups by Pierantoni et al. (2004), following the characterization of more than 100 apple microsatellites in four cultivars of pear. For practical purposes, comparative mapping could help to transfer genomic information from apple to the less-studied pear. As a first example, Yamamoto et al. (2005) located the *Vnk* locus for the resistance to pear scab (*Venturia nashicola*) on LG 1 of the Japanese pear cultivar Kinchaku, 33 cM from the map position of CH-Vf2, a close marker for the *Vf* resistance to apple scab in *Malus* (Vinatzer et al. 2004). These studies indicate that further, more detailed map alignment between apple and pear could help pear researchers.

1.2.4.2 *Malus* and *Prunus*

In the first comparison between genomes within the Rosaceae family, 30 loci in the *Prunus* almond × peach (Texas × Earlygold) reference map were found to have homologous counterparts in the Prima × Fiesta apple reference map (Dirlewanger et al. 2004). Generally one linkage group of *Prunus* corresponds to two homeologous apple linkage groups because of the allotetraploid origin of the apple genome, e.g. *Prunus* LG 4 with apple LG 5 and LG 10; half of *Prunus* LG 1 with apple LG 13 and LG 16. As well as these large collinear blocks, major genome rearrangements were identified, e.g. the rearrangement between LG 1 of *Prunus* and LG 8, LG 13 and LG 16 of *Malus*. However the synteny seems conserved between the two species. Nevertheless, considering the high economic importance of *Malus* and *Prunus* species, and in regard to the complementarity of the genomic resources for the two systems (i.e. large characterized EST datasets in apple compared with physical map and small genome in peach), a high-density alignment of the two genomes should be a priority in the next few years, in order to consider the Rosaceae genome as a single system, as has been done for cereals (Keller and Feuillet 2000). Consideration needs to be given to the type of mark-

ers to be used. Dirlewanger et al. (2004) found that microsatellite markers were not as useful as RFLPs and isoenzymes for map comparisons, because only a small proportion of microsatellites mapped had more than one copy.

1.3 Gene Mapping

The task of identification of genetic markers for resistances to the economically significant pests and diseases of apple (i.e. apple scab, powdery mildew and rosy and woolly apple aphid) has been simplified by the large number of resistances to these pathogens that are controlled by major genes (Table 2). A number of major apple resistance genes have now been assigned to linkage groups; *Vf* (Maliepaard et al. 1998) - to LG 1, *Vm* to LG 17 (Patocchi et al. 2005), *Vr2*, *Vh2, Vh4, Vt57, Vbj* and *Vh8* to LG 2 (Bus et al. 2004; Gygax et al. 2004; Patocchi et al. 2004; Bus et al. 2005a,b), *Sd-1* and *Sd-2* to LG 7 (Maliepaard et al. 1998; Cevik and King 2002a), *Pl-w* plus *Er-1* and *Er-3* to LG 8 (Maliepaard et al. 1998; James and Evans 2004; Chagné, Gardiner and Durel, unpublished), *Vd* to LG 10 (Tartarini et al. 2004), *Pl-2* to LG 11 (Seglias and Gessler 1997), *Vg* plus *Pl-d* and *Vb* to LG 12 (Durel et al. 1999; James et al. 2004; Erdin et al. 2006).

1.3.1 Methods Used to Map Major Genes in Apple

For major gene resistances, the relatively speedy process of BSA (Bulked Segregant Analysis) suffices, rather than the time-consuming development of a complete map for the variety in question. This involves identification of markers using pooled DNA from a number of resistant and susceptible plants (Michelmore et al. 1991), in order to develop partial maps around resistance loci. The earliest markers for apple resistance genes were obtained using BSA with RAPDs and the method is still widely utilized by some groups. The efficiency of gene tagging with anonymous markers has been further facilitated by the introduction of automation for DNA extraction from plant tissue, setting up PCR reactions and loading of agarose gels (Cook et al. 2002; Cook and Gardiner 2004). Reproducibility of reactions using RAPD primers is enhanced by automation, and a laboratory throughput of 1,200 samples in a 24-hour period is now possible. RAPD markers are normally converted to more robust sequence specific markers (e.g. SCARs, sequence characterized amplified region), CAPS (cleaved amplified polymorphic sequence) for final map construction and use for MAS.

The publication of the comprehensive microsatellite-based framework map of Liebhard et al. (2003b) has opened up the way to whole genome scanning in apple. Patocchi et al. (2004, 2005 and Erdin et al. 2006) first utilized this approach in their identification of a microsatellite marker linked to *Vr2* and later to locate *Vm* to LG 17 and *Vb* to LG 12. It was successfully modified by James and Evans (2004) in a screen of bulked DNA from a population segregating for the *Pl-w* resistance to enable location of this gene at the top of LG 8. Later, Rusholme (unpublished) confirmed the location of *Pl-2* on LG 11 in a similar approach, utilizing screening of bulked DNA of extreme phenotypes with 3-4 microsatellite markers/linkage group. Patocchi et al. (2005) discuss the significant parameters in setting up a whole genome scanning experiment. These include the interdependent parameters of number of plants and number of microsatellite markers per linkage group, plus the degree of detail in the microsatellite map, and polymorphism in the markers. The recent development of 157 new microsatellite markers brings the total published markers available to over 300 and subset of 86 highly polymorphic microsatellite markers covering 85% of the apple genome with an average density of one marker per 15 cM have been selected as a resource for whole genome scanning (Silfverberg-Dilworth et al.; http://www/hidras.unimi.it). EST databases 2006 are being currently used as a resource for further microsatellite marker development (Gardiner and Korban, unpublished).

A drawback of microsatellites is that they are generally anonymous markers that can be located at large distances from the resistance genes, and therefore they may not be the most suitable for MAS in breeding programs. Another, more recent approach consists of identifying candidates for the gene, or even the polymorphism that is directly responsible for the observed phenotype. This approach is commonly termed the candidate gene approach. Candidate resistance genes identified by searching EST databases with sequence or protein motifs from known resistance genes from model plant systems have proved to be a rich source of genetic markers for resistance genes in apple. Candidates from all known classes of resistance genes

have proved to generate good markers for apple R genes (Gardiner et al. 2003). To date candidate gene markers have been identified for 13 different resistances to apple scab, powdery mildew and woolly apple aphid. Candidate genes are most economically screened initially as RFLPs across mini-populations, and markers mapping as RFLPs close to specific genes are then converted to PCR-based markers such as SNPs or SCARs for mapping in large segregating populations (Gardiner et al. 2003). Other workers have found that NBS-LRR homologues generated by PCR using degenerate primers (Baldi et al. 2004) or from the new methodology of NBS-profiling (Calenge et al. 2005a) generate effective markers. These frequently map in the vicinity of known major resistance loci as well as QTL, accelerating the identification of the genomic regions where functional resistances are located.

1.3.2 Target Traits

1.3.2.1 Apple Scab Resistance Genes

Apple scab resistance genes have received the most attention by genetic mapping groups, because of the significance of the economic impact of apple scab on production. It is also relatively easy to identify markers for major resistances to apple scab in comparison with other resistances. This is because of the relative reliability of phenotypes obtained from glasshouse screening of very young seedlings from mapping populations for response to infection by *Venturia inaequalis*, compared with other pathogens.

1.3.2.1.1 *Vf* The first report of a marker linked to *Vf* was that of the isoenzyme *Pgm-1* (Manganaris et al. 1994) (Table 4). In the same year use of bulked segregant analysis (BSA) enabled speedy identification of a number of RAPD markers linked to this gene (Durham and Korban (1994) (OPA15), Koller et al. (1994) (OPU01, OPM18), Tartarini (1996) (OPC09) and Yang and Kruger (1994) (OPD20)). DNA for BSA was extracted from phenotypic extremes, either from segregating populations, or from varieties. Further markers linked to *Vf* were identified by the same technique and mapped either directly as RAPDs, or after conversion to more robust SCAR or CAPS markers (Gardiner et al. (1996) (OPH01, OPR16); Gianfranceschi et al. (1996) (OPUO1, OPM18); Tartarini (1996) (OPAM19, OPAL07); Yang et al. (1997a) (OPAR4); Yang et al. (1997b) (OPK16); and Hemmat et al. (1998) (S5, B505, S29, P198, B398)). *Vf* was mapped to LG 1 of Prima on the reference map of Maliepaard et al. (1998).

King et al. (1998) and Patocchi et al. (1999a) developed fine maps around *Vf*, locating this resistance in a short interval between OPM18 and OPAL07, thus resolving discrepancies among earlier maps concerning the relationship of these two markers and *Vf*. Later, the colocation of OPAM19 and OPAL07 reported by King et al. (1998) was confirmed by Tartarini et al. (1999). Xu and Korban (2000) constructed a highly saturated AFLP map around *Vf* using a 'narrow down' bulked segregant strategy and converted these markers to SCARs (Xu et al. 2001a). These were later employed to develop a revised higher order fine map around *Vf* (Huaracha et al. 2004). The closest SCAR markers to *Vf* (ACS 3, ACS 7 and ACS 9) are extremely reliable for MAS. The first physical map of the *Vf* region, constructed by Vinatzer et al. (2001), located four homologs of the tomato *Cf* gene family to a 350 kb region around *Vf*. Xu and Korban (2004) have performed detailed pairwise sequence comparisons among these and concluded that the 4 paralogs have arisen by divergent selection on 4 original somatic variations.

Recently, bacterial artificial chromosome (BAC) clones within the contig encompassing *Vf* have been successfully employed as a source of multiallelic microsatellite markers (Vinatzer et al. 2004), termed CHVf-1 and CHVf-2. Analysis of linkage of the *Vf* – coupled alleles, plus *Vf* markers OPM18 and AL07SCARs indicates that a clone of *M. micromalus*, SA573-3, Golden Gem, *M. prunifolia* 19651 and M.A. 16 all carry *Vf*. The use of these markers in combination will enable breeders to predict quickly and economically in germplasm collections, which scab resistant plants carry resistances other than *Vf*.

Gardiner et al. (2003) employed RFLP screening of a mini-population of Royal Gala × A172-2 to identifiy very close linkage to *Vf* of a candidate apple EST (GenBank accession DR033891) derived from a Royal Gala (susceptible) library. This EST possesses homology to the *Hcr-Vf* resistance genes and mapped to the same region on LG 1 of A172-2 as markers derived from the candidate genes of Xu and Korban (2002b). Several other apple candidate ESTs mapped to *Vf* more distantly.

Table 4. Mapping of *Vf* resistance to apple scab

	Progeny	No. sdlgs	Method	Markers (marker/distance) (cM from *Vf*)	Marker class	Reference
A. Initial mapping	Jonathan × A849-7	37	Segregation analysis	*Pgm-1*/8	isoenzyme	Manganaris et al. 1994
	Idared × A679-12	58	–	–	–	–
	Prima × Spartan	63	–	–	–	–
	Liberty × Royal Gala	39	–	–	–	–
	COOP selections, commercial cultivars	15	BSA	$OPA15_{900}$/n.d.	RAPD	Durham and Korban 1994
	M. floribunda 821	7	–	–	–	–
	Idared × *M. floribunda* 821	59	BSA	$OPU01_{400}$/19.7	RAPD	Koller et al. 1994
	–	–		$OPM18_{900}$/10.6	RAPD	–
	Susceptible cultivars plus:	10	BSA	$OPD20_{600}$/n.d.	RAPD	Yang and Kruger 1994
	Prima × A142/5 (resistant sdlgs), *M.floribunda*, Pillnitz (for BSA)	5	–	(25.0, 20.0% recombination frequency)	–	–
	81/19-35 × Margol (mapping)	28	–	–	–	–
	81/19-35 × 87/7-10 (mapping)	158	–	–	–	–
	Granny Smith × A679-2	98	BSA	$OPH01_{1100}$/10	RAPD	Gardiner et al. 1996
	Royal Gala × A172-2	160	–	$OPR16_{400}$/14, 13	–	–
	Florina × Nova Easygro	500	Cloning/sequencing	$OPU01_{400}$/4	RAPD → SCAR	Gianfranceschi et al. 1996
	Florina × Golden Delicious	100	–	$OPM18_{450,230,170}$/1.9	RAPD → CAPS	–
	Prima × Golden Delicious	40	BSA	$OPM19_{2200}$/0.9	RAPD	Tartarini 1996
	–	–	–	$OPAL07_{580}$/0.9	RAPD	–
	–	–	–	$OC09_{900}$/8.8	RAPD	–
	–	–	–	$OPC08_{1100}$/15.5	RAPD	–
	–	–	–	$OPAB19_{1430}$/13.4	RAPD	–
	COOP selections, commercial cultivars	10	BSA	$OPAR4_{1400}$/3.6	RAPD	Yang et al. 1997a
	81/19-35 × 87/7-10	10	–	–	–	–
	–	138	Cloning/sequencing	$OPAR4_{1400}$/3.6	SCAR	
	COOP selections, commercial cultivars	10	BSA	$OPK16_{1300}$	RAPD	Yang et al. 1997b
	81/19-35 × 87/7-10	10	–	–	–	–
	–	138	Cloning/sequencing	$OPK16_{1300}$/4.3	SCAR	–
	Prima × Spartan (for BSA)	38	BSA based on *Pgm-1* genotype	$S5_{2500}$/1.3	RAPD	Hemmat et al. 1998
				$B505_{1700}$/7.8	RAPD	
	Golden Delicious × Prima (mapping)	73	–	P198 $_{750}$/26.8	RAPD	–
				B398 $_{480}$/10.8	RAPD	

Table 4. (continued)

	Progeny	No. sdlgs	Method	Markers (marker/distance) (cM from *Vf*)	Marker class	Reference
B. Fine mapping	Prima × Fiesta	155	Genetic mapping (JoinMap 2.0, Stam and van Ooijen, 1995)	$OPM19_{2200}$/0.7, $OPAL07_{580}$/0.7, $OPM18_{900}$/0	RAPD RAPD RAPD, RFLP	King et al. 1998
	Florina × Nova Easygro	491	Genetic mapping	$OPAL07_{466}$/0.9	SCAR	Tartarini et al. 1999
	5 other seedling progenies	125	(JoinMap 1.4, Stam, 1993)	$OPM19_{526}$/0.9	SCAR	
	Florina × Nova Easygro	521	Genetic mapping	$OPAL07_{466}$/1.1	SCAR	Patocchi et al. 1999a
	Braeburn × FAW 167	279	(JoinMap 1.4, Stam, 1993)	$OPM18_{900}$/0.2	CAPS	
	Fuji × Ariwa	409				
	Co-op selections 1-38	38	Narrow-down BSA	$OPM18_{450}$/0.4	CAPS	Xu and Korban 2000
	Commercial cultivars	10	–	ET9MC3-1/0.4	AFLP	–
	M. floribunda 821	–	–	EA2G11-1, EA12MG16-1,	AFLPs	–
	Resistant Co-op selections	38	Genetic mapping (CRI-MAP v 2.4, Green et al. 1990)	EA11MG4-1, ET2MC8-1, ET3MG10-1, ET8MG1-1, ET8MG7-1/0	–	–
	Co-op 17 × Co-op 16 (resistant seedlings)	203	–	$OPM19_{526}$, $OPAL07_{466}$/0.2 EA9MC15-1, EA4MG1-1	SCARs AFLPs	–
	Jonafree × III. Del.no.1 (resistant seedlings)	227	–	EA16MG2-1, ET4MC14-1, ET8MG16-1, ET3MG10-2, ET10MG8-1/0.2	–	–
	–	–	Physical mapping	*HcrVf1*, *HcrVf2*, *HcrVf3*, *HcrVf4* mapped to 350 kb interval around *Vf*	Gene homologs	Vinatzer et al. 2001
	Resistant Co-op selections	38	Genetic mapping	$OPM18_{450}$/0.4	CAPS	Xu et al. 2001a
	Co-op 17 × Co-op 16 (resistant seedlings)	203	(CRI-MAP v 2.4, Green et al. 1990)	ACS-6/0.4, ACS-3, ACS-7, ACS-9/0	SCAR SCARs	
	Jonafree × III. Del.no.1 (resistant seedlings)	227	–	$OPM19_{526}$, $OPAL07_{466}$/0.2 ACS-1, ACS-2, ACS-4, ACS-5, ACS-8, ACS-10, ACS-11/0.2	SCARs SCARs	–
	–	–	Physical mapping	*Vfa 1*, *Vfa2*, *Vfa3*, *Vfa4* mapped to 200kb interval around *Vf*	Gene paralogs	Xu and Korban 2002b
	Royal Gala × A172-2	160	Screening of ESTs with homology to R genes	2 bands from DR033891 mapped between *Vfa* 1, 2 and *Vfa* 3, 4	RFLP	Gardiner et al. 2003
	16 Crosses	1412	Genetic mapping	ACS-6/0.2 ACS-7, ACS-9/0 ACS-3/0.1	SCAR SCARs SCAR	Huaracha et al. 2004

1.3.2.1.2 *Vm* Bulked segregant analysis with RAPD markers was employed to identify the OPB12 marker for *Vm*, using a population three generations removed from *M.* x *atrosanguinea* 804. OPB12RAPD was converted to a SCAR marker and verified in a second population derived from a selection of *M. x atrosanguinea* 804. Joint segregation analysis on the combined data indicated a distance of 6 cM between *Vm* and OPB12 (Cheng et al. 1998) (Table 5). OPB12 has proved recently to be linked to *Vm* in the breeding parent X2225 derived from *M. micromalus* (Richards, Rikkerink, Bassett and Plummer, unpublished). Recently, Patocchi et al. (2005) performed a whole genome scan with three selected microsatellite markers per linkage group to locate *Vm* at the end of LG 17, the first major resistance gene in this genomic region. A new microsatellite marker that co-segregated with the resistance phenotype (Hi07h02) will be invaluable for MAS.

OPB12 is also linked to apple scab resistances derived from Red Sauce o.p. F91-135 A002-100 and Zelenovka Sotchnaya o.p. F91-184 A003-020 (Rusholme et al. unpublished). The same group used bulked segregant analysis of a Red Sauce o.p. population to identify linkage of OPAY5 to apple scab resistance and this marker was transferable into *M. x atrosanguinea* 804 and Zelenovka Sotchnaya o.p. populations. Microsatellite Hi07h02 has recently been mapped to the resistance in the *M. micromalus*, Zelenovka Sotchnaya o.p and Red Sauce o.p. populations, indicating a close relationship, if not the same gene, between *Vm* and the resistances from Red Sauce o.p. and Zelenovka Sotchnaya o.p. (Chagné et al. unpublished).

M. micromalus has been reported to carry both *Vm* and a 'masked gene' (Shay et al. 1953), that has been demonstrated to be allelic to *Vf* (Dayton and Williams 1968, 1970). This second gene is likely to be that identified by amplification of the *Vf* specific alleles of the microsatellite markers CHVf-1 and CHVf-2 in DNA from *M. micromalus* (Vinatzer et al. 2004).

1.3.2.1.3 Apple Scab Resistances from Differential Hosts 2 and 4 that Map to Linkage Group 2

Several streams of research have contributed to the knowledge of markers linked to apple scab resistances mapping to LG 2 in differential hosts 2 and 4 derived from Russian apple R12740-7A (conditioning stellate necrotic and hypersensitive reactions, respectively).

1.3.2.1.3.1 Vh2 Bulked segregation with RAPDs was used to identify marker OPL19 that mapped close to *Vh2* from differential host 2 (Gardiner et al. 1999a; Bus et al. 2000) (Table 5). The use of its derivative OPL19SCAR for MAS in a second population was reported by Bus et al. (2002). At this stage, the host 2 was mistakenly identified in several publications as accession TSR34T132 from the Purdue-Rutgers-Illinois apple breeding program (Bus et al. 2005b). However, the correct identification number is now known to be TSR34T15 (Lespinasse, personal communication). Hemmat et al. (2002) reported that a gene they identified as *Vr* was closely flanked by a SCAR marker $OPB18_{620bp}$ and a more distant microsatellite CH02b10 reported by (Gianfranceschi et al. 1998). More recent mapping using $CH02b10_{121bp}$, $OPL19_{433bp}$SCAR and $OPZ13_{900bp}$SCAR (Gygax et al. 2004) in the population Royal Gala × TSR34T15 has indicated that *Vr* and *Vh2* are identical and that the gene maps to LG 2 (Bus et al. 2005b). This was confirmed in a second population Sciglo × A68R03T057 derived from a non-differential accession of Russian apple R12740-7A. It was therefore proposed that the gene conditioning stellate necrotic reactions from Russian apple R12740-7A be known as *Vh2*, and that the name *Vr* be reserved for the original race-non specific gene from this accession.

1.3.2.1.3.2 Vt57 *Vt57* was identified in the Sciglo × A68R03T057 population through the use of differential screening of the population with several isolates of *V. inaequalis* (Bus et al. 2005b). It conditions a chlorotic resistance reaction, and maps 3 cM from OPL19SCAR on LG 2 (versus 1 cM for *Vh2*) (Table 5).

1.3.2.1.3.3 Vh4 Similarly, research has been performed in parallel by teams internationally on the host 4 derivative of Russian apple conditioning a hypersensitive response. (Hemmat et al. 2002) reported the identification of a RAPD marker from primer S22 that they converted to S22SCAR and mapped in R12740-7A within 9 cM of *Vx* (Table 5).

In New Zealand, S22SCAR was mapped at a similar distance or closer to *Vh4* in Royal Gala × TSR33T239 populations (Bus et al. 2002; 2005b). This group also reported a distant linkage of *Vh4* to OPB10RAPD (Bus et al. 2000) and OPB10SCAR (Bus et al. 2005b), but had been unable to identify new markers closer to the gene. Boudichevskaia et al. (2004) identified the linkage of $OPAD13_{950bp}$ to a gene they termed *Vr1* that segregated in three Regia progenies. However consideration of pedigree, resistance phenotype and linkage information for all markers, including $OPAD13_{950bp}$ in the Royal Gala × TSR33T239 population (Gardiner et al. unpublished) suggests that this gene is iden-

Table 5. Mapping of major scab resistance genes, apart from *Vf*, in apple

Gene	Progeny	No. sdlgs	Method	Markers marker/distance (cM from gene)	Marker class	Linkage group assignment	Reference
Vm	Empire × NY74828-12 (3 generations from *M.* x *atrosanguinea*)	59	BSA	$OPB12_{687}$/6.0	SCAR	–	Cheng et al. 1998
–	Royal Gala × OR45T132 (selection of *M.* x *atrosanguinea* 804)	184	Verification by genetic mapping	–	–	–	–
Vm	Golden Delicious × Murray	142	Whole genome scan	Hi07h02/0 CH05d08y/3.5	microsatellite microsatellite	LG 17	Patocchi et al. in press
Vh2	Royal Gala × TSR34T15 (F2 of R12740-7A)	192	BSA	$OPL19_{550}$/2.5	RAPD	–	Gardiner et al. 1999a, Bus et al. 2000
Vh2	Golden Delicious × TSR34T15	122	Population screen	$OPL19_{433}$/n.d. (8.2% recombination frequency [r.f.])	SCAR	–	Bus et al. 2002
Vh2	Empire × R12740-7A	315	BSA	$OPB18_{620}$/n.d. (0.8% r.f.)	SCAR	–	Hemmat et al. 2002
	–		Population screen	$CH02b10_{122}$/n.d. (7.8% r.f.)	microsatellite	–	–
	–	–	–	–	(ex Gianfranceschi et al. 1998)	–	–
Vh2	Royal Gala × TSR34T15	192	Comparative mapping (JoinMap v. 3.0, Van Ooijen and Voorrips, 2001)	$CH02b10_{121}$/8.8 $OPZ13_{900}$/5.0 (ex Gygax et al. 2004) $OPL19_{433}$/1.0 $CH05e03_{165}$/10.0	microsatellite SCAR SCAR microsatellite	LG 2	Bus et al. 2005b
Vh2	Sciglo × A68R03T057	111	As above	$CH02b10_{121}$/9.0	microsatellite	–	Bus et al. 2005b
	–		–	$OPL19_{433}$/5.0	SCAR	–	–
	–		–	$CH05e03_{165}$/4.0	microsatellite	–	–
VT57	Sciglo × A68R03T057	111	Single strain inoculation, comparative mapping (JoinMap v. 3.0, Van Ooijen and Voorrips, 2001)	$CH02b10_{126}$/2.0 $OPL19_{433}$/3.0 $CH05e03_{166}$/4.0	microsatellite SCAR microsatellite	LG 2	Bus et al. 2005b
Vh4	Royal Gala × TSR33T239	242	BSA	$OPB10_{>2000}$/22.1	RAPD	–	Bus et al. 2000
Vh4	Empire × R12740-7A	315	BSA	$S22_{1300}$/n.d. (9.8% r.f.) $S6_{800}$/n.d. (23% r.f.)	SCAR RAPD	– –	Hemmat et al. 2002

Table 5. (continued)

Gene	Progeny	No. sdlgs	Method	Markers marker/distance (cM from gene)	Marker class	Linkage group assignment	Reference
Vh4	Royal Gala × TSR33T239	154	Population screen	$S22_{1300}$/n.d. (9.8% r.f.)	SCAR	–	Bus et al. 2002
	Regia × Pingo	191	BSA	$OPAD13_{950}$ n.d. (15% r.f.)	SCAR	–	Boudichevskaia
	Regia × Pinova	188	–	–	–	–	et al. 2004
	Regia × Delbarestivale	97	–	–	–	–	
Vh4	Royal Gala × TSR33T239	242	Genetic mapping	$S22_{1300}$/4.0	SCAR	LG 2	Bus et al. 2005b
	–	–	(JoinMap v. 3.0,	$CH02c02a_{170}$/5.0	Microsatellite	–	–
			Van Ooijen and Voorrips, 2001)	$OPB10_{>2000}$/19.0	SCAR		
	–	–	–	$OPAD13_{950}$/7.0	SCAR	–	Gardiner et al. unpublished
Vr2	GMAL 2473 × Idared	377	BSA	$EA35MA41_{262}$/0	AFLP	LG 2	Patocchi et al. 2004
	–	–	–	$EA37MA39_{188}$/0	AFLP	–	–
	–	–	–	$CH02c02a_{176}$/0	microsatellite	–	–
	–	–	–	$CH02f06_{146}$/6.9	microsatellite	–	–
Vr2	Fiesta × Discovery	44	Comparative mapping	ARGH37/3.5 ARGH17 3.5	RGA RGA	–	Baldi et al. 2004
Vr2	Discovery × TN10-8	149	Whole genome scan	NBS2M9/2.0	NBS marker	–	Calenge et al. 2005
–	–	–	Comparative mapping	NBS2M10/2.0	NBS marker	–	–
			on another framework map	NBS2R9/1.0	NBS marker		
				NBS3M3/1.0	NBS marker		
–	–	–	–	NBS2M4/2.0	NBS marker	–	–
–	–	–	–	NBS3M1b/3.0	NBS marker	–	–
Vbj	A722-7 × Golden Delicious	148	BSA	$OPZ13_{773}$/0	SCAR	LG 2	Gygax et al. 2004
		–	–	$OPT06_{410}$/5.8	SCAR	–	–
–	–	–	–	$OPK08_{743}$/10.2	SCAR	–	–
–	–	–	Comparative mapping	$CH2c06_{248}$/0	microsatellite	–	–
–	–	–	(JoinMap v 2.0,	$CH5e03_{150}$/2.1	microsatellite	–	–
			Stam and van Ooijen, 1995)	$CH3d01_{115}$/8.3	microsatellite		
Vh8	Royal Gala × *M. sieversii* W193B	152	Comparative mapping	$OPL19_{433}$/1.3	SCAR	LG 2	Bus et al. 2005a
			(JoinMap v. 3.0,	$OPB18_{628}$/4.3	SCAR		
			Van Ooijen and Voorrips, 2001)	OPB 18_{799} (*Vh8* SCAR)/5.1	SCAR		
				$CH3d01_{124}$/18.5	microsatellite		

Table 5. (continued)

Gene	Progeny	No. sdlgs	Method	Markers marker/distance (cM from gene)	Marker class	Linkage group assignment	Reference
Va	Fortune × PRI 1841-11 and	120	BSA	$P136_{700}$/n.d. (18% r.f)	RAPD; SCAR primers unpubl.	LG 1?	Hemmat et al. 2003
	NY489 × PRI 1841-11	–	–	$B398_{480}$/n.d. (16% r.f)	RAPD	–	–
				ACS-6/n.d. (24% r.f.)	SCAR	–	–
Vb	Empire × Hansen's baccata #2	140	BSA	$B220_{700}$/n.d. (12% r.f)	RAPD; SCAR primers unpubl.	LG 1?	Hemmat et al. 2003
	–	–	–	$OPAM19_{450}$/n.d. (24% r.f)	SCAR	–	–
–	–	–	–	ACS-1; $OPU01_{400}$/26% r.f.)	SCARs	–	–
Vb	Golden Delicious × Hansen's baccata #2	149	Whole genome scan	Hi02d05/7.8	microsatellite	LG 12, not LG 1	Erdin et al. 2006
				Hi07f01/9.7	microsatellite		
Vd	Durello di Forlì × Fiesta	146	Genetic mapping	$OPAF07_{880bp}$/2.0	RAPD	LG 10	Tartarini et al. 2004
–	–	–	–	G63Tru91a/2.0	microsatellite	–	
–	Discovery × TN10-8	149	Comparative mapping on another framework map	NBS3M13/2.0	NBS marker	–	Calenge et al. 2004
				NBS2M18/1.0	NBS marker		
				NBS2M12/1.0	NBS marker		
				NBS3M8/3.0	NBS marker		
Vg	Prima × Fiesta	149	Single strain inoculation; mapping	MC105/3.0	RFLP	LG 12	Durel et al. 1999
Vg	Discovery × TN10-8	149	Genetic mapping	CH01d03/0.5	microsatellite	–	Calenge et al. 2004
Vg	Discovery × TN10-8	149	Genetic mapping	NBS2M14/5.0	NBS marker	–	Calenge et al. 2005
				NBS3M11/0.7	NBS marker	–	
Vmis	Splendour × MIS o.p. 93.051 G01-048	155	BSA	$OPAS11_{760}$/<8.0	RAPD	Not determined	Gardiner et al. 2001
				$OPAS07_{690}$/<17.5	RAPD		

tical to *Vh4*, and that since the first naming of the gene takes precedence over later namings, it should be identified as *Vh4*. *Vh4* has been mapped to LG 2 (Bus et al. 2005b). Screening of EST candidate R genes using RFLP analysis, followed by conversion to SNPs has indicated that screening of candidate genes will be a useful route for developing further markers for both *Vh2* and *Vh4* (Gardiner et al. unpublished).

1.3.2.1.4 Other Major Apple Scab Resistances Mapping to Linkage Group 2 Described below are three other major apple scab resistances that have been mapped to LG 2, which possesses the largest number of resistances to apple scab of any linkage group. QTL for scab and mildew resistance have also been identified there – see Sect. 1.4.2, (Calenge et al. 2004; Calenge and Durel, in preparation) as well as numerous RGAs (Baldi et al. 2004; Calenge et al. 2005a) -see below. This high concentration of active and potential resistances makes LG 2 of high priority for an apple genome sequencing initiative.

1.3.2.1.4.1 Vr2 *Vr2* from GMAL 2473 was reported by Patocchi et al. (2004). Four markers were obtained by BSA using both RAPDs and AFLPs. Two of these markers segregated with the resistance (EA35MA41 and EA37MA39), making their future SCAR derivatives excellent tools for MAS (Table 5). A fifth marker, a microsatellite that also co-segregated with the resistance, was identified by a whole genome scan using selected markers from the map of Liebhard et al. (2002). This marker (CH02c02a) enabled the location of *Vr2* on LG 2 at about 43 cM from *Vh2*, which excludes any possibility that these two resistance genes are identical. However, the question of the relationship between *Vr2* and *Vh4* is not yet completely resolved. Uncertainty about the origin of the Russian apple accession used, the low number of seedlings with distinctive HR, the difference in their distances to CH02c02a, and the absence of data for S22SCAR suggest that they are different. On the other hand, since 65% of the seedlings of the GMAL2473 × Idared family were resistant (Patocchi et al. 2004), GMAL2473 may well carry two, possibly linked, scab resistance genes, one of which may be *Vh4*, while the other gene is the true *Vr2* gene. The two-gene hypothesis is supported by the distinct phenotypes of HR (for *Vh4*) and chlorotic/necrotic (for *Vr2*) resistance reaction. Another reason for the segregation of *Vr2* not fitting a clear R:S ratio may be segregation distortions, since LG 2 is well-known for these (Maliepaard et al. 1998; Liebhard et al. 2003b; Bus et al. 2005a).

Baldi et al. (2004) located two RGAs within 3.5 cM of the putative location of *Vr2*, while Calenge et al. (2005a) mapped six markers identified by NBS profiling to a genomic region corresponding to 5 cM around *Vr2* and seven more in the next 3 cM in the direction of *Vh4* confirming that the region around *Vr2* and *Vh4* is extremely rich in potential resistance genes and certainly warrants further analysis.

1.3.2.1.4.2 Vbj RAPD markers for *Vbj* (OPZ13 and OPK08) have been identified by BSA and converted to SCAR markers (Gygax et al. 2004). These were mapped around *Vbj* together with three microsatellite markers (CH02c06, CH05e03 and CH03d01) previously defined as members of LG 2 by (Liebhard et al. 2002) (Table 5).

1.3.2.1.4.3 Vh8 A new scab resistance from *M. sieversii* accession W193B was identified and distinguished from *Vh2* with the aid of a new race of *V. inaequalis*, race 8. Although no distinction between *Vh2* and *Vh8* could be made on the basis of genetic marker studies with the original markers for *Vh2* (OPL19 and $OPB18_{628bp}$), the latter marker produced a second band of 799 bp that was specific to the *Vh8* gene (Bus et al. 2005a). Sequencing of the products of the OB18 PCR reaction from both resistant parents enabled the development of a second marker (*Vh8*SCAR) that could distinguish the two genes as that also exhibited a band in the presence of *Vh8* that was not exhibited by *Vh2*. Microsatellite CH03d01 from LG 2 maps 5 cM from *Vh8* (Table 5). Sequencing of the products of the OPB18 PCR reaction from both resistant parents enabled the development of a second marker (*Vh8*SCAR) that could distinguish the two genes.

Consideration of the mapping data for the *Vh2* and *Vh4* genes from Russian apple R12740-7A, plus the information on markers for *Vh8* and *Vbj* and *Vr2*, enabled Bus et al. (2004) to use the then available markers to delineate a map of LG 2 that locates *Vbj*, *Vh2* and *Vh8* close to each other, and at a distance from *Vr2* and *Vh4* (Fig 2).

1.3.2.1.5 *Va* Hemmat et al. (2003) employed BSA to identify linkage of P136RAPD to the hypersensitive *Va* resistance from Antonovka PI1762623 and then developed a SCAR marker (sequence not published) (Table 5). Screening of their mapping population with a range of markers for *Vf* demonstrated that many markers for this gene map on the opposite side to P136. These markers include those reported to map with *Vf* in a high resolution map (Xu et al. 2001a)

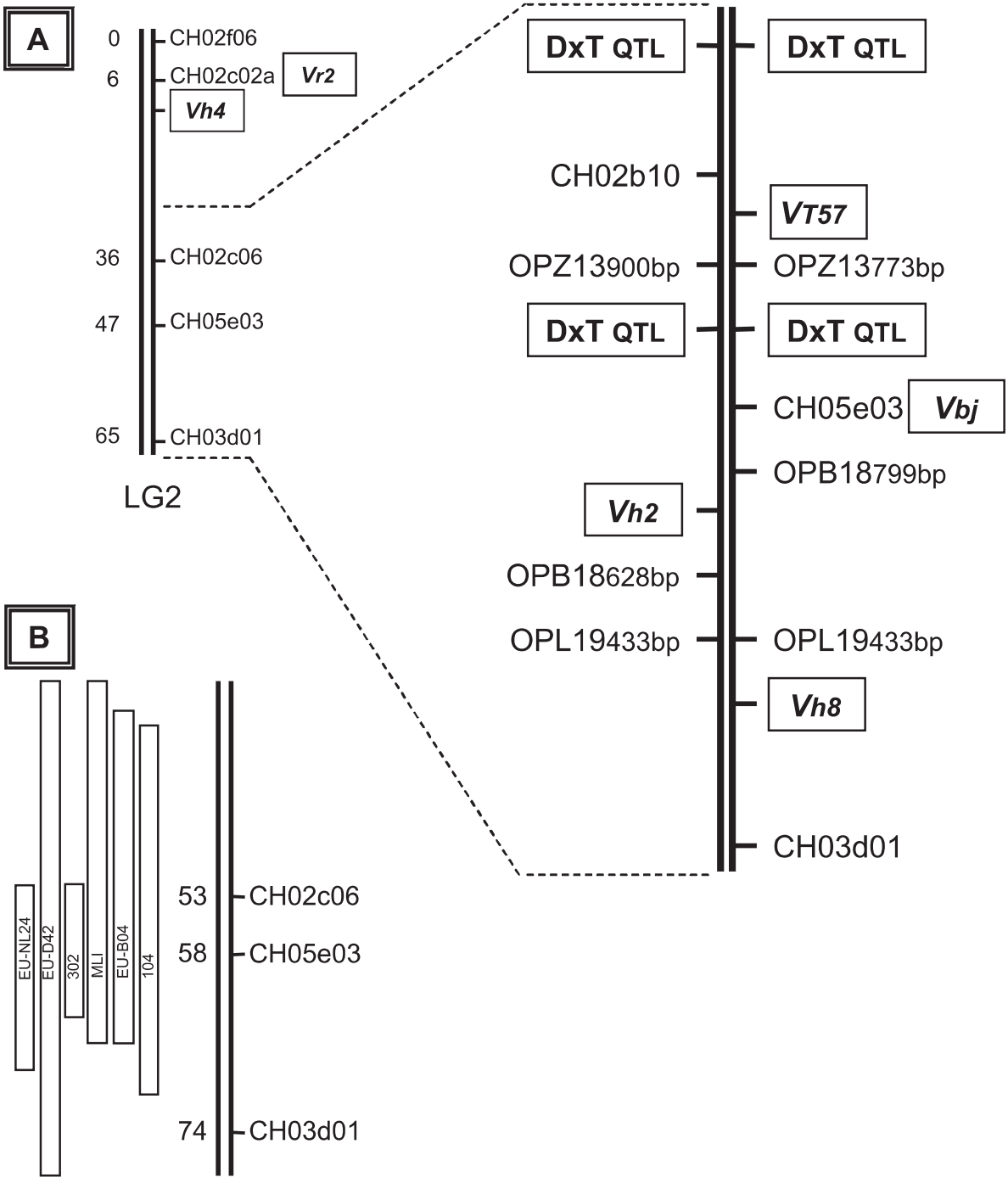

Fig. 2. Delineation of a scab resistance gene cluster on LG 2 of apple based on the genetic maps for the individual major genes (**A**). The diagram on the right is a higher magnification of the diagram on the top left in the area containing several QTL and major genes. The chromosome regions of LG 2 identified as carrying QTLs for scab resistance in a Discovery × TN10-8 family (**B**) have been mapped according to their QTL peaks. (Adapted from Bus et al. 2005b)

but not all *Vf* markers mapped in this population. This indicates that *Va* may be located on LG 1, with a recombination frequency of 27% between the *Va* and *Vf* loci. This agrees with Dayton and Williams (1968), who had earlier concluded that *Va* and *Vf* were not allelic.

1.3.2.1.6 *Vb* In the same paper as their *Va* study, Hemmat et al. (2003) reported the linkage of B220RAPD to *Vb* (chlorotic resistance reaction from Hansen's baccata #2) and that several markers for *Vf* mapped on the opposite side to B220SCAR (primer sequence unpublished), but in a different order from that found around *Vf* (Table 5). Test crosses had indicated that *Vb* and *Vf* were not allelic (Dayton and Williams 1968). B220SCAR mapped in repulsion to resistance phenotype in the *Va* population (above), and also in material not related to Hansen's baccata #2, including *M. floribunda, M. prunifolia, M. zumi* calocarpa, David, Liset, Prairiefire, Carmine Crab, D95-295 Redleaf Crab and AV Redleaf Crab. Hansen's *baccata* #1 amplified fragments with 11 of the 16 *Vf* primers tested. *M. baccata jackii* amplified fragments from four of the primers. Rusholme and Gardiner (unpublished) mapped B220RAPD distantly, at 30 cM, to *Vb* in a population derived from the GMAL2477 accession of Hansen's baccata #2 and identified a new RAPD marker (OPAJ03) mapping inside B220 at 25 cM from *Vb*. They confirmed the finding of M. Hemmat and S. Brown (personal communication) that a marker for *Vbj* ($OPZ13_{773bp}$SCAR) mapped outside B220RAPD. However, the *Vf* markers reported by Hemmat et al. (2003) were not exhibited by Hansen's *baccata* #2 GMAL2477. A recent conference report (Erdin et al. 2006) clarifies the issue. A whole genome scan of plants in a Golden Delicious × Hansen's *baccata* #2 progeny using microsatellite markers demonstrated that *Vb* maps to the distal end of LG 12 and not to LG 1. This result is consistent with the early test cross results (Dayton and Williams 1968), and demonstrates the power of the whole genome scan for mapping of major resistances to linkage group.

1.3.2.1.7 *Vd* The resistance from the old Italian apple cultivar Durello di Forli that has been described as conferring high field tolerance to apple scab (3B type reaction) and a stellate necrotic reaction in glasshouse grown seedlings exposed to the EU-D-42 race 6 reference strain of *Venturia inaequalis* has been mapped to one end of LG 10 (Tartarini et al. 2004) using the microsatellite map developed by this team (Tartarini et al. unpublished). The markers $OPAF07_{880bp}$RAPD and G63Tru91a, that flank *Vd* closely (Table 5), are in repulsion phase to the resistance and will need to be converted to markers linked to the presence of a fragment, before becoming useful for MAS. Four markers identified by NBS profiling mapped to a genomic region corresponding to 5 cM around *Vd* in another apple progeny (Calenge et al. 2005a). The high level of resistance to race 6 conferred by *Vd* would make it a useful reinforcement to the otherwise effective *Vf* resistance that has been broken by race 6 (Bénaouf and Parisi 2000).

1.3.2.1.8 *Vg* *Vg*, the major gene derived from Golden Delicious that confers resistance to apple scab incited by race 7 of *V. inaequalis*, breaker of the *Vf* resistance from *Malus floribunda* 821 was first described by Bénaouf et al. (1997). Screening of the Prima × Fiesta framework mapping population of Maliepaard et al. (1998) with differential strains of *V. inaequalis* enabled Durel et al. (1999) to map *Vg* 3 cM from a new RFLP marker on LG 12 (Table 5). Mapping in a second framework mapping population (Discovery × TN10-8) enabled location of the gene 0.5 cM from the microsatellite CH01d03 (Calenge et al. 2004). NBS profiling identified two more markers close to *Vg*, one 5 cM upstream of *Vg* and the other flanking the resistance at 0.7 cM (Calenge et al. 2005a).

1.3.2.1.9 *Vmis* Scab resistance segregates from an open pollinated seedling (93.051 G01-048) of the mildew immune seedling described by Dayton (1977) as being the product of a pollination of a domestic apple with an unknown crab apple. Initial analysis by bulked segregant analysis resulted in identification of two RAPD markers, OPAS07 and OPAS11 (Gardiner et al. 2001) (Table 5). As the phenotype segregation data suggested the presence of a second gene, this analysis has been carried into the second generation, resulting in the identification of new markers with flanking markers located in a 15 cM span around *Vmis* (Gardiner et al. unpublished).

1.3.2.2 Powdery Mildew Resistance Genes (Table 6)

Mapping of resistances to powdery mildew is much more time consuming than mapping apple scab resistances, because of the need to phenotype seedling populations over several years to ensure that the adult resistance phenotype has been attained and that this

Table 6. Mapping of major genes for powdery mildew resistance in apple

Gene	Progeny	No. sdlgs	Method	Markers marker/distance (cM from gene)	Marker class	Linkage group assignment	Reference
Pl-w	Jester × White Angel Gloster 69 × White Angel	40 80	*LAP* isoenzyme analysis	*Lap-2an*/n.d. (2.6 – 3.1% recombination frequency)	Isoenzyme		Batlle and Aston 1996
Pl-w	Prima × Fiesta	152	Mapping of *LAP-2*			LG 8	Maliepaard et al. 1998
Pl-w	Katja × White Angel	80	BSA	EM M02 /6.4 EM M01/4.6	AFLP → SCAR AFLP → SCAR	–	Evans and James 2003,
	Fiesta × (Gloster 69 × White Angel)	267	Whole genome scan	CH01e12/10 CH05a02y/13	microsatellite microsatellite	–	James and Evans 2004
Pl-d	Fiesta × A871-14	272	Whole genome scan	CH03c02/8.0	microsatellite	LG 12	James et al. 2004
	–	–	BSA	$OPA01_{900}$/4.0 (repulsion)	RAPD	–	
	–	–	–	ETA-CTC/5.0	AFLP	–	
	–	–	–	EM DM01/9.0	AFLP; SCAR	–	–
	–	–	Whole genome scan	Ch01d03/13.0	microsatellite	–	–
Pl-1	85/23-2 × 81/19-35	64	BSA	$OPAT20_{450}$/4.0 $OPD02_{1000}$/5.0	SCAR RAPD	–	Markussen et al. 1995
Pl-1	Idared × 78/18-4	233	Population screen	$OPAT20_{450}$/7.0	SCAR	–	Dunemann
	–	–	BSA	$AU_{?bp}$SCAR/3-4	AFLP; SCAR	–	et al. 2004
	–	–	–	$AU_{<100}$CAPS /3-4	–	–	–
Pl-1	–	150	Whole genome scan	–	–	LG 12	Lesemann and Dunemann 2006
Pl-n	X3191 × Novosibirski Sweet o.p. 91.117 A01-003	200	Screen of 76 phenotype extremes	$OPAT20_{450}$/9 $AU_{<100}$CAPS/9.5 AU_{600}SNP trans/9.5	SCAR CAPS SNP	Not determined	Dunemann et al. 2004
Pl-2	A679-2 × Iduna	358	QTL analysis	$OPN18_{1000}$/ $OPO04_{1800}$/ $OPK15_{1400}$/ $OPAG02_{450}$/	RAPDs spanning a QTL of 28 cM	–	Seglias and Gessler 1997 and Gianfranceschi et al. 1999
Pl-2	Fiesta × SA572-2	61	Population screen	$OPAT20_{900}$/6	RAPD	–	Dunemann et
–	–	–	Genetic map construction	OPAJ13600/11	RAPD	–	al. 1999
Pl-2	Royal Gala × A689-24	190	Population screen	$OPN18_{950}$/7	RAPD	–	Gardiner et al. 1999a
Pl-2	Fiesta × Discovery and A679-2 × Iduna	112	Comparative microsatellite mapping	–	–	LG 11	Liebhard et al. 2002

Table 6. (continued)

Gene	Progeny	No. sdlgs	Method	Markers marker/distance (cM from gene)	Marker class	Linkage group assignment	Reference
Pl-2	Royal Gala × A689-24	190	BSA	$OPU02_{1700}$/8	RAPD → SCAR	–	Gardiner
–	–	–	BSA	$OPAY17AB16a_{1100}$/5.9	RAPD → SCAR	–	et al. 2003
–	–	–	BSA	$OPAY17AB16b_{400}$/7.1	RAPD → SCAR	–	–
–	–	–	EST screening	GenBank DR033891/closest band 0.9	RFLP	–	–
–	–	–	–	GenBank DR033886/closest band 1.1	RFLP	–	–
–	–	–	–	GenBank DR033892 /closest band 2.9	RFLP	–	–
–	–	–	–	GenBank DR033893/4.1	RFLP	–	–
–	–	–	–	GenBank DR033888/5.4	SCAR	–	–
Pl-m	Fuji × MIS o.p. 93.051 G02-054	174	BSA	$OPAC20_{1800}$/14.4	RAPD	Not determined	Gardiner et al. 1999a
Pl-m	Fuji × MIS o.p. 93.051 G02-054	–	Genetic mapping	$OPAC20_{1800}$/0.7	SCAR	–	Gardiner et al. 2003
			(JoinMap v.2.0, Stam and van Ooijen 1995)	$OPN18_{1000}$/13.5	SCAR		
				$OPAY17AB16a_{1100}$/5.5	SCAR		
–	–	–	–	$OPAY17AB16b_{400}$/7.3	SCAR	–	–
–	–	–	–	$OPU02_{2000}$/8.1	SCAR	–	–
–	–	–	EST screening	GenBank DR033892/closest band 1.1	RFLP	–	–
–	–	–	–	GenBank DR033886/closest band 1.6	RFLP	–	–
–	–	–	–	GenBank DR033888 /5.8	SCAR	–	–
Pl-a	M9 × Aotea	277	Marker screening	$OPN18_{1000}$/11.5	SCAR	Not determined	Gardiner et al. 2004

phenotype is stable. It is necessary to expose the seedlings to the inciting organism *Podosphaera leucotricha* in the field, as it cannot be cultured in vitro.

1.3.2.2.1 *Pl-w* The first markers reported to be linked to a major resistance to powdery mildew were isoenzymes linked to the *Pl-w* gene derived from the crab apple White Angel (Manganaris 1989; Manganaris and Alston 1992; Hemmat et al. 1994; Batlle and Alston 1996). The closest was *Lap-2* (Table 6). Batlle and Alston also used segregation analysis to identify a complementary but unlinked gene *Rw* that was required for expression of resistance by *Pl-w*.

A whole genome scan using phenotype bulks demonstrated that the microsatellites CH01e12 (locus 1) and CH05a02y flank *Pl-w*, at positions 10 and 12 cM, respectively from the gene (James and Evans 2004). These microsatellites both map to LG 8 (Liebhard et al. 2002) confirming the assignment of *Pl-w* to this linkage group that was first indicated by the mapping of *Lap-2* to *LG* 8 by (Maliepaard et al. 1998). A study employing bulked segregant analysis using amplified fragment length polymorphisms (AFLPs) enabled the identification of two markers that map inside CH01e12 at 4.6 and 6.4 cM (EM M01 and EM M02, respectively) (Evans and James 2003; James and Evans 2004). Baldi et al. (2004) located an RGA marker 7 cM from the putative position of *Pl-w* in the Fiesta × Discovery population and Calenge et al. (2005a) mapped three NBS-LRR homologs within 2 cM of the putative position of *Pl-w* in the Discovery × TN10-8 population.

1.3.2.2.2 *Pl-d* This strong mildew resistance is derived from an open pollinated crab apple from the South Tyrol, Italy (Visser and Verhaegh 1976). Bulked segregant analysis identified AFLP and RAPD markers mapping to *Pl-d* (James et al. 2004) (Table 6). One of the AFLPs has been converted to a SCAR marker that maps to one side of *Pl-d*. In the course of the same study, two flanking microsatellite markers that were identified in a whole genome scan located *Pl-d* on the bottom of LG 12, a region where other disease resistance genes have been identified, including *Vg* (Durel et al. 1999), and NBS markers (Calenge et al. 2005a).

1.3.2.2.3 *Pl-1* Markussen et al. (1995) first reported $OPAT20_{450}$SCAR as a close marker for the *Pl-1* mildew resistance from *M. robusta* (Table 6). *Pl-1* was later verified to map at a distance of about 7 cM from the gene in a separate population (Dunemann et al. 2004). Two more markers have been identified using BSA with AFLPs. One was converted to a SCAR (AU-SCAR) and also a CAPs marker AU-CAPs (Urbanietz 2002; Dunemann et al. 2004) that maps 3-4 cM from *Pl-1*, making this marker a very valuable tool for selection purposes. *Pl-1* has been mapped to LG 12 in the vicinity of *Vg* and *Pl-d* following a whole genome scan (Lesemann and Dunemann 2006).

1.3.2.2.4 *Pl-n* Screening of a segregating population derived from a cross between the susceptible breeding parent X3191 and mildew resistant Novosibirski Sweet o.p. (91.117 A01- 003) demonstrated clearly that the *Pl-1* marker $AT20_{450bp}$SCAR is linked to resistance to powdery mildew infection derived from Novosibirski Sweet o.p. (Table 6). Development of a new SNP marker for this population from AU-CAPs enabled the mapping of this second *Pl-1* marker to the mildew resistance (Dunemann et al. 2004). The relationship between *Pl-1* and *Pl-n* is currently unclear; *Pl-n* may be a new gene, perhaps allelic to *Pl-1*.

1.3.2.2.5 *Pl-2* The earliest molecular markers for the *Pl-2* resistance from *M. zumi* were RAPDs obtained using a QTL mapping approach (Seglias and Gessler 1997; Gianfranceschi et al. 1999) (Table 6) but the gene clearly segregated as a single gene in a Royal Gala × A689-24 family in New Zealand (Bus et al. 2000). Dunemann et al. (1999) screened the markers that Markussen et al. (1995) had identified for *Pl-1* and found that $AT20_{900bp}$RAPD mapped at a similar distance from *Pl-2* as the 450 bp band had mapped to *Pl-1*. Gardiner et al. (1999a) utilized the OPN18 RAPD marker from the first study to construct DNA bulks on the basis of genotype as well as phenotype and identified a second, flanking marker for *Pl-2* (OPU02). They reported that the markers mapped in absence of phenotype were located less than 12 cM apart, making them useful for MAS. Gardiner et al. (2003) reported two more SCARs mapping inside OPN18SCAR, both derived from BSA with a combination of OPAY17RAPD and OPAB16RAPD primers (OPAY17/OPAB16a and b SCARs). They also mapped OPAC15/OPAZ16SCAR distal to OPU02SCAR and used this map of anonymous markers as a framework to locate EST markers (Gardiner et al. 2003). One hundred and ten ESTs were mined from an EST database of 30,000 unigene sets on the basis of sequence homology to recognized resistance genes from other plants and screened as RFLP probes over mini-populations segregating for a range of resistances. This enabled the detection of putative

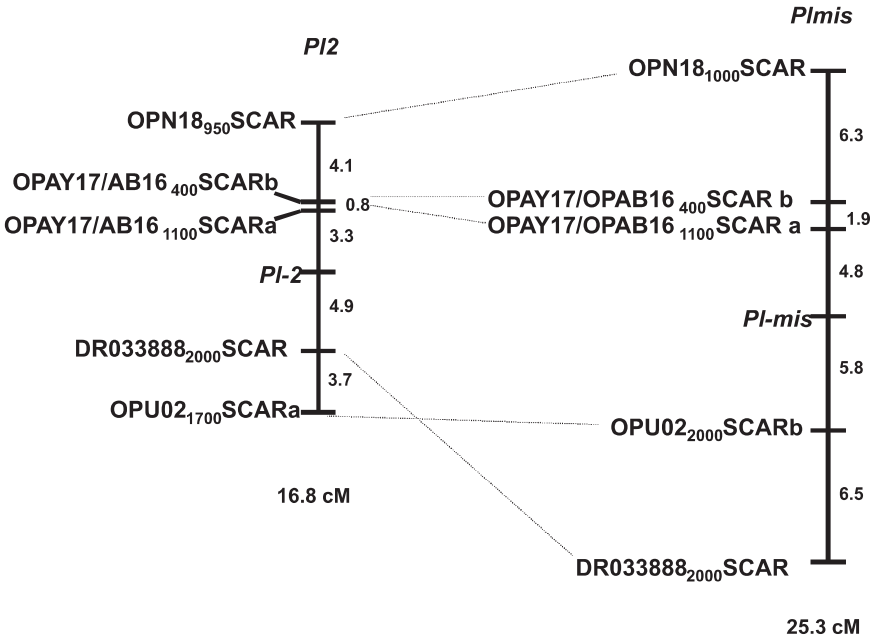

Fig. 3. Comparison of *Pl-2* and *Pl-m* genetic linkage maps using PCR based markers only. The *vertical line* represents the linkage group with marker loci *on the left*, interval sizes (in cM) *on the right* and the total length (in cM) *at the base* of the maps (after Gardiner et al. 2003)

linkages to *Pl-2* and other genes. These were then confirmed in larger subsets of the mapping population.

Loci characterized by ESTs mapped to either side of *Pl-2* in regions where it had previously proved difficult to locate anonymous SCAR markers. Of ESTs mapped as RFLPs within 3 cM of *Pl-2*, a band from GenBank accession DR033891 showing homology to HcrVf2 mapped 0.9 cM from *Pl-2*. The closest bands from GenBank accession DR033886 and GenBank accession DR033892 (no homology to known R gene classes) mapped at 1.1 and 2.9 cM, respectively on the other side of *Pl-2*. A second NBS-LRR homolog (GenBank accession DR033893) mapped just outside of GenBank accession DR033892. An EST exhibiting homology to the NBS-LRR class of R genes (GenBank accession DR033888) was converted to a PCR based marker that mapped at 5.4 cM from *Pl-2*, inside OPUO2SCAR.

It has been noted that GenBank accession DR033891 also maps close to *Vf* (Sect. 1.3.2.1.1). It is interesting to speculate that it might be involved in the scab resistance QTL located close to *Pl-2* that was reported by Liebhard et al. (2003c).

Mapping of a common microsatellite in the A679-2 × Iduna population of (Seglias and Gessler 1997) and the Fiesta × Discovery population (Liebhard et al. 2002) enabled assignment of *Pl-2* to LG 11. This has been confirmed by Rusholme et al. (unpublished) in a whole genome scan of DNA bulks with 74 microsatellite markers, as well as the framework map under construction by Rikkerink et al. (unpublished).

1.3.2.2.6 *Pl-m* This strong mildew resistance segregates as a major gene from an open pollinated seedling (93.051 G07-062) of the mildew-immune seedling described by Dayton (1977) as being the product of a pollination of a domestic apple with an unknown crab apple. The first marker reported for *Pl-m*, OPAC20, was obtained using BSA with RAPDs (Gardiner et al. 2001) (Table 6) and the SCAR derivative mapped very closely to the resistance at 0.7 cM (Gardiner et al. 2003). Four SCAR markers previously mapped around *Pl-2* also mapped to *Pl-m* and the marker order around the resistance phenotype was conserved. Fragment length was conserved for three of these anonymous markers and for the NBS-LRR marker derived from GenBank accession DR033888.

Figure 3 shows simplified maps around *Pl-m* and *Pl-2*, constructed with the PCR-based markers in common only. Two other ESTs with no known sequence homology to R genes (GenBank accessions DR033886 and DR033892) also identified markers for both mildew resistances when mapped as RFLPs. Whole genome scanning with the same 74 microsatellites used to assign *Pl-2* to LG 11 did not enable as-

signment of *Pl-m* to a linkage group, and therefore the possibility that *Pl-2* and *Pl-m* may not map to the same group (Rusholme et al. unpublished) and that the genomic regions around these two genes may be homeologous rather than homologous cannot yet be ruled out. As *Pl-2* and *Pl-m* have already been pyramided in a resistance breeding population (Bus et al. unpublished), test crosses from seedlings with both genes would provide information on this question, as would more intensive microsatellite mapping of *Pl-m*.

1.3.2.2.7 *Pl-a* The rootstock Aotea 1 derived from *M. sieboldii* (Taylor 1981) has been observed to carry resistance to powdery mildew, as well as to apple scab (*Vat*) and woolly apple aphid (*Er-3*). When markers for *Pl-2* and *Pl-m* were screened across a population phenotyped for three years for mildew resistance, it was noted that two of the markers (OPN18SCAR and $OPUO2_{2100bp}$SCARc) mapped to *Pl-a* (Table 6), indicating that *Pl-a* may share a positional relationship with *Pl-2* and *Pl-m* (Gardiner et al. 2004).

1.3.2.3 Rosy Leaf Curling Aphid Resistance Genes

1.3.2.3.1 *Sd-1* Three very close RFLP markers (MC06a, 2B12a and MC029b) mapped within 2 cM from the *Sd-1* gene for resistance to biotypes 1 and 2 of *Dysaphis devecta* from Cox's Orange Pippin (Roche et al. 1997a) in a Prima × Fiesta population (Table 7). Four RAPD markers mapped more distantly. The RFLP 2B12a was later converted into $2b12a_{196bp}$SCAR (Roche et al. 1997b) and its linkage with *Sd-1* was confirmed through pedigree analysis. The original mapping population was later employed to construct the detailed genetic map of Maliepaard et al. (1998), which located *Sd-1* at the top of LG 7. Repeated phenotyping, plus fine mapping in over 700 seedlings from six more families later enabled the co-location of MC064a with *Sd-1* in a 1.3 cM interval between $2b12a_{196bp}$SCAR and microsatellite SdSSRa (Cevik and King 2002a).

1.3.2.3.2 *Sd-2* Co-segregation of $2b12a_{196}$SCAR and SdSSRa in a small population segregating for the *Sd-2* gene from Double Red Northern Spy that confers resistance to *D. devecta* biotype 1 only, indicated that *Sd-2* is tightly linked to *Sd-1*, and is probably allelic to *Sd-1* (Cevik and King 2002a) (Table 7).

1.3.2.4 Woolly Apple Aphid Resistance Genes

1.3.2.4.1 *Er-1* Markers G327 and OPC20RAPD that flanked the *Er-1* resistance from Northern Spy at 12 and 8 cM, respectively were identified by bulked segregant analysis (Gardiner et al. 1999a; Bus et al. 2000) (Table 7) and converted to SCARs for use in MAS. OP05SCAR, a marker close to *Er-3* is linked more distantly to *Er-1* (Gardiner et al. 2004). More recently, mapping of two microsatellite markers (CH01c06 and CH02g09) from Liebhard et al. (2002) made it possible to assign *Er-1* to LG 8. Interestingly, CH01c06 was located only 2 cM from *Er-1* (Chagné, Durel and Gardiner unpublished), which makes it a suitable marker for marker-assisted selection.

1.3.2.4.2 *Er-3* A novel resistance to woolly apple aphid identified in the rootstock Aotea 1 (Taylor 1981) has been studied in a segregating population (M9 × Aotea 1) and named *Er-3* (Gardiner et al. 1997; Bus et al. 2000). The close linkage of $OP05_{1700bp}$ SCAR to this resistance (0.8 cM) (Table 7) indicated that it would be useful for MAS. This was confirmed by the detection of only four recombinants in a 2nd generation population of 121 plants, using the modified marker $OP05_{880}$SCAR constructed to segregate in this particular population (Bus et al. 2000). Candidate genes developed from ESTs that mapped to *Er-3* include two NBS LRR homologs (GenBank accession DR033890 and GenBank accession DR033887), a receptor protein kinase (GenBank accession DR033889) and a leucine rich repeat EST with homology to extensin and tomato *Cf-2* (GenBank accession DR033885). GenBank accessions DR033885 and DR033887 have been converted to PCR based markers and mapped to *Er-1* as well as *Er-3* (Gardiner et al. unpublished). Screening of these two markers in a Discovery × TN10-8 population enabled the assignment of *Er-1* and *Er-3* to LG 8 (Chagné, Durel and Gardiner unpublished). This has been confirmed for *Er-3* by mapping of microsatellite CH02g09 12 cM (Liebhard et al. 2003c) from *Er3* in the M9 × Aotea 1 population (Chagné, Durel and Gardiner unpublished).

1.3.2.4.3 *Er-m* The same pollinated seedling (93.051 G07-062) that is the source of the *Pl-m* resistance (see above) also carries a novel resistance to woolly

Table 7. Mapping of major genes for resistance to aphids in apple

Trait Resistance to	Gene	Progeny	No. sdlgs	Method	Markers (marker/distance) (cM from gene)	Marker class	Linkage group assignment	Reference
1. Rosy leaf curling aphid (*Dysaphis divecta*)	*Sd-1*	Prima × Fiesta	141	Linkage analysis (JoinMap v.2.0, Stam and van Ooijen, 1995)	MC029b/2 MC064a/1 2B12a/1	RFLP RFLP RFLP	–	Roche et al. 1997a
	Sd-1	Prima × Fiesta	77	Marker conversion, pedigree analysis	$DdARM_{196}$	SCAR	–	Roche et al. 1997b
–	*Sd-1*	Prima × Fiesta	152	Mapping	–	–	LG 7	Maliepaard et al. 1998
–	*Sd-1*	Resistant cultivars Susceptible cultivars	9 8	BSA	ETC/MCTT-1/1.4	AFLP → SCAR		Cervik and King 2002a
–	–	6 segregating families	759	Linkage analysis	MC064a/0 *Sd-1* located in 1.3 cM	RFLP	–	–
–	–	–	–	–	interval between $2B12a_{196}$	SCAR	–	–
–		–	–	–	and SdSSRa	AFLP; microsatellite	–	–
–	*Sd-2*	Double Red Northern Spy × Totem	47	Linkage analysis	$2B12a_{196}$/0 SdSSRa/0	SCAR microsatellite	LG 7	Cervik and King 2002a
2. Woolly apple aphid (*Eriosoma lanigerum*)	*Er-1*	Sciglo x Northern Spy	132	BSA	$G327_{1600}$/11.6 $OPC20_{2000}$/7.9	RAPD; SCAR RAPD; SCAR	–	Gardiner et al. 1999a, Bus et al. 2000
	Er-1	Sciglo × Northern Spy	132	Linkage analysis	$OPO05_{1700}$/9.6 $DR033885_{1500}$/23 trans $DR033887_{900}$/23	SCAR SNP SCAR	–	Gardiner et al. 2004
–	*Er-1*	Discovery × TN10-8	149	Marker screening	$DR033885_{1300}$ $DR033887_{750}$	SNP SCAR	LG 8	Chagné et al. unpublished
–	*Er-1*	Sciglo x Northern Spy	94	Marker screening	CH01c06/2	microsatellite	LG 8	Chagné et al. unpublished
–	*Er-3*	M.9 × Aotea	131	BSA	$OPO05_{1700}$/0.8	RAPD; SCAR	–	Gardiner et al. 1997
–					$OPA01_{1250}$/3.3	RAPD (trans)	–	Bus et al. 2000
–	*Er-3*	M.9 × Aotea	277	EST screening	$DR033885_{500}$/9.8; 7.0	RFLP; SNP	–	Gardiner et al. 2004
–	–	–	–	–	DR033890 /closest band 5.7 $DR033887_{750}$/7.8; 7.0 DR033889 /closest band 8.0	RFLP RFLP; SCAR RFLP	–	and Gardiner et al. unpublished
–	*Er3*	M.9 × Aotea	277	Marker screening	CH02g09/12	microsatellite	LG 8	Chagné et al. unpublished
–	*Er-m*	Fuji × MIS o.p. 93.051 G02-054	153	BSA	$OPA04_{950}$/7 $OPZ20_{1200}$/6	RAPD → SCAR RAPD	Not determined	Gardiner et al. unpublished
–	*Er-l*	Prima × Longfield o.p. 93.043 G07-062	144	BSA	$OPAD01_{630}$/13	RAPD; SCAR	Not determined	Gardiner et al. 2001

apple aphid that does not map to markers for *Er-1* and *Er-3*. Flanking markers OPA4SCAR and OPZ20RAPD have been identified by BSA (Table 7) (Gardiner et al. unpublished).

1.3.2.4.4 *Er-l* A single RAPD marker, OPA01, has been identified for a novel woolly apple aphid resistance derived from an open pollinated seedling of Longfield (93.043 G07-062) (Gardiner et al. 2001). Markers identified to date for other woolly apple aphid resistances do not map to *Erl* (Table 7) (Gardiner et al. unpublished).

1.3.2.5 Other Major Gene Traits

1.3.2.5.1 Self-Incompatibility (*SI*) Maliepaard et al. (1998) mapped the self-incompatibility locus as an allele-specific marker (Janssens et al. 1995) in the Prima × Fiesta framework map (Table 8). It is closely associated with AAT-1 isoenzyme and the RFLP MC038b on the lower end of LG 17.

1.3.2.5.2 Rootsuckers (*Rs*) Joint segregation analysis of root sucker formation with RAPD markers segregating from White Angel identified linkage of RAPD P124e with a single locus determining the formation of root suckers (*Rs*) (Weeden et al. 1994) (Table 8). This locus was assigned to a US linkage group that corresponds to LG 17 in the European numbering system (Maliepaard et al. 1998; Hemmat et al. 2003).

1.3.2.5.3 Fruit skin color (*Rf*) Weeden et al. (1994) found that the isoenzyme marker *Idh-2* was closely linked to red skin color in a Rome Beauty × White Angel population. Study of segregation of BC226SCAR (identified by BSA) in the same Rome Beauty × White Angel population indicated that the basis for control of red/yellow skin color was simple (Cheng et al. 1996) (Table 8). Fruit of progeny with the dominant 1160 bp fragment from Rome Beauty or the 1180 bp fragment from White Angel (or both) were red skinned, while the recessive 1230 bp fragment inherited from both parents segregated with yellow skin. In three other progenies, only the 1160 bp fragment segregated with red skin. A second fragment of 1320 bp could be associated with yellow skin, in addition to the 1230 bp fragment. Screening of 56 other cultivars indicated that this marker system could be used to predict skin color in most cases. BC226 mapped to the US LG 3 (Conner et al. 1997) and to European LG 9 between two RFLP markers in the Prima × Fiesta framework map (Maliepaard et al. 1998). Comparative DNA sequencing would have to be carried out to confirm that the BC226 locus in the Rome Beauty × White Angel cross is allelic to the BC226 locus amplified in the Prima × Fiesta framework population, and the putative LG 9 assignment.

1.3.2.5.4 Fruit Juice pH (*Ma*) Acidity in apple is mainly due to malic acid and Nybom (1959) demonstrated that low acid fruit (pH 3.8 and above) was determined by the presence of homozygous recessive alleles for the *Ma* gene, *ma ma*. Mapping of fruit juice pH <3.7 in a Wijcik McIntosh × NY75441-58 population enabled the identification of a RAPD marker for *ma* ($S65_{600}$) (Table 8) (Conner et al. 1997). Acid-fruited progeny were assumed to have at least one copy of *Ma* and both parents (acid fruited) were heterozygous *Ma ma*. Using similar criteria, Maliepaard et al. (1998) located *Ma* on the distal end of LG 16, co-segregating with the RAPD $OPT16_{1000}$.

1.3.2.5.5 Columnar habit (*Co*) The *Co* gene in apple that was identified in a mutant of McIntosh (Fisher 1970) decreases branching, internode length and plant height, while increasing spur formation. The columnar character is believed to be controlled by a dominant allele, but modifiers may be involved (Lapins 1976). Bulked segregant analysis was used to identify a RAPD marker for the columnar habit that contained a repeat of $(GA)_{17}$ (Table 8) (Hemmat et al. 1997). This RAPD fragment was converted to a microsatellite marker (SSR^{CO}), where the null allele was linked to *Co*. Conner et al. (1997) then mapped two further RAPDs, B347 and B318 to a 5 cM interval around *Co* on the US linkage group corresponding to LG 10 in the EU framework map. SSR^{CO} (designated USASSR11) was later mapped directly to LG 10 in Fiesta (Maliepaard et al. 1998).

1.3.2.5.6 Fruit Allergens (*Mal d*) Allergies to fresh apples in Northern and Central European populations sensitized to birch pollen arise from four allergens identified to date: Mal d 1 (a Bet v 1 homologous protein) belonging to a group of pathogenesis related (PR10) proteins, Mal d 2 (apple thaumatin-like protein), Mal d 3 (apple non-specific lipid transfer protein) and Mal d 4 (apple profiling) (see Gao et al. 2005a). Knowledge of the genetics of allergenicity caused by healthy apple fruit will enable breeding for low allergen

Table 8. Mapping of major genes not involved in resistance, in apple

Gene	Progeny	No. sdlgs	Method	Markers marker/distance (cM from gene)	Marker class	Linkage group assignment	Reference
SI	Prima × Fiesta	152	Map construction	AAT-1/<1 MC038b/1	Isoenzyme RFLP	LG 17	Maliepaard et al. 1998
Rs	Rome Beauty × White Angel	56	Joint segregation analysis	P124e/not determined	RAPD	LG 17	Weeden et al. 1994
Rf	Rome Beauty × White Angel	56	Joint segregation analysis	*Idh-2*/not determined	Isoenzyme	–	Weeden et al. 1994
Rf	Rome Beauty × White Angel	72	BSA, mapping	$BC226_{1180}$/<2 *Idh-2*/<2	RAPD; SCAR Isoenzyme	–	Cheng et al. 1996
Rf	Wijcik McIntosh × NY75441-58	172	Linkage analysis	$BC226_{1175}$/0	RAPD (= 1,160 bp SCAR fragment above)	–	Conner et al. 1997
Rf	Prima × Fiesta	152	Mapping of BC226 SCAR			LG 9	Maliepaard et al. 1998
Ma	Wijcik McIntosh × NY75441-58	172	Map construction	$S65_{600}$/7.1	RAPD	–	Conner et al. 1997
Ma	Prima × Fiesta	152	Map construction	$OPT16_{1000}$/0	RAPD	LG 16	Maliepaard et al. 1998
Co	Wijcik McIntosh × NY5441-67	126	BSA	$OPA11_{1000}$/? → SSR^{CO}	RAPD, trans microsatellite	–	Hemmat et al. 1997
–	Wijcik McIntosh × NY75441-58	172	Linkage analysis	SSR^{CO}/6	microsatellite, trans	–	
Co	Wijcik McIntosh × NY75441-58	172	Map construction $B318_{440}$/3.2	$B347_{890}$/1.8 RAPD	RAPD	–	Conner et al. 1997
Co	Prima × Fiesta	152	Mapping of SSR^{co}	–	microsatellite	LG 10	Maliepaard et al. 1998
Mal d 1 gene family	Prima × Fiesta plus Jonathan × Prima	144 196	PCR based cloning; mapping of allele specific markers for each member of family (18 members)	–	–	–	Gao et al. 2005b
Subfamilies I, IV	–	–	–	Gene specific markers	SNP, microsatellite	LG 13, LG 16	–
Subfamilies II, III	–	–	–	Gene specific markers	SNP, microsatellite	LG 16	–
Mal d 1.05	–	–	–	Gene specific marker	SNP, microsatellite	LG 6	–
Mal d 2.01A	Prima × Fiesta	141	PCR based cloning; mapping of allele specific markers	Gene specific marker	SNP	LG 9	van de Weg, personal communication
Mal d 2.01B	–	–	–	Gene specific marker	SNP	–	–
Mal d 3.01	Jonathan × Prima	196	PCR based cloning; mapping of allele specific markers	Mal d 3.0101a-JO/0,	SNP	LG 12	Gao et al. 2005a

Table 8. (continued)

Gene	Progeny	No. sdlgs	Method	Markers marker/distance (cM from gene)	Marker class	Linkage group assignment	Reference
Mal d 3.02	Prima × Fiesta plus Jonathan × Prima	144 196	PCR based cloning; mapping of allele specific markers	Mal d 3.0201c-PM/0	SNP	LG 4	Gao et al. 2005a
Mal d 4.01	Prima × Fiesta	141	PCR based cloning; Gene specific marker		SNP	LG 9	van de Weg, personal communication
-2 copies	plus Jonathan × Prima	175	Mapping of allele specific markers	–	–	–	–
Mal d 4.2A	–	–	–	Gene specific marker	SNP	LG 2	–
Mal d 4.3A	–	–	–	Gene specific marker	SNP, SSR	LG 8	–
Md-ACS1	Prima × Fiesta	144	Mapping of gene specific molecular marker		SCAR	LG 15	Costa et al. 2005
Md-AC01	Prima × Fiesta Fuji × Mondial Gala	144 ?	Mapping of gene specific molecular marker	–	SCAR	LG 10	Costa et al. 2005
sl-1	6 progenies segregating for *Vf*		Mapping with *Vf* markers	–	± 14 cM from *Vf*	LG 1	van de Weg, personal communication
sl-2	6 progenies segregating for *Vf*		Mapping with *Vf* markers	–	1–8 cM from *Vf*	LG 1	van de Weg, personal communication

cultivars for European consumers with this food allergy.

1.3.2.5.6.1 Mal d 1 A number of conserved and specific primers were designed to obtain all possible *Mal d 1* sequences from Prima and Fiesta. PCR cloning of fragments, sequencing and genome walking towards the ends of genes enabled construction of allele-specific SNP and microsatellite primers for 18 *Mal d 1* genes that could be classified into four sub-families according to intron size and presence/absence. These genes were mapped directly in the Prima × Fiesta population, as well as a Jonathan × Prima progeny (Table 8). Seven genes from sub-families I and IV mapped in a 30–35 cM interval between the RFLP markers MC001 and MC041 on LG 13, and nine on the homeologous LG 16. One gene mapped to LG 6 and one has not yet been mapped (Gao et al. 2005b).

1.3.2.5.6.2 Mal d 3 PCR-based cloning and sequencing of DNA from the parents of the Prima × Fiesta framework mapping population of Maliepaard et al. (1998) resulted in the identification of two distinct genes, each with several sequence variants, that encode Mal d 3 proteins. SNP markers were constructed for each of the genes, *Mal d 3*.01 and *Mal d 3.0*, and mapped to homeologous segments of LG 12 and 4 between two RFLP markers shared in common (MC127 and MC105) (Gao et al. 2005a) (Table 8).

1.3.2.5.6.3 Mal d 2 Similar techniques have been employed to map two copies of *Mal d 2* to an identical position on LG 9 (Table 8) (Gao et al. 2005c)

1.3.2.5.6.4 Mal d 4 Two copies of a *Mal d 4* gene mapped to LG 9 and two single copy genes mapped to LG 2 and LG 8 respectively (Table 8) (Gao et al. 2005c).

1.3.2.5.7 Ethylene Production Shelf life in apple is a significant factor in determining the economic value of an apple cultivar, particularly in countries that rely on shipping of product to distant markets. In the course of a study examining the role of enzymes involved in the biosynthesis of ethylene and shelf life of apple fruit stored at room temperature after harvest, two key genes of the ethylene biosynthesis pathway were mapped. Both have proved to be candidates for marker-assisted breeding, as homozygotes for alleles *Md-AC01-1* and *MdACS1-2* yield fruit with lowest ethylene production and superior shelf life (Costa et al. 2005).

1.3.2.5.7.1 Md-ACS1 The *Md-ACS1* marker developed by Harada et al. (2000) mapped to LG 15 in the Prima × Fiesta framework mapping population (Costa et al. 2005) (Table 8). Its location is distant from a known QTL for fruit firmess that was previously identified in this population (King et al. 2000; Maliepaard et al. 2001). *Md-ACS1* exhibited a relatively large effect on ethylene content and apple fruit shelf life.

1.3.2.5.7.2 Md-AC01 A codominant gene specific marker for *Md-AC01* developed from full-length gene sequences derived from apple gDNA and mRNA mapped to LG 10 in two populations (Table 8). The clear, although small effect of *Md-AC01* on ethylene production, coupled with its location at the border of the 5% interval for fruit firmness QTL (King et al. 2000; Maliepaard et al. 2001), indicates that the role of *Md-AC01* in determining shelf life requires further examination (Costa et al. 2005).

1.3.2.5.8 Sub-lethal Genes (*sl*) Distorted segregation ratios have frequently been reported in populations segregating for the *Vf* gene (e.g., Yang and Kruger 1994; Tartarini 1996; Conner et al. 1997; Gardiner et al. 1999b; Tartarini et al. 1999; Bus et al. 2002). Analysis of this phenomenon in six progenies has indicated that these distortions could be explained by three homozygous recessive sub-lethal genes (*sl-1, sl-2, sl-3*). *Sl-1* mapped about 14 cM from *Vf*, and *sl-2* between 1–8 cM from *Vf*. Both genes required the presence of an unlinked gene, *sl-3*, for expression (Van de Weg, personal communication). A good understanding of the role of sub-lethal genes will facilitate strategic choice of parents by breeders to provide progenies with optimal proportion of seedlings with *Vf*.

1.4 QTL Trait Mapping

1.4.1 QTL Identification and Mapping in Apple

'Quantitative trait' describes a character for which the observed variation is due to the segregation of several genes and where, for each gene, the effects of the allelic differences on phenotype are generally small compared with the effects of the environment for each gene (Kearsey and Poon 1996). Genetic mapping of quantitative trait loci (QTL) involves identifying and determining the degree of association between continuous quantitative traits and sets of genetic markers.

The ability to assess complex phenotypes in apple at the seedling stage, such as tree architecture, fruit texture, fruit size and susceptibility to storage disorders using genetic markers would greatly accelerate new variety development. In addition to the selection of advantageous traits, markers linked to complex traits could be used to select against negative characteristics, and could even be used to select the combination of parents that would give rise to progeny with the desired genotype.

An essential requisite for accurate QTL identification in any plant species is a saturated genetic map covering the entire genome. If certain regions of the genome are not adequately represented by genetic markers, then QTLs located in such regions will not be reliably mapped, because it will be difficult to determine if the QTL has a genuinely small phenotypic effect, or is merely weakly linked to flanking markers (Lander and Botstein 1988). Several genetic maps have been constructed for apple using a range of genetic markers, such as random amplified polymorphism (RAPDs), restriction fragment length polymorphisms (RFLPs), amplified length polymorphism (AFLPs) and isozymes (Hemmat et al. 1994; Conner et al. 1997; Seglias and Gessler 1997; Maliepaard et al. 1998) (see Sect. 1.2.2). More recently, codominant microsatellite markers have been employed, and the development of 115 new microsatellite markers, mapped in the Fiesta × Discovery population (Liebhard et al. 2002; Liebhard et al. 2003b) has established a saturated, robust apple map comprising 1,140 cM and 1,450 cM in Fiesta and Discovery, respectively. This genetic map has been aligned with the Iduna × A679/2 map (Gianfranceschi et al. 1998) using common microsatellite loci, demonstrating that existing linkage maps, such as those published by Conner et al. (1997) and Hemmat et al. (1994) could easily be enriched and subsequently aligned and integrated with the Fiesta × Discovery map (Liebhard et al. 2002, 2003b), providing a valuable tool for QTL detection and analysis in apple. Map alignment with a consensus, saturated map will enable the detailed comparison of QTL positions between populations (King et al. 2000; Durel et al. 2003; Liebhard et al. 2003a, b, c; Calenge et al. 2004).

In addition to the classical QTL mapping approaches, there are other resources that can be utilised in the identification of QTLs. For example, genetic markers based on the sequence homology between the NBS domain in plant resistance genes identified loci which co-segregated with apple scab and powdery mildew resistance QTLs previously detected in a Discovery × TN10-8 population (Calenge et al. 2005a). Since such QTLs could sometimes be the result of residual resistance encoded by defeated major resistance genes, this is effectively a candidate gene mapping approach. Genome synteny between related species has also been exploited in QTL detection, with comparative mapping approaches used to identify QTLs conserved between maize and rice (Chardon et al. 2004) maize and sorghum (McIntyre et al. 2004) and maritime and loblolly pines (Chagné et al. 2003), suggesting that QTLs detected in genomes of other species belonging to the Rosaceae family, could be used to aid the identification of QTLs in apple.

1.4.2 Mapping QTLs for Disease Resistance

Most of the disease resistance genes characterized to date in apple are single dominant genes (see Sect. 1.3). Such genes commonly confer resistance to the pathogen in a gene-for-gene manner and are therefore in theory, easily overcome by the pathogen's ability to mutate to virulence (Crute and Pink 1996). In view of the ease with which a pathogen can break down single gene resistances, illustrated by the two recently discovered races of apple scab, able to overcome *Vf* (Parisi et al. 1993; Roberts and Crute 1994), it is likely that durable resistance to apple pathogens will be established through the pyramiding of different resistance genes with different resistance specificities into a single cultivar. Pathogen resistance conferred by QTLs would be a valuable addition to breeding portfolios of major resistance genes, as incorporating QTLs into a single cultivar is likely to be more effective than the combining of major genes alone (Parlevliet and Zadoks 1977). However, it is likely that several QTLs with significant phenotypic effects would be required to achieve a level of resistance comparable to that controlled by major genes (Liebhard et al. 2003c).

1.4.2.1 QTLs for Resistance to Powdery Mildew

Kellerhals et al. (2000) identified two major QTLs for powdery mildew resistance in the accession A 679-2 (Table 9). One of these QTLs originates from *M. zumi*, (*Pl-2* parent) and the other is linked to the *Vf* locus (approximately 16 cM from *Vf*) and is likely to origi-

Table 9. Summary of QTLs for resistance to powdery mildew

Parents Female	Male	No. indv.[a] P/T	G/T	Method of phenotypic assessment	Number of QTL detected Linkage group (LG or G), LOD score and percentage of variance explained by QTL[b]	Reference
Iduna	A 679-2	450	189	Artificial infection in field Scored: 5 point scale	**A 679-2 map:** LG 3, 5, 16 **Iduna map:** LG 2, 3, 5, 7, 8, 9	Kellerhals et al. 2000[c]
Idared	U 211	98	98	Assessed: 3 years in nursery 2 years in orchard Scored: 5 point scale	**U 211 map:** G 2: LOD 12.0; 72.1% and LOD 12.1; 71.5% G 3: LOD 2.6; 37.5% and LOD 8.9; 72.4% G 4 = LG 12: LOD 9.3; 64.9% and LOD 8.8; 71.9% and LOD 10.8; 72.0% **Idared map:** G 3 = LG 2: LOD 3.3; 40.6% and LOD 2.4; 39.4% G 5 = LG 15: LOD 7.9; 61.0%	Stankiewicz-Kosyl et al. 2005[d]
Discovery	TN10-8	149	149	Natural infection in field Assessed over 5 seasons Scored: 10 point scale	**Discovery × TN10-8 map:** LG 2: LOD 3.0–9.01; 7.4%–22.5% LG 13: LOD 3.74–9.73; 7.5%–27.4% LG 1: LOD 3.0; 7.4% LG 8: LOD 5.27–8.45; 8.9%–19.5% LG 10: LOD 3.99–4.02; 7.9%–8.3% LG 14: LOD 3.45; 5.7% LG 17: LOD 4.36–4.64; 8.8%–10.5%	Calenge and Durel 2006[e]

[a] Number of individuals used in QTL detection, either in phenotyping population (P/T) or in map construction and genetic marker analysis (G/T)

[b] Linkage groups containing QTL are listed with the prefix LG or G. Where possible, the LOD score of the associated QTL and the percentage of phenotypic variance (PVE) it explains are included, with the LOD score first. Where several linkage groups are included, the range of associated LOD score and PVE have been included.

[c] Linkage maps not aligned with adopted consensus map (Liebhard et al. 2003b), thus standard linkage group nomenclature not used.

[d] Where possible linkage groups have been aligned with those of the consensus map (Liebhard et al. 2003b) and are annotated as such.

[e] Linkage maps have been aligned with the consensus map (Liebhard et al. 2003b), and standard nomenclature is used.

nate from *Malus floribunda* 821. Eight other putative QTLs were also identified, six of which were located on the genome of the susceptible parent, Iduna. Mildew infection was assessed in the field using a 5-point classification scale, and the non-parametric Kruskal-Wallis test was used to determine the association of mapped RAPD markers with powdery mildew resistance. Stankiewicz-Kosyl et al. (2005) used MAPMAKER/EXP 3.0 and MAPMAKER/QTL 1.1, to analyse data from a limited number of 98 individuals derived from an Idared × U211 cross and map 10 QTLs involved in powdery mildew resistance (Table 9). Although the genetic maps of Idared and U211 span only five and four linkage groups, respectively, these maps were specifically constructed around genomic regions of interest. Several of the markers used made it possible to align some of these linkage groups with those of Maliepaard et al. (1998).

Stankiewicz-Kosyl et al. (2005) assessed trees over a period of three years in the nursery and two years in the field. Of the 10 QTLs detected five of them were associated with powdery mildew resistance in only one year. QTL U7, mapped on the equivalent of LG 12, was associated with powdery mildew resistance over four years and explained between 47.8% and 72.0% of phenotypic variation. Apple scab resistance genes such as *Vg* (Durel et al. 1999) and *Pl-d* (James et al. 2004) have already been mapped to LG 12, suggesting the possibility of resistance QTL/gene clusters in this region.

Calenge and Durel (2006) also assessed the occurrence of powdery mildew resistance in a population of 149 individuals derived from a Discovery × TN10-8 cross (Calenge et al. 2004) across five seasons over four years (Table 9). Using MapQTL software, two QTLs on LG 2 and LG 13 were consistently identified over all five seasons and explained between 7.5 and 27.4% of the phenotypic variation depending upon the season, making them ideal candidates to select for in marker-assisted breeding programs. Five other QTLs were also identified during either one or more seasons on LG 1, LG 8, LG 10, LG 14 and LG 17 and several of these QTLs were mapped to the same region as previously identified major resistance genes, or resistance gene clusters. Calenge and Durel (2006) hypothesised that the detection of a range of QTLs over the five seasons could be the result of environmental effects, such as climate, tree growth and development, or changes to the *P. leucotricha* local populations. The fluctuation in the presence/absence of these five QTLs over the four seasons indicates that to explain this powdery mildew resistance fully, assessments over more years will be necessary. Indeed, Calenge and Durel (2006) plan to maintain and continue to assess this population beyond this study, to determine a key set of QTL that control resistance to powdery mildew.

1.4.2.2 QTLs for Resistance to Apple Scab

QTLs have been identified for apple scab resistance using the reference genetic maps (Sect. 1.2.2) constructed in the following populations; Prima × Fiesta (Durel et al. 2003), Fiesta × Discovery (Liebhard et al. 2003c), Discovery × TN10-8 (Calenge et al. 2004) (Table 10). A summary of these results plus those from progenies of Discovery × Prima and Durello de Forli x Fiesta is presented in Durel et al. (2004).

Durel et al. (2003) used two monoconidial strains of race 6 to identify QTLs controlling resistance in both Fiesta and Prima. Detailed QTL analysis using both MCQTL (Jourjon et al. 2000) and MapQTL (Van Ooijen 2004) software identified four genomic regions that were significantly involved in partial resistance, characterized by a reduction in sporulation (Table 10). One of these regions was located close to the original *Vf* gene and it is possible that the observed partial resistance was due to a closely linked gene, or a result of a residual effect of the overcome *Vf* gene (Durel et al. 2003). The remaining three additional regions identified on LG 15, 11 and 17 were novel locations for association with scab resistance.

Liebhard et al. (2003c) carried out extensive assessment of field resistance to apple scab over a three-year period involving three different geographical sites. Using MapQTL, eight QTLs were identified that contributed to apple scab resistance; six for leaf scab and two for fruit scab (Table 10). Interestingly, although Discovery demonstrated a greater degree of resistance, most of the identified QTLs were attributed to Fiesta, the more susceptible parent, indicating a high degree of homozygosity at the resistance gene loci in Discovery that prevented their detection in the progeny because of the lack of segregation. The high levels of resistance observed in individuals during the study confirmed that Discovery was a strong resistant parent for breeding (Liebhard et al. 2003c). The strongest scab resistance QTL from Prima × Fiesta mapped to LG 17 (Liebhard et al. 2003c), coinciding with a scab resistance QTL that Durel et al. (2003) identified, and similarly LG 11 was identified in both studies as possessing a region of interest. One of the QTLs detected by Liebhard et al. (2003c) that

Table 10. Summary of QTLs for apple scab resistance

Parents Female	Male	No. indv.[a] P/T	G/T	Method of phenotypic assessment	Number of QTL detected Linkage group (LG or G), LOD score and percentage of variance explained by QTL[b]	Reference
Prima	Fiesta	143	143	Glasshouse inoculated: Race 6 strains (strain 302, EU-D-42)	**QTLs in four main genomic regions identified:** LG 1 (isolate specific): LOD 8.2–8.9; 16.0–17.8% LG 11: LOD 5.4–9.5; 16.5–22.8% LG 15 (isolate specific): LOD 3.1–3.3; 5.6–6.0% LG 17: LOD 3.6–5.9; 9.4–13.4%	Durel et al. 2003[c]
Fiesta	Discovery	251	251	Infected leaves placed in all locations and artificial inoculations at Wädenswil and Conthey Detached leaf assay and 9 point scale 3–4 times/season Fruit assessed using 4 point scale	**Fiesta × Discovery map:** **Leaf scab:** LG 6, 7, 10, 11, 12, 17: LOD 2.3–13.2; 4.0–23% **Fruit scab:** LG 15, 17: LOD 2.8–4.9; 7.0–9.0%	Liebhard et al. 2003c[c]
Discovery	TN10-8	149	149	Glasshouse tested Scored: Infection on 6 point scale Sporulation on 8 point scale	**Discovery:** LG 2, 5, 12, 13, 15, 17 **TN10-8:** LG 1, 2 **Discovery × TN10-8 map:** 3 major QTL for partial resistance to most isolates: LG 1, 2, 17: LOD 3.16–26.59; 5.1–51.1% QTL for single isolate resistance LG 5: LOD 5.4–12.57; 12.5–20.8%	Calenge et al. 2004[c]

[a] Number of individuals used in QTL detection, either in phenotyping population (P/T) or in map construction and genetic marker analysis (G/T)

[b] Linkage groups containing QTL are listed with the prefix LG. Where possible, the LOD score of the associated QTL and the percentage of phenotypic variance (PVE) it explains are included, with the LOD score first. Where several linkage groups are included, the range of associated LOD score and PVE have been included.

[c] Linkage maps have been aligned with the consensus map (Liebhard et al. 2003b), and standard nomenclature is used.

accounted for 4% of the phenotypic variability was located on LG 12 in a position comparable to *Vg* (Van de Weg unpublished data).

Calenge et al. (2004) used a panel of eight monoconidial isolates to inoculate replicated progeny from a Discovery × TN10-8 cross, resulting in the identification of numerous QTLs across seven linkage groups (with MapQTL), depending upon the isolate used (Table 10). Combining QTLs with overlapping confidence intervals and close likelihood peaks revealed three major QTLs on LG 1, LG 2 and LG 17. The region identified on LG 1 corresponds to the region around *Vf* that Durel et al. (2003) identified as contributing between 16.0% and 17.8% of phenotypic variation, and the QTL identified on LG 17 (Calenge et al. 2004) is also in agreement with a QTL mapped in both Fiesta and Discovery that explained 23% of the observed phenotypic variability. Calenge et al. (2004) also detected additional QTLs on LG 5, 13 and 15 to only one or two isolates and a QTL on LG 2 that appeared to control more broad-spectrum resistance to apple scab. This QTL spans a region around the major scab resistances *Vbj*, *Vh2* and *Vh8* (Calenge et al. 2004; Durel et al. 2004). The identification of isolate-specific QTL indicates that some partial resistance QTL could be involved in a pathogen-mediated recognition response, similar to major genes.

These reports of detecting QTLs contributing to disease resistance highlight the importance of phenotyping segregating populations over several years and in different environments. Different infection pressures in different years, especially years with low disease incidence, can lead to high within-genotype variability (Liebhard et al. 2003c). Location-specific pathogen populations are another possible cause of increased variability, expressed as genotype-location interaction. This occurs particularly with pathogens such as apple scab, where the first wave of infection in a season is often due to ascospores originating from crosses between fungal strains from the previous growing season and where it is likely that particularly effective ascospore-derived progeny can then multiply asexually as the season progresses. An example of this is the QTL detected by Liebhard et al. (2003c) on LG 10 for leaf scab, as this QTL was only associated with data gathered at the Wädenswill location, over three years. Stankiewicz-Kosyl et al. (2005) identified five QTLs for powdery mildew resistance that were only associated with resistance for a single year. A QTL that is detected in a single year is dependent upon the allelic difference at a particular locus, the interaction of the QTL with environmental factors and/or the alteration of the expression of the QTL over time with plant development (Stankiewicz-Kosyl et al. 2005). Assessment of QTL detection over several years is one way of independently verifying the presence of QTL in the same genetic background, which can in turn minimise additional sources of variation.

1.4.2.3 QTL for Resistance to Fire Blight

Although accessions of apple displaying resistance to fire blight have been identified, the genetic control of this resistance is not well understood, and is thought to have a quantitative, polygenic aspect (Lespinasse and Aldwinckle 2000). Using two populations derived from crosses between Prima × Fiesta and Fiesta × Discovery respectively, Calenge et al. (2005b) described the first comprehensive identification of QTL controlling fire blight resistance in apple (Table 11). Several QTL were detected in both progenies, with one QTL on LG 7, derived from the common parent Fiesta explaining 34.3–46.6% of the resistance. The identification of this major QTLs in both populations demonstrated its robustness in two different genetic backgrounds. Four minor QTL were also identified on LG 3 (Prima and Fiesta), LG 12 (Discovery) and LG 13 (Discovery), each explaining 4.4–7.9% of the variation. Using a different strain of fire blight, and a different Fiesta × Discovery progeny, Khan et al. (2006) confirmed a QTL for resistance on LG 7 of Fiesta and demonstrated the stability of this QTL. The minor QTL identified by Calenge et al. (2005b) were not detected in this study.

In addition, Calenge et al. (2005b) utilized a number of microsatellite markers in common between apple and pear to compare the location of the QTLs they detected in apple with QTLs for resistance to fire blight that had been previously mapped in pear (Dondini et al. 2004). In two cases, the microsatellite markers detecting loci close to identified fire blight resistance QTL in apple, also identified loci in pear that mapped close to fire blight resistance QTLs. Further investigation of these potentially homologous fire blight resistance QTLs could aid the identification of potential new resistance QTLs candidates in both crops, and enhance our understanding of the synteny between pear and apple. Calenge et al. (2005b) also compared inter-loci interactions for all possible two-way combinations of markers to identify potential epistatic QTLs. The recurrent involvement of certain genomic

Table 11. Summary of QTLs for resistance to fire blight

Parents		No. indv.[a]		Method of phenotypic assessment	Number of QTL detected	Reference
Female	Male	P/T	G/T		Linkage group (LG or G), LOD score and percentage of variance explained by QTL[b]	
Prima	Fiesta	144	144	Artificial inoculation in glasshouse with strain CFBP 1430 Assessed 7 and 14 dpi using a multipoint scale	**Fiesta × Prima:** **Fiesta map:** LG 7: 7 dpi, LOD 18.43; 43.2% 14 dpi, LOD 19.14; 46.6% **Prima map:** LG 3: 14 dpi, LOD 4.09; 7.5%	Calenge et al. 2005[c]
Fiesta	Discovery	188	188	–	**Fiesta × Discovery:** **Fiesta map:** LG 3: 7 dpi, LOD 3.57; 4.4% LG 7: 7 dpi, LOD 26.82; 42.6% 14 dpi, LOD 13.39, 34.3% **Discovery map:** LG 12: 7 dpi, LOD 3.53; 5.4% LG 13: 17 dpi, LOD 4.87; 7.9%	
Fiesta	Discovery	86	251	Artificial inoculation in glasshouse with strain Ea610 Assessed 6,13,20 and 27 dpi using a multipoint scale	**Fiesta × Discovery:** **Fiesta map:** LG 7; 13, 20, 27 dpi, LOD 7.5–8.1; 37.5–38.6%	Khan et al. 2006[c]
Idared	*M.* x *robusta*	150	150	Artificial inoculation	***M.* x *robusta* map:** LG 5	Peil et al. 2006[c]

[a] Number of individuals used in QTLs detection, either in phenotyping population (P/T) or in map construction and genetic marker analysis (G/T)
[b] Linkage groups containing QTLs are listed with the prefix LG or G. Where possible, the LOD score of the associated QTL and the percentage of phenotypic variance (PVE) it explains are included in parentheses, with the LOD score first. Where several linkage groups are included, the range of associated LOD score and PVE have been included.
[c] Linkage maps have been aligned with the consensus map (Liebhard et al. 2003b), and standard nomenclature is used.

regions indicated putative epistatic QTLs that need to be confirmed in more detailed future analyses utilizing larger progeny sets.

A recent report by Peil et al. (2006) has identified a QTL controlling fireblight resistance on LG 5 of *M.* x *robusta*.

1.4.3 Mapping QTLs for Tree Growth and Development

Many morphological and developmental traits in apple are perceived to be under complex genetic control. When Lawson et al. (1995) compared marker data of Rome Beauty × White Angel progeny with phenotypic data, single loci controlling branching type, reproductive budbreak and root suckering were identified using the Microsoft Excel macro, QUIKMAP (designed by N. F. Weeden and J. Barnard) and the program LINKAGE-1 (Suiter et al. 1983). However, of these three traits only root sucker formation appeared to be under simple Mendelian genetic control (See Sect. 1.3.2.5.2). Branching type and reproductive budbreak gave a range of phenotypes for which the genetic basis was not immediately apparent (Table 12). Using only the phenotypic extremes of these traits, initial genetic analysis enabled loci contributing major portion of the variation to be identified, but it became obvious that other genetic loci were probably involved in the expression of branching and budbreak (Lawson et al. 1995). However, the ability to detect other genes in this study would have been limited, because, although data was available for over 400 markers (isozymes, RAPDs and RFLPs), segregation data was only available from 56 individuals, and thus only major gene effects could have been examined in this population. Limited population size in QTL detection exercises may lead to an underestimation of QTL number, overestimation of QTL effect, and a failure to accurately quantify QTL interactions. Vales et al. (2004) explored the effect of population size in the estimation of barley stripe rust QTLs and showed that as population size increased, so did the number of QTLs detected and that the overestimation of the percentage of variance explained by the QTLs was reduced.

Further work by Conner et al. (1998) used a population derived from a cross between the columnar mutant Wijcik McIntosh and accession (NY 75441-58) to position additional QTLs influencing tree growth and development. Maps were constructed for each parent, consisting of approximately 180 RAPD and isozyme loci. These maps were aligned using markers heterozygous in both parents. The positions of these putative QTLs were established by a range of statistical analyses of marker and phenotype data using MINITAB. One to eight QTLs were identified as involved in the control of height increment, internode number and length, base diameter, branch number and leaf break (Table 12). Most of the regions identified were associated with a specific trait for one year and many of the traits assessed were related to each other, and when mapped appeared to be clustered on linkage groups. The largest cluster was identified on LG 10, close to the position of the *Co* gene (Conner et al. 1997). Conner et al. (1998) suggested that other large clusters of marker trait associations could be the result of single loci with pleiotropic effects. Previously, Lawson et al. (1995) also hypothesised that vegetative budbreak, which correlated with the segregation of the terminal bearing characteristic could be the result of the pleiotropic effect of this gene.

Using the extensive Fiesta × Discovery linkage map, consisting of 804 genetic markers (a significant proportion of these microsatellites) and covering all 17 apple chromosomes, Liebhard et al. (2003a) undertook a more comprehensive analysis of several quantitative physiological traits in apple (Table 12). Both single parent linkage maps and the integrated map were used in MapQTL analysis to identify the contributor of the effective allele, map position and effect. For some traits, data was collected from own rooted seedling populations as well as grafted individuals. Three QTLs were detected for seedling stem diameter and two QTLs for seedling leaf size, but these could not be detected using the grafted plants, where independent QTLs were identified. Liebhard et al. (2003a) also positioned six QTLs for tree height increment. As found by others (Lawson et al. 1995; Conner et al. 1998), there were associations among some of these different growth and development traits. Of the six regions (Liebhard et al. 2003a) identified for height increment, four coincided with QTLs for stem diameter, indicating that these traits are related or clustered in some way. Conner et al. (1998) also reported a correlation between height increment and 'base diameter increment' identifying single markers on two linkage groups associated with this trait. Investigations into blooming traits identified five QTLs associated with blooming characters, located on five different linkage groups (Liebhard et al. 2003a). Similarly one of these QTLs for number of flower bunches was located very

Table 12. Summary of QTLs for tree growth and development

Parents Female	Male	No. indv.[a] P/T	G/T	Method of phenotypic assessment	Number of QTL detected Linkage group (LG or G), LOD score and percentage of variance explained by QTL[b]	Reference
Rome Beauty (RB)	White Angel (WA)	82	56	Branching habit: 3 point scale and with and without spurs Vegetative budbreak: 5 point scale, over 3 years Reproductive budbreak, used phenological categories of Chapman and Catlin (1976), over 2 years Bloom time: 2 dates used	**White Angel:** **Terminal bearing (*Tb*):** LG 6, possible QTL or masked by spurring **Bloom time:** LG 1	Lawson et al. 1995[c]
Wijcik McIntosh	NY75441-58	172	172	Tree vigour assessed by tree height and base diam (3 years plus again in year 9) Branch no. scored after 3rd year of growth Leaf break scored: 5 times at weekly intervals, 6 point scale Columnar form: visual assessment	**Wijcik McIntosh × NY75441-58 map:** **Height increment:** LG 6, 7, 9, 10, 11, 12, 21 (3.9–7.9%) **Internode length:** LG 5, 6, 9, 10 (4.6–23.1%) **Internode no:** LG 1, 5, 7, 10, 12, 21 (4.3–16.8%) **Base diameter increment:** LG 2, 7, 10, 14, 16, 21 (4.0–8.5%) **Base diameter:** LG 7, 9 (5.5–7.5%) **Branch number:** LG 7, 10 (7.1–24.3%) **Leaf break:** LG 3, 6, 7, 9, 11, 12, 15 (3.9–7.3%)	Conner et al. 1998[c]

[a] Number of individuals used in QTL detection, either in phenotyping population (P/T) or in map construction and genetic marker analysis (G/T)

[b] Linkage groups containing QTL are listed with the prefix LG. Where possible, the LOD score of the associated QTL and the percentage of phenotypic variance (PVE) it explains are included in parentheses, with the LOD score first; except for the Wijcik McIntosh × NY75441-58 map where only PVE are listed. Where several linkage groups are included, the range of associated LOD score and PVE have been included.

[c] Linkage maps not aligned with adopted consensus map (Liebhard et al. 2003b), thus standard linkage group nomenclature not used.

Table 12. (continued)

Parents Female	Male	No. indv.[a] P/T	G/T	Method of phenotypic assessment	Number of QTL detected Linkage group (LG or G), LOD score and percentage of variance explained by QTL[b]	Reference
Fiesta	Discovery	251	251	Leaf area, stem diameter measured on seedlings at one site over 2 years. Stem diam, height increment, blooming, no. measured over next 3 years in all locations	**Fiesta × Discovery map:** **Seedling:** **Stem diam:** LG 2, 15, 17 (LOD 3.1-4.8; 6.0–10.0%) **Leaf size:** LG 9, 17 (LOD 3.0–4.2; 6.0–8.0%) **Tree:** **Height increment:** LG 3, 5, 8, 11, 13, 17 (LOD 2.4-6.2; 5.0–11.0%) **Stem diam:** LG 1, 2, 3, 8, 11, 13, 14, 15, 17 (LOD 1.7-6.5; 4.0–13%) **Blooming time:** LG 7, 10, 17 (LOD 2.5-3.6; 5–13%) **No. bunches:** LG 8, 15 (LOD 3.6-5.1; 7.0–10.0%) **Juvenile phase length:** LG 3, 15 (LOD 3.2-4.0; 6.0–8.0%) **Fruit harv. date:** LG 3 (LOD 4.7; 16.0%)	Liebhard et al. 2003a[d]

[d] Linkage maps have been aligned with the consensus map (Liebhard et al. 2003b), and standard nomenclature is used.

close to another QTL for juvenile phase length on LG 15, but on the alternate linkage group of the Fiesta parent.

QTLs or major genes have been identified for a variety of growth and development characteristics in apple with a view to using marker-assisted selection to develop new varieties with shorter juvenile phase, later blooming to avoid frost injury, and desired branching patterns. Controlling the growth of the grafted apple scion, through the use of a dwarfing rootstock, such as Malling 9 (M.9) permits a higher planting density, which in turn results in an increased yield per hectare. However, the precise physiological or genetic mechanism by which a rootstock induces dwarfing is not well understood. In a similar approach to that of Lawson et al. (1995), Rusholme et al. (2004) used bulked segregant analysis (BSA) of the phenotypic extremes of a segregating population to identify genetic markers flanking a single gene, *Dw-1*, that contributed to the dwarfing effect of M.9. *Dw-1* has been mapped to LG 5 with microsatellite markers (Celton et al. 2006).This single major effect gene did not explain all of the variation observed in the segregating population, indicating that additional genes could be involved in the control of dwarfing. However, this initial genetic analysis was based on individuals that had been assigned to one of four simplified phenotypic classes. QTL analysis with more detailed phenotyping on a larger population is required to determine how many loci are involved in addition to the one identified. Such a whole genome-based approach, in addition to identifying QTL involved in dwarfing, will also enable additional QTL involved in the control of flowering to be identified, and hence the postulated relationship between the dwarfing ability of rootstocks and grafted scion precocity to be determined.

When identifying genetic markers for traits controlled by major, simply inherited genes, it is possible to use straightforward, accelerated approaches that are designed to target specific regions of the genome, such as BSA (Michelmore et al. 1991) or candidate gene screening (Gardiner et al. 2003) as well as more detailed genetic analysis of the whole genome. In addition to identifying markers for major genes, BSA has also been used to identify QTL for increased yield in soybean (Yuan et al. 2002) and drought tolerance in maize (Quarrie et al. 1999) through the analysis of recombinant inbred lines. In more genetically diverse species, such as apple, it is possible that significantly more individuals would be required in each bulked DNA sample, to ensure that each allele is represented in the bulks at the same frequency as in the population, as several marker alleles are likely to be present (Quarrie et al. 1999). Although BSA has been employed as a cost-effective approach to identify genetic markers for a major locus contributing to dwarfing of apple scions by the rootstock M.9, genetic mapping of QTL using whole population analysis is a more precise method, likely to identify additional smaller QTL that also impact on phenotype, enabling full characterization and understanding of the dwarfing trait.

1.4.4 Mapping QTLs for Fruit Quality

In addition to positioning QTLs for a range of growth characteristics, Liebhard et al. (2003a) also assessed a range of traits associated with fruit development and quality. The development of genetic markers linked to key physiological traits in apple would significantly accelerate and improve the efficiency of new cultivar development in apple. Such rapid and non-destructive marker-based assessment of young seedlings for fruit characteristics would greatly reduce the number of generations required for cultivar development, an invaluable benefit in a crop with such a long generation time. Liebhard et al. (2003a) identified a QTL on LG 3 of the variety Discovery that explained 16% of the variability associated with fruit 'harvest date' and eight QTLs controlling 'fruit weight' (Table 13). It could be predicted that some blooming traits and fruit traits would exhibit co-segregation. Indeed one of the three QTLs identified for the 'number of fruit' coincided with a QTLs for 'number of flower bunches', yet poor correlation was detected (Liebhard et al. 2003a) between the phenotypes of these traits, which was attributed to other potential QTL affecting the same traits, or changes in tree behavior with time. It is recognised that as the tree enters different phases of development and growth, the expression of certain traits can change, making accurate phenotypic assessments difficult. Continued assessment of such populations is essential to establish true phenotypes and subsequently identify the genetic loci involved accurately.

Fruit texture is also a complex character and is of key importance in the development of new apple varieties that comply with consumer preference. Several quite different aspects of fruit composition can be assessed to determine fruit texture, such as fruit

Table 13. Summary of QTL for fruit quality

Parents Female	Male	No. indv.[a] P/T	G/T	Method of phenotypic assessment	Number of QTL detected Linkage group (LG or G), LOD score and percentage of variance explained by QTL[b]	Reference
Fiesta	Discovery	251	251	No. fruit, fruit weight, flesh firmness (penetrometer), sugar content, acidity measured over next 3 years in all locations	**Fiesta × Discovery map:** **Tree:** **No. fruit:** LG 5, 15, 16 (LOD 3.2–4.5; 8.0–10.0%) **Fruit weight:** LG 1, 3, 6, 8, 10, 12, 15, 16 (LOD 2.5–17.0; 7.0–31.0%) **Flesh firmness:** LG 6, 11, 12, 14 (LOD 3.6–12.3; 6.0–27.0%) **Sugar content:** LG 3, 6, 8, 9, 14 (LOD 3.1–5.1; 3.6–12%) **Fruit acidity:** LG 8, 16 (LOD 4.7–6.2; 42.0–46.0%) (*Ma* locus on LG 16)	Liebhard et al. 2003a[c]
Prima	Fiesta	152	152	Fruit firmness (2 penetrometer readings) Stiffness by acoustic resonance Sensory descriptors (hardness, crispness, granularity, spongy texture, slow breakdown, juiciness, overall liking) scored on scale of 0–100:	**Prima × Fiesta map:** **Fruit firmness:** LG 1, 8, 10 (LOD 4.7–7.4; 16.0–22.0%) **Resonant freq:** LG 10 (LOD 4.6; 21.0%) **Hardness:** LG 10 **Crispness:** LG 1, 5, 10, 12, 13, 16 (LG 16, LOD 6.0; 24%) **Juiciness:** LG 1, 12, 16 (LG 16, LOD 14.8; 46%) **Granularity:** LG 2 (LOD 5.1; 24%)	King et al. 2000[c]

[a] Number of individuals used in QTL detection, either in phenotyping population (P/T) or in map construction and genetic marker analysis (G/T)

[b] Linkage groups containing QTL are listed with the prefix LG. Where possible, the LOD score of the associated QTL and the percentage of phenotypic variance (PVE) it explains are included in parentheses, with the LOD score first. Where several linkage groups are included, the range of associated LOD score and PVE have been included.

[c] Linkage maps have been aligned with the consensus map (Liebhard et al. 2003b), and standard nomenclature is used.

Table 13. (continued)

Parents		No. indv.[a]		Method of phenotypic assessment	Number of QTL detected	Reference
Female	Male	P/T	G/T		Linkage group (LG or G), LOD score and percentage of variance explained by QTL[b]	
					Slow breakdown: LG 1 **Sponginess:** LG 1, 5, 6, 16 (LG 16, LOD 7.7; 30.0%) **Overall liking:** LG 12, 16 (LG 16, LOD 11.3; 38.0%)	
Prima	Fiesta	130	152	6 fruit per tree sampled, texture: wedge fracture test, compression test, cells in fruit cortex analysed	**Prima × Fiesta map:** **Compression:** LG 1, 6, 8, 12, 15 (LOD 4.09–8.62; 16.0–27%) **Wedge measures:** LG 1, 7, 15, 16 (LOD 4.51–9.83; 15.0–32.0%) **Specific gravity:** LG 6 (LOD 7.99; 28.0%), 16 (LOD 4.54; 15.0%) **Fruit weight:** LG 4 (LOD 4.53; 25.0%) **Stress at first failure (compression):** LG 13 (LOD 3.51) **Work of fracture (wedge fracture):** LG 7 (LOD 4.51) **Circularity of cells:** LG 3 (LOD 3.3)	King et al. 2001[c]

firmness, crispness and juiciness. These characteristics are in turn determined by several variables such as cell size or cell wall strength. It is therefore particularly complicated to accurately identify QTLs controlling fruit texture, making fruit texture a challenging candidate for marker-assisted breeding.

King et al. (2000, 2001) carried out a detailed genetic dissection of fruit textural attributes using a population derived from the Prima × Fiesta cross that had been used earlier for the construction of the first reference genetic map (Maliepaard et al. 1998) (See Sect. 1.2.2). Fruit was assessed using a range of mechanical measurements and sensory parameters. Using MapQTL, QTLs accounting for differing degrees of variation for firmness, stiffness and a number of sensory attributes were identified on seven linkage groups (Table 13). This research also provided insight into the relationship between some of the mechanical measurements and sensory perceptions (King et al. 2000). Further work (King et al. 2001) extended the range of mechanical measurements to include compression and wedge fracture tests. The wedge fracture tests identified significant QTLs on LG 16 and LG 1. The QTL on LG 16 was located in the same region as QTL identified for certain sensory textural attributes, such as crispness and juiciness. Linkage group 16 has also been shown to contain the *Ma* acidity locus (Maliepaard et al. 1998). King et al. (2001) determined that the apparent association of the *Ma* gene with regions contributing to sensory assessments was unlikely to be the result of 'perceptual interactions' with the *Ma* locus. Four QTLs controlling fruit firmness, measured by penetrometer, were also identified by Liebhard et al. (2003a) in the Fiesta × Discovery reference population. The results of this study can be compared with the previous studies (King et al. 2000, 2001), as both genetic maps were constructed in part using codominant markers and have the Fiesta parent in common. Liebhard et al. (2003a) and King et al. (2000, 2001) identified three QTLs for fruit firmness (using penetrometer readings) on the common linkage groups LG 3, LG 12 and LG 16. However King et al. (2000) also identified additional QTLs for fruit firmness, (measured by penetrometer) across four more linkage groups. Investigations with the related Fiesta × Discovery population did not detect this range of QTLs, indicating that expression of Fiesta alleles contributing to fruit firmness could be different in different genetic backgrounds, or that certain alleles may only be expressed in certain environments (Liebhard et al. 2003a).

1.4.5 Conclusions

There are many favorable complex traits that would be desirable to select for in the development of new apple varieties. The studies detailed in this Section, where many QTLs have been identified for disease resistance, tree architecture and fruit quality traits, are the initial steps that will lead to the unravelling of such complex traits, and the development of molecular markers linked to major QTLs that can be deployed in the marker-assisted selection of parents and progeny in apple breeding programs. These broad-ranging studies emphasise that to develop robust genetic markers that will be useful to apple breeders, it is essential that QTL are accurately positioned on the apple genome. There are many factors that can influence and further enhance reliable QTL identification, such as the use of dense genetic maps constructed with codominant markers and robust, objective phenotyping methods. Estimates of variance for site-to-site and occasion-to-occasion variance can be accurately determined by the inter- and intra-site replication of only a relatively small proportion of the population (Lynn 1998), and maximising the number of recombinant individuals in a population can also enable greater genetic resolution. If marker-assisted selection is to be successfully applied to traits controlled by QTLs, it is important to remember that QTL analysis of a population can detect only differences between the inherited parental alleles (Liebhard et al. 2003a). Therefore, to fully estimate the phenotypic effect of identified QTL alleles, they should also be compared with the equivalent and ineffective allele at the same locus (Liebhard et al. 2003a). Once QTL and associated markers have been identified, their conserved position in different genetic backgrounds, such as other cultivars or genetically more diverse germplasm accessions, should be established to ensure that the genetic markers developed have the widest applicability to new variety development.

The continued development of new QTL mapping models and algorithms designed to extract the maximum amount of information about QTL positions and effects will also aid in more accurate positioning of QTLs on the apple genome. Unfortunately, a précis of methods of QTLs detection is beyond the scope of this review, but of interest is a study by Maliepaard et al. (2001) that recently compared the more traditional simple interval mapping and approximate multiple QTL model mapping with Bayesian multiple QTL

analysis, in the context of experimental data derived from a large full-sib family in apple.

New statistical tools and methods are also currently being assessed in a pedigree-analysis based approach to QTLs mapping in apple (Van de Weg et al. 2004). Pedigree-analysis is an ideal approach to identify QTL in data gathered from more complex and diverse populations, such as those derived from multiple founders or collected from ongoing breeding programs. This method of QTL identification, originally used for detecting QTLs in human populations, has several advantages for plant geneticists, namely: detailed data on QTL variation within relevant breeding populations will be generated, since multiple alleles will be present; the context of identified QTL alleles can be examined; and the cost efficiency of the QTL mapping exercise will be improved as existing selection experiments can be utilised (Bink et al. 2002).

1.5 Marker-Assisted Breeding

The advantages and limitations of conventional versus molecular breeding have recently been discussed in general terms in a review article by Oraguzie et al. (2004). A major advantage of the use of markers is that they increase the breeding efficiency by enabling early selection for adult traits; simultaneous selection for multiple traits, including resistance gene pyramids; and selection for traits that are expensive to phenotype. Strategies can be developed for the efficient marker-assisted introgression of a range of traits into one cultivar (Servin et al. 2004). In this chapter, we will discuss marker-assisted breeding (MAB) using examples of its application, and the potential of whole-genome selection as part of a fast-breeding strategy combined with the reduction of the juvenile phase.

1.5.1 Germplasm Screening

Genetic markers linked to a specific gene are an efficient modern alternative for the screening of germplasm for the distribution of that gene, compared with the traditional cumbersome allelism tests (MacHardy 1996). For example, evaluation of selected germplasm with the OPB12SCAR marker for *Vm* showed that it was present in two other species only, apart from *M. micromalus* and *M.* x *atrosanguinea* which are the two primary allelic sources for this gene (Dayton and Williams 1970), out of 28 species tested (Cheng et al. 1998). However, such marker data needs to be interpreted with caution, as presence of a marker does not necessarily mean that the gene is present, or *vice versa*, since even if the marker and gene are in linkage disequilibrium, rare recombination events can uncouple the association between the particular alleles of the marker and gene of interest. Furthermore, as resistance loci in plants are frequently located in clusters (Michelmore and Meyers 1998), a marker may well be expected to be linked to more than one gene. This was demonstrated with the OPL19SCAR marker, which could not distinguish the *Vh2* and *Vh8* genes for scab resistance (Bus et al. 2005a). As microsatellite markers are very polymorphic, there is more opportunity for a specific allele to be linked to a resistance gene than for SCAR markers. While there are many germplasm sources with resistances allelic to *Vf* (Williams et al. 1966; Williams and Dayton 1968; Dayton and Williams 1970; Dayton et al. 1970), only *M. micromalus*, *M. prunifolia* 19651, and M.A.16 have been suggested to carry this gene, because all three amplify the same allele for the two very closely linked CHVf-1 and CHVf-2 microsatellite markers (Vinatzer et al. 2004). In the same study, it was shown that the F_2 selections 26829-2-2 and 26830-2 are not descendants of the original cross between *M. floribunda* and Rome Beauty (Hough et al. 1953), because a CHVf-1 allele of 137 bp amplified from both accessions was not present in either of the parents of this original cross.

1.5.2 Marker-Assisted Selection

Genetic markers enable the selection of combinations of both specific genes and QTLs, which cannot be identified through phenotypic selection, as epistatic effects are usually involved. Using the example of resistance breeding, in the past such combinations would have been eroded during the breeding process and eventually led to a loss of the quantitative resistances, which in turn put the major gene resistances under pressure. This sometimes had disastrous consequences, as was shown with the 'Vertifolia effect' of *P. infestans* on potato (*Solanum tuberosum*) in spite of this cultivar carrying two major genes for late blight resistance (Vanderplank 1984). Hence, the monetary value of genetic markers to a breeding program goes

well beyond the replacement cost of traditional phenotypic selection techniques (Luby and Shaw 2001) and should include an estimation of their contribution to realizing the potential value of durable resistances in food production. The cost of marker assisted selection (MAS) can be reduced in cases where an initial phenotypic selection can be performed prior to MAS. An example can be seen in a glasshouse screen for scab resistance in a family segregating for genes conditioning distinctly different resistance reactions, e.g. Liberty (*Vf* conditioning chlorotic resistance reactions) × TSR33T239 (*Vh4* conditioning HR resistance reactions epistatic to *Vf*). Since we know now from marker analysis that one of these resistance reactions is epistatic to the other, the number of marker analyses could be halved by discarding the susceptible seedlings and the seedlings showing chlorosis (Bus et al. 2000). Further cost efficiency of MAS will be achieved through the development of (semi-) automated DNA extraction and marker analysis systems. Robotic systems and the use of marker multiplexes (Cook and Gardiner 2004; Frey et al. 2004) will reduce the costs of both labour and consumables.

The success of MAS with one marker is determined mostly by the linkage distance of the marker to the gene of interest. If one assumes a recombination rate of 5% between a marker and a gene, a rate not met in many breeding families (Bus et al. 2000), MAS would result in 14.3% of the selected seedlings not carrying the desired combination of resistances in the case of three pyramided genes, and 26.5% in the case of six genes. A recombination rate of 1% for each marker would see the levels of inaccurate selection drop to 3.0% and 5.9%, respectively. However, when using flanking markers, the distance of the markers becomes much less of an issue, as the rate of inaccurate selection declines to less than 1%, even if the recombination rate is 5% for both markers for each of the three genes, and selection is carried out for one or both markers for each gene. With recombination rates of 1%, inaccurate selection becomes negligible. One issue for some types of markers, such as SCARs, is that their transportability may be limited, hence it is prudent to check linkages in a new parent prior to developing progenies intended for MAS. Once reliable markers have been developed, a strategy can be developed to efficiently pyramid the resistance genes from a number of breeding parents (Servin et al. 2004). One can also utilise more transportable markers from previously published apple maps if the position of the gene of interest in the genome (or failing that the SCAR marker) has been confirmed. On the other hand these transportable markers (mainly microsatellites and RFLPs) are more labour intensive than SCARs.

The application of MAS has been shown to be successful in several examples involving epistatic interactions between resistance genes (Bus et al. 2000, 2002). In the case of the A163-42 × TSR34T15 family, where *Vf* (conditioning chlorotic resistance reactions) and *Vh2* (conditioning stellate necrotic resistance reactions) were combined, a number of seedlings showed an unexpected hypersensitive response. Marker analysis with the OPL19SCAR and AL07SCAR revealed that in 91% of the cases these pin-point lesions were the result of a synergistic effect between the two genes (Bus et al. 2002) (Table 14). A similar effect was shown for *Vf* with relatively more seedlings showing no or Class 2 symptoms (*sensu* Chevalier et al. 1991) when carrying the gene in homozygous state, than those carrying it in heterozygous state (Tartarini et al. 2000).

Genetic markers are also an important tool in understanding the segregation of traits involving segregation distortions. For example, the naming of the *Er-3* gene for woolly apple aphid resistance consistent with a single gene was based on the R:S = 1:1 segregation in one M.9 × Aotea family (Bus et al. 2000). When a progeny of this family was crossed with Royal Gala, only 17% percent of the seedlings were resistant, which suggested that the single gene model was not correct. However, MAS with the OPO05SCAR developed for *Er-3* confirmed that there had been a segregation distortion (Table 15) as the marker was also not segregating 1:1 as expected. In contrast, the consistent discrepancies between the AT20SCAR marker segregating 1:1, while the phenotypes for the *Pl-1* powdery mildew resistance gene do not (Dunemann et al. 2004), is consistent with the two-gene hypothesis proposed for this resistance (Alston 1977).

1.5.3 Marker-Aided Introgression

The term MAS usually refers to the introgression of a gene, or a limited number of genes, e.g. in the case of pyramiding resistance genes. A step up from MAS is the use of genetic markers to select for a wide range of traits within one breeding cycle, or for "whole genome selection" (Pradhan et al. 2003), i.e. the selection of the genome resembling one of the parents. For example, the number of progeny of a backcross between a crabapple selected for a specific resistance gene and

Table 14. The segregation data for the *Vf* and *Vh2* apple scab resistance genes and AL07SCAR and OPL19SCAR markers in an A163-42 × TSR34T15 family. Adapted from Bus et al. (2002)

Marker		Phenotype[z]					Dead	Total
L19	AL07	HR	SN	3A	3B	S		
+	+	20	162	13	45	29	7	276
+	–	1	156	0	5	60	37	259
–	+	1	11	3	48	15	4	82
–	–	0	3	1	1	37	22	64
		22	332	17	99	141	70	681

[z] *HR* = hypersensitive response, *SN* = stellate necrosis, *3A* and *3B* = chlorosis with sporulation, *S* = susceptible (scale adapted from (Chevalier et al. 1991))

Table 15. The segregation data for the *Er3* woolly apple aphid resistance gene and PO05SCAR marker in a Royal Gala × S26-E290 family. Adapted from Bus et al. (2000)

O05	Woolly apple aphid phenotype						Segregation[z]		Total
Marker	0	1	2	3	4	5	R	S	
+	18	2	0	0	0	1	18	3	21
–	2	0	0	0	13	84	2	97	99
	20	2	0	0	13	85	20	100	120

[z] *R* = immune (Class 0); *S* = susceptible (Classes 1–5)

a high quality apple cultivar can not only be reduced to the resistant progeny, but to the number of seedlings carrying genomes most resembling that of the quality grandparent(s) as well as the desired resistance gene. In a crop with a long juvenile period, such as apple, considerable cost savings in the breeding program may be achieved by not having to grow seedlings until fruiting and to perform fruit evaluations on them. Obviously, the larger the number of markers used in the selection, the more effective this approach will be. However, the optimum number will be determined by balancing the extent of selection achieved with the cost of achieving it. As with MAS, the economics of this technology is determined for a large part of the market value of the character(s) of interest (Moreau et al. 2000).

At HortResearch, the whole genome selection approach is being investigated in combination with a technique of reducing the juvenile period in order to develop "fast breeding". It has been shown that the juvenile period in apple can be reduced from on average five years from seed germination, to about one to one and a half year by growing the seedlings continuously in the glasshouse (Zimmerman 1971; Aldwinckle 1975a). The aim of the HortResearch fast breeding program is to reduce the breeding cycle from cross to cross from the current six years on average, to two years. In initial studies involving growing the seedlings under optimal conditions in a phytotron, inducing flower bud formation, providing sufficient chilling, and forcing the seedlings to flower, have to date shown a success rate of only 15% within 10 months from germination (Bus et al. 2001) compared with over 68% for glasshouse grown seedlings (Aldwinckle 1975a). Further research is being carried out to increase the efficiency of this breeding strategy.

1.6 Map-Based Cloning

In the 1990s several disease resistance genes (Martin et al. 1993; Bent et al. 1994; Jones et al. 1994; Lawrence et al. 1995) and a few pest resistance genes (Milligan et al. 1998; Rossi et al. 1998) were cloned from tomato, tobacco, *Arabidopsis* and flax. Because of their dominant nature and major effect, resistance genes have become the most common plant genes to be targeted by map-based cloning techniques in recent years and

apple is no exception to this rule. In fact, all known apple map-based cloning projects embarked upon to date have been for disease or pest resistance genes. By the late 1990s, as detailed in Sect. 1.3, several partial maps had been constructed around disease resistance genes in apple with the most detailed maps being around the *Vf* locus conferring resistance to apple scab (Sect. 1.3.2.1 and Table 4). Map-based cloning projects to isolate the *Vf* gene were initiated in Europe by a Swiss/Italian consortium and later in the USA by the University of Illinois (see references below).

1.6.1 *Vf*

Considerable progress has been made in the last few years in identifying the *Vf* gene, the first apple disease resistance gene to be cloned. Apple BAC libraries have been developed from at least four different sources containing *Vf*. Vinatzer et al.(1998) developed the first BAC library of almost 37,000 clones from Florina, a cultivar containing the *Vf* locus. It has an average insert size of 120 kb and is expected to cover about five haploid genomes. Xu et al. (2001b) developed a 31,000 clone library from *Malus floribunda* clone 821, the original source of the *Vf* locus. This library has an average insert size of 125 kb and is also expected to cover about five haploid genomes. Subsequently Xu and Korban (2002a) constructed a 35,000 clone BAC library with an average insert size of 110 kb from the *Vf* containing cultivar GoldRush. This again represents approximately five haploid genome equivalents. The present authors have developed a 56,000 clone BAC library and a 168,000 clone cosmid library from an apple breeding parent containing both the *Vf* apple scab resistance locus and the powdery mildew resistance locus *Pl-2* (Rikkerink et al. unpublished). These two libraries are each expected to cover nearly seven haploid genome equivalents.

In reality, the map-based cloning projects that utilise these BAC resources have relied on a mixture of pure map-based techniques and candidate gene approaches made possible by information provided by other plant systems. Patocchi et al. (1999b) identified a 550 kb minimal BAC tiling path containing the *Vf* locus, based on detailed analysis of markers around the gene and new markers generated from the BAC clones in the region. A similar but somewhat smaller contig of 290 kb around the gene was later developed by Xu and Korban (2002b), assisted by previous saturation mapping with AFLPs (Xu et al. 2000, 2001a). Vinatzer et al. (2001) continued the work of Vinatzer et al. (1998) and Patocchi et al. (1999a), using BAC inserts to probe a large cDNA library combined with partial cDNA sequencing to identify three putative genes in the *Vf* region that showed homology to the cloned *Cladosporium fulvum* (*Cf)* resistance genes from tomato. These cDNA clones were then used to identify the portions of the BACs that needed to be sequenced to derive the sequence of the entire open reading frame corresponding to each of these cDNAs. They also derived partial sequence data from a fourth candidate. These candidates were named *HcrVf1 to HcrVf4*. Xu and Korban (2002b) used a slightly different approach, based on screening BAC subclones with labelled total cDNA. The BAC subclones containing transcribed regions were then partially sequenced and a full gene sequence was obtained by a combination of RACE (rapid amplification of complementary DNA ends) and further sequencing of clones containing resistance-like sequences. The first candidates identified were also used to develop additional PCR-based screens for further (similar) genes. This yielded an almost identical set of four candidate genes to that of Vinatzer et al. (2001) near *Vf* that they labelled *Vfa1* to *Vfa4*. Based on available sequence data *Vfa1* = *HcrVf1, Vfa2* = *HcrVf2* and *Vfa4* = *HcrVf3*. It is reasonable to assume, in the absence of full sequence data, that *Vfa3* may be the same as *HcrVf4*, although the relative location of these four genes does not quite agree between the two contigs with *Vfa4* (i.e. *HcrVf3*) being the furthest clone from *Vfa1* (i.e. *HcrVf1*) in one contig, but *HcrVf4* (i.e. presumably *Vfa3*) being the furthest in the other contig. This could also be explained if there are in fact five candidates, and *Vfa3* is not the same as *HcrVf4*. Recently Belfanti et al. (2004) expressed one of their candidate genes (*HcrVf2*) in a susceptible apple (Gala) under a 35S promoter and demonstrated that this construct confers resistance against apple scab. This result may indicate that this candidate is the *Vf* gene. However, the interpretation of this result could be complicated by the substitution of the native promoter with the 35S promoter that (presumably) drives higher expression. The same group reported recently (Silfverberg-Dilworth et al. 2005) that the resistance in these *HcrVf2* transformants could be overcome by a scab race that specifically overcomes the *Vf* resistance. This result strengthens the conclusion that *HcrVf2* is actually the *Vf* gene. A contribution to *Vf* resistance by another gene in the cluster cannot be formally ruled out without similar transformation

data for all the candidates and indeed there is now transgenic evidence to suggest that in fact two of the candidates at the *Vf* locus can each (independently) confer a degree of resistance to scab (Malnoy et al. 2006).

1.6.2 *Sd-1*

Cevik and King (2002a) developed a high-resolution map around the *Sd-1* aphid *Dysaphis devecta* resistance locus. Subsequently, Cevik and King (2002b) used the abovementioned 'Florina' library to develop a BAC contig around the *Sd-1* locus. They identified several putative NBS-LRR resistance-like gene sequences within a BAC in this contig (Genbank AM167520), suggesting the presence of a cluster of these genes. This library is however not expected to contain the gene encoding this resistance as the *Sd-1* resistance gene is derived from Cox's Orange Pippin, which does not feature in the ancestry of Florina. Cevik and King (2002b) also confirmed that Florina is susceptible to the aphid. More work using libraries from an aphid resistant host will therefore be required to identify the gene(s) responsible for conferring the resistance present at this locus.

1.7 Advanced Work

1.7.1 Tools Developed: Transformation, ESTs, Microarrays and Functional Genomics

1.7.1.1 Transformation

Agrobacterium-based apple transformation was first demonstrated by James et al. (1989) using a disarmed Ti-binary vector and has since been demonstrated in a number of laboratories around the world using several different apple cultivars (Sriskandarajah et al. 1994; Yao et al. 1995; Puite and Schaart 1998). These transformation events can be stably maintained (James et al. 1995) in the plant. Initially transformation was used to introduce various heterologous (trans)genes largely aimed at providing pathogen or pest protection, including the attacin family (Norelli et al. 1994) and T4 lysozyme (Ko et al. 2002) lytic proteins, chitinases (Bolar et al. 2000, 2001) and avidin or streptavidin (Markwick et al. 2003). More recently, it has been used to deliver endogenous genes in order to identify their function or effect on pathogen resistance. These studies have included *Vf* gene candidate *HcrVf2* (Belfanti et al. 2004) mentioned above, introducing an apple homologue of the *Arabidopsis* regulatory gene *NPR1* as well as apple proteins that are known to interact with the *E. amylovora* secreted type III effector protein DspE (Aldwinckle et al. 2003). Another strategy for functional analysis attempts to turn off the genes in order to either identify function (Dandekar et al. 2004), or modify the plant's development to create a novel phenotype or mimic a useful phenotype such as dwarfing (Bulley et al. 2005).

Methods for gene knock-down using RNA interference technology (Wesley et al. 2001) have also begun to be applied in apple (Gilissen et al. 2005). There is significant scope to extend this list to further genes, such as other disease resistance genes that are likely to be identified in the near future, as well as genes that may play a role in the various defense pathways that can now be identified in apple EST databases (see below). For example, we have also started investigating the function of members of several key protein families identified in the EST sequencing effort (Crowhurst et al. 2005, Newcomb et al. 2006).

Introducing genes by transformation is not only a useful analytical tool, but also is a way to circumvent the difficulty of introgressing useful single genes into new varieties by pseudo-backcrossing. This effectively becomes equivalent to the true backcrossing-based introgression that can be performed in selfing species to recreate existing varieties with new characteristics. The introgression of useful simply inherited characters such as disease resistance from wild germplasm into commercial varieties is a relatively standard breeding strategy for autogamous crops. In such crops, a variety that is very similar to the original but containing the introgressed character can be recreated by repeated backcrossing. Introgression is not as simple in non-autogamous crops like apple. The re-creation of a variety with a single introgressed gene (or more correctly a small region in linkage disequilibrium with this gene) in non-autogamous crops such as apple by traditional backcrossing is rendered impractical by the low success rate with traditional backcrossing, because the resultant apple progeny are either unviable or much less vigorous. The combination of various gene cloning/identification methods and gene transformation now make this possible in apple. Precautions are required, because of the possi-

bility of somaclonal variation in plant tissue culture (Courtial et al. 2001), but the essential character of the existing variety can be maintained and the new variety could be marketed essentially as a new sport of the variety, with an added advantage (e.g. a scab resistant Gala). In the case of apple, characterization of transformants has only been carried out in containment. It would be interesting to see whether most transformed apples perform true to type when they are grown under field conditions for extensive periods of time.

1.7.1.2 EST and Candidate Gene Sequencing

In the last five years a considerable effort has gone into developing more advanced genomics resources for apple, in addition to the large insert library resources discussed in Sect. 1.6. An extensive public domain EST resource now exists for apple. This is largely derived from cDNA based single pass sequencing carried out by HortResearch (Newcomb et al. 2006) and more recently, from a program initiated by a US consortium (Korban et al. 2005). Over 250,000 apple ESTs are in the public domain at the time of writing and these probably represent a substantial proportion of the expressed genes in apple, since they form well over 30,000 non-redundant clusters of sequence. A bioinformatic analysis of the combined sequencing efforts is still needed to get a more accurate measure of the number of non-redundant sequences that these ESTs represent. Since they are largely based on single pass sequencing from one end, it is likely a number will end up falling into the same non-redundant contig once complete cDNA sequence becomes available. There has also been considerable progress in identifying candidate genes of particular classes by PCR based approaches. We have recently used this approach to sequence parts of over 350 candidate disease resistance genes (Rikkerink et al. submitted manuscript) and build on the smaller datasets of these genes already available from apple (Lee et al. 2003, Baldi et al. 2004).

1.7.1.3 Microarrays

Microarrays are now becoming an important tool in the global characterization of gene expression in plants (e.g., Liu 2005). They consist of high density arrays on glass slides using either PCR-amplified cDNAs, or long oligonucleotides complementary to the transcribed part of genes. The expression of a large number of genes can be simultaneously assayed by hybridizing these slides with labeled RNA prepared from plants subjected to different treatments or from different tissue types, and looking for hybridization patterns that suggest a significant change in expression. HortResearch has also gone on to develop a 5,000 oligonucleotide pilot and a 16,000 oligonucleotide microarray from their EST data (Crowhurst et al. 2005). These arrays are based on oligonucleotides of approximately 50 bases in length with a Tm near 74 °C and have started yielding information on RNA expression profiles of the corresponding ESTs (Janssen and Schaffer, personal communication)

1.7.1.4 Functional Genomics

Some of the other tools required for any comprehensive functional analysis have also been developed in apple. These include RNA interference, expression in apple cell lines, the use of model plant species such as *Arabidopsis thaliana* and micro-organisms such as *E. coli* or yeasts to express apple genes in order to develop assays for their biochemical function. Expression of candidate genes in *Arabidopsis thaliana* has helped narrow down candidates for SNP marker development and subsequent genetic mapping.

1.7.2 Third-Generation Maps: Physical

Currently the only physical maps of apple that exist are around specific resistance genes that have been targets for map-based cloning (see Sect. 1.6). Other novel technologies such as radiation hybrid mapping have not been developed or applied to accelerate map development. Given that the apple genome is modest in size and that the price of whole genome sequencing efforts are decreasing, it is likely that genome sequencing (see below) will overtake such strategies and make them more or less obsolete.

1.8 Future Scope of Work

1.8.1 Association Mapping and Other Ways to Link Genotype to Phenotype

A major goal of research in the future will consist of developing faster and better methods to link genotype information to both desirable and undesirable phenotype information. Association mapping, which

utilises the phenomenon of linkage drag (disequilibrium) to identify candidate genome regions (and at its extreme, candidate genes) that show statistically significant associations between phenotypes and markers, is likely to be one of these methods. To improve the chance of identifying candidate genes for any given phenotype by whole genome scans will require methodology that can generate very dense genetic maps – since the region that stays in linkage disequilibrium (LD) with the phenotype is expected to rapidly decay because of recombination. Microarray technology in partnership with SNPs could potentially generate enough markers and the methodology to simultaneously assay a large number of SNPs in a single genotype. The recently developed EST databases can be used to identify many of these SNPs. In order to be able to effectively utilise this tool and to identify the most appropriate germplasm, we will also need to develop knowledge about the rate of LD decay in various apple populations and how uniform (or non-uniform) this decay is across the genome. Other methodologies such as targeting local lesions in genomes (TILLING) can now utilise EST sequence data and could potentially rapidly generate much greater variation in plant phenotypes than exists in the wild (Slade et al. 2005).

1.8.2 Structural Genomics and Whole Genome Sequencing

When compared with our understanding of the genome of the model plant species *Arabidopsis* and rice, it is clear that apple still lags a long way behind these plant genomes. Although a certain degree of lag with respect to these crops is inevitable, the range of resources identified above indicate that this gap could be closed significantly during the next decade. More detailed maps, or the construction of proper whole chromosome physical maps will be required before the apple community can realistically contemplate whole genome sequencing in apple. It is likely that the existing sequencing effort in the related genus *Prunus* will yield both an interesting start point for comparative analysis of these two important Rosaceae genera, and some actual leads to help construct physical maps in apple. As might be reasonably expected, there is already some indication of significant levels of synteny between apple and other Rosaceae genera (particularly with *Pyrus*, another member of the Maloideae) (see Sect. 1.2.4).

Even without a whole genome analysis, a pilot comparative study sequencing BACs around one or a few homologous loci in several members of the Rosaceae would be informative in terms of the level of microsynteny. This sequencing would probably also yield other useful information, such as the nature of transposable elements, the identity of transposon families present in several of the Rosaceae, and the gene density in these species. Information about transcribed transposons could also be deduced from the EST sequencing efforts. HortResearch has already identified ESTs in its database that appear to be interrupted by transposon-like sequences (E. Rikkerink unpublished), but a more comprehensive analysis might identify many more of the active transposons in apple. Comparative BAC sequencing might also begin to cast some additional light on the origin of the Maloideae as a subfamily within the Rosaceae (see also Sect. 1.1.1). While genome resources for apple have come a long way in the last decade, much remains to be done.

There is a commitment now within the Rosaceae research community to support the complete genome sequencing of peach as the first crop in the family. However, the world-wide economic importance of apple means that it should follow reasonably quickly as the next logical Rosaceae member to sequence in full. This would generate very significant amounts of comparative data in regions outside the immediate (transcribed) gene-space, which could play important regulatory roles. The haploid genome size of members of the Rosaceae is not unreasonably large (262–743 Mb, Table 16) when compared with the genome size of other plants like *Arabidopsis* (145 Mb), rice (420 Mb) and poplar (550 Mb). Moreover, even if little is known about the structure of the apple genome, its relatively small size compared with the complex and highly-repeated sequence-rich genomes of maize and pine, and the fact that it is probably an ancient polyploid suggests that it may have a true haploid size close to that of peach and be of relatively low complexity. The average physical/genetic ratio for apple (estimated at 0.51 Mb/cM using the most complete maps available) is lower than that observed in tomato (0.77 Mb/cM). Since positional cloning of QTL has been successfully carried out in tomato, this suggests that map-based chromosome walking is feasible in apple. Therefore, whole genome sequencing of many of the major Rosaceae genomes is well within the realms of possibility in the next decade. Indeed, the technical limitations that were encountered by pioneer whole

Table 16. Genome size and physical/genetic correlation of distance in several plant species

Species	Common name	Physical size (Mb/C)	Maximum genetic length (cM)	Ratio (Mb/cM)[1]	Status of genome sequencing
Arabidopsis thaliana	*Arabidopsis*	145	675	0.21	Completed
Populus deltoides	Poplar	550	2,300	0.24	Completed
Oryza sativa	Rice	420	1,490	0.28	Completed
Lycopersicum esculentum	Tomato	980	1,280	0.77	In progress[2]
Zea mays	Maize	2,300	1,860	1.24	In progress
Pinus pinaster	Maritime pine	25,700	1,850	13.89	Still impractical
Rubus ideaus	Raspberry	280	789	0.35	–
Prunus persica	Peach	262	712	0.37	Physical mapping in progress[3]
Fragaria spp	Strawberry	392	445	0.88	Limited BAC resources
Pyrus communis	Pear	496	949	0.52	–
Malus x *domestica*	Apple	743	1,454	0.51	BAC and EST resources[4]

[1] Average physical/genetic ratio based on most complete genetic maps
[2] Gene space sequencing
[3] Physical mapping partially completed and several thousand ESTs also exist
[4] see above for details

genome sequencing projects in the past (e.g. human and *Arabidopsis* genomes) can be easily circumvented now by the progress made in terms of sequencing methods and bioinformatics analysis, as well as the availability of large sequencing facilities. One striking example is the sequencing of the poplar genome in less than two years (Brunner et al. 2004).

The alternative (and complementary) way for accessing genomic information relies on the use of cDNA libraries instead of BAC genomic libraries. As detailed above, this approach has been extensively used in apple where over a quarter of a million of ESTs have been produced recently. However, as EST data can only partially compensate for complete genome data, whole genome sequencing is therefore still required. ESTs are proving to be a good source for the microsatellite markers and SNPs (Newcomb et al. 2006) that should help to generate more detailed maps. Even though most of the ESTs developed by HortResearch are derived from a single variety (Royal Gala) SNPs can still be identified at a reasonable frequency (presumably because of the highly heterozygous nature of apple). These more detailed maps can then act as a springboard for developing whole chromosome physical maps. Alternatively, this could be done by a random strategy relying on BAC fingerprinting to develop contigs, using new methods such as overlapping oligonucleotide (OVERGO) probes (Wesley et al. 2001) or a combination of these approaches.

If the cost of sequencing goes down significantly, then the option of assembly from deep sequencing using a whole genome shotgun (WGS) library approach, as opposed to a hierarchical shotgun sequencing approach, becomes feasible for apple. Another alternative strategy could be to concentrate initial sequencing efforts on transcribed regions of the genome. In some species such as tomato there is evidence of reasonably sharp demarcation lines between transcriptionally active and more silent portions of the genome, the euchromatic regions making up the former perhaps constituting less than one-quarter of the total genome (Van der Hoeven et al. 2002). Van der Hoeven et al. (2002) based these deductions on sequence data from a set of BACs biased by being selected because they contained transcribed genes.

There is anecdotal evidence for a higher gene density on BACs than might be expected by chance based on the number of times random BAC sequencing identifies ESTs in the HortResearch database (Rikkerink et al. unpublished). Sequence analysis of several complete BACs would indicate if concentrating on euchromatic regions is a viable strategy for sequencing the "more important" parts of the apple genome. Of course this strategy also suffers from the major drawback that it assumes the non-euchromatic regions are

less important. It is possible that this assumption is based largely on inability to make sense of the function of DNA in these regions. This is particularly pertinent to remember, now that there is increasing evidence that short transcribed RNAs in fact may sometimes contain very significant regulatory information and tie in with endogenous RNA interference-based methods of gene control. While there are many disadvantages to apple lagging behind the model crops in terms of genomics efforts, some of the advantages include learning important lessons from these model systems about paying closer attention to the less-well characterized parts of plant genomes. In these may well lie the secret to many of the interesting and useful properties of our own favorite plant system.

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2 Grape

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2.1 Introduction

The *Vitis vinifera* L. grape is one of the oldest cultivated plants, and is thought to have originated in the region between the Mediterranean basin and the Caspian Sea (Olmo 1976). Grapevines are climbing perennial plants with coiled tendrils. Under cultivation they generally require trellising to increase productivity and optimize growth and quality. They are pruned during the dormant and growing season to enable cultivation and promote fruitfulness and fruit quality. The fruit, a berry, is essentially an independent biochemical factory. It is primarily composed of water, sugars, amino acids, minerals, and micronutrients. The berry has the ability to synthesize other berry flavor and aroma components that define a particular berry or wine character. The berry is a commercial source of tartaric acid and is also rich in malic acid. Cultivation is easiest in a Mediterranean type climate with hot dry summers and cool rainy winters, however grapevines are grown throughout the world's temperate climates. *Vitis vinifera* cultivars are heterozygous and are therefore propagated clonally in order to maintain their distinctive and economically significant individual characteristics. These cultivars are typically grown on rootstocks to resist soil-borne pests and to adapt to adverse soil conditions, but there are areas of the world where they can be grown without rootstocks.

Grapes are grown in more than 80 countries of the world with a total of 7,572,237 hectares devoted primarily to wine grapes, but also including table and raisin grapes. The countries with the greatest acreage are Spain, France, Italy, Turkey, China and the United States of America (FAOSTAT data 2005). Wine production adds at least $2 for each $1 of farm gate value. The leading countries for production of table grapes consumed as fresh fruit are China, Turkey, Italy, Chili, the USA, RSA, Spain and Greece (www.fas.usda.gov/psd/complete_tables/HTP-table6-104.htm). The leading countries in the production of raisins, largely sun dried fruit of seedless cultivars, are the USA, Turkey, Greece and Australia.

2.1.1 Origin and Early History of Domestication

A single Eurasian grape species (*V. vinifera*) is the source of the estimated 10,000 cultivars that produce 99% of the world's wine and table grapes today. This species has tremendous genetic diversity and an extremely wide range of variants have been selected over the millennia. Grape cultivation is a very ancient art. Legend and tradition favor ancient Armenia as the home of the first grape (Olmo 1976). Figure 1 indicates the principle areas of the Old World where viniculture began. Levadoux (1956) summarized the distribution of wild and domesticated varieties of *V. vinifera* as follows:

- *Vitis vinifera* was in existence during the final stages of the tertiary period as evidenced by the fossils in many locations of Western Europe and the Mediterranean basin.
- During the Pleistocene period fossil evidence suggests that *V. vinifera* survived in the forests circling the Mediterranean and south shores of the Caspian Sea.
- In the Neolithic period, *V. vinifera* occupied the same distribution, as at present, however, primitive polymorphism and dioecious nature remained intact because of heterozygosity.

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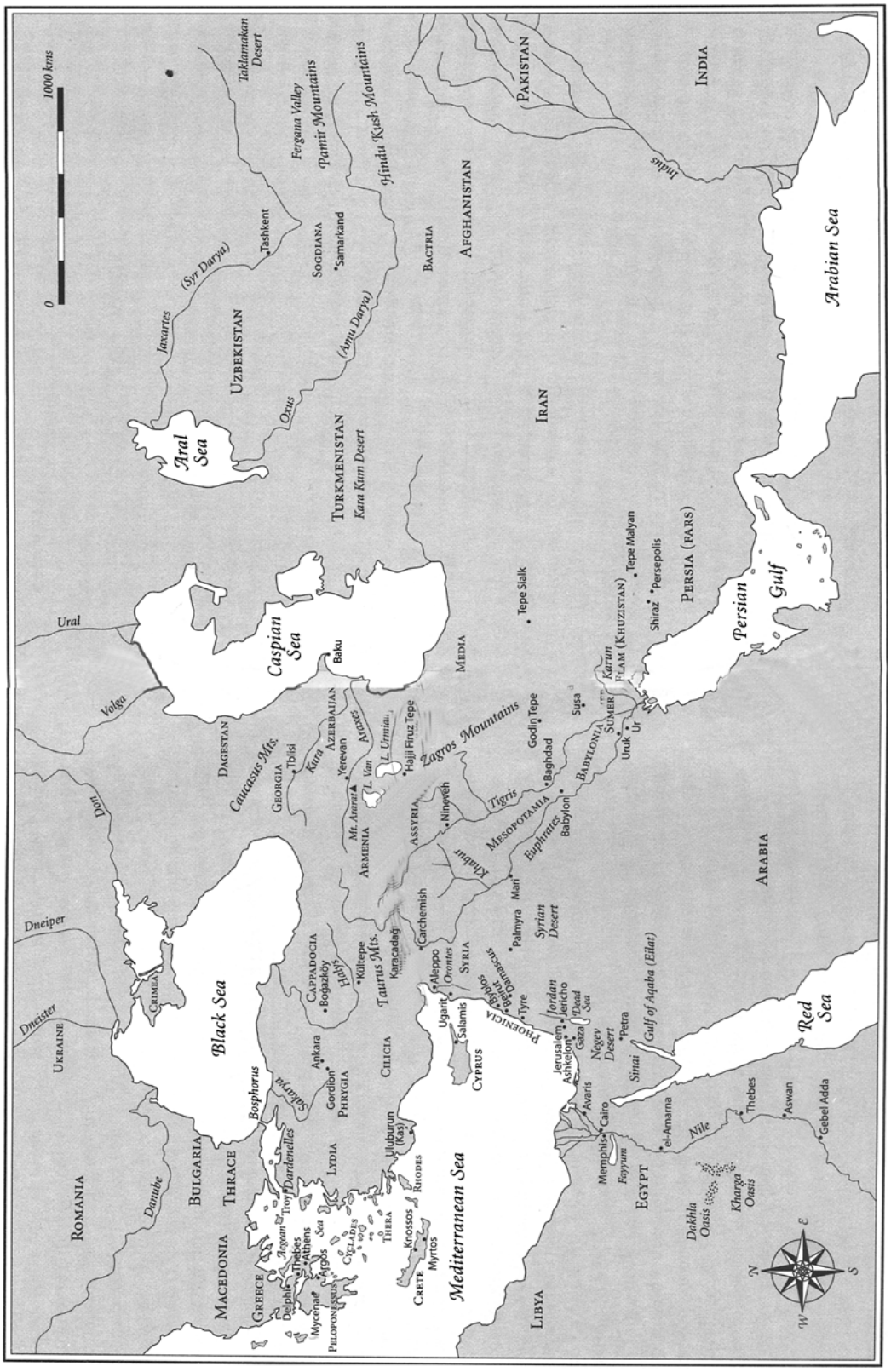


Fig. 1. The principal areas of the Old World where viniculture began

- The domestication of *V. vinifera* began ca. 8000–6000 BC in Transcaucasia.
- Toward the end of fifth millennium BC, grape culture began to spread around the Mediterranean.

Although there is no written record describing the process, there has been sufficient archaeological evidence uncovered to demonstrate the transformation from wild to domesticated forms (Olmo 1995). Domestication likely started when nomads marked forest trees that supported particularly fruitful vines. Grapevines grow most successfully in areas where water is readily available. As sedentary agriculture developed and forests were cleared, fruit trees and vines were kept in areas where water was available and plants were protected from the reach of grazing animals by building mud walls around what became vineyards and orchards. Neolithic communities of the ancient Near East and Egypt were permanent, year-round settlements made possible by domesticated plants and animals. Given a more secure food supply and a stable base of operations than nomadic groups possessed, a Neolithic culture and "cuisine" emerged. Using a variety of food processing techniques – fermentation, soaking, heating, spicing – Neolithic peoples are credited with first producing bread, beer, and an array of meat and grain entrées we continue to enjoy today. A major advance in the development of winemaking was the creation of pottery vessels about 6000 BC that allowed the production and storage of wine. Confirmation of the evolution of winemaking comes from yellowish residue inside a wine storage jar excavated by Mary M. Voigt at the site of Hajji Firuz Tepe in the northern Zagros Mountains of Iran (McGovern 2003, see Plate 1 and 2). The jar, with a volume of about 10 liters was found together with five similar jars embedded in the earthen floor along one wall of a "kitchen" of a Neolithic mud brick building, and was dated to ca. 5400–5000 BC (McGovern 2003, see Map 2). Infrared, liquid chromatographic, and wet chemical analyses were conducted and detected the presence of calcium tartrate in the jars. Grapes are the only fruit in which tartaric acid occurs in large amounts.

Archeological evidence indicates that organized cultivation of wine grapes was underway in the near east as early as fourth millennium BC and in Greece during the first millennium BC (Helbaek 1959). The westward movement of viticulture fanned out from Asia Minor and Greece, following the Phoenician sea routes. Religion was strongly associated with viticulture and winemaking. The Egyptians ascribed wine to the god Osiris, the Greeks had Dionysus, the Romans Bacchus, and the Babylonians the goddess Siduri (McGovern 2003). Wine was associated with the Christian faith as a necessary ingredient in the consecration of the Mass during the Roman period. With the decline of the Roman Empire, Europe plunged into the Dark Ages, wine's influence waned, and vineyards became relegated to monasteries and churchyard plots. Wine's influence was revived in 800 AD, and vineyards were planted along the major river valleys of the Danube, Rhône, Rhine, Tiber and Douro. Records document vineyards along the Moselle valley in Germany by 55 AD. In the fifteenth century viticulture became established in Madeira and the Canary Islands. Later it spread to South Africa, Australia and South America. The *V. vinifera* grape was introduced to the new World by Portuguese and Spanish explorers and settlers in the 1500s. The first recorded introduction of grape into the USA was in 1621 (Olmo 1976). Grapes were moved from Mexico into California in the mid 1700s, and expanded rapidly during the 1850s.

2.1.2 Genetic Diversity

The botanical family Vitaceae is made up of 15 genera (http://www.ars-grin.gov/) and about 1,000 species. Only the genus *Vitis* contains species with edible fruit. There are about 60 *Vitis* species in the world, with the greatest concentration in Asia and North America. The number of *Vitis* species is in taxonomic dispute due to the interfertility of all the species, their sympatric nature and the resulting high degree of hybridity. Some authors separate the species *V. rotundifolia* and its related subspecies and species (*V. rotundifolia* var. *munsoniana* and *V. popenoei*) into a separate genus *Muscadinia* (Small 1913). *Muscadinia* species have 40 somatic chromosomes and are restricted to the southeastern USA and northeastern Mexico (Winkler et al. 1974; Einset and Pratt 1975).

Domestication of *V. rotundifolia*, the muscadine grape, pre-dates the arrival of Europeans in the USA in the 1600s. The rest of the *Vitis* species contain 38 very small somatic chromosomes that form 19 bivalents at meiosis and fertile hybrids with the muscadine species are rare and do not occur naturally (Jelenkovic and Olmo 1969). Estimates of the number of *Vitis* species varies widely depending upon taxonomic opinion. De Lattin (1939) grouped the species

into nine sections, and included 18 North American species. Bailey (1934) included 28 American species and his grouping and designation differed from that of De Lattin. Galet (1956, Vol 1) states that about 20 species of *Vitis* can be found in America but later reports that there are 28 (Galet 1988). A literature search covering published reports from 1753 to 1940 revealed 155 species names for American grapes, adding to the confusion (Rogers and Rogers 1978). This confusion is largely based on disagreements as to what constitutes good species, extreme variants and hybrid forms (Levadoux et al. 1962; Barrett et al. 1969; Comeaux et al. 1987). Additional summaries of the family Vitaceae are found elsewhere (Galet 1988; Alleweldt et al. 1990; Mullins et al. 1992 Chap. 2). The United States Department of Agriculture Germplasm Resources Information Network (GRIN) (http://www.ars-grin.gov/) is an accepted listing of crop plant germplasm. This list describes the 15 genera and 43 species, 5 natural hybrids and 15 varieties of species in *Vitis*. Molecular techniques are being applied to taxonomic relationships within Vitaceae (Rossetto et al. 2001), but more work is needed. The North American species, including *V. aestivalis, V. cinerea var. helleri, V. labrusca, V. riparia* and *V. rupestris*, have been extensively used to produce rootstocks and fruiting cultivars with fungal resistance. Among the Asian species, only *V. amurensis* has been domesticated and used for fresh fruit, juice, wine and jelly production (Huang 1980). *Vitis vinifera* is the most successfully used grape species with thousands of wine, table and raisin grape cultivars grown throughout the world's temperate zones (Alleweldt et al. 1990).

2.1.3 Advanced Breeding Objectives

The common objectives of most breeding programs are to produce locally adapted, high yielding and quality cultivars adapted to environmental and pest stresses. In practice these objectives are complex given the different characteristics needed for table, raisin and wine grape production. In addition, other desirable qualities are considered when breeding rootstocks.

Grapes are generally grown in the Northern hemisphere between 20 and 51°N latitude. The most northern extent of *V. vinifera* cultivation is in Germany's Rhine Valley and British Columbia, Canada. The southern ranges extend into India, but most cultivation occurs between 20 and 40°S latitude. The major limiting factors to *V. vinifera* cultivation are the length of the growing season and water availability, which must allow both fruit and cane maturation, and winter cold. Tropical viticulture is practiced with both *V. vinifera* cultivars, and with hybrids based on American grape species, in areas where dormancy can be enforced by a dry season, by withholding water or by planting at high elevation. Grapes are grown beyond the winter cold limits of *V. vinifera*. These cultivars are hybrids based on northern species particularly *V. riparia*, *V. amurensis* and *V. labrusca.*

Grape is subject to an array of diseases caused by bacteria, fungi, mycoplasmas, nematodes and viruses (Pearson and Goheen 1988). The most damaging grape pests are indigenous to North America and, because *V. vinifera* cultivars have no or little inherent resistance, they created havoc when introduced into Europe during the nineteenth century. Insects and nematodes can also act as vectors for diseases such as Pierce's disease, flavescence dorée and fanleaf degeneration, and cause serious vine decline or death as in the case of grape phylloxera and root borer. The most common fungal disease in the world's grape growing regions is powdery mildew, caused by *Uncinula necator*. This fungus was unintentionally introduced to Europe from North America before the 1850s (Reisch and Pratt 1996). About 20 years later downy mildew caused by another fungus, *Plasmospora viticola*, became a serious problem. Soon after, black rot (*Guignardia bidwellii*) appeared in European vineyards. These diseases were all introduced from North America. A serious disease of warmer climate is anthracnose, *Elsinoë ampelina*, perhaps the first North American grape disease to be imported to Europe. Sources of resistance to these diseases are found in many North American grape species. Barrett (1955) reported that resistance to black rot is quantitatively controlled. A few forms of resistance to this disease have been identified. Some genotypes of *V. rupestris* and *V. cinerea* transmit high levels of resistance, however, there is great variability among different clones suggesting quantitative inheritance is likely, as reported by Barrett earlier (McGrew 1976).

Several plant parasitic nematodes attack grape roots, and many commonly used commercial rootstocks are susceptible (Raski et al. 1965). The root-knot nematode (*Meloidogyne* spp.) and dagger nematode (*Xiphinema index*) cause serious damage to grape roots and reduce vigor and productivity of the

plant. *Xiphinema index* also acts as a vector for grape fanleaf virus, and this virus/nematode complex cause one of the most severe grape viral diseases – fanleaf degeneration. This disease interferes with normal fertilization of the flowers, disrupting berry set and resulting in severe yield losses. Nematode problems become more severe with time as growers replant grapes on vineyard sites without regard to fallow or crop rotation, or plant vineyards on agricultural soils with high nematode populations. Resistance to nematodes is found in a number of North American grape species particularly *V. arizonica, V. candicans* and its hybrids, *V. cinerea* and *Muscadinia rotundifolia*. Table 1 summarizes known sources of resistances to different pests and diseases in grapes.

2.1.4 Classical Breeding Efforts: Obstacles and Achievements

French scientists, nurserymen and viticulturists first initiated well-documented grape breeding, when phylloxera and fungal diseases created havoc in European grape growing regions. Table 1 presents the main genetic resources used by European and North American breeders to incorporate disease, pest and abiotic stress resistance into *V. vinifera* cultivars. Some of these hybrids, Hybrid Direct Producers or French Hybrids, are still used to combat fungal diseases and cold winter weather, however they are generally considered to have inferior fruit quality compared to *V. vinifera* cultivars. Breeding of these interspecific hybrids ceased in Europe after the creation and utilization of phylloxera resistant rootstocks took hold. Progress on limiting the expression of undesirable flavor compounds was limited because most of them are inherited as complex polygenic traits (Alleweldt and Possingham 1988). However, new *V. vinifera* cultivars continue to be developed. The most successful of these are seedless table grapes, while wine grapes have been less successful since their wide utilization is greatly limited by the demands of winemakers and marketers to have traditional varieties with well-documented quality and historical acceptance. New *V. vinifera* varieties continue to be released in a number of countries including Argentina, Australia, France, Germany, Hungary, South Africa, USA and Chile (Antcliff 1978).

There are several main constraints to grapevine improvement. Grape is a relatively long-lived perennial and requires time and space for adequate evaluation. It can also be slow to come into bloom resulting in a relatively long generation time. In the case of wine grapes vinification and wine evaluation must be carried out which further complicates and delays selection. Most wine grape cultivars are extremely heterozygous and old varieties carry deleterious alleles that exhibit pronounced inbreeding depression after selfing or sibling mating, although inbreeding affects can vary among cultivars (Winkler et al. 1974). The grape breeding efficiency depends on the screening methods used for fruit quality, yield, disease resistance, winter hardiness and tolerance to other abiotic stresses. Field and laboratory procedures are often performed in order to select for horticultural traits prior to determining enological potential. Wine grape evaluation is again more complex because single seedling vines produce very small amounts of fruit, adding to the difficulty of judging wine making potential. Finally, little is known about the inheritance of wine quality components, which are likely to be quantitatively inherited and under environmental influence.

Improvement of crops through breeding is greatly facilitated by genetic knowledge of traits under selection. Such genetic information can be used to calculate heritability estimates, which help breeders to select parents for controlled crosses. Heritability estimates could be derived from parameters of covariance among relatives. One method of covariance estimation is through factorial sib analysis, a mating system that is less biased by environmental covariances than other methods (Fehr 1991; Falconer and Mackay 1996). The design II mating system consists of a series of male parents each mated to a series of female parents. To make the calculations simple, selected females are not mated to each other, selected males are not mated to each other, and there are no reciprocal or selfing crosses. Such factorial designs are particularly well-suited to a dioecious species such as the wild species and rootstocks of grape. This design has been used with wild grape species to study the inheritance of Pierce's disease resistance (Krivanek et al. 2005) and has been used with grape rootstocks to study root-knot nematode resistance (Cousins and Walker 2002).

Table 1. Native american species as sources of resistance or tolerance to diseases and biotic stress

Stress factor	Causal agent	Sources of resistance or tolerance	References
Fungal Diseases			
Anthracnose	*Elsinoe ampelina* [de Bary] shear	*V. simpsoni* Mun.	Mortensen (1981)
		V. smalliana Bailey	Olmo (1986b)
		V. shuttleworthii House.	
		V. labrusca L.	
		V. rotundifolia Michx	
		V. munsoniana Simp ex Mun	
Botrytis bunch rot	*Botrytis cinerea* Pers.	*V. vinifera* L.	Alleweldt et al. (1990)
		V. riparia Michx	
		V. rupestris Scheele	
Black rot	*Guignardia bidwellii* [Ellis]Viala & Ravaz	*V. riparia* Michx	Alleweldt et al. (1990)
		V. rupestris Scheele	
		V. candicans Engelm	Jabco et al. (1985)
		V. rotundifolia Michx	McGrew (1976)
		V. cinerea Engelm	
Downy mildew	*Plasmopara viticola* Berl. and Toni	*V. riparia* Michx	Alleweldt et al. (1990)
		V. rupestris Scheele	Eibach et al. (1989)
		V. lincecumii Buckl.	He and Wang (1986)
		V. labrusca L.	
		V. amurennsis Rupr.	
		V. rotundifolia Michx	
		V. yenshanesis	
		V. aestivalis	
		V. cinerea Engelm	
		V. berlandieri	
Powdery mildew	*Oidium*, *Uncinula necator* (schw.) Burr.	*V. aestivalis* Michx	Alleweldt et al. (1990)
		V. cinerea Engelm	Pearson and Goheen (1988)
		V. riparia Michx	
		V. berlandieri	
		V. rotundifolia Michx	
		V. labrusca L.	
Rust	*Physopella ampelopsidis*	*V. shuttleworthii* House.	Fennell (1948)
		V. simpsoni Mun.	
		V. rotundifolia Michx	
Bacterial Diseases			
Crown gall	*Agrobacterium tumefaciens*	*V. amurennsis* Rupr.	Alleweldt et al. (1990)
		V. labrusca L.	Pearson and Goheen (1988)
Pierce's disease	*Xylella fastidiosa* Wells et al.	*V. rotundifolia* Michx	Mortensen et al. (1977)
		V. candicans Engelm	Olmo (1986b)
		V. champinii Pl	Stover (1960)
		V. vulpina L.	
		V. shuttleworthii House.	
		V. simpsoni Mun.	
		V. smalliana Bailey	
		V. arizonica	
Flavescence doree	Mycoplasma like organism suspected	*V. labrusca* L.	
		V. rupestris Scheele	Pearson and Goheen (1988)

Table 1. (continued)

Stress factor	Causal agent	Sources of resistance or tolerance	References
Viral diseases			
grapevine fan leaf virus		*V. arizonica*	
		V. rotundifolia Michx	Walker et al. (1985)
		V. vinifera L.	Walker and Meredith (1990)
		V. rufotomentosa Small	
		V. candicans Engelm	
		V. riparia Michx	
Insects			
Rootknot nematodes	*Meloidogyne* Goeldi spp	*V. champinii* Pl	Lider (1954)
		V. candicans Engelm	
		V. rotundifolia Michx	Olmo (1986b)
Dagger nematodes	*Xiphinema index*	*V. rufotomentosa* Small	Alleweldt et al. (1990)
		V. arizonica	
		V. rotundifolia Michx	Bouquet and Danglot (1983)
		V. cinerea Engelm	Meredith et al. (1982)
Phylloxera	*Dakyulosphaira vitifolia* [Fitch]	*V. riparia* Michx	Alleweldt et al. (1990)
		V. rupestris Scheele	Olmo (1986a)
		V. berlandieri	
		V. rotundifolia Michx	
		V. cinerea Engelm	
		V. champinii Pl	

2.1.5 New Genetic Tools for Grape Improvement

2.1.5.1 In Vitro Culture

Tissue culture has greatly increased our knowledge of plant biology from the cellular (metabolism, differentiation) to the plant level (organogenesis, host-parasite relationships). Successful tissue culture also led to unconventional methods for genetic improvement. Since early 1960s, grapevine has been the subject of research aimed at defining the best procedures for micropropagation.

In vitro culture starts with the excision of a small piece of contaminant-free plant tissue followed by its establishment in sterile culture. The choice of plant material and preparation of sterile explants are critical, since the tissue must be able to survive the initial culture and produce expected or experimental responses. Environmental conditions and the physiological state of the mother plant also need to be considered. Once the plant material is cleaned with surface disinfectants [common surface disinfectants and procedures are reviewed by Street (1977) and Hu and Wang (1983)], the tissue is placed in an appropriate culture media. The major functions of culture media are (i) to supply the basal nutrients for continued growth of the isolated explants and its subsequent propagules; and (ii) to manipulate growth and development through the balance of growth regulators. In vitro development is commonly controlled by the kind of growth regulator, its concentration and combination with other growth regulators, and the sequence in which growth regulators are supplied. Auxins and cytokinins are most typically used, but gibberellins and abscisic acid have also been used in specific situations.

Techniques of in vitro culture are commonly classified as standard techniques using pre-existing meristems, and those requiring neoformation of buds or meristem like structure. The standard method uses explants bearing intact apical or axillary buds cultured on a growth regulator-free media containing sucrose, macro and micronutrients with vitamins, and solidified with a gelling agent. Depending on the genotype and environmental conditions, an ax-

illary bud gives rise to a single rooted plant. Subculturing of these plants can generate yearly multiplication rates of 10^4 to 10^6. Such techniques are widely used because of their operational feasibility and ease of plantlet transfer to greenhouse conditions. In addition the culture of small meristems can often give rise to virus-free plantlets and thus these methods are specifically used for virus elimination programs.

Neo-formation techniques require the stimulation of axillary bud proliferation through the use of cytokinins, plant growth regulators with the ability to overcome the apical dominance of axillary buds. Cytokinins in the culture medium induce intense shoot proliferation by the enhanced release of axillary buds. Axillary bud proliferation is currently considered one of the most convenient and reliable regeneration techniques for shoot multiplication in many plants, herbaceous and woody crop species, and grapevine (Hu and Wang 1983). Yearly production rates can theoretically reach 10^8 buds per initial explant. Many research groups have adapted and improved these techniques with a wide range of *Vitis* genotypes (Table 2).

Progress in cell, tissue and organ culture of grapevine led to the development of other technologies with great potential for grape improvement (Mullins et al. 1992; Torregrosa and Bouquet 1993). Major advances in genetic engineering of grapevine have been made through the coupling of recombinant DNA technologies with regeneration from plant tissue cultures. A brief overview of uses of in vitro culture in grapevine is present below.

Generation of Virus-Free Grapevines Virus and virus-like entities greatly hinder grape cultivation by reducing vine vigor and yields, delaying and arresting berry ripening, changing must composition and aromatic profiles, and affecting graft compatibility (Walter and Martelli 1996). Many viruses affect grape including fanleaf (GFLV), leafroll (GLRaV), fleck (GFkV), stem pitting (RSPaV), stem grooving (GVA-closely associated) and corky bark (GVB-closely associated) and are considered to be of major importance to growers, nurseries and winemakers. In vitro meristem, shoot apex cultures, and one node explant culture were developed to eliminate viruses from grapevines (Barlass et al. 1982; Hatzinikolakis and Roubelakis-Angelakis 1993; Staudt and Kassemeyer 1994). In recent years, micrografting of scion graft meristems on hypocotyls of germinating embryos resulted in the advantage of simultaneous virus indexing (Tanne et al. 1993, 1996). Somatic embryogenesis became a useful tool to eliminate harmful viruses after methods were developed to establish long-term regeneration of somatic embryos in different grape genotypes (Torregrosa 1995). When combined with heat therapy, somatic embryogenesis successfully eliminated viruses from vascular and non-vascular tissues (Goussard and Wiid 1992). Researchers in South Africa have used somatic embryogenesis to establish *V. vinifera* cultivars since 1990. It was judged to be more effective and less expensive than conventional techniques at virus elimination and has not resulted in somaclonal variation or virus contamination, as judged by ISEM and ELISA (Goussard and Wiid 1995).

Establishment of Germplasm Repositories Grape germplasm is currently maintained in field collections where two or more plants of each genotype (species, hybrid, variety and clone) are cultivated. Management of germplasm in the filed is expensive and subject to environmental hazards and funding shortages. There are three basic types of in vitro storage modes for conservation: (i) standard micropropagation, (ii) in vitro culture combined with reduced growth rate, and (iii) suspension of growth (Withers 1992). Because of the cost and risk of genotype instability, the first method is unsuitable for long-term conservation of grapevine. Reducing the growth rate of in vitro cultures increases the time between subcultures, reducing upkeep costs and risk of subculture mistakes. Galzy et al. (1990) reported that grapevine plantlets could adapt to a number of different culture conditions. When culture conditions encourage growth, plant behavior depends on a number of variables such as nutrients, carbohydrate source and concentration, and light, but dry matter remains stable. Conversely, when growth is restricted by lowering temperature, dry matter content increases significantly in response to stress. To compensate for this effect, Galzy et al. (1990) suggested reducing the carbohydrate content of the medium. The nutrient content of media has a strong impact on growth (Torregrosa 1994), and restricting nutrients, especially nitrogen and potassium, can alter plantlet growth (Moriguchi and Yamaki 1989).

Grapevine cryopreservation studies have been conducted on latent buds taken from in situ canes. Ezawa et al. (1989) obtained high survival rate with *V. labrusca (V. X. labruscana*), and low to no success with several *Vitis* species and *V. vinifera* cv Riesling,

Table 2. Axillary bud proliferation studies in grapevine

Species	Studied factor	Reference
V. vinifera	Culture vessel size	Monette (1983)
Vitis hybrids	Vitamins, amino acids, BAP/Kin/Picloram	Chee and Pool (1985)
V. labrusca	Adenine/MS strength	Reisch (1986)
Vitis hybrids	Light spectrum, Mn and KI	Chee (1986)
Vitis hybrids	Salt formulation	Chee and Pool (1987)
V. rotundifolia	BAP/IBA	Lee and Wetztein (1990)
V. vinifera	TDZ	Gribaudo and Fronda (1991)
V. rotundifolia	BAP/TDZ/Kin/NAA/ explant length	Gray and Benton (1991)
V. vinifera and *Vitis* hybrids	MS strength, vitamins	Zlenko et al. (1995)
Vitis × *muscadinia* hybrids	Mg, Ca, BAP, salt formulation	Torregrosa and Bouquet (1995)
Vtis hybrids, *V. vinifera*	BAP/2iP/NAA, darkness	Molina et al. (1998)

respectively (Esensee et al. 1990). Plessis (1994) described the most comprehensive work while adapting cryopreservation techniques developed for pear. In this process, axillary buds from in vitro grown plantlets, composed of the prompt (lateral) bud with several leaf primordia and a rudimentary latent bud are encapsulated in calcium alginate and soaked in a liquid medium containing 1 M sucrose to reduce the water content of beads. The coated buds are then partially dehydrated under sterile airflow and frozen through two immersion steps in liquid nitrogen. Using this process, it was found that 24% of frozen buds from *V. vinifera* cv. Chardonnay were capable of producing viable plants (Plessis 1991).

Utilization of cryopreservation techniques to conserve germplasm is an appealing alternative to field culture. However, cryopreservation of large collections of genotypes is expensive and time consuming. Moreover the possibility, even if remote, of propagating plants with genotypic alterations undetectable under in vitro conditions is problematic. The primary goal of cryopreservation is to back-up working collections for short and long terms, but they are not likely to replace field collections.

In Vitro Embryo Rescue In many table-grape growing countries, consumers favor seedless table grapes. In the USA, seedless cultivars make up more than 80% of the total table grape production, and only one seeded table grape, Redglobe, is a commercial success. Table grape breeding has been pursued intensively for more than 70 years in California, and a large number of new seedless cultivars have been released (Ledbetter and Ramming 1989). Traditional breeding methods are based on hybridization between seeded female parents and seedless male parents. The seedlessness is stenospermocarpic (where fertilization occurs, embryo is viable, but seed development aborts at various stages, leading to quantitative variation of seed trace size) with low proportion of seedless plants in the progenies. Since seedlessness is only one of a number of important traits, the selection process requires growing a large number of plants. In addition, since grape seedlings often take 3–4 years to produce fruit after planting, selection for seedless progeny is further delayed.

Through the use of in ovulo and in vitro culture techniques, it is possible to rescue viable embryos from seedless × seedless crosses and greatly increase the number of seedless progeny (Emershad and Ramming 1984, Spiegel-Roy et al. 1985; Bouquet and Davis 1989; Gray et al. 1990; Gribaudo et al. 1993; Garcia et al. 2000; Ponce et al. 2000). Fertilized ovules are extracted and placed on media with $GA_3$and IAA followed (although not in all cases) by the excision of the embryos. The success of embryo rescue depends on many factors, the most important being the variety used as the female parent, and the harvest time of the berries and ovules after pollination (Bouquet and Davis 1989; Ponce et al. 2000). Low temperatures and treatments with growth retardants have been shown to improve embryo germination (Agüero et al. 1995, 1996).

Emershad and Ramming (1994a) showed that proliferative somatic embryogenesis could be initiated from in ovulo cultured zygotic embryos of seed-

less grapes. This phenomenon was later shown to be a demonstration of direct somatic embryogenesis occurring from epidermal cells of larger embryos (Margosan et al. 1994), and was proposed as a system to facilitate gene transfer technology in seedless grapes (Emershad and Ramming 1994b). However, the seedless character cannot be controlled in the genotypes of such embryos. Higher proportions of seedless plants can be recovered through in ovulo embryo culture (Ramming et al. 1990; Spiegel-Roy et al. 1990; Bouquet and Danglot 1996). The limitation of these procedures is their labor-intensive nature, and the size of progeny populations must therefore be limited.

2.1.5.2 Genetic Engineering

Over the last 20 years, advances in plant biotechnology have produced new tools for genetically improving crops. The general aim of molecular grapevine breeding programs is to develop and apply novel gene technologies capable of introducing genes in a careful targeted manner. The transfer of a single trait into a grape variety is almost impossible by classical methods due to grape's heterozygous nature. The potential of genetic engineering would be to make directed and specific changes in existing grape cultivars, thus modifying disease or pest resistance and perhaps regulating fruit and wine quality factors. The use of genetic engineering in the wine, table and raisin grape industries has high potential because grapevines are vegetatively propagated. Thus, modifications to established cultivars by genetic transformation should, in theory, leave intact the essential characteristics that make each cultivar unique. This is especially important in the wine industry, due to the dependence of wine sales on the use of established and historic cultivars names. New cultivars resulting from classical breeding are assigned new names, which contributes to their slow acceptance in the marketplace.

Agrobacterium-mediated transformation of grape began with the use of leaf disks, petioles, and other shoot/root explants in the 1980s (reviewed by Grey and Meredith 1992; Reisch and Pratt 1996). These efforts produced transformed cells, but not transgenic plants, due to the tissue type used, the competency of the cells, and difficulties with regeneration. However, by the mid 1990s, many groups had reported development of transgenic grapes including rootstocks and scion cultivars (Table 3). These successes derived from advances in embryogenesis, regeneration, and transformation and biolistic methods. The production of transgenic vines has now become routine in both public and private laboratories (Table 3).

Many projects have focused upon pest resistance including fungal resistance in scion varieties (powdery mildew: Kikkert et al. 2000) and virus resistance in rootstocks (fanleaf degeneration: Mauro et al. 1995). Other studies have also focused on product quality: changing seeded grapes into seedless grapes (Perl et al. 2000a, b), and reducing the browning of raisins (Thomas et al. 2000). While potentially improved forms of important cultivars have been produced, years of field and product testing are still required before genetically engineered grapes will reach the marketplace. Although it may become possible to target gene incorporation and expression, at this point transformation events are independent of each other and require the same evaluation strategies, as would classically bred grapes.

Field trials in most countries require approval from the relevant authorities. In France, transgenic research is controlled by two authorities: the Commission de Génie Génétique (CGG), which oversees research in confined environments such as laboratories and glasshouses; and the Commission d'étude de la dissémination des produits issus du Génie Biomoléculaire (CGB), which is responsible for field releases. In Germany, license from the Robert Koch Institute is required for field trials and the "Gene Technology law" controls transgenic research. In Australia, the office of the Gene Technology Regulator (OGTR) established by the Federal Government oversees the deliberate release of transgenic plants for field trials. A legislative basis for the regulation of GMO's in Australia came into force following passage of the *Gene Technology Bill 2000*. In the USA, authority to regulate transgenic plant research resides within the Animal and Plant Health Inspection Service (APHIS)– Biotechnology Permits Unit of the United States Department of Agriculture (USDA). The web database of field releases in the United States (http://www.nbiap.vt.edu/cfdocs/fieldtests1.cfm) lists 33 separate release notifications and permits for grape from both private companies and universities dating from 1995. Most of these were later withdrawn. Any plant engineered for fungal, viral or herbicide resistance would also undergo a required review by the Environmental Protection Agency, which assesses the impact upon the environment.

Table 3. Summary of transgenic plants of Vitis scion and rootstocks

	Cultivar	Selectable marker	Trait of interest	Reference
Rootstocks	110R	NPTII	Coat protein (GCMV resistance)	Le Gall et al. 1994
	110R	NPTII	Coat protein (GFLV)	Krastanova et al. 1995
	41B	NPTII	Coat protein, replicase protein	Mauro et al. 1995
	SO4	NPTII	Coat protein (GFLV)	Mauro et al. 1995
	V. rupestris	NPTII	Coat protein (GFLV)	Krastanova et al. 1995
	Freedom	NPTII	GNA (homopeteran insect resistance)	Viss and Driver 1996
	MGT101-14			
	5C Teleki			
	V. rupestris, 110R	NPTII	Coat protein, antifreeze protein	Tsvetkov and Atanassov 2000
	V. rupestris	NPTII	Anti-sense movement protein	Martinelli et al. 2000
	110R	NPTII	Coat protein	Gölles et al. 2000
		NPTII	Replicase (virus resistance)	Barbier et al. 2000
		na	Eutypa toxin resistance	Legrand et al. 2000
	3309C	NPTII	Virus resistance	Krastanova et al. 2000
	V. riparia	NPTII	Virus resistance	Krastanova et al. 2000
	MGT101-14	NPTII	Virus resistance	Krastanova et al. 2000
	5C Teleki	NPTII	Virus resistance	Krastanova et al. 2000
Scion cultivars	Chardonnay	NPTII	Coat protein (GFLV)	Mauro et al. 1995
	Sultana	NPTII	Shiva-1 (disease resistance)	Scorza et al. 1996
	Superior seedless	Bar	Basta herbicide resistance	Perl et al. 1996
	Cabernet Franc	NPTII	Fe-superoxide dismutase (freeze tolerance)	Rojas et al. 1997
	Chardonnay	NPTII	Chitinase (disease resistance)	Kikkert et al. 2000
	Sultana	NPTII, HPT	Silencing of polyphenol oxidase to reduce browning	Thomas et al. 2000
	Merlot	NPTII	Chitinase (disease resistance)	Kikkert et al. 2000
	Riesling, Dornfelder	NPTII	Glucanase, chitinase (disease resistance)	Harst et al. 2000a
	Red Globe	na	Barnase gene (seedlessness)	Perl et al. 2000a
	Red Globe	NPTII, HPT	Seedlessness	Perl et al. 2000b
	Neo Muscat	NPTII	Class I chitinase (disease resistance)	Yamamoto et al. 2000

Public perception Education about the environmental and health benefits likely to derive through the use of gene technology for crop improvement appears to be the key to public acceptance of transgenic plants. The year 1999 saw increased media attention paid to consumer and environmentalist groups opposed to the use of genetic engineering for the production of food items. This opposition was particularly strong in Europe where the matter quickly became a political and economic issue. Most of ongoing field trials were discontinued or put on halt in France and Germany. However, in Australia and the USA, public opposition to field trials of transgenic grapevines has been much less vocal. Perhaps an advantage of working on transgenic grapes, at least wine grapes is that many years of field evaluation and wine quality tests are required before release. Thus, there will be more time for public education and awareness before transgenic grapes are used commercially, compared with transgenic cereals, grains and vegetables.

2.2 Genome Mapping

The genome size of *Vitis* is 475 Mbp, 96% of which is non-coding (Lodhi et al. 1995a). It is about half the size of the tomato genome (950 Mbp) and equivalent to the rice genome (450 Mbp). There is no significant variation for DNA content among cultivars of *V. labrusca, V. vinifera* and diploid *Vitis* hybrids (Lodhi et al. 1995b). Knowledge of an organism's DNA content is essential to allow correlation of genetic and physical mapping distances. In grapes, 1 cM represents on average 300 kb in physical distance.

A genetic linkage map of an organism is an abstract model of the linear arrangement of a group of genes and markers. The gene can be a traditionally defined Mendelian factor or a piece of DNA identified by a known function or by means of a biochemical assay. The marker can be a cytological marker, a protein, or a piece of DNA without known function. Because a genetic map is based on homologous recombination during meiosis, this map is also a meiotic map.

In plants, some traits are controlled by a single gene (major gene). The location of the gene controlling a trait of interest is deduced by following the inheritance of the trait relative to the inheritance of linked molecular markers. Markers that are located very close to the DNA region controlling the trait are identified by virtue of co-inheritance with the trait in the progeny of a cross between two plants differing in the trait (but not necessarily in heterozygote species). By identifying two such markers that are very close and flank the trait of interest (fine-mapping), a small DNA fragment that contains the gene can be isolated (positional or map-based cloning). Once isolated, the DNA sequence can be determined and the function and organization of the gene can be studied.

Map-based cloning has been used to isolate disease resistance genes in many crop plants, for example the gene controlling resistance to bacterial pathogen, *Pseudomonas syringae*, in tomato (Martin et al. 1993). This gene product was determined to be a protein kinase, and when this gene was transferred to susceptible plants, they became resistant. A rice gene controlling resistance to *Xanthomonas oryza* was also identified with the map-based cloning approach (Song et al. 1995). Genome maps have also been used to find genes controlling various aspects of plant composition and development that have not been previously described or isolated. For example, map-based cloning of *Arabidopsis* has been used to find a gene controlling fatty acid composition, as well as several genes controlling developmental response to ethylene and abscisic acid (Arondel et al. 1992; Chang et al. 1993; Leung et al. 1994).

In plants, many traits exhibit continuous variation resulting from the action of multiple genes that are subject to environmental modification, a quantitative trait loci (QTL). Determining the location and number of genes that condition such quantitative traits and estimating the magnitude of individual gene effects is the focus of quantitative geneticists. Before interval mapping, QTL detection could be done by variance analysis at individual markers: Lander and Botstein (1989) provided the theoretical basis for QTL analysis. The availability of detailed linkage maps composed of molecular markers and major genes for traits of interest provided the framework for manipulation of QTL. Once a large number of markers are available, segregating loci can be chosen to mark most regions of a genome. QTL mapping has been used to locate genomic regions controlling aroma in corn (Azanza et al. 1996) and clone sugar content QTLs from the wild tomato species *Lycopersicon pennellii* (Zamir et al. 2000). In the latter case, the *L. pennellii* introgression IL9-2-5 improves sugar content by 22% by increasing fructose and glucose compared to the controls. This partially dominant QTL (designated as Brix9-2-5) enhanced total soluble solids of the fruit in different years, environments and genetic backgrounds. In a similar study, another QTL fw2.2 was found to be responsible for approximately 30% of the difference in fruit size between large, domesticated tomatoes and their small-fruited wild relatives. The gene underlying this QTL was cloned and shown to be associated with altered cell division in ovaries (Frary et al. 2000). Many QTLs were detected but only few identified.

Genome maps also provide tools to plant breeders for marker-aided selection (MAS), allowing them to optimize selection for a desirable trait. If seedlings are screened for the presence of a closely linked molecular marker, there is high probability that the seedlings carrying the marker will also carry the desirable trait, allowing them to be selected at a much earlier stage than would otherwise have been possible.

2.2.1 History and Current Status of Grape Genetic Linkage Mapping

Linkage maps in most plants are obtained from segregating populations derived from crosses between pure or inbred lines. Because grapes are extremely heterozygous, their mapping populations are usually F_1 and the pseudo-testcross mapping strategy is used to construct genetic linkage maps of both parents, which can be then be integrated into a consensus map with the use of multialleleic codominant markers with alleles that segregate in both parents (Grattapaglia and Sederoff 1994). In the last decade, several groups have initiated programs to develop molecular marker linkage maps in grapes. Table 4 summarizes all published maps in grapes. Initial efforts by Weeden et al. (1988) and Mauro et al. (1992) reported linkage groups in grape using isozyme and RFLP (restriction fragment length polymorphism) markers. However, these molecular markers are limited; isozymes are restricted to genes encoding soluble proteins, and RFLP markers are mostly limited to coding regions of the genome. In 1995, Lodhi et al. reported the first detailed genetic linkage map of grape based on a seedling population from a cross of 'Cayuga White' (a complex hybrid of *V. vinifera, V. labrusca, V. rupestris* and *V. aestivalis*) and 'Aurore' (a complex hybrid of *V. vinifera, V. rupestris* and *V. aestivalis*). The parental maps were based on 422 randomly amplified polymorphic DNA (RAPD) and 16 RFLP and isozyme markers. These maps were developed by using the double pseudo-testcross strategy with an average distance of 6.1 cM between markers. The 'Cayuga White' map consisted qof 20 linkage groups with 214 markers covering 1,196 cM and that of 'Aurore' map consisted of 22 linkage groups with 255 markers spanning 1,477 cM. This mapping population segregated for disease resistance and other important traits.

A second grape map utilizing interspecific hybrids was developed by Dalbò et al. (2000), using the progeny from 'Horizon' ('Seyval' × 'Schuyler') × Illinois 547-1 (*V. cinerea* B9 × *V. rupestris* B38). Parental maps were constructed with 277 RAPD, 25 microsatellite, 4 CAPS (cleaved amplified polymorphic sequences), and 12 amplified fragment length polymorphism (AFLP) markers This map also used the double pseudo-testcross strategy, and consisted of 153 markers covering 1,199 cM, with an average distance of 7.6 cM between markers on the Horizon map and 179 markers covering 1,470 cM with an average distance of 8.1 cM on the Illinois 547-1 map.

In 2002, Doligez et al. reported the first *V. vinifera*-based genetic linkage map. The map was constructed using a F_1 progeny of 139 individuals from a cross between two partially seedless genotypes [MTP2223-2 (Dattier de Beyrouth × Pirovano 75) × MTP2121-30 (Alphonse Lavallée × Sultanina)]. All the progeny were produced via embryo rescue (Bouquet and Davis 1989). This consensus map consisted of 301 markers [AFLP, simple sequence repeat (SSR), RAPD, SCAR (sequence characterized amplified region)]. This map consisted of 20 linkage groups and covered 1,002 cM. In 2003, Grando et al. reported on a map of a *Vitis* inter-specific hybrid population from 81 progeny of *V. vinifera* 'Moscato bianco' × *V. riparia* Wr63. Three types of markers were used to construct this map, AFLP, SSR and SSCP (single strand conformation polymorphism). A total of 338 markers were assembled in 20 linkage groups covering 1,639 cM for the maternal map, and 429 loci defined the 19 linkage groups of the paternal map, which covered 1,518 cM.

In 1998, the grape genetics research community formed the International Grape Genome Program (IGGP) for the purpose of cooperation and coordination in increasing knowledge of the grape genome (http://www.vitaceae.org). The cooperative effort resulted in the Vitis Microsatellite Consortium (VMC), established to generate a large number of codominant SSR markers, an effort coordinated by Agro-Gene S.A. in France. Among the goals of the IGGP is the creation of reference linkage maps to harmonize linkage groups resulting from individual mapping projects, and to serve as a resource for physical mapping. This map would also be useful for targeting genomic regions for more intensive mapping efforts, such as localizing QTLs and optimizing MAS.

The first reference map was based on only codominant SSR markers and used *V. vinifera* 'Riesling' (prime name 'Riesling weiss') × *V. vinifera* 'Cabernet Sauvignon'. Riesling is one of the world's most important white wine varieties and is especially important in cool climates, such as Germany, Canada and the northeastern United States. Cabernet Sauvignon is the world's most widely distributed red wine variety; it has also been selected by the IGGP as the target cultivar for cooperative efforts on physical mapping. This reference mapping population consisted of 153

Table 4. A list of all published maps in grapes

Population	Marker system	No. of Genotypes	Average marker distance (cM)	Reference
Cayuga White (Hybrid of *V. vinifera*, *V. labrusca*, *V. rupestris* and *V. aestivalis*) × Aurora (Hybrid of *V. vinifera*, *V. rupestris* and *V. aestivalis)*	RAPD, RFLP, Isozyme	60	6.1	Lodhi et al. 1995
Horizon ('Seyval' × 'Schuyler') × Illinois 547-1 (*V. rupestris* × *V. cinerea*)	RAPD, SSR, CAPS, AFLP	58	7.8	Dalbo et al. 2000
MTP2223-2 (Dattier de Beyrouth × Pirovano 75) × MTP2121-30 (Alphonse Lavallée × Sultanina)	AFLP, SSR, RAPD, SCAR, Isozymes	139	6.2	Doligez et al. 2002
Moscato bianco (*V. vinifera* L.) × *V. riparia* Mchx	SSR, AFLP, SSCP	81	8.1	Grando et al. 2003
Riesling × Cabernet Sauvignon	SSR, EST	153	11	Riaz et al. 2004
V. rupestris and *V.arizonica* hybrids	AFLP, SSR, RAPD, ISSR	116	10.2	Doucleff et al. 2004
Syrah × Grenache	SSR	96	6.4	Adam-Blondon et al. 2004
Regent × Lemberger	AFLP, RAPD, SSR, SCARs/CAPS	153	5.9	Fisscher et al. 2004
Riesling Self	SSR	96	6.4	Adam-Blondon et al. 2004

progeny plants. A total of 152 SSR markers and one polymorphic expressed sequence tag (EST) marker mapped to 20 linkage groups (Riaz et al. 2004). An integrated linkage analysis was performed to obtain the consensus map. The map covered 1,728 cM with an average distance of 11.0 cM between markers (Fig. 2).

As part of the IGGP an international grape genomics initiative (IGGI) was proposed to generate an international consensus genetic linkage map to integrate the codominant marker data from different mapping populations. This effort will combine information from different genetic backgrounds into one framework map for use in MAS and the physical mapping of genes. Five different populations have been chosen for this purpose. The first population (A1) of 95 full-sib progeny is the Syrah × Grenache map mentioned above (Adam-Blondon et al. 2004). The second population (A2) is the population of 114 progeny obtained by selfing Riesling as mentioned above (Adam-Blondon et al. 2004). The third population of 46 full-sib progeny (DG) is from a cross between Chardonnay and Bianca cultivars (Di Gaspero et al. 2005). The fourth population (D) consists of 139 full-sib progeny from the cross MTP2223-27 × MTP2121-30 mentioned above (Bouquet and Danglot 1996). The fifth population (R) consists of 153 full-sib progeny from the Riesling × Cabernet Sauvignon cross, mentioned above (Riaz et al 2004). The first two and the fourth population are being maintained at INRA, France, the third population is maintained at the University of Udine (Italy), and the National Clonal Germplasm Repository, Davis, USA, maintains the last population.

2.2.2 Mapping and Tagging of Major Genes

Relatively few genes have been isolated in grapes compared to the other major agronomic crop plants and model organism *Arabidopsis thaliana*. Two strategies from "phenotype to gene" and from "gene to phenotype" (reverse genetics) have been used to isolate

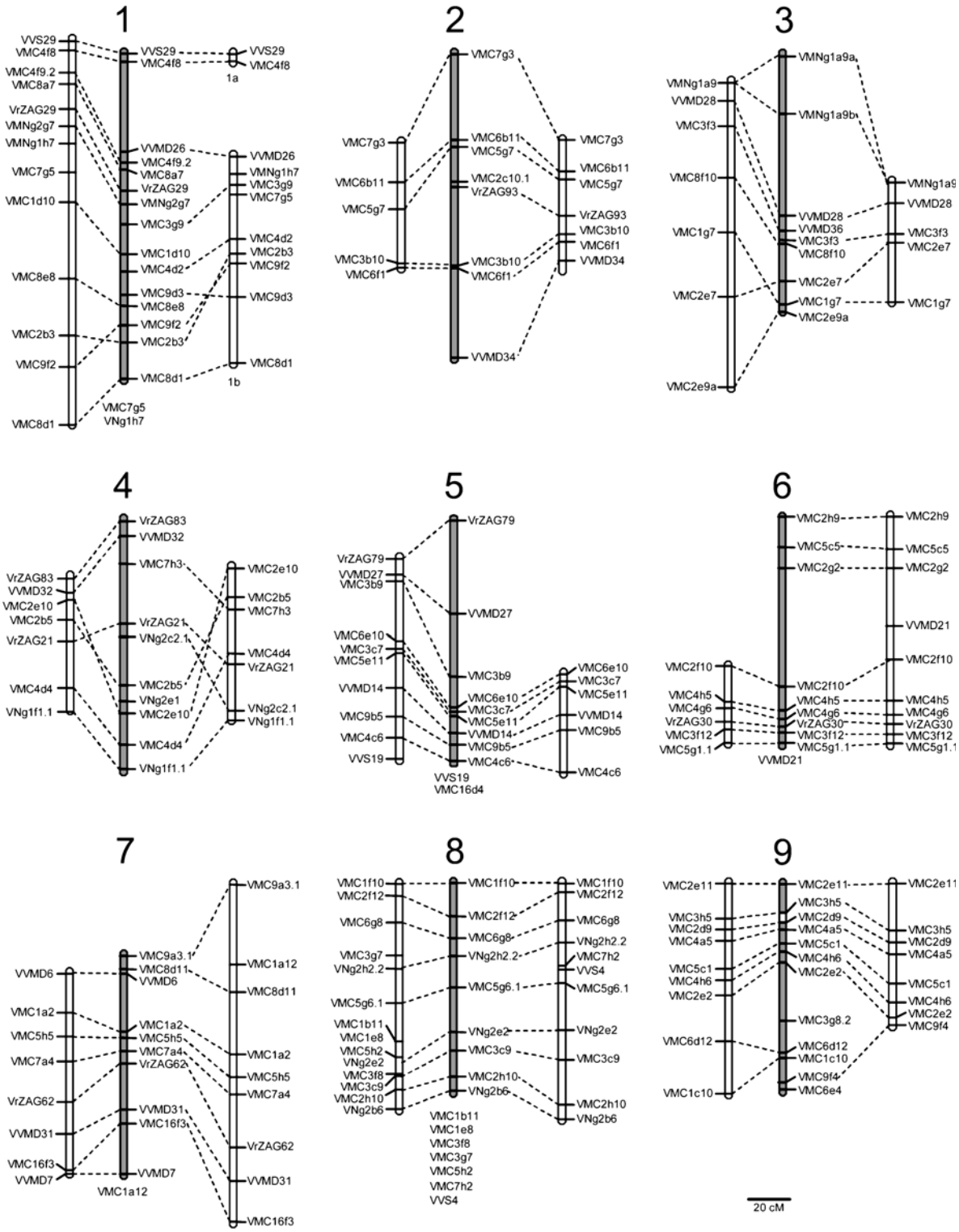


Fig. 2. Linkage map of *Vitis vinifera* 'Riesling' × 'Cabernet Sauvignon'. For each linkage group, parental maps are shown on the *left* ('Riesling') and *right* ('Cabernet Sauvignon') and consensus map is in the *center* (Riaz et al. 2004)

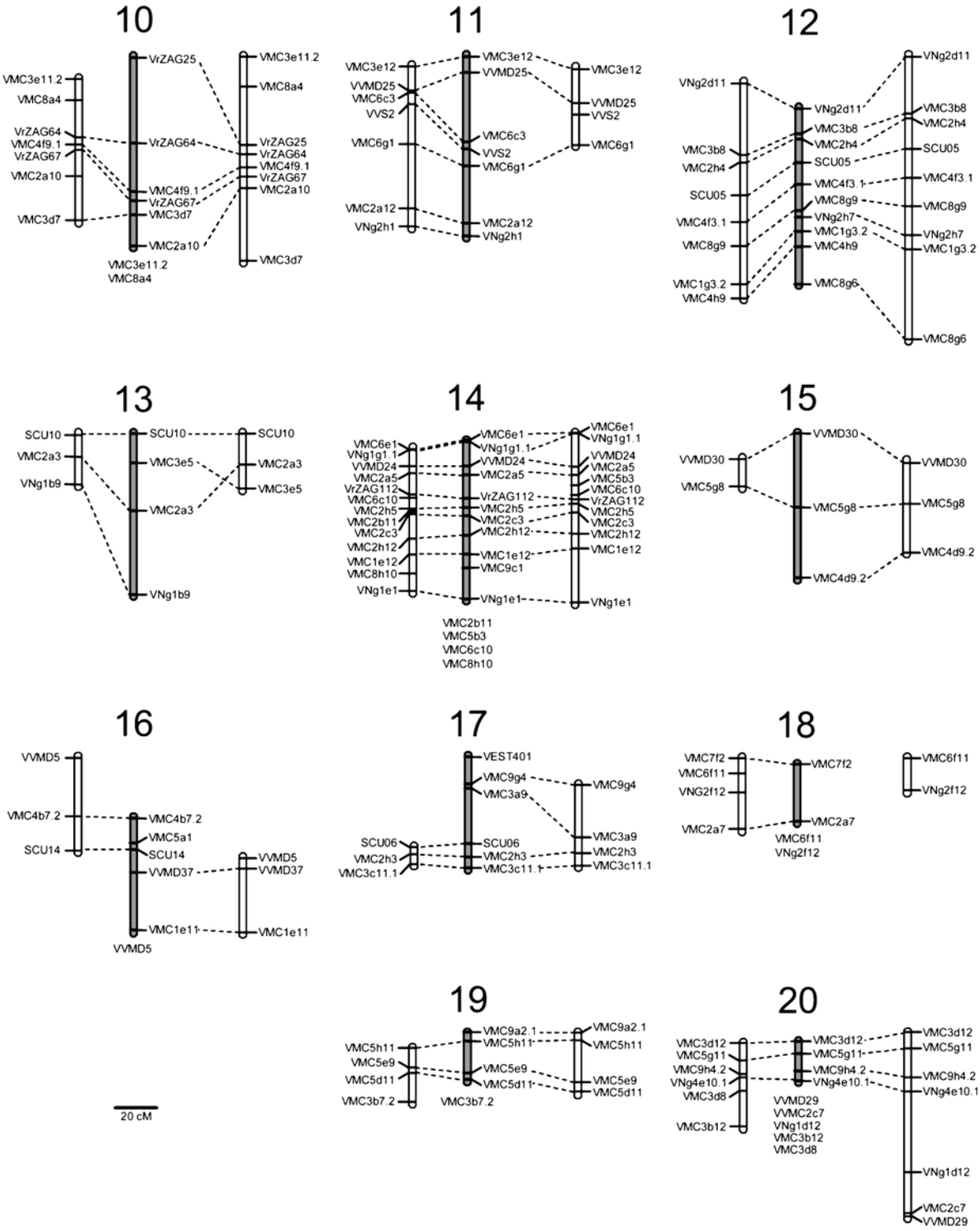


Fig. 2. (continued)

and characterize genes. In *Arabidopsis*, several combined approaches, such as positional cloning, candidate gene approach, and insertional mutagenesis with either transposons or T-DNA vectors have been used successfully to isolate genes identified by the phenotype of their mutant alleles. In grape, it is very difficult to use reverse genetic approach to tag and isolate genes. Multiple genes control most horticultural traits and no information is available about gene function and expression. With the availability of molecular markers, it became possible to map traits of interest on genetic linkage maps of segregating populations. The main focus is on disease resistance for different pests and diseases.

2.2.2.1 Fungal Diseases

Bouquet (1986) introduced a dominant resistance gene for powdery mildew from *Muscadinia rotundifolia*, *Run1*, into the *Vitis vinifera* genome over five backcross generations (Bouquet 1986). *Run1* confers total resistance to the populations of this fungus naturally occurring in Montpellier, France. The segregating population was created in 1995 by crossing a resistant hybrid carrying *Run1* in the heterozygous state (VRH 3082-1-42) with Cabernet Sauvignon. They used the bulked segregant analysis (BSA) approach with AFLP markers tightly linked to the *Run1* locus to develop a local map around the gene. Pauquet et al. (2001) later reported a local map of AFLP markers around the *Run1* gene (Fig. 4a). A BC_5population of 157 genotypes was used to select AFLP markers linked to the resistance gene. A total of 13 markers were used to develop this local map and 10 of them co-segregated with the resistance gene. They also studied the usefulness of these 13 AFLP markers in a wider set of resistant and susceptible genotypes. Three markers out of 13 analyzed were absent in all susceptible genotypes and present in all resistant genotypes.

Doucleff et al. (2004) reported on a map of *V. rupestris* × *V. arizonica.* This mapping population segregates for resistance to the dagger nematode (*Xiphinema index*) and to Pierce's disease (PD), a bacterial disease caused by *Xylella fastidiosa*. A total of 475 DNA markers [mostly AFLP, inter simple sequence repeat (ISSR), RAPD and SSR)] were used to construct the parental maps with PGRI (Plant Genome Research Initiative) mapping program. Maternal and paternal maps covered 756 and 1,082 cM, respectively. Currently, this population is being re-mapped with SSR, EST-SSR and EST markers. A total of 240 markers have been mapped to 19 linkage groups. The main focus is to develop a high density linkage map around the nematode and PD resistance loci, and use these tightly linked markers for MAS in an ongoing grape scion and rootstock breeding program and initiate map-based positional cloning of resistance genes.

Fischer et al. (2004) reported on a map of full sib F_1 population consisting of 153 genotypes from the cross of 'Regent' × 'Lemberger'. Parental maps were constructed with AFLP, RAPD, SSR and SCARs/CAPS markers. The Regent map covered a total of 1,277.3 cM with an average marker distance of 4.8 cM. The Lemberger map extends over 1,157.7 cM with an average marker distance of 7.0 cM.

A second international grape reference map solely based on SSR markers was published in 2004 (Adam-Blondon et al. 2004). It mapped 96 progeny from *V. vinifera* 'Syrah' × *V. vinifera* 'Grenache'. The Syrah map was constructed from 177 markers (many VMC, and newly developed VVI within Genoplante, see Merdinoglu et al 2005) into 19 linkage groups (1,172.2 cM) and the Grenache map was constructed of 178 markers into 18 linkage groups (1,360.6 cM). The consensus map consisted of 220 markers ordered in 19 linkage groups covering 1,406.1 cM. This was the first published map that represented the 19 chromosomes of genus *Vitis* (Fig. 3). In the same study, a map based on progeny from a selfed Riesling population consisting of 110 SSR and covering 1,191.7 cM was also reported. Using these maps, the genome length was estimated to be around 2,200 cM, which was comparable to genome length estimates from the first published reference map (Riaz et al. 2004).

A new PCR-based approach for rapid generation of genetic markers capable of tagging disease resistance genes has been developed and effectively used in other crops. This approach is based on the observation that genes conferring resistance from a diverse range of host-pathogen interactions have a high degree of structural and amino acid sequence conservation. In particular, the majority of cloned resistant genes, "R genes", contain a nucleotide binding site (NBS) and a leucine rich repeat (LRR) domain (Meyers et al. 1999; Young 2000). The NBS sequences of these genes are characterized by the presence of up to seven conserved domains including the P-loop, Kinase-2, and GLPL motifs. The presence of these conserved domains has facilitated the cloning of resistance gene

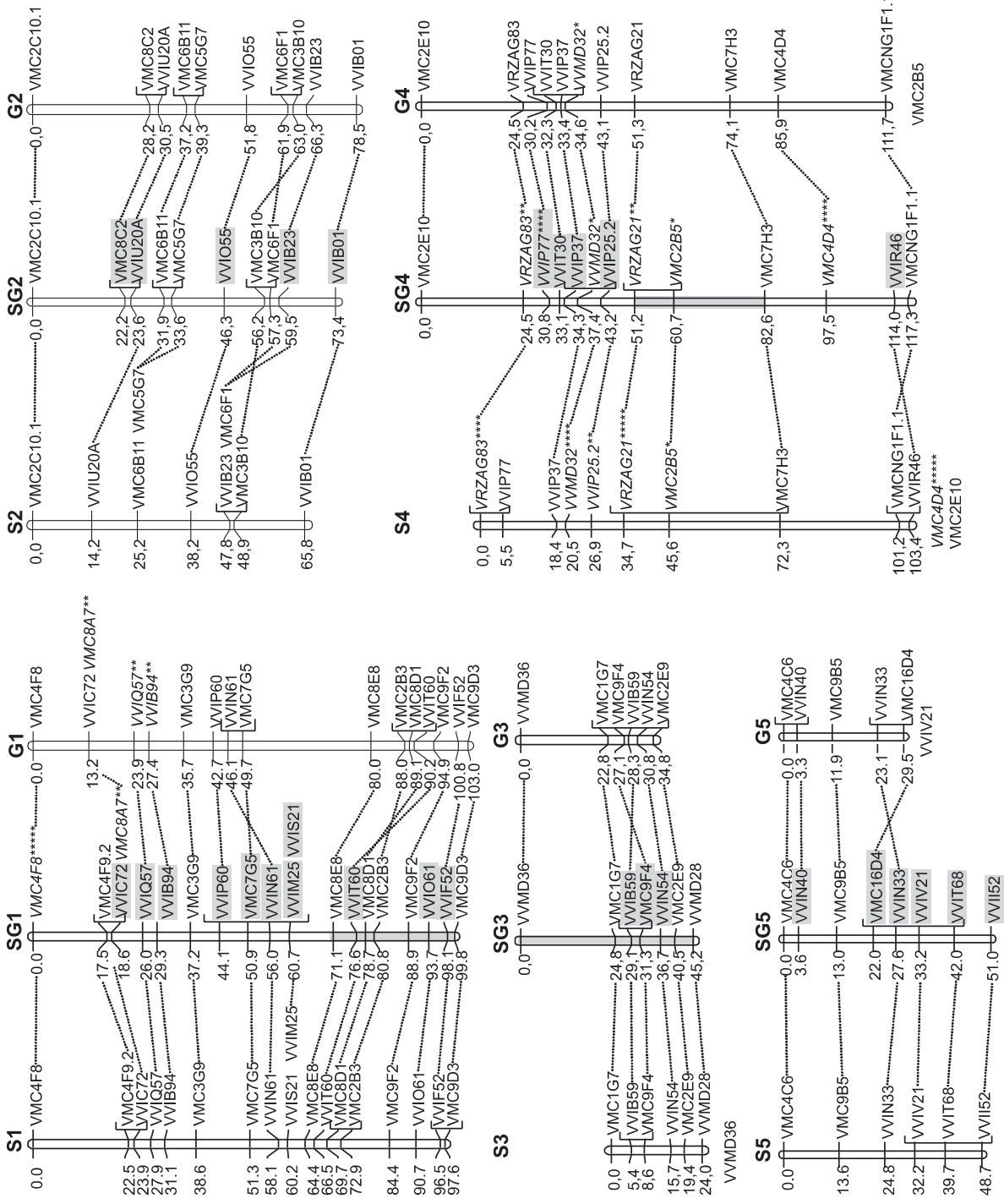


Fig. 3. Syrah, Grenache and consensus S × G maps. Linkage groups are respectively numbered from 1 to 19 with the prefix *S*, *G* and *SG*. Distorted markers are in *italics* with an *asterisk* indicating the level of distortion (* $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$; **** $P < 0.001$; ***** $P < 0.0001$). Markers that were ordered at LOD = 2.0 are indicated with the *bracket* on the *left*. Markers present only in S × G map and not in Riaz et al. (2004) are indicated with *grey boxes*. *Grey zones* in the linkage groups are indicating the markers with a different order in this map and the three maps published by Riaz et al. (2004). (Adam-Blondon et al. 2004)

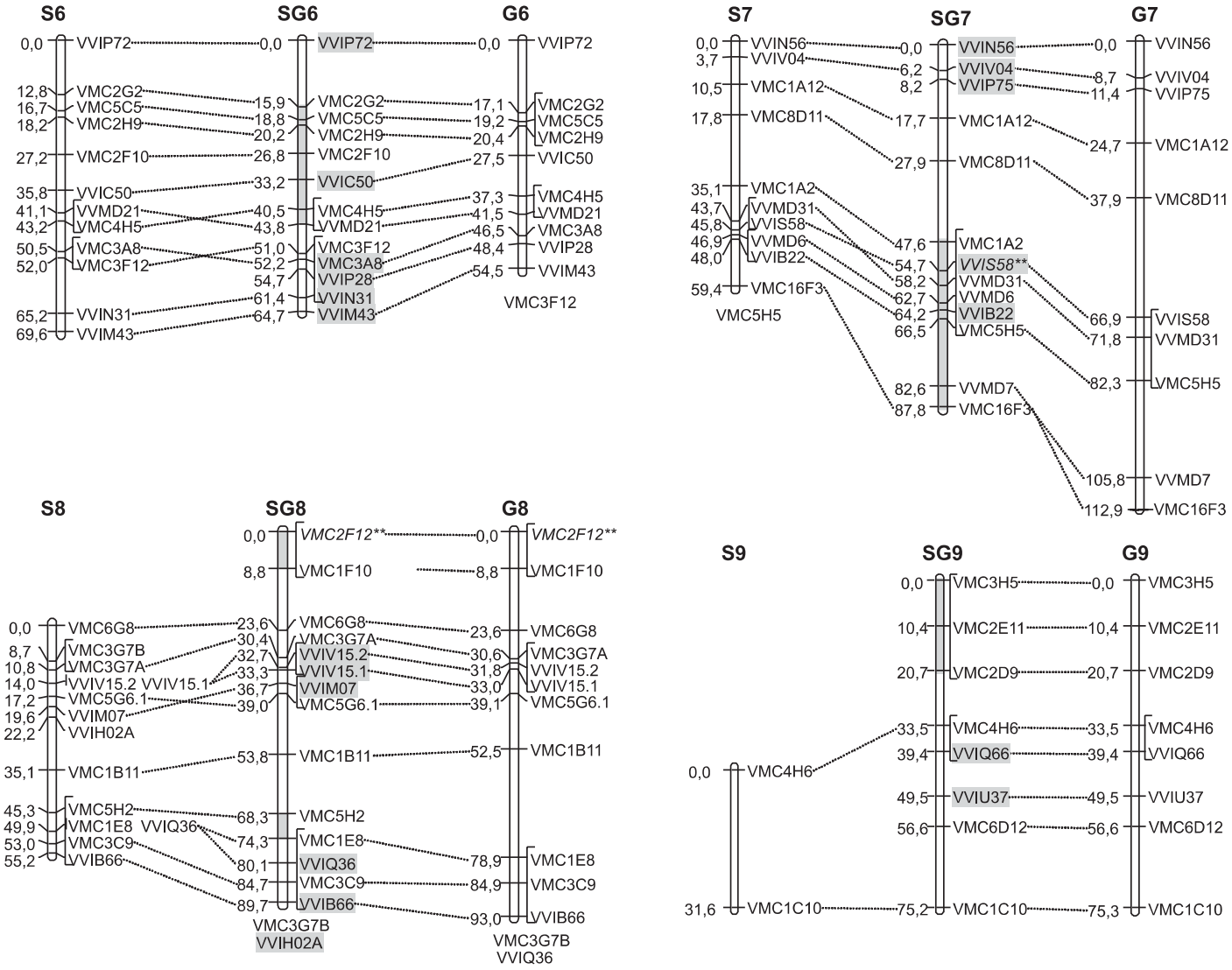
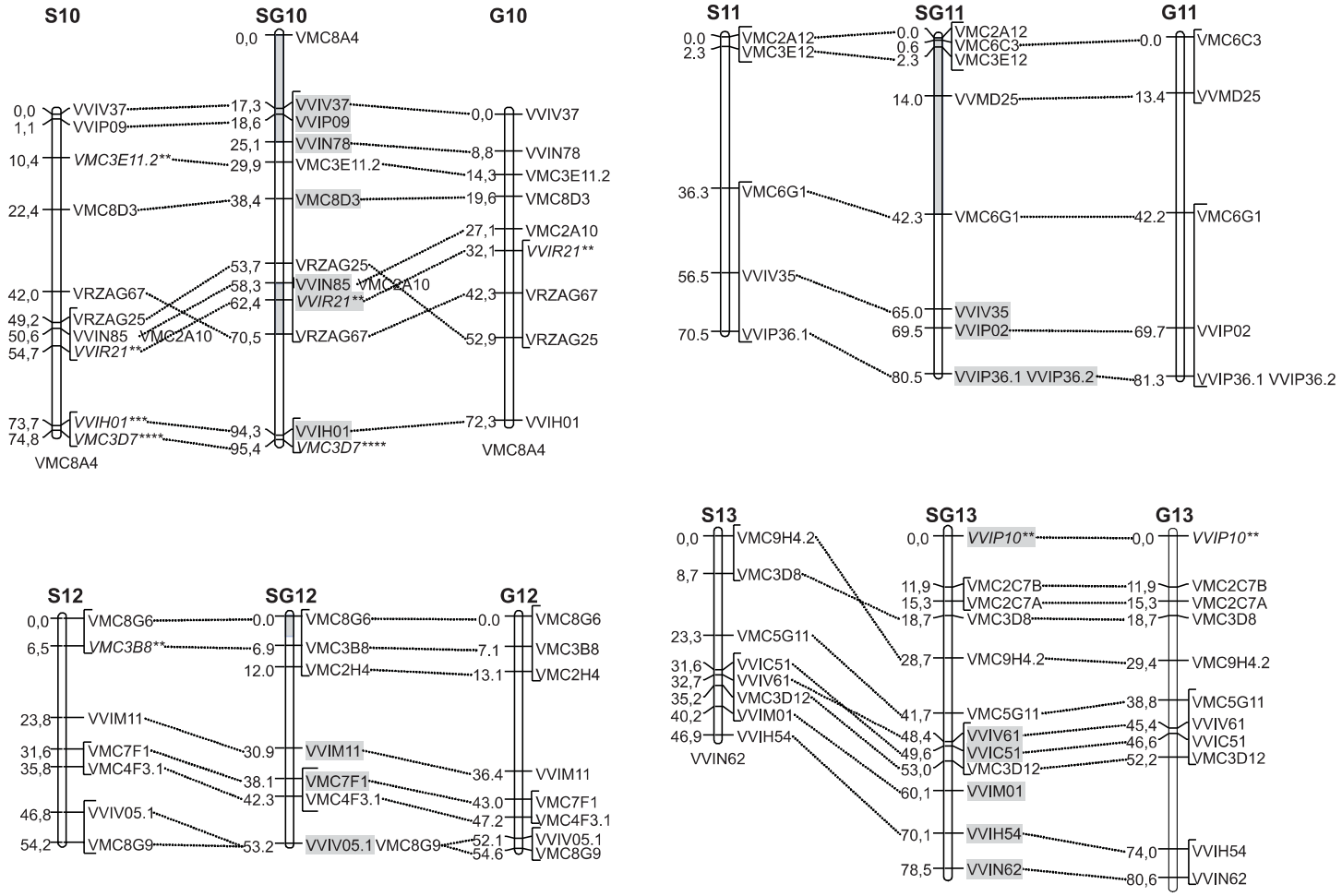


Fig. 3. (continued)



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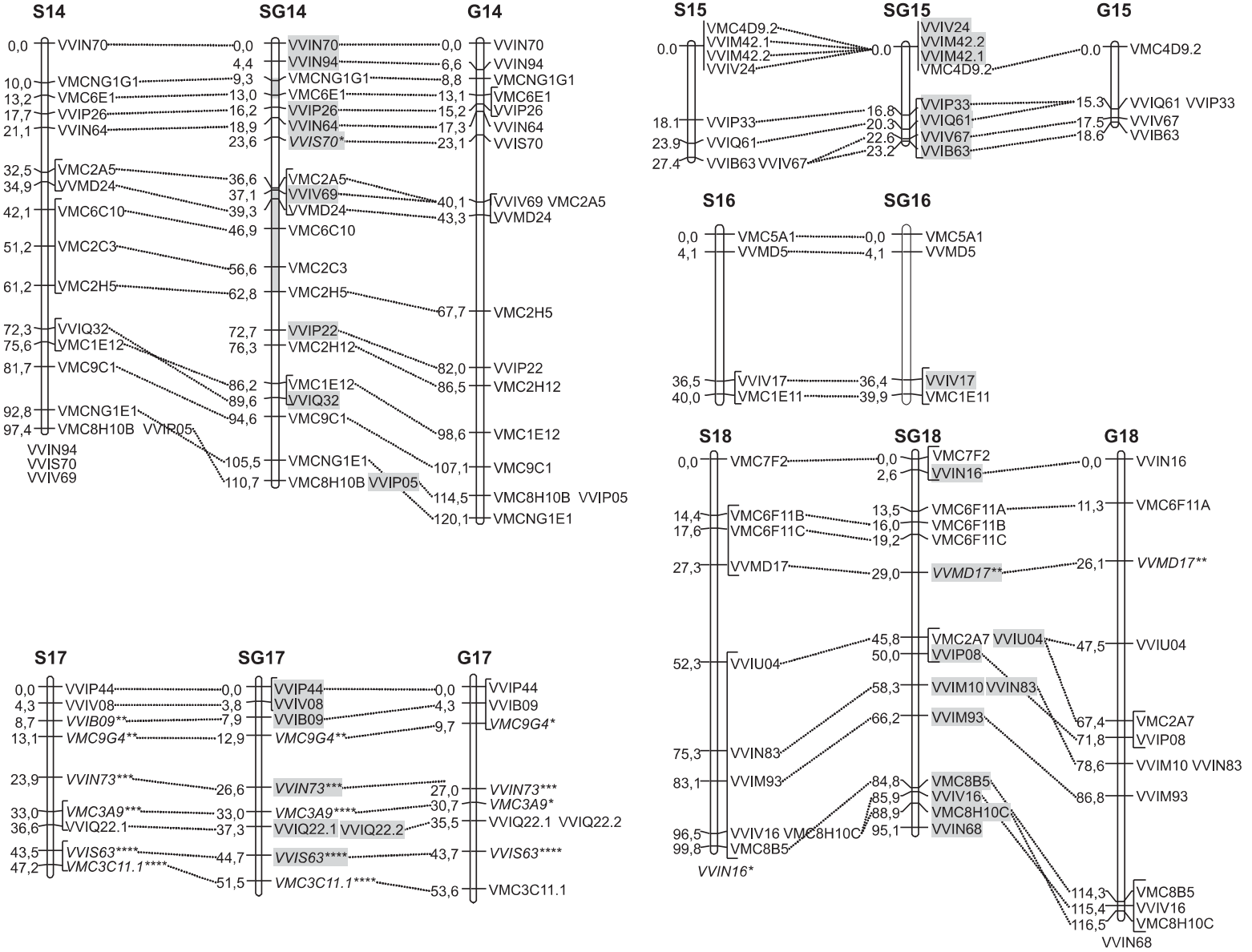

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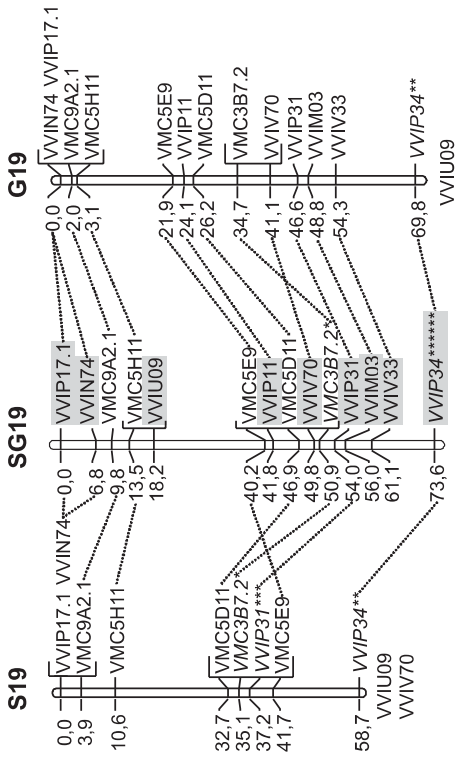

Fig. 3. (continued)

analogs (RGA) from diverse species using PCR and degenerate oligonucleotide primers. NBS encoding sequences tend to be clustered in the genome and, in accordance with this, isolated RGAs are frequently genetically located at, or near, previously identified resistance loci (Aarts et al. 1998; Collins et al. 1998; Leister et al. 1999; Mago et al. 1999; Pan et al. 2000). Therefore, the identification of RGAs represents a potentially powerful strategy to develop new markers around resistance genes and a good aid for map-based positional cloning of genes.

In a continuation of the previous work on the *Run1* locus, Donald et al. (2002) were the first grape researchers to utilize the RGA approach in grapes. They used degenerate primers designed to conserved regions of NBS motifs within previously cloned pathogen resistance genes, to amplify RGAs from grape. Twenty-eight unique grapevine RGA sequences were identified and subdivided into 22 groups on the basis of a nucleic acid sequence identity of approximately 70% or greater. Three RGA markers were tightly linked to the *Run1* locus. Of these markers, two RGA (GLP1-12 and MHD 145) co-segregated with the resistance phenotype in the 167 tested genotypes of BC5 population, and the RGA marker MHD98 was mapped to a position 2.4 cM from the *Run1* locus (Fig. 4b). As part of the continuing effort to tag the *Run1* gene, Barker et al. (2005) recently published a genetic and physical map of the gene using a BAC library constructed using genomic DNA from a resistant *V. vinifera* individual carrying *Run1* within an introgression. This is the first published report of physical mapping of any gene in grape. The BAC contig assembly also allowed the generation of new genetic markers that are closely linked to the *Run1* gene. Initial analysis indicates that region containing *Run1* gene contains two multigene families of RGA.

Luo et al. (2001) also employed BSA with RAPD and sequence characterized amplified region (SCAR) molecular markers to tag the downy mildew-resistance genes of grape derived from *V. quinquangularis*. The parents and 60 selected progeny were tested. Among 280 Operon RAPD primers tested, 160 gave distinct banding patterns and one marker, OPO06-1500, was tightly linked to a major gene for resistance to *Plasmopara viticola* (RPv-1). Linkage analysis with Mapmaker determined the distance between RPv-1 and OPO06-1500 to be 1.7 cM. Marker OPO06-1500 was cloned and sequenced to develop a SCAR marker (SCO06-1500),

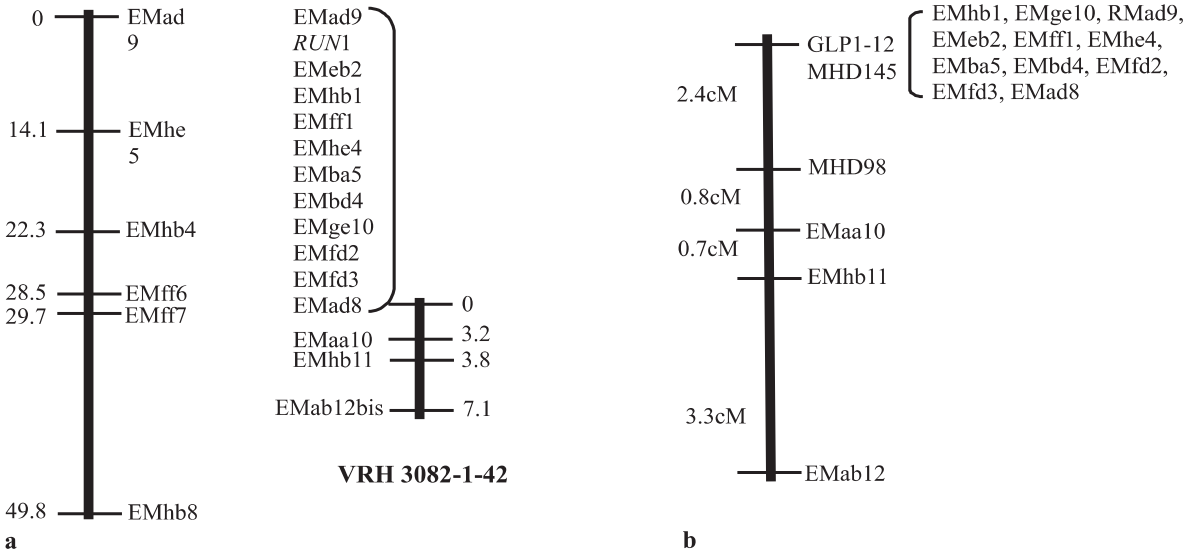

Fig. 4. Local map of the resistant genotype VRH3082-1-42 and in Cabernet Sauvignon of the RUN1 region. The 11 loci in *brackets* together with ***RUN***1 at the top of the VRH3082-1-42 linkage groups are all co-segregate. Figure 3b shows linkage map of the resistant locus RUN1 and RGA markers GLP1-12, MHD145 and MHD98 (Pauquet et al. 2001; Donald et al. 2002)

which produces a single band only in resistant plants.

2.2.2.2 Bacterial Diseases

In spite of the fact that bacterial diseases cause serious losses in grape (Pierce's disease and bacterial blight of grape), there has been little information available for incorporation of bacterial resistance from wild species into *V. vinifera* except for the case of Pierce's disease (PD). All *V. vinifera* varieties are highly susceptible to PD, which is caused by the bacterium *Xylella fastidiosa*. In grape growing areas, where the bacterium is endemic, the disease severely limits the cultivation of *V. vinifera* cultivars. Symptoms of PD include: leaf scorching, fruit cluster dehydration, uneven maturation of infected canes, stunting and death within 3–4 years. Resistance to PD exists in American *Vitis* species and has been introgressed into many hybrid cultivars, but very little is known about the genetics of resistance. Krivanek et al. (2005) reported that a single gene *PdR1* with a dominant allele is responsible for PD resistance originating from a *V. arizonica* background. An extensive, grape-breeding program is underway to incorporate this resistance gene into improved wine, table and raisin grapes. The *PdR1* locus has been localized on chromosome 14 of a genetic linkage map resulting from a cross of D8909-15 [*V. rupestris* 'A. de Serres' × 'b42-26' (*V. arizonica*)] and F8909-17 [*V. rupestris* 'A. de Serres' × b43-17 (*V. arizonica*/*V. candicans*)] (Krivanek et al. 2006).

2.2.2.3 Insects and Nematodes

Grape phylloxera (*Daktulosphaira vitifoliae* Fitch) is the most important insect pest of grape and continues to impact the world's vineyards. It is native to North America but has spread to every region where grapes are grown and caused billions of dollars in damage by its destructive feeding on *V. vinifera* roots. Native American grape species are resistant to phylloxera and rootstocks have been bred and used to control phylloxera for over 100 years. Very little is known about the mechanism of resistance or the number of resistance genes available from the Native American grape species.

Researchers at the Department of Viticulture and Enology, University of California, Davis, are attempting to position phylloxera resistance on a genetic linkage map of a population from a cross between the resistant *V. rupestris* and the susceptible *V. vinifera* grape (Roush et al. 2004). It has been hypothesized that the number and type of root galls formed in response to phylloxera should be associated with a plant's level of resistance. In this study, plants from

the F_1 generation were selected for a series of sibling crosses among resistant and susceptible individuals as well as backcrosses to *V. vinifera* 'Aramon' and *V. rupestris* 'Ganzin'. A subset of the resulting F_2 progeny was selected from these crosses and infested to identify resistant and susceptible plants and to determine the inheritance of gall formation. Preliminary data suggests that more than one gene is likely responsible for gall formation, and hence resistance or susceptibility.

The Department of Viticulture and Enology is also studying resistance to root-knot (*Meloidogyne incognita*) and dagger (*Xiphinema index*) nematodes. Two mapping populations have been developed from progeny segregating for resistance to these pests. The first is a *V. riparia* 'Riparia Gloire' × *V. champinii* 'Ramsey' population created to map resistance to root-knot nematode and locate QTLs for salt tolerance and rooting angle (Lowe and Walker 2004). The second is the 9621 population mentioned above on which *X. index* resistance is being mapped. Both nematode resistances seem to map as a single dominant gene, but to different linkage groups.

2.2.2.4 Other Morphological Traits

Grape, being relatively large, perennial, and heterozygous, is not a good system for classical developmental genetic efforts to map and tag important horticultural traits that affect plant form, cluster architecture, factors affecting fruit composition and yield. Thus, only a few morphological traits have been mapped. Dalbo et al. (2000) mapped a locus controlling flower type on linkage group 14 of parental map of 'Illinois 547-1' that corresponds to linkage groups 2 of reference map (Adam-Blondon et al. 2004; Riaz et al. 2004). The probable parental genotypes were homozygous hermaphrodite, HH ('Horizon'), and heterozygous male, MF (III.547-1). The resulted progeny indicated 1:1 segregation of male and hermaphrodite types. This confirmed that a single gene controls sex expression in grapes as previously suggested by Olmo (1976).

In another study, Doligez et al. (2002) mapped a major gene for berry color to linkage group 3 that now corresponds to linkage group 2 of the international reference maps (Adam-Blondon et al. 2004; Riaz et al. 2004). Fischer et al. (2004) also reported that berry color segregated as a simple trait and it mapped on the linkage group 13 of their population, which also corresponded to linkage group 2 of international consensus map.

Seedlessness is another important trait that was tagged in a study by Bouquet and Danglot (1996). Analysis of progenies obtained by crossing seedless genotypes led to a proposed model for the inheritance of seedlessness (Bouquet and Danglot 1996) and to the identification of a SCAR marker linked to the seedless character (Lahogue et al. 1998). The efficiency of obtaining seedless progeny can be greatly improved by the use of molecular markers tightly linked to seedlessness and these markers will also help in optimizing parental selections.

2.2.3 Detection of QTLs

Genetic linkage maps have facilitated mapping of agriculturally important QTLs in grapes, including QTLs for disease resistance, seedlessness and berry weight. Using QTL mapping, resistance loci whose alleles exert smaller effects on the phenotype may be manipulated more effectively (Young 1996). In the case of disease resistance, an obvious goal would be to develop grape cultivars with resistance alleles at all QTLs of interest. Establishment of generalized genomic regions that affect a particular trait within inter- and intra-species grape mapping populations with common markers will help to clarify the relationships of QTLs in different genetic backgrounds, and promote marker assisted selection and breeding.

2.2.3.1 QTLs for Disease and Pest Resistance

There are only a few published reports of QTL studies in grape and the main focus is powdery (*Uncinula necator*) and downy mildew (*Plasmopora viticola*). Dalbo et al. (1997) studied the inheritance of powdery mildew resistance in the cross Horizon × Illinois 547-1. Genetic maps based on RAPD markers were constructed for each parent with a mean distance between markers of 5.5 cM. A major QTL was found in the resistant parent Illinois 547-1. BSA was used to screen 203 primers to find additional linked RAPD markers. A single marker (S25b; LOD = 6.9) explained 44% of the variation. The same marker was present in *V. cinerea* B9, one of the parents of Illinois 547-1 and the likely source of resistance. Two other regions on the Horizon map were associated with powdery mildew resistance. The markers S25b (from Illinois 547-1) and S35a (from Horizon) could be used to correctly classify resistance in all but 9 of 60 seedlings.

Zyprian et al. (2002) reported on the tagging of resistance to powdery and downy mildew from the cultivar Regent. An F_1 population on about 153 individuals was derived from the cross of the fungus-resistant Regent × the fungus-susceptible Lemberger. This population segregates for resistance to both diseases as well as other agronomic traits in a quantitative manner and AFLP, RAPD and SSR markers were used in the map. In continuation of this work, Fisher et al. (2004) reported major QTLs for resistance to powdery and downy mildew on linkage groups 9, 10 and 16 of the Regent map that corresponds to group 7 and 11 of international consensus map. These QTLs explained up to 69% variation in the tested population.

2.2.3.2 QTLs for Other Traits

Doligez et al. (2002) reported on the detection of QTLs for traits involved in seed production with the goal of characterizing seedlessness sub-traits (seed number, seed total fresh and dry weights, seed percent dry matter and seed mean and fresh dry weights) and berry weight in an F_1 progeny obtained by crossing two partially seedless genotypes (MTP2223-2 × MTP2121-30, mentioned above) and embryo rescuing the progeny. QTL detection was performed with two methods: the non-parametric Kruskal-Wallis rank-sum test, and composite interval mapping. QTLs with large effects (R^2 up to 51%) were detected for all traits and years at the same location on linkage group X (which now corresponds with linkage group 18 of the international reference map, Riaz et al. 2004). Three QTLs with small effects (R^2 from 6% to 11%) were found on three other groups.

Riaz (2001) genetically analyzed different components of the grape cluster. Compact cluster architecture is closely associated with bunch rot (Vail et al. 1998), and small berries contribute to loose clusters. Small berries are also desirable for red wine production because the higher skin to pulp ratio is thought to increase wine color intensity. Cluster form was divided into different components (rachis length, number of laterals, length of laterals, total cluster weight, number of berries, berry weight, and cluster density) in order to study their individual contribution to cluster architecture and compactness and their relationship to each other. The QTL analysis was carried out on a consensus framework linkage map based on 154 SSR and one EST marker on 153 progeny of Riesling × Cabernet Sauvignon. QTLs were identified with two different methods (Interval mapping and Kruskal-Wallis rank sum test). They were identified for total cluster weight, average weight of one berry, berry number per cluster, rachis length, number of laterals per cluster, average lateral length and cluster density, as well as for fruit composition and young shoot morphology. Most of the traits that markedly affected cluster architecture showed strong correlation to each other and QTLs were identified with overlapping intervals. These were preliminary results based on three years of data on a single plant of each genotype and it is very important to validate data with multi-vine replicates of progeny and parents in different environments. Thus far, three studies on QTLs associated with berry size have been reported (Riaz 2001; Doligez et al. 2002; Fischer et al. 2004), however they mapped to different linkage groups in first two studies (group 17 and group, 18 respectively) and mapped to linkage groups 3 and 10 of Regent map (Fischer et al. 2004). Neither of these linkage groups had SSR markers common to the international reference map (Riaz et al. 2004). Fischer et al. (2004) also reported QTLs for veraison and axillary shoot growth.

2.3 Whole Genome Projects

The completion of the human, *Arabidopsis* and rice genome sequences in the last five years stimulated rapid development of genomic technologies and applications. The functional information accumulating in *Arabidopsis* also offers a model system for the functional analyses of grape genes. These possibilities provide a framework for a concerted effort to efficiently identify and functionally analyze important grape genes. The International Grape Genome Program was formally announced in January 2002 at the Plant, Animal, and Microbe Genome X Conference, in San Diego, California. The main objectives are: (1) Coordinate the Grape Genome Program. (2) Facilitate exchange of information and collaboration with the wider viticulture and enology research communities. (3) Monitor, summarize and communicate progress of scientific activities of participating laboratories. (4) Identify research areas of benefit to grape improvement and plant biology and communicate them to funding agencies of participating nations. (5) Periodically up-date the goals of the program. (6) Serve as a primary contact with other plant genome projects. (7) Interact with an Industry Advi-

sory Committee to ensure relevance of the research to industry problems. (8) Act on recommendations received from the various working groups.

In addition to the International Grape Genome Initiative, individual genomic efforts are also underway in grape growing countries and are briefly described below.

2.3.1 Australia

Current research involves a wide range of techniques from functional characterization of single genes to genomic approaches including genetic mapping, physical mapping, gene discovery using ESTs, and gene expression analysis using microarrays and transgenic plants. Beneficial outcomes from this research are expected to increase our knowledge of grape biology, improved berry and wine quality, and provide resistant or tolerant plants to powdery mildew, botrytis, nematodes and phylloxera.

Recently, Dupont Genome Sciences in conjunction with Southern Cross University initiated a large-scale grape DNA sequencing project. The main focus of this project is to obtain genetic information to allow research in areas such as dormancy and bud burst; fruit quality including sugar content, flavor and color, and tendril development. The technologies include large-scale expressed sequence tag (EST) analysis (Ablett et al. 2000), a 16× BAC library (Tomkins et al. 2001), and functional analysis of grape genes in *Arabidopsis* to advance gene discovery in these areas. The BAC library was constructed from the cultivar Syrah and consisted of 55,296 clones with average insert size of 144 kb.

To date, this project has produced over 45,000 grape (*V. vinifera*) ESTs or partial gene sequences from a range of tissues and cultivars. These represent nearly 19,000 distinct ESTs covering an estimated two-thirds to three-quarters of the grape genes (based on an estimated number of 25,000 to 28,000 genes). New SSR markers with a high degree of transferability have been developed from the ESTs (Scott et al. 2000). This was the first report of SSR identification from grape ESTs. This approach has been used widely in other plant species. Phenotypic changes produced by over-expression of novel grape transcription factors in *Arabidopsis*, are being analyzed, and cDNA grids are being used to study gene expression during budburst and berry development.

2.3.2 France

The development of grape genomic resources in France has been greatly aided since 1999 through financial support from the Génoplante consortium (www.genoplante.org) and INRA (www.inra.fr). BAC libraries have been constructed and will be used in the development of a physical map of the *V. vinifera* genome in collaboration with members of the IGGP (Chalhoub et al. 2002; Adam-Blondon et al. 2005). The URGV (Unité de recherche en génomique Végétale) has been set-up at INRA to work on several BAC libraries from different cultivated plants of importance to France and Europe. They have developed three grape BAC libraries: Cabernet Sauvignon (13×, International Grape Genome Program reference library, www.vitaceae.org), Syrah (8×) and Pinot noir (15×), with about 70,000 BAC clones each. The average size of inserts is 150 Kb. Further work was carried out to develop physical map with the Cabernet Sauvignon BAC library (http://www.evry.inra.fr/public/projects/genome/grape). It involved development of 3D pools from a 6× subset of the Cabernet Sauvignon BAC library to anchor with PCR 237 SSR markers (Adam-Blondon et al. 2004) and 565 ESTs (from the unigene set used in the Génoplante project CI2001003). An additional set of 592 ESTs from the NCBI Vitis Unigene set # 11 was anchored in silico on the BAC end sequences. These results are providing access to regulatory regions of genes of interest and to the position of about 50 new genes on the genetic map. The fingerprinting of 30,000 BAC clones is now underway in collaboration with Génoscope and the University of Udine (M. Morgante).

Recently, emphasis was put on the development of SSR markers and on a reference genetic map as a tool for QTL detection of traits such as berry characters and pathogen resistance. The production of ESTs by Terrier et al. (2001) will contribute to the development of microarrays for the study of the expression, regulation and signaling control of berry development genes. In parallel, INRA has been developing methods for grapevine transformation (transient & stable). A database for grape genetic resources is available at INRA and at the European level and several other databases are under development (EST management and processing, genetic maps, BAC) (See lists below). This knowledge should help in the development of high quality grape varieties resistant to pathogens and also lead to a better understanding and management

of grape-environment interactions and their effect on fruit and wine quality.

2.3.3 Germany

In Germany grapevine genomics started in the early 1990s with the application of molecular marker technology to questions of cultivar identification, pedigree analysis, evaluation of genetic resources, and genetic mapping (also in France). The major focus is on localization and long-term molecular characterization of genes involved in pest resistance and fruit quality traits with the aim of understanding their complex genetic basis. Different marker systems are being employed, including SSR markers developed by the Vitis Microsatellite Consortium (VMC) allowing integration with the results from other international mapping projects.

2.3.4 Italy

Since the early 1990s molecular biologists have been using molecular tools for variety characterization, disease diagnosis, phylogenetic studies, and genetic transformation of *Vitis* species. In the last few years, the interest in grape genomics has increased enormously and research involves marker-assisted selection, molecular mapping, and large EST sequencing, establishing BAC libraries for map-based positional cloning of genes of economical interest, pest resistance, and fruit quality. Italy has two large genomic projects: the first is headed by a public institution, The University of Udine, focused on developing tools for molecular breeding and map-based positional cloning of genes approaches; and the second is a collaborative project among several universities and headed by S. Grando, with a focus on the functional genomics of berry maturation phases.

2.3.5 South Africa

Grapevine genomics research in South Africa started with participation in the Vitis Microsatellite Consortium (VMC) in 1998. The Institute for Wine Biotechnology (IWBT) and the Institute for Plant Biotechnology (IPB), are the two major sites for grape genomic research. Genomics efforts include genetic transformation, and development of cDNA libraries. The IWBT generated genomic libraries for the *V. vinifera* cultivars Sultana (correctly Sultanina) and Pinotage, and cDNA libraries from young expanded leaves of the same two cultivars. Genomic libraries for Chardonnay and Merlot as well as cDNA libraries from early and late berry developmental stages of these cultivars were made at the IPB. A consortium including the Genetics Department, the IWBT, the Department of Molecular and Cellular Biology at the University of Cape Town, and the Biotechnology Department of the University of the Western Cape, have an interest in studying molecular interactions between grape and fungal pathogens using microarray technology.

2.3.6 USA

In the USA, grape genomics work commenced in the early 1990s. Several groups have developed molecular marker based maps in both *V. vinifera* and interspecific hybrid populations (Lodhi et al. 1995; Dalbo et al. 2000; Doucleff et al. 2004; Riaz et al. 2004). There are several labs involved with research on functional genomics of *V. vinifera*. The main focus of research at the University of Nevada, Reno (GR Cramer and JC Cushman) is to study the effect of abiotic stresses (e.g. cold, heat, salinity, drought) on grape. They have initiated an EST-based gene discovery program focused solely on stressed vines by constructing cDNA libraries from mRNA isolated from leaf, root, and berry tissues of *V. vinifera* cv. Chardonnay. The growing database of EST sequence information will allow large-scale gene expression profiling using microarray technology.

At the Department of Plant Pathology, University of California, Davis (DR Cook) another EST project is focused on identifying the transcriptional pathways correlated with susceptibility or resistance in *V. vinifera* to Pierce's disease (PD) and with genes involved in berry ripening (http://cgf.ucdavis.edu/). This database contains an analysis of all public ESTs from *Vitis*, and ESTs are grouped as contigs or singletons and analyzed for homology to the NCBI Non-Redundant (NR) database by means of BLASTX. All contigs and singleton ESTs were also analyzed for the presence of SSRs and 1000 EST-SSR markers were developed that are available to grape genetic research community. The GeneChip® *Vitis vinifera* Genome

Array developed by Affymetrix is the first commercially available array to provide comprehensive coverage of the *V. vinifera* genome. Convenient one-array views of 14,000 *V. vinifera* transcripts and 1,700 transcripts from other *Vitis* species can be examined (http://www.vitaceae.org/).

A collaborative research project between the USDA/ARS – Parlier, CA and the Department of Viticulture and Enology, University of California, Davis (H. Lin and M.A. Walker) is studying resistance to PD and developing new tools for grape improvement (Lin and Walker 2004). The goal of this project is to characterize the molecular events in grape/*Xylella fastidiosa* interaction and develop a functional genomic approach to specifically identify the PD-related gene expression profiles from susceptible and resistant responses. About 5000 expressed genes have been sequenced from PD resistant and susceptible grape plants. These expression profiles derived from stem, leaf and shoot of resistant and susceptible genotypes throughout the course of disease development will provide informative details of molecular basis of PD responses.

Lin et al. (2005) used a cDNA-AFLP technique to analyze the gene expression profile of PD infected grapevines. In this study, they compared gene expression of highly susceptible and resistant siblings selected from a segregating population of *V. rupestris* × *V. arizonica*. Comparing the profiles of resistant and susceptible genotypes identified fragments representing up- and down-regulated genes. About 100 differentially expressed cDNA-AFLP fragments were collected, sequenced and annotated. These fragments reflect the differentially expressed genes from various tissues at different stages of PD development. To further study the genes involved in the host-pathogen interaction at different stages of disease development, a Taq-Man gene expression assay was developed to analyze selected genes for their spatial and temporal expression in response to PD infection. This study will help identify genes involved in the defense response and signaling/recognition cascade in PD susceptible genotypes.

2.4 Marker-Aided Selection and Breeding

In the last 15 years, the development of molecular markers has stimulated advances in breeding, since these markers directly reveal genetic variability through DNA analysis (Staub et al. 1996), and environmental effects do not influence their detection. The primary use of these molecular markers is in marker-assisted selection (MAS) (Paterson et al. 1991). The main objective of crop breeding is to obtain new cultivars exhibiting better yield, quality traits, and resistance to biotic and abiotic stresses. In many cases, these useful traits come from wild and distantly related species. The traditional approach is based on interspecific hybridization to transfer genes from wild to cultivated species, followed by selection of hybrids that combine the "new wild" trait with the cultivated genetic background. This breeding strategy is primarily achieved by generational backcrossing in which the selected hybrids at each generation are crossed back to the cultivated genotype (although the cultivated genotype may vary in grape to avoid inbreeding depression) with the aim of reducing the wild genome and its undesirable traits.

Marker-aided selection is one of the most efficient applications of biotechnology to plant breeding. It is an effective and efficient breeding tool for detecting, tracking, retaining, combining, and pyramiding disease resistance genes in crop species (Kelly and Miklas 1998, 1999). The essential requirements for MAS in a plant breeding program are: 1) the marker(s) should be closely linked (1 cM or less is probably sufficient for MAS) with the desired trait; 2) an efficient means of screening large populations for the molecular marker(s) is key; 3) the screening technique should have high reproducibility across laboratories, be economical to use and user-friendly. Molecular markers closely linked to the gene controlling the trait to be transferred allow precocious screening on the DNA extracted from young leaves without waiting for the specific developmental stage at which the trait is expressed. This precocious screening results in large savings in time and space, and becomes far more valuable when multiple traits and many progeny are under testing. Choosing the most suitable markers for MAS depends on the ease of their detection, the possibility of revealing single or multiple loci, their dominant or co-dominant nature, and their expense. The most widely used markers for MAS are RFLP, RAPD, AFLP, and SSR. Their polymorphic nature is based on point mutations or chromosome rearrangements that accumulate during the evolution of the species without negatively influencing survival and reproduction. The recent surge of development of grape genetic linkage maps with molecular markers has the potential to greatly expand use of MAS in grape breeding pro-

grams. However, until additional work is completed that tags phenotypic traits with molecular markers, progress will be limited.

The first published effort to utilize MAS with grape was tagging QTLs for powdery mildew and black rot resistance with RAPD and AFLP markers (Dalbo et al. 2001). As mentioned above this effort utilized a segregating population from a cross of Horizon × Illinois 547-1. A major QTL (LOD 6.6) was found for powdery mildew resistance in the Illinois 547-1 (resistant parent) map and two other QTLs with a smaller effect were found in the Horizon map. When black rot resistance was mapped, four QTLs were detected, two in each parent. The three most important QTLs were located in the same linkage groups as the ones for powdery mildew. One was also associated with a QTL for production of the phytoalexin resveratrol. Two markers (a RAPD and an AFLP) linked to this QTL were obtained by BSA and then converted into CAPS markers for testing in four different crosses. In all cases, the markers were strongly associated with resistance to powdery mildew. A similar approach was used to find markers that are tightly linked to the powdery mildew resistance gene *Run1* in a BC_5population with AFLP markers (Pauquet et al. 2001).

In another study, Lahogue et al. (1998) used BSA to identify two RAPD markers tightly linked to the seedlessness gene *SdI*, a dominant gene that controls three recessive complementary genes for seedlessness in the Sultanina (Thompson Seedless in California, and Sultana in Australia) grape. The closest marker was used to develop a co-dominant SCAR named SCC8. This latter marker was used to exclude seeded progeny (scc8-scc8-) and to select seedless individuals (SCC8+SCC8+). The SCC8 marker accounted for at least 65% of the phenotypic variation of the seed fresh weight, and for at least 79% of the phenotypic variation of the seed dry matter. SCC8 was further checked by Adam-Blondon et al. (2001) in a grape germplasm collection and in other seedling populations and found out that seeded individuals can be heterozygous at SSC8 marker. This observation indicated that more understanding of the genetic determination of stenospermocarpic seedlessness is required to allow use of molecular markers for efficient MAS for seedlessness.

Mejía and Hinrichsen (2003) also chose the BSA approach with RAPD markers to identify markers linked to seedlessness. They studied a Ruby (Ruby Seedless) × Sultanina population for different stenospermocarpy sub-traits. Of the 336 RAPD primers tested, six fragments were seedless-specific and one was related to the seeded phenotype. A RAPD fragment named WF27-2000 was cloned and sequenced, and then converted into a SCAR marker. This SCAR, designated SCF27, generated a specific amplicon of 2.0 kb that was present in all of the seedless individuals, and segregated 3:1 in the population suggesting both parents were heterozygous for this locus.

2.5 Cultivar Identity

Ampelography is the traditional method of identifying grape cultivars based on morphological differences of the foliage and fruit. It is accurate and reliable, but requires years of training and practice, and few individuals are sufficiently skilled. Ampelography is also influenced by environmental conditions, which can alter leaf and cluster size and influence characters such as the degree of tomentum, vine vigor and shoot tip coloration. In addition, the most reliable leaves for identification are formed in the mid-shoot region and they may not be available for observation or shipment. In the case of rootstocks, once they are grafted they rarely form shoots from below the union. Finally, there are many thousands of cultivars in germplasm collections around the world and few have been described in readily accessible forms, and ampelographers tend to be experts on cultivars grown within the region they reside. Thus, alternative identification methods based on tissue DNA have been developed to overcome these limitations and produce DNA fingerprints of grape cultivars.

Molecular identification efforts began with the use of isozymes (Stavrakakis and Loukas 1983; Benin et al. 1988; Calo et al. 1989; Walker and Liu 1995), but this system had limitations. The primary disadvantage was that expression of certain enzymes was dependent on developmental and environmental influences, which restricted the number of available markers and the consistency of their polymorphisms.

In the early 1990s, it became possible to analyze grape DNA. The main advantage of the techniques that were developed was that DNA could be obtained from all plant material, in any environment and at any time of year. Restriction fragment length polymorphism (RFLP) analysis was used successfully to detect cultivar specific DNA fingerprints for grapevine and rootstock varieties (Bourquin et al. 1991, 1992, 1993,

1995; Thomas and Scott 1993). However, the RFLP fingerprinting method was limited by the nature of their complex banding patterns, low level of polymorphism in the coding regions of the genome, and time consuming and costly development of probes for analysis. With the advent of PCR technology, RAPD and later AFLP systems became popular in efforts to fingerprint grape cultivars (Jean-Jacques et al. 1993; Moreno et al. 1995; Xu and Bakalinsky 1996; This et al. 1997; Hinrichsen et al. 2000). Finally, the development of co-dominant SSR markers surpassed all other marker systems. The establishment and development of SSR markers was expensive and time consuming because of the construction and screening of the required genomic libraries, prior to design and optimization of PCR primers. However reproducibility, standardization, and transfer and comparison of data among different labs made SSR markers the choice for fingerprinting and cultivar identification.

Thomas and Scott (1993) were the first to report on the use of SSR markers to identify grape cultivars. Their work demonstrated that SSR sequences are abundant in the grape genome and primer sequences are conserved among *Vitis* species and other genera in Vitaceae. These results generated immense interest in grapevine SSR markers, leading to the development of many more markers (Bowers et al. 1996, 1999b; Sefc et al. 1999) culminating in the development of the Vitis Microsatellite Consortium (VMC) consisting of 21 different grape research groups from 12 countries. The VMC effort resulted in development of 333 new markers from SSR enriched genomic libraries. Most of these markers were later used to develop genetic linkage maps (Doligez et al. 2002; Adam-Blondon et al. 2004; Riaz et al. 2004).

Many studies made use of SSR markers to fingerprint and genotype wine, table, raisin grape and rootstock cultivars (Thomas and Scott 1993; Cipriani et al. 1994; Thomas et al. 1994; Botta et al. 1995; Bowers et al. 1996; Sefc et al. 1998a, 1998b, 1998c, 1998d, 1999; Grando and Frisinghelli 1998; Lin and Walker 1998; Bowers et al. 1999; Lefort et al. 2000; Sefc et al. 2000). SSR-based fingerprinting has been used to correct synonyms (Cipriani et al. 1994; Bowers et al. 1996; Sefc et al. 1998a; Lopes et al. 1999; Lefort et al. 2000), detect clonal polymorphism (Riaz et al. 2002), and construct pedigrees for old grape cultivars (Bowers and Meredith 1997; Sefc et al. 1998; Bowers et al. 1999a; Lopes et al. 1999; Meredith et al. 1999; Vouillamoz et al. 2004). There are three public databases that provide information of grapevine genetic fingerprint with SSR markers: the grape microsatellite collection (GMC) database (http://relay.ismaa.it:12164/genetica/gmc.html) was developed to permit an easy retrieval of grape nuclear microsatellite profiles and related information, the Greek Vitis database (http://www.biology.uch.gr/gvd/) contains nuclear as well as chloroplast SSR profiles of Greek grapevine cultivars, rootstocks, *Vitis* species and hybrids used as rootstocks.

2.6 Conclusions and Future Prospects

To date grape improvement has been based on classical breeding and the incorporation of advances in viticulture and enology to optimize vine growth and wine production. However, we are now poised to make rapid advances in grape improvement through the utilization of molecular genetic tools. The development of genomic technologies and their application in other crops like *Arabidopsis* and rice is now providing the necessary tools and comparative information for grape biologists to begin understanding the genetic and molecular basis of pest resistance, tolerance to abiotic stresses, and fruit ripening and quality. The potential of grape genomic research has been recognized by both the public and private sector in many countries of the European Union, Australia, the USA, South Africa, and many other grape growing countries. The coordinated efforts of the Vitis Microsatellite Consortium have generated a large set of SSR markers, which continues to be expanded, refined and utilized. Research groups in many countries are involved in developing genetic linkage maps focused on resistance and tolerance to biotic and abiotic stress and fruit and vine quality. These efforts have resulted in two international reference genetic maps based on SSR markers, and efforts are underway to develop consensus map utilizing populations of different backgrounds. Coordination of these maps will greatly aid researchers to identify set of markers linked to traits of interests (single major genes and QTLs) for use in MAS breeding programs and gene identification efforts.

The next phase of genetic research will be the initiation of the grape genome project to identify key grapevine genes and understand their functions. Grape researchers in Europe, Australia, Canada and

the USA have begun this effort with public and private sector funding. Most of these projects are combining a number of technologies including large-scale EST analysis, BAC libraries, physical map construction, and functional analysis of grape genes in *Arabidopsis*. The development of EST libraries will greatly aid the characterization of genes and allow researchers to study gene expression profiles. Finally, sequencing of the grape genome would be a quantum leap for the grape research community and is critical for the realization of molecular genetics potential on grape and wine production.

2.7 Grape Research Resources on the Web

1. The American Vineyard Foundation (AVF): (http://www.avf.org/).
2. Bioinformatics.Org: (http://bioinformatics.org/). Bioinformatics.Org is a non-profit, academe-based organization committed to opening access to bioinformatics research projects, providing Open Source software for bioinformatics by hosting its development, and keeping biological information freely available.
3. Grapevine Genomics at the Centre for Plant Conservation Genetics: (http://bioinformatics.org/). Grapevine Genome database is a result of a large-scale sequencing project carried out at the Centre for Plant Conservation Genetics.
4. The Cooperative Research Centre for Viticulture (CRCV): (http://www.crcv.com.au/). The Cooperative Research Centre for Viticulture is a joint venture between Australia's viticulture industry and leading research and education organizations
5. Grapevine Breeding and Genetics Program: (http://www.nysaes.cornell.edu/hort/faculty/reisch/grapeinfo.html)
6. CSIRO Plant Industry, Australia - Research Programs: (http://www.csiro.au/). CSIRO applies strategic research in the plant sciences to promote profitable and sustainable agri-food, fiber and horticultural industries, develop novel plant products and improve natural resource management.
7. French Institute for Agrononomical Research: (http://www.inra.fr/gap/departement/especes/vigne.htm). INRA (Institut National de Recherche Agronomique) (*site is in French*)
8. Grapevine Biotechnology at the Institute for Wine Biotechnology (IWBT) (http://academic.sun.ac.za/wine_biotechnology/research_programmes.htm): University of Stellenbosch, South Africa – The IWBT is a member of the "Vitis Microsatellite Consortium" consisting of 20 laboratories world-wide to develop genetic markers, primers and probes for the genetic fingerprinting of *Vitis vinifera* varieties.
9. International Grape Genomics Initiative (http://grapegenomics.ucdavis.edu) – The site (utilizing frames) provides information in the categories: Meetings and Conferences, Grape Experts, Grape Websites, and the Phone Book.
10. Institute for Grapevine Breeding, Geilweilerhof, Germany (http://www.bafz.de/baz99_e/baz_orte/sdg/irz/irz_frmd.htm): The institute's research concentrates on: Development of disease-resistant grapevine varieties in consideration of the wide diversity of varieties in German viticulture; Selection methods to assess characteristics such as resistance to noxious agents, resistance to stress factors (e.g. drought, frost), and the flavor and taste-determining aroma components.
11. International Grape Genome Program (http://www.vitaceae.org/): The primary research focus is grapevine genomics carried out within the framework of the International Grape Genome Program (IGGP).
12. National Clonal Germplasm Repository for Fruit and Nut Crops at Davis, California (http://www.ars-grin.gov/ars/PacWest/Davis/): is one of over two dozen facilities in the National Plant Germplasm System (NPGS) which collect, maintain, characterize, document and distribute plant germplasm from all over the world.
13. Pomology & Viticulture Program at the University of Udine, Italy (http://www.dpvta.uniud.it/arb/Arb_ric.htm#grape): The grape research group manages a grape germplasm repository, which includes wild species, international and local cultivars and breeding lines carrying disease resistance genes.
14. The Institute for Genomic Research (TIGR) (http://www.tigr.org/): The TIGR databases are a collection of curated databases containing DNA and protein sequence, gene expression, cellular role, protein family, and taxonomic data for microbes, plants and humans.
15. Vitis Gene Discovery Program: A Mission to Explore the Genetic Resources of Native North

American Grape Species. (http://mtngrv.smsu.edu/vgdp/). Wild grapes (*Vitis* species) are able to thrive in harsh environments and under high disease and pest pressure conditions. They are natural sources for genes that confer tolerance to adverse biotic and environmental conditions.

2.8 Databases

1. EST Database of Grape from Genomics Facility, College of Agricultural and Environmental Sciences, University of California, Davis. - This database contains an analysis of all public expressed sequence tags (ESTs) from grape. ESTs are grouped as contigs or singletons and analyzed for homology to the NCBI Non-Redundant (NR) database by means of BLASTX.
2. European Network for Grapevine Genetic Resources Conservation and Characterization (http://www.genres.de/vitis/vitis.htm): The database is collection-oriented, i.e. the same cultivar/variety appears in the database as many times as there are participating collections containing it. Data (IPGRI passport data, primary and secondary descriptor data) refer to an individual accession (cultivar) only.
3. Grape Microsatellite Collection (GMC) – A web-backed database of genotypes at SSR loci obtained from IASMA analysis and literature. GMC is a database developed to permit an easy retrieval of grape nuclear microsatellite profiles and related information. Each record has 8 fields: locus (name of the locus), allele 1 and allele 2 (allele size in bp), cultivar (name of the accession) and finally 3 fields providing information about authors, references and fragment analysis method of collected data.
4. Grapevine Genome Database (http://www.scu.edu.au/research/cpcg/genomics/index.php): The Grapevine Genome database is a result of a large-scale sequencing project carried out at the Centre for Plant Conservation Genetics. A number of objectives were achieved including the development of SSR markers from grape ESTs, micropropagation of table and wine grape varieties and an analysis of the grape genome based on 5000 EST sequences.
5. The Greek Vitis Database (http://www.biology.uch.gr/gvd/contents/index.htm): A multimedia web-backed genetic database for germplasm management of *Vitis* resources in Greece. By Francois Lefort and Kalliopi A. Roubelakis-Angelakis, Laboratory of Plant Physiology and Biotechnology, Department of Biology, University of Crete, Haralson, Crete, Greece.
6. TIGR *Grape* Gene Index (VvGI) (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=grape): The TIGR *Grape* Gene Index integrates research data from international *Grape* EST sequencing and gene research projects. The ultimate goal of the TIGR Gene Index projects, including VvGI, is to represent a non-redundant view of all *Grape* genes and data on their expression patterns, cellular roles, functions, and evolutionary relationships.
7. Vitis International Variety Catalogue (http://www.genres.de/idb/vitis/): All available information has been condensed for each cultivar/variety, i.e. each variety makes a single data set. Data (IPGRI passport data, bibliography, morphological and resistance characteristics

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3 Cherry

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3.1 Introduction

The cherry is one of the most popular temperate fruit crops despite of its relatively high price. The fruits are attractive in appearance, because of their bright, shiny skin color, and their subtle flavor and sweetness are appreciated by most consumers. Compared to other temperate fruits, such as apple and peach, breeding improvements for cherries have been slow. The long generation time and the large plant size of cherry trees severely limit classical breeding. Thus, the integration of molecular markers in breeding programs should be a powerful tool. Only a few genetic linkage maps are available for sweet or sour cherry and quantitative trait loci (QTLs) were reported only for sour cherry. Until now, most of the efforts were concentrated on the use of molecular markers in order to (i) identify the *S*-alleles controlling gametophytic self-incompatibility, (ii) characterize cultivars, and (iii) assess genetic diversity.

3.1.1 Brief History of the Crop

Prunus avium L. includes sweet cherry trees cultivated for human consumption and wild cherry trees used for their wood, also called mazzards (Webster 1996). The sweet cherry is indigenous to parts of Asia, especially northern Iran, Ukraine, and countries south of the Caucasus mountains. In Europe, the Romanian and Georgian wild cherry trees appeared to be very differentiated from those of central and western Europe (Tavaud 2002). The Georgian wild cherry trees were the most genetically diverse suggesting that this area could have been a main glacial refuge. The ancestors of the modern cultivated sweet cherries are believed to have originated around the Caspian and Black Seas, from where they have slowly spread. This phenomenon was driven initially by birds. Sweet cherries are now cultivated commercially in more than 40 countries around the world, in temperate, Mediterranean, and even subtropical regions. Its natural range covers the temperate regions of Europe, from the North part of Spain to the Southeastern part of Russia (Hedrick et al. 1915). They prefer regions with warm and dry summers, but require adequate rainfall or irrigation during the growing season for production of fruit with appropriate size for marketing. Rainfall at harvest time may reduce the commercial potential of the production by inducing fruit cracking.

Fruit of *Prunus cerasus* L., the sour cherry tree, are mainly used for processed products such as pies jam or liquor. Sour cherry originated from an area very similar to that of sweet cherry, around the Caspian Sea and close to Istanbul. While sour cherry is less widely cultivated than sweet cherry, large quantities of sour cherries are produced in many European countries and in the USA. Most of these are used in processing and processed cherry products are sold worldwide.

Prunus fruticosa Pall., the ground cherry tree, is sometimes used as rootstocks for other *Prunus* species. This species is widespread over the major part of central Europe, Siberia and Northern Asia (Hedrick et al. 1915).

The duke cherries, which result from crosses between *P. avium* and *P. cerasus*, are cultivated at a much smaller scale. Different names have been given to this species like *Prunus acida* Dum, *Cerasus regalis*, *Prunus avium* ssp. *regalis*, but the name used today is *P. x gondouinii* Rehd. (Faust and Suranyi 1997; Saunier and Claverie 2001). Duke cherry trees are intermediate for their tree and fruit characteristics compared to their progenitors.

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3.1.2 Botanical Descriptions

All cherry species belong to the *Cerasus* subgenus of the *Prunus* genus, part of the *Rosaceae* family. The majority of cultivated cherry trees belong to *Prunus avium* L. and *Prunus cerasus* L. species. Together with *Prunus fruticosa* Pall., these species and their interspecific hybrids constitute the *Eucerasus* section of the *Cerasus* subgenus, based on morphological criteria (Rehder 1947; Krussmann 1978). This classification and the monophyletic origin of the *Eucerasus* clade have been confirmed by chloroplast DNA variation analysis (Badenes and Parfitt 1995).

A large amount of morphological polymorphism is observed among *P. avium*, *P. fruticosa* and *P. cerasus* species. Multivariate analysis on sour cherry revealed continuous variation between the *P. avium* and *P. fruticosa* traits throughout the geographic distribution of the species. In Western Europe, *P. cerasus* trees look like *P. avium* whereas in Eastern Europe, *P. cerasus* is closer to *P. fruticosa* (Hillig and Iezzoni 1988; Krahl et al. 1991). This continuum of morphological characteristics makes the species assignation difficult when considering only phenotypic traits. The sweet cherry is a deciduous tree of large stature, occasionally reaching almost 20 meters in height, with attractive peeling bark. The sour cherry is a small tree, or more often a deciduous bush, which suckers profusely from the base. It has smaller leaves and flowers than the sweet cherry. Concerning the fruits, sweet cherries fruits are usually split into three groups: Mazzards, often wild types with small inferior fruits of various shapes and colors: Guignes, Hearts or Geans, with soft-fleshed fruits and the Bigarreaux with hard-fleshed, heart-shaped, light-colored fruits. Sour cherries cultivars are generally classified as Amarelles (or Kentishand) and as Griottes (or Morellos). Amarelles have pale red fruits flattened at the ends and uncolored juice. Griottes have, in contrast, dark spherical fruits and dark-colored juice. A third group of sour cherry cultivars, called Marasca, are characterized by small, very black-red colored and bitter fruit whose juice is of the best quality for making maraschino liquor. Marasca cultivars are sufficiently distinct to have been classified by early botanists as a subspecies of *P. cerasus* (*Prunus cerasus* Marásca (Reichb.) Schneid, Redhder 1947).

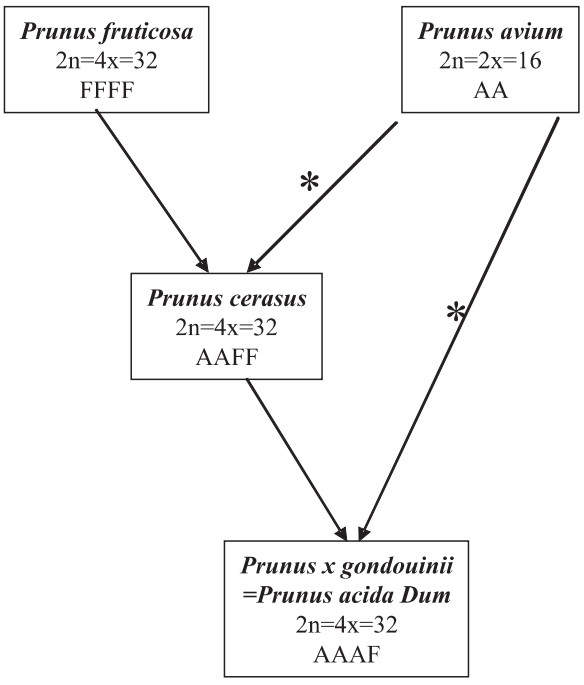

Fig. 1. Relationships and genome constitution among the species of the Eucerasus section. * *P. avium* is thought to produce diploid gametes. A and F are haploid genomes coming from *P. avium* and *P. fructicosa* respectively

3.1.3 Genome Contents

Prunus avium has a diploid genome (AA, $2n = 2x = 16$) and small haploid genome size (338 Mb) (Arumuganathan and Earle 1991) bigger than the genome of peach (290 Mb) which is the smallest *Prunus* genome evaluated to date.

Prunus fruticosa, the ground cherry tree, is a tetraploid wild species ($2n = 4x = 32$) believed to be (FFFF). The genome size is still unknown.

Prunus cerasus is an allotetraploid species (AAFF, $2n = 4x = 32$), with a genome size of 599 Mb, supposed to result from natural hybridization between *P. avium* (producing unreduced gametes) and *P. fruticosa* (Fig. 1). This origin was first suggested by Olden and Nybom (1968) who observed that artificial hybrids between tetraploid *P. avium* and *P. fruticosa* were very similar to *P. cerasus*. Isozyme analysis, genomic in situ hybridization and karyotype analysis further confirmed the hybrid origin of *P. cerasus* (Hancock and Iezzoni 1988; Santi and Lemoine 1990; Schuster and Schreiber 2000). The patterns of inheritance of seven isozymes in different crosses of sour cherry indicated that *P. cerasus* might be a segmental allopolyploid (Beaver and Iezzoni 1993; Beaver et al. 1995).

Studies based on cpDNA markers detected two distinct chlorotypes in *P. cerasus* which strongly suggest that crosses between *P. avium* and *P. fruticosa* have occurred at least twice to produce sour cherry (Badenes and Parfitt 1995; Iezzoni and Hancock 1996; Brettin et al. 2000). Moreover, these works showed that, most of the time, *P. fruticosa* was the female progenitor of *P. cerasus,* but in few cases, *P. avium* was the female parent due to the formation of unreduced ovules. Tavaud et al. (2004) demonstrated that specific alleles in *P. cerasus* were not present in the A genome of *P. avium* and probably came from the F genome of *P. cerasus*. Recent analysis with cpDNA and microsatellite markers show that some *P. cerasus* share the same chloroplastic haplotype as some *P. fructicosa*, and that some microsatellite markers are shared by both species (A. Horvath, personal communication). Triploid hybrids through the fusion of normal gametes of *P. avium* and *P. fruticosa* occur naturally but remain sterile. Due to this sterility and many unfavorable *P. fruticosa* traits, these triploids are not clonally propagated by humans (Olden and Nybom 1968).

P. x gondouinii Rehd is an allotetraploid (AAAF, $2n = 4x = 32$) species stemming from the pollinization of sour cherry by unreduced gametes of sweet cherry (Iezzoni et al. 1990). These hybrids are often sterile, due to disturbances during meiosis, but they are clonally propagated by human.

3.1.4 Economic Importance

Worldwide, 375,000 Ha of sweet cherry and 248,000 Ha of sour cherry are cultivated giving a total production of 1,896,000 Mt and 1,035,000 Mt respectively (FAO 2005). The main production areas in the world for sweet and sour cherries are located in Europe (953,000 Mt and 711,000 Mt), Asia (653,000 Mt and 208,000 Mt) and North America (228,000 Mt for sweet cherry and 115,000 Mt for sour cherry) (FAO 2005). However, a huge increase in sweet cherry hectares in production occurred 10 years ago in the Southern hemisphere especially in Chile and Argentina. In Chile, the cultivated area increased by four times in two years and nearly all the production is exported to the USA and Europe. In the Northern hemisphere, sweet cherry production is mainly located in Europe but major shifts are occurring in European production. France which was one of the main producers in Europe (100 to 120,000 tons) reduced its production by two in 2003 and 2004 (57,000 tons), and at the same time Spain doubled its production, especially with early maturing varieties. In the next following years, Turkey may become the leading world producer of sweet cherries.

3.1.5 Breeding Objectives

The main breeding objectives for sweet cherry are:

- large, attractive and good-flavored fruits,
- short juvenile phase,
- large and constant yields,
- reduced susceptibility to fruit cracking,
- self-compatibility,
- improved resistance or tolerance to diseases, especially bacterial canker induced by *Pseudomonas* mors pv. *prunorum* and *P. syringae*.

Regular yields and superior fruit quality are the two main objectives of sour cherry breeding programs. Breeding for disease resistance in sour cherry is concentrated on resistance to cherry leaf spot caused by *Blumeriella japii.*

Yields per hectare vary by the country of production, the commercial use (for fresh market or for industry) and the training system. The average yield ranges from 8 to 10 t/ha in classical orchards but can reach 30 to 40 t/ha for an intensive industrial orchard. The highest limitation to the development of the cherry culture is the high cost required to manually pick the fruit as manual picking can account for 70% of the production price. The yield of the pick up can be 6 to 8 Kg/ha and by person in a traditional orchard and can be 30 Kg/ha in intensive orchards. Several breeding programs led to the selection of new varieties that can be harvested partially with machines, such as 'Sweetheart' and 'Van' cultivars that can be harvested without the stem. In the same time, a better knowledge of the architecture of the tree led to new ways of orchards training.

Thanks to classical breeding programs, a large number of cultivars are now available. Within the last 10 years, 20 new varieties are gaining wide interest internationally such as 'Earlise' (early season), 'Summit' (middle season) and 'Sweetheart' (late season). Each of them should be widely cultivated in the next 15 to 20 years.

Classical breeding programs are time consuming, especially for cherry that requires a minimum of 3–5 years of growth before flowering and fruit pro-

duction. Prior knowledge of linkage relationships between marker loci and important flower and fruit characteristics will facilitate and shorten the selection of promising individuals. Consequently, marker-assisted selection would be especially beneficial for sweet and sour cherry breeding.

3.2 Construction of Genetic Maps

The construction of genetic maps is useful for localisation of important genes controlling both qualitative and quantitative traits in numerous plant species and, then, for improving and shortening breeding selection (Tanksley et al. 1989). In *Prunus*, many mapping studies were done on peach (Belthoff et al. 1993; Chaparro et al. 1994; Rajapakse et al. 1995; Dirlewanger et al. 1998; Lu et al. 1998; Dettori et al. 2001; Yamamoto et al. 2001) or on interspecific crosses between peach and other *Prunus* species (Foolad et al. 1995; Joobeur et al. 1998; Jauregui et al. 2001; Bliss et al. 2002; Dirlewanger et al. 2004a; Quilot et al. 2004). An highly saturated linkage map including 562 markers, based on segregation analyses of an almond (cv. 'Texas') × peach cv. ('Earlygold') F_2 population serves as a reference map for the *Prunus* scientific community (Dirlewanger et al. 2004b). Several genetic linkage maps were also obtained for other *Prunus* such as almond (Viruel et al. 1995; Joobeur et al. 2000) and apricot (Hurtado et al. 2002; Lambert et al. 2004). Despite the potential usefulness of genetic linkage maps for sweet or sour cherry, saturated cherry linkage maps have not yet been constructed.

In the subgenus *Cerasus*, several maps have been published using five segregating populations (Table 1). Until now, only partial maps for sweet or sour cherry are available. The earliest of them was constructed in a sweet cherry using random amplified polymorphic DNA (RAPD) and allozyme analysis of 56 microspore-derived callus culture individuals of the cv. 'Emperor Francis' (Stockinger et al. 1996). Two allozymes and 89 RAPD markers were mapped to 10 linkage groups totalling 503 cM. Interestingly, another map integrating isozyme genes exclusively, was obtained using data from two interspecific F_1 cherry progenies: *P. avium* 'Emperor Francis' × *P. incisa* E621 and *P. avium* 'Emperor Francis' × *P. nipponica* F1292 (Bošković and Tobutt 1998). This map, one of the most exhaustive ever made with isozyme markers in the Plant Kingdom, included a total of 47 segregating isozyme genes, from which 34 were aligned into seven linkage groups.

Another genetic linkage map is in progress in the INRA of Bordeaux (France) for sweet cherry using an intraspecific F_1 progeny including 133 individuals from a cross between cultivars 'Regina' and 'Lapins'. These cultivars were chosen as parents for their distinct agronomic characters and especially because they differ for resistance to fruit cracking which is a limiting factor in sweet cherry production. 'Regina' is resistant and 'Lapins' is susceptible to fruit cracking. 'Lapins' is a self-compatible cultivar as opposed to 'Regina'. Moreover, they differ for several other characters: blooming and maturity dates, peduncle length, and fruit color, weight, firmness, titratable acidity and refractive index. Preliminary maps of each parent and their comparison with the referenced *Prunus* map 'Texas' × 'Earlygold' (T×E) is described by Dirlewanger et al. (2004b). These maps include microsatellite markers, 30 of which are located in the 'Régina' map are anchors marker with T×E map, 28 located in the 'Lapins' map are anchors with T×E map. Only one non-collinear marker was detected but for all other markers the location in the maps were in the homologous linkage group. These results are in agreement with the high level of synteny among the *Prunus* genus (Arús et al. 2005). The two sweet cherry maps will be used for detection of QTLs involved in fruit quality as soon as the progeny produces fruits, in 2006.

A sweet cherry genetic linkage map is also in progress at Michigan State University (US) from a F_1 progeny from a cross between a wild forest cherry with small (~2 g) highly acidic dark-red colored fruit (NY54) and a domesticated variety with large (~6 g), yellow/pink, sub-acid fruit 'Emperor Francis' (EF). The F_1 population is composed of approximately 700 individuals, 200 of them will be used for map construction and initial QTL analysis. The remaining progeny will be used for fine mapping major QTL identified. The objective of the study is to identify QTLs that control fruit quality traits that have been improved during domestication. In addition, this cross is fully compatible and progeny segregation for the *S*-locus fits the expected 1:1:1:1 ratio (Ikeda et al. 2005). This population will be used to fine map the *S*-locus region due to the large family size and the absence of skewed segregation that exists in many of the *Prunus* mapping populations.

Table 1. Cerasus linkage maps

Population	Type (nb. of individuals)	Nb. of markers in the map	Marker type	Linkage groups	Total distance (cM)	Longest gap (cM)	Unlinked markers	References
P. avium 'Emperor Francis'	Microspore-derived calli	89	RAPD (90), isozyme (2)	10	503	27	3	Stockinger et al. 1996
P. avium 'Napoleon' × ***P. incisa*** E621	F1 (63)	34	Isozymes	7	174 r.u.[1]	24 r.u.	13	Bošković and Tobutt 1998
P. avium 'Napoleon' × ***P. nipponica*** F1292	F1 (47)							
P. avium 'Régina' (R) × 'Lapins' (L)	F1 (133)	R: 68 L: 54	SSRs	11 9	639 495	26 30	1 10	Dirlewanger et al. 2004b
P. avium NY54 × 'Emperor Francis'	F1 (200)	in progress						Iezzoni 2004
P. cerasus 'Rheinische Schattenmorelle' ('RS') × 'Erdi Botermo' ('EB')	F1(86)	RS: 126 EB: 95 Consensus: 160	RFLPs RFLPs RFLPs (144) SSRs (16)	19 16 19	461 279 442	19 20 17	17 23	Wang et al. 1998 Canli 2004a

[1] Distance is measured in recombination units (r.u.)

In sour cherry, linkage maps were constructed at Michigan State University (US) from 86 individuals from the cross of two cultivars; 'Rheinische Schattenmorelle' (RS) and 'Erdi Botermo' (EB). Since sour cherry is a tetraploid, informative restriction fragment length polymorphisms (RFLPs) were scored as single-dose restriction fragments (SDRF) according to Wu et al. (1992). A genetic linkage map was constructed for RS that consists of 126 SDRF markers assigned to 19 linkage groups covering 461 cM (Wang et al. 1998). The EB linkage map had 95 SDRF markers assigned to 16 linkage groups covering 279 cM (Wang et al. 1998). Due to the limited number of shared markers between the RS × EB map compared to other *Prunus* maps, putative homologous linkage groups could only be identified in for the *Prunus* LGs 2, 4, 6, and 7. The other linkage groups were arbitrarily numbered from the longest to shortest and therefore the sour cherry linkage groups numbers have not been rigorously aligned with that of the *Prunus* consensus map. The RS × EB population was subsequently scored using 10 *Prunus* microsatellite primer pairs (Canli 2004a) and a consensus map of 442 cM, less than the previously reported RS map of 461 cM, was constructed. A total of 16 microsatellite markers were added to 10 of the 19 linkage groups; however, the linkage groups were not re-numbered to reflect these markers. In addition, four of the microsatellite primer pairs identified duplicate linked markers. This "double mapping" of a marker is due to the inclusion of progeny individuals exhibiting tetrasomic inheritance for that linkage group. If this correction had been done by Canli (2004a), it is likely that the number of microsatellite markers added to the map would be reduced to twelve.

The difficulty of identifying SDRFs and eliminating progeny that resulted from non-homologous pairing for the linkage group under study, illustrate the complexity of linkage mapping in a segmental allopolyploid. Therefore, future work at Michigan State University will concentrate on linkage map construction in the diploid sweet cherry.

3.3 Gene Mapping and QTLs Detected

In sour or sweet cherries most of the agronomically important traits have complex inheritance. Only self-incompatibility (SI) is controlled by a single locus (*S*) with multiple alleles, and fertilization only takes

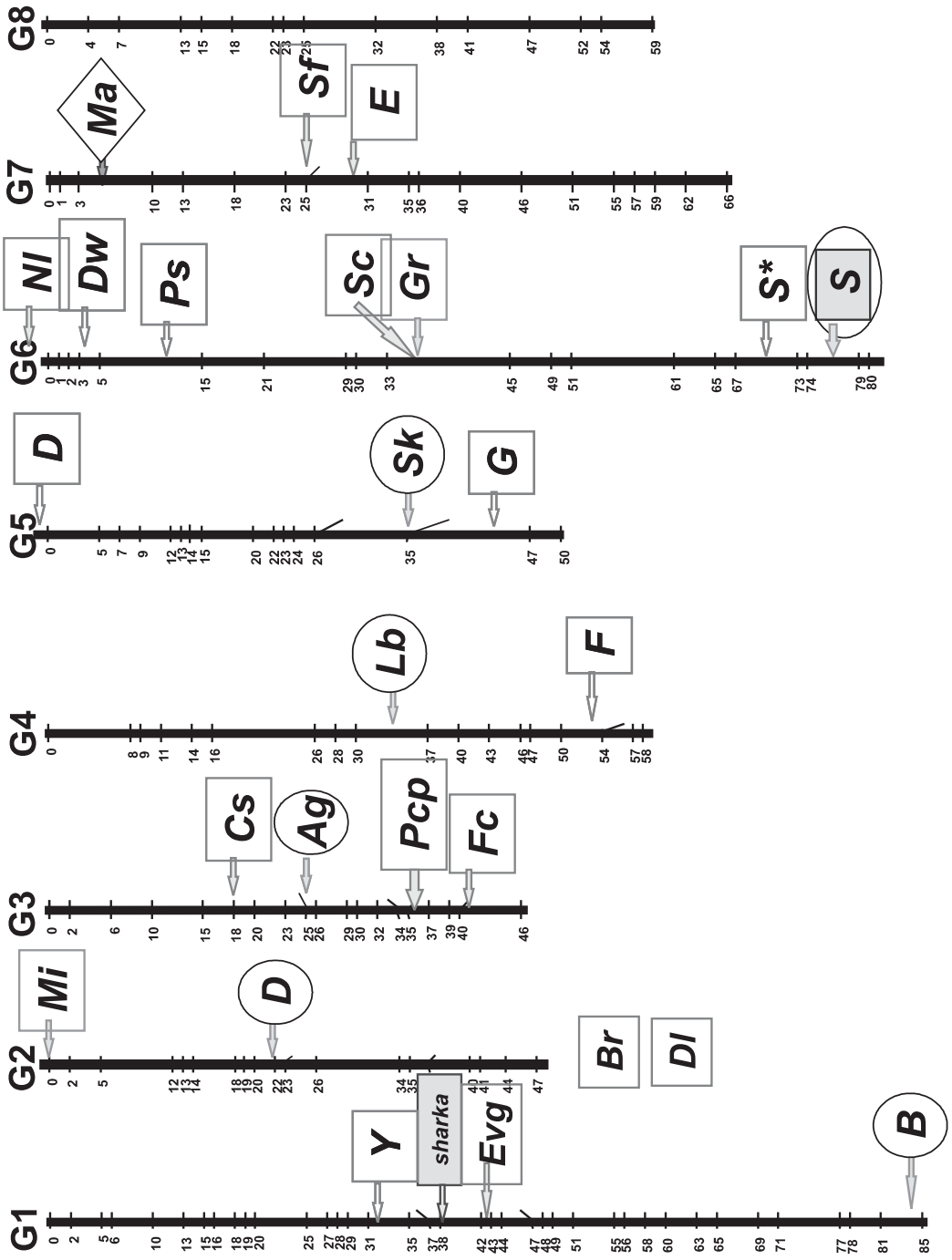

Fig. 2. Approximate position of 28 major genes mapped in different populations of apricot (gray background), peach (square), almond or almond × peach (ring), and Myrobalan plum (rhombus) on the framework of the *Prunus* reference map (Dirlewanger et al. 2004b). Gene abbreviations correspond to: *Y*, peach flesh color; *B*, almond/peach petal color; *sharka*, plum pox virus resistance; *B*, flower color in almond × peach; *Mi*, nematode resistance from peach; *D*, almond shell hardness; *Br*, broomy plant habit; *Dl*, double flower; *Cs*, flesh color around the stone; *Ag*, anther color; *Pcp*, polycarpel; *Fc*, flower color; *Lb*, blooming date; *F*, flesh adherence to stone; *D*, non-acid fruit in peach, *Sk*, bitter kernel; *G*, fruit skin pubescence; *Nl*, leaf shape; *Dw*, dwarf plant; *Ps*, male sterility; *Sc*, fruit skin color; *Gr*, leaf color; *S**, fruit shape; *S*, self-incompatibility (almond and apricot); *Ma*, nematode resistance from Myrobalan plum; *E*, leaf gland shape; *Sf*, resistance to powdery mildew. Genes *Dl* and *Br* are located on an unknown position of G2

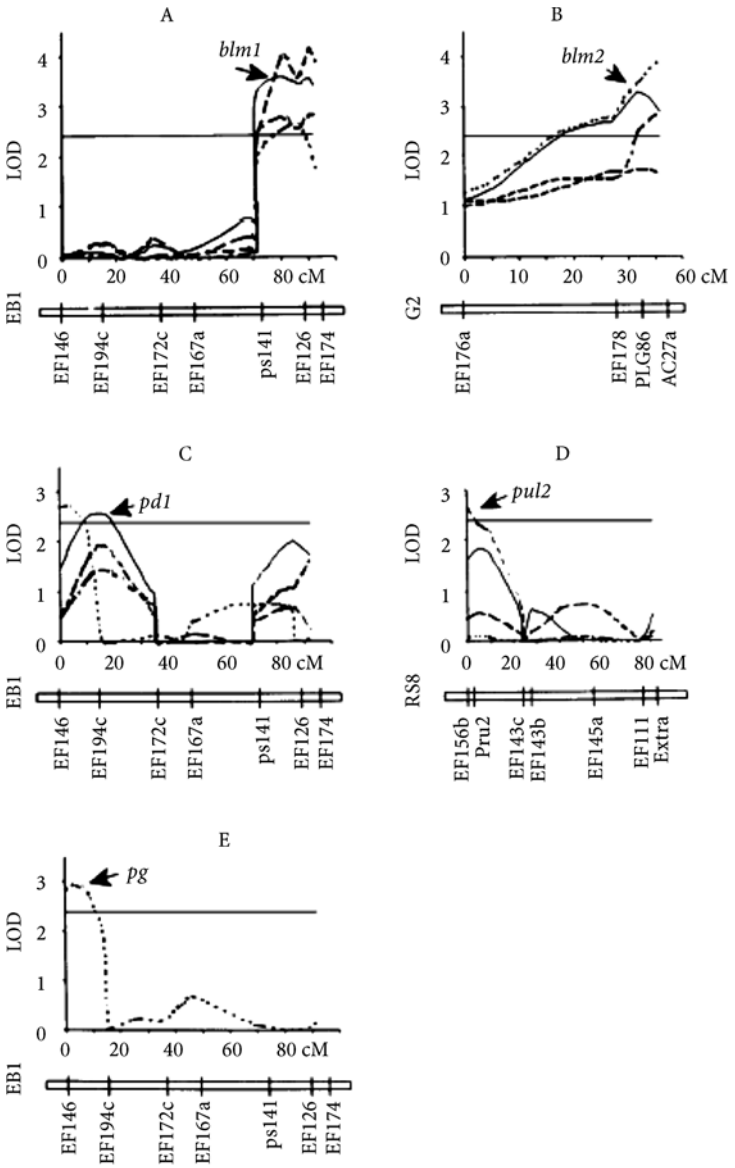

Fig. 3. QTLs detected for flower and fruit traits in sour cherry (Wang et al. 2000). LOD scores for bloom date on linkage groups EB 1 (*blm*1) (**A**) and Group 2 (*blm*2) (**B**); pistil death (*pd*) on linkage groups EB 1 (**C**) and RS 8 (**D**); pollen germination percentage (*pg*) on linkage group EB 1 (**E**). Peak LOD scores for each trait are indicated by *arrows*. Linkage groups are shown below the *x-axes*. The *horizontal line* indicates the level of significance at LOD = 2.4. *Curves* represents results from individual years of 1995 (· · ·), 1996 (- - -), 1997 (– –) and over years (—)

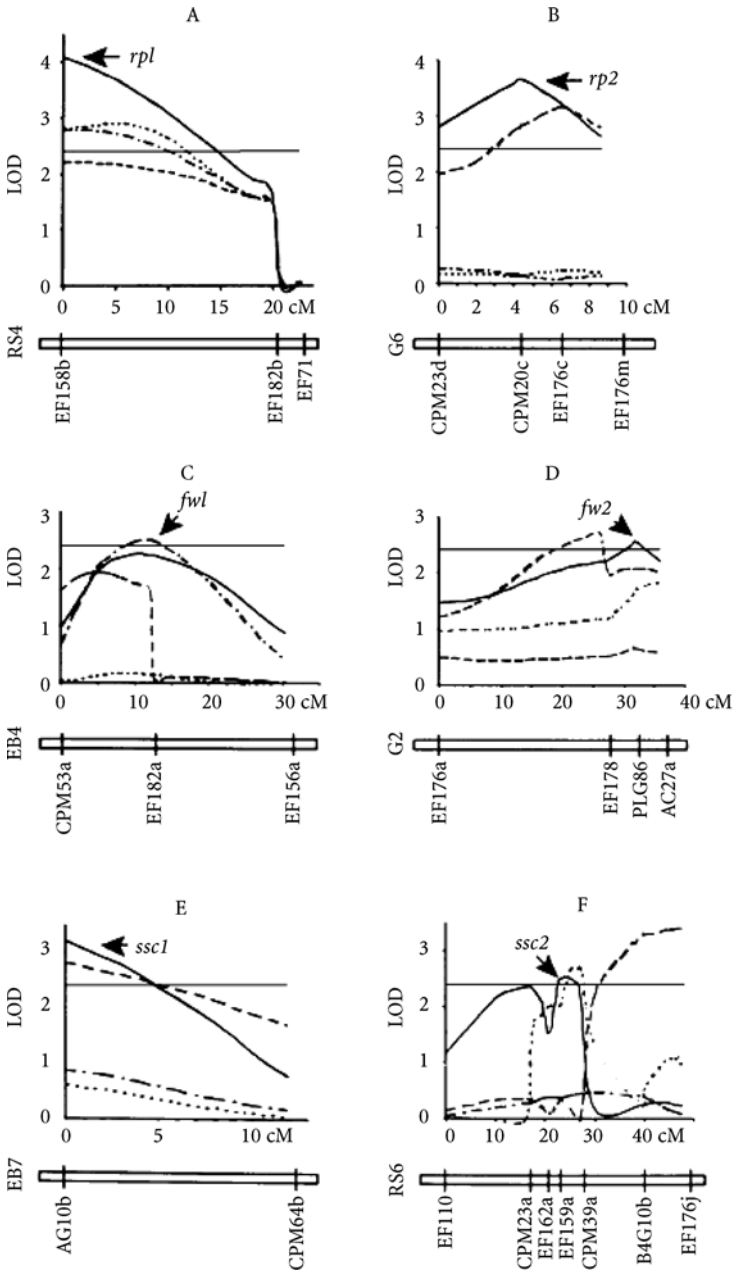

Fig. 3. (continued) LOD scores for ripening date on linkage groups RS 4 (*rp1*) (**A**) and Group 6 (*rp2*) (**B**); fruit weight on linkage groups EB 4 (*fw1*) (**C**) and Group 2 (*fw2*) (**D**); soluble solids concentration on linkage groups EB 7 (*ssc1*) (**E**) and RS 6 (*ssc2*) (**F**)

place when the *S* allele in the haploid genome of the pollen is different from the two *S* alleles in the diploid tissue of the style. In contrast, blooming and ripening time, flower bud and pistil death and characters controlling fruit quality are quantitative traits. The self-incompatibility locus is located in the distal part of the linkage group 6 in almond (Ballester et al. 1998; Bliss et al. 2002) and in apricot (Vilanova et al. 2003) on the same area (Fig. 2; Dirlewanger et al. 2004b). According to the high level of synteny within *Prunus* (Arús et al. 2005), the gene S may be located on the same place in cherry.

Linkage relationships between molecular markers and agronomically important quantitative traits have been extensively studied in many tree fruit crops. In peach many QTLs involved in fruit quality (Dirlewanger et al. 1999; Etienne et al. 2002; Quilot et al. 2004) and diseases resistance (Quarta et al. 1998; Viruel et al. 1998; Foulongne et al. 2003) have been reported. However, the only QTL study published to date in cherry is a QTL analysis of flower and fruit traits using the sour cherry RS × EB linkage mapping population (Wang et al. 1998). Eleven QTLs (LOD > 2.4) were identified for six traits (bloom time, ripening time, % pistil death, % pollen germination, fruit weight, and soluble solids concentration) (Wang et al. 2000, Fig. 3). The percentage of phenotypic variation explained by a single QTL ranged from 12.9% to 25.9% (Wang et al. 2000). Subsequently, three microsatellite markers were identified that mapped within the putative location of the previously described QTLs (Wang et al. 2000) for bloom time (*blm2*), pistil death (*pd1*) and fruit weight (*fw2*), respectively (Canli 2004a). Unfortunately these three microsatellite markers were not used in QTL analyses to determine their location relative to the previously published QTLs.

The identification of bloom time QTL is of particular interest for cherry breeding as the development of new cultivars with late bloom would significantly reduce the probability of spring freeze damage to the pistils (Iezzoni 1996). Sour cherry exhibits extreme diversity for bloom time with many cultivars blooming exceedingly late in the spring (Iezzoni and Hamilton 1985; Iezzoni and Mulinix 1992). This late bloom character in sour cherry is likely due to the hybridization and continued introgression with the very late blooming ground cherry, *P. fruticosa*.

Bloom time in cherry is a quantitative trait; however its high broad sense heritability (0.91) led to the identification of two bloom time QTL, *blm*1 and *blm*2, in the RS × EB population (Wang et al. 2000). Unfortunately the genetic effects of these two QTL alleles from EB were to induce early bloom. To identify QTL with alleles conferring late bloom time, a second mapping population between the mid-season blooming 'Balaton®' and late blooming 'Surefire' was developed at Michigan State University (US). The population exhibited transgressive segregation for bloom time permitting a bulked segregant approach to identify markers linked to bloom time QTL (Bond 2004). To date, a third QTL for late bloom, named *blm*3, was identified using AFLP markers that is significantly associated with late bloom using Single Marker QTL analysis (Bond 2004). This QTL allele is present in Surefire and confers late bloom time. We are in the process of determining the linkage map location of this QTL. Using this same mapping population, two AFLP markers were identified that differed between the early and late bulks (Canli 2004b). However these markers were never scored on the 'Balaton' × 'Surefire' progeny population and the marker results described could not be repeated.

3.4 Marker-Assisted Breeding for Self-Incompatibility and Molecular Cloning

3.4.1 Self-Incompatibility

Sweet cherry, like in other Rosaceae species, operates a strict self-incompatibility system that has been naturally selected to promote out-breeding (De Nettancourt 2001). This mechanism avoids the fertilization of flowers of one genotype by its own pollen. As a consequence, commercial fruit set in this species depends upon the presence of other compatible pollinating genotypes or on the introduction of self-compatible cultivars. In sour cherry, self-incompatible as well as self-compatible genotypes have been identified (Lansari and Iezzoni 1990; Yamane et al. 2001; Hauck et al. 2002). Sour cherry is a tetraploid hybrid of diploid sweet cherry and tetraploid ground cherry, and thus the self-incompatibility mechanism seems to be conserved only in some genotypes.

The type of self-incompatibility operating in the Rosaceae is called gametophytic self-incompatibility (GSI) (De Nettancourt 2001), and it is shared by

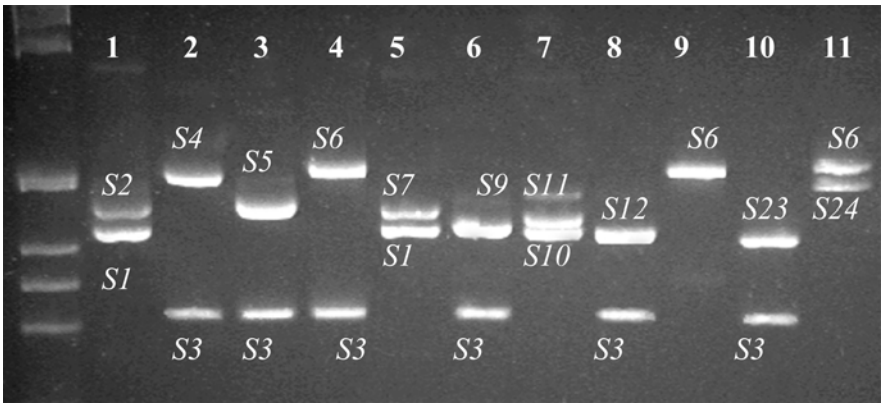

Fig. 4. PCR amplification with primers PruT2-SI32, of cultivars: *1*: Summit (S_1S_2); *2*: Bing (S_3S_4); *3*: Hedelfingen (S_3S_5); *4*: Hartland (S_3S_6); *5*: Charger (S_1S_7); *6*: Burlat (S_3S_9); *7*: Orleans 171 ($S_{10}S_{11}$); *8*: Schneiders (S_3S_{12}); *9*: Noble (S_6S_{13}); *10*: Vittoria (S_3S_{23}); *11*: Pico Colorado (S_6S_{24})

other plant families like the Solanaceae and Scrophuliaraceae. Self-incompatibility has been extensively studied at the molecular level (Kao and Tsukamoto 2004). It is now known that GSI is controlled by different genes of one polymorphic locus (S) that determine the incompatibility response of the pollen and the style (McCubbin and Kao 2000). The incompatibility phenotype of the style in sweet and sour cherry is determined by a ribonuclease called *S*-RNase (Boskovic and Tobutt 1996; Tao et al. 1999c; Yamane et al. 2001) and the specificity of the pollen is now believed to be determined by the product of the recently identified F-box gene *SFB* (Yamane et al. 2003; Ikeda et al. 2004a; Ushijima et al. 2004). These two factors would interact in an allele specific manner to give rise to the self-incompatibility reaction. The mechanism of this reaction is such that the growth of the pollen tube is inhibited in the style when the *S*-allele of the pollen factor matches either of the two *S*-alleles of the *S*-RNases expressed in the diploid style tissue. Several models have been proposed to explain in which manner these factors mediate to produce the incompatibility reaction (Luu et al. 2001; Kao and Tsukamoto 2004; Ushijima et al. 2004). In sour cherry there is evidence that a similar mechanism takes place to inhibit the growth of pollen tubes, but self-compatibility seems to be caused by different mutations in each genotype, either in the *S*-RNase, in SFB or in additional factors involved in the reaction (Hauck et al. 2002). The progress made in the knowledge of the genetic and molecular basis of the self-incompatibility reaction has allowed the application of molecular techniques in two main aspects of sweet cherry breeding, the identification of cross-compatible combinations of different varieties by the identification their S-alleles and the selection of self-compatibility.

3.4.2 S-Allele Typing

Self-incompatibility in sweet cherry prevents inbreeding but the same mechanism also prevents cross-pollination among varieties with the same *S*-alleles. This situation makes it necessary to know the *S*-haplotypes of each variety to be able to establish which cultivar combinations are compatible and, thus, to select which varieties can be inter-planted. Varieties with the same incompatibility alleles and, therefore, cross-incompatible, form an incompatibility group. Until the molecular basis of self-incompatibility were known, *S*-allele typing and incompatibility group assignment was carried out by controlled pollinations followed by the recording fruit set (Crane and Brown 1937; Matthews and Dow 1969) or by the observance of pollen tube growth in the style by fluorescent microscopy. Since the style *S*-factor in GSI was known to be a ribonuclease in Solanaceae (McClure et al. 1989), it was possible to identify *S*-alleles in sweet cherry by correlating known *S*-alleles with bands obtained from stylar proteins separated by isoelectric focusing and stained for ribonuclease activity (Boskovic and Tobutt 1996). Subsequently different bands were correlated to new incompatibility alleles (Boskovic et al. 1997).

The cloning and sequence characterization of the *S-RNases* of sweet cherry (Tao et al. 1999a, b) allowed the development of PCR and RFLP based methods to type the sweet cherry *S*-alleles. Tao et al. (1999c) developed an *S*-allele typing method based in the utilization of two pairs of PCR primers, designed in the conserved regions of the sweet cherry *S-RNase* sequences. These *S-RNase* sequences have two introns varying in length for each different allele and, consequently, PCR amplification with those primers allows to distinguish the different *S*-alleles according to the

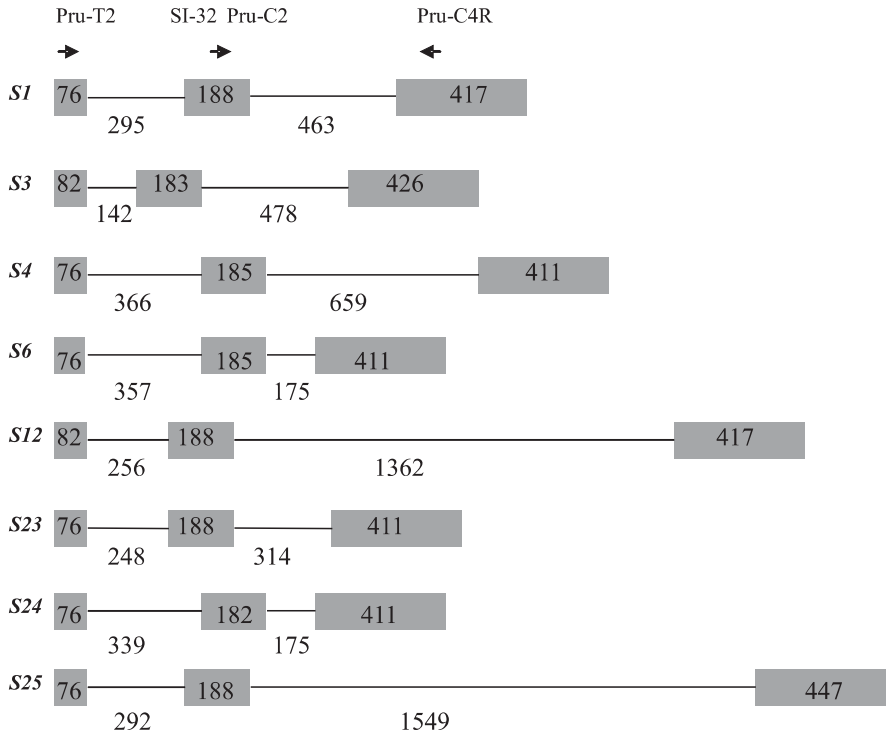

Fig. 5. Schematic representation of genomic DNA of 8 sweet cherry *S*-RNases. *Boxes* represent exons, *lines* represent introns and *arrows* represent PCR primers. PCR primers shown Pru-T2, Pru-C2 and Pru-C4R from Tao et al. (1999c), SI-32 from Wiersma et al. (2001)

size of the amplified fragments (Figs. 4 and 5). Subsequently, other sweet cherry *S*-RNases were cloned and other PCR methods based in conserved sequence primers (Wiersma et al. 2001), allele specific primers (Sonneveld et al. 2001; Sonneveld et al. 2003), and PCR followed by restriction fragment analysis (Yamane et al. 2000b) have been developed. Simultaneously RFLP profiles have also been used to assign self-incompatibility alleles to different sweet cherry genotypes (Hauck et al. 2001). The introduction of molecular methods in sweet cherry *S*-allele typing has allowed a rapid confirmation of the *S*-alleles and incompatibility groups of different cultivars reported previously, the identification of the *S*-genotype of new varieties and the identification of putative new *S* alleles by their correlation with new PCR products (Table 2; Tao et al. 1999; Yamane et al. 2000a, b; Hauck et al. 2001; Sonneveld et al. 2001; Wiersma et al. 2001; Choi et al. 2002; Zhou et al. 2002; Sonneveld et al. 2003; Wunsch and Hormaza 2004a, c, d; De Cuyper et al. 2005; Iezzoni et al. 2005).

3.4.3 Self-Compatibility

The use of self-compatible varieties in sweet cherry orchards can avoid some of the problems derived from self-incompatibility, such as the cost derived from the need to use pollinator varieties and a more erratic production (Teherani and Brown 1992). As a consequence, obtaining and introducing self-compatible varieties has been one of the main objectives of sweet cherry breeding (Brown et al. 1996). Self-compatibility was induced in sweet cherry by X-radiation, giving rise to several self-compatible seedlings (Lewis 1949). The variety 'Stella' (Lapins 1970), descendent of one of these seedlings (JI2420), is self-compatible and has been widely used as a progenitor in self-compatible sweet cherry breeding. Most of the self-compatible varieties currently used derive from 'Stella'. Self-compatibility in these genotypes is caused by a pollen function mutation in the *S4′* allele (*S4′* standing for mutated *S*4 allele), (Boskovic et al. 2000). To carry on selection of self-compatible seedlings derived from these genotypes it is necessary to differentiate the genotypes that inherited the *S4′* allele. However, since the *S4-RNase* in these genotypes is intact, it was not possible to differentiate genotypes that presented the *S4′* mutant allele from genotypes with a 'normal' *S*4 allele, by using *S*-allele typing methods based on S-RNase sequence allele diversity. It was not until the recent finding of the pollen determinant of GSI in *Prunus* (Yamane et al. 2003; Ushijima et al.

Table 2. Incompatibility groups and *S*-allele genotype of some of the most widely used sweet cherry cultivars. Nomenclature according to Tobutt et al. (2001). For extensive reviews in sweet cherry *S*-allele genotypes see Iezzoni et al. (in press) and Tobutt et al. (2001 and 2004)

Inocomp. Group	*S*-Genotype	Cultivar
I	S_1S_2	Black Tartarian, Early Rivers, Sparkle, Starking Hardy Giant, Summit
II	S_1S_3	Cristalina, Gil Peck, Lamida, Regina, Samba, Sumele, Van, Venus
III	S_3S_4	Bing, Emperor Francis, Kristin, Lambert, Napoleon, Sommerset, Star, Ulster
IV	S_2S_3	Merton Premier, Sue, Vega, Velvet, Victor, Viva, Vogue
V	S_4S_5	Late Black Bigarreau
VI	S_3S_6	Elton Heart, Governor Wood, Hartland, Satonishiki, Ambrunesa, Duroni 3
VII	S_3S_5	Hedelfingen
VIII	S_2S_5	Vista
IX	S_1S_4	Black Republican, Chinook, Merton Late, Rainier, Sylvia, Garnet, Viscount*
X	S_6S_9	Early Lyons, Black Tartarian, Ramon Oliva*
XII	S_6S_{13}	Noble*
XIII	S_2S_4	Corum, Deacon, Merchant*, Peggy Rivers, Royalton, Sam, Schmidt, Vic
XIV	S_1S_5	Valera
XV	S_5S_6	Colney
XVI	S_3S_9	Burlat, Moreau, Chelan, Tieton
XVII	S_4S_6	Elton Heart, Merton Glory, Larian
XVIII	S_1S_9	Brooks, Marvin, Earlise
XIX	S_3S_{13}	Reverchon
XXI	S_4S_9	Inge
XXII	S_3S_{12}	Princess, Schneiders
XXV	S_2S_6	Arcina
SC/O	$S_3S'_4$	Newstar, Sonata, Stella, Sunburst, Staccato, Sweetheart
SC/O	$S_1S'_4$	Celeste, Lapins, Santina, Skeena

SC: Self-compatible cultivar. O: Universal donor. *: Cultivars also reported with another *S*-allele genotype

2004;) that has been possible to establish a method that allows to determine genotypes carrying the mutated *S4′* allele (Ikeda et al. 2004b). This method is based in the identification of a 4 bp deletion in the *SFB* sequence of the *S4′* allele when compared with the normal *S*4 allele. This deletion has been used to design molecular markers that identify the *S4′* allele by PCR followed by polyacrylamide gel electrophoresis or restriction digestion (Ikeda et al. 2004b). Additional sources of self compatibility, that can broaden the genetic base of cultivated germplasm and that can also be highly useful to understand the mechanism of GSI, are also being studied (Wunsch and Hormaza 2004b; Sonneveld et al. 2005).

3.5 Conclusion and Future Scope of Works

3.5.1 Genome Mapping and QTL Detection

Genetic mapping and QTL detection efforts will be continued especially in sweet cherry. Since sweet cherry is diploid, it is much easier to develop a linkage map as it avoids the difficulties associated with tetraploidy in sour cherry, e.g. partial disomic inheritance, with occasional intergenomic pairing and pre- or post-zygotic selection. According to the high level of synteny already demonstrated within the *Prunus*, results obtained in sweet cherry will be useful for sour cherry. For the same reason, we can expect that cherry will benefit from knowledge generated for a multitude of Rosaceae genera. A Rosaceae database (www.genome.clemson.edu/gdr) has recently been created with the objective of assembling all this

information and making it available worldwide to researchers working in this group of species. An international consortium led by Albert Abbott at Clemson University (Clemson, SC) has developed tools for the characterization of the *Prunus* genome. The enormous progress made during the last decade on genetic knowledge of the cultivated species of the Rosaceae, and particularly of peach as its more logical model, can be exploited for cherry.

3.5.2 Self-(in)compatibility: Molecular Cloning and MAS

The identification and characterization in the late 90s of the *S-RNase* gene in sweet cherry has accelerated *S*-allele genotyping and incompatibility group assignment, as this information can now be obtained using molecular tools like PCR. Since then, the incompatibility group of a great number of varieties has been confirmed, and the S-genotype of the most widely used cultivars has been identified. Additionally the screening of more exotic germplasm has allowed the rapid identification of new *S*-alleles. On the other side, the more recent finding of the *SFB* gene has led to the design of PCR markers for the early screening of self-compatible seedlings carrying *S*4′.

In sweet cherry self-compatibility is a priority in commercial varieties and thus the investigation of new sources of self-compatibility will allow the development of molecular markers that permit a more rapid introduction of this character in elite germplasm. This is of special importance in this species, where breeding for self-compatibility has been mostly done from the same source, with the consequent narrowing of the genetic base. Additionally, the study of self-compatibility in sweet cherry and the knowledge of how the mechanism is operating in tetraploid sour cherry, will help to understand the gametophytic self-incompatibility reaction, a mechanism, which molecular and biochemical basis are still not fully understood.

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4 Plum

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4.1 Introduction

4.1.1 History, Diversity, Domestication, Ploidy Level

According to Rehder (1947), *Prunus* are divided into three major subgenera: Prunophora (plums and apricots), Amygdalus (peaches and almonds) and Cerasus (sweet and sour cherries). The subgenus Prunophora is divided into two main sections: Euprunus which groups the plum species and Armeniaca which contains the apricot species. Plums have been domesticated independently in Europe, Asia and America (Weinberger 1975; Shaw and Small 2004). In Europe, *P. domestica* L. is the most important source of fruit cultivars and has been grown for over 2,000 years. Nevertheless, seeds of another European plum, *P. insititia* L., have been recovered in antiquity ruins and might be of a more ancient origin. The Myrobalan plum *P. cerasifera* Ehrh. probably originated in the Caucasus and Crimea regions (Eremin 1978). In Asia, the Japanese plum *P. salicina* Lindl. originated from China where it has been cultivated since very ancient times. Two to four centuries ago, it has been brought to Japan from where it has been spread all around the world as Japanese plum (Hedrick 1911). In North America, the third plum domestication source, a wide range of native species such as *P. americana* Marsh., *P. hortulana* Bailey, *P. munsoniana* Wight & Hedr., *P. angustifolia* Marsh. and *P. maritima* Marsh. (Okie 1987) are present.

Within the *Prunus* genus, plums are the most taxonomically diverse and are adapted to a broad range of climatic and edaphic conditions (Ramming and Cociu 1991; Salesses et al. 1993). Morphological taxonomy has long been difficult because species boundaries are blurred by interspecific similarities and hybridizations and intraspecific variations. Some plum species are used for their fruits but a majority is being used as rootstocks for plum and other stone fruits. As all the *Prunus* species, plums have a basic chromosomic number of 8 and range from diploid ($2n = 2x = 16$) to hexaploid ($2n = 6x = 48$). Most commercial varieties of plums belong to the European plums *P. domestica* and *P. insititia*, which both are hexaploid, and to the Japanese plum *P. salicina* which is diploid. The diploid Myrobalan plum *P. cerasifera* is widely used as a rootstock (Salesses et al. 1994) and is supposed to have been one of the genomic components of *P. domestica* ($6x$) in association with *P. spinosa* (blackthorn or sloe; $4x$) and might also be one of the components of this latter species (Salesses 1975; Reynders-Aloisi and Grellet 1994). As the peach genome size (diploid) is estimated of 280 Mbp/1C, diploid plums are expected to have equivalent genome sizes (what corresponds to twice the value of the *Arabidopsis* genome) while *P. domestica* genome size is estimated of 883 Mbp/1C (Arumuganathan and Earle 1991).

4.1.2 Economic Importance

In 2004, approx. ten billion tons of plums have been produced in the world of which ca. 3 and 5 billion tons are grown in Europe and Asia, respectively. After peaches and nectarines, this represents the second production among *Prunus* crops at the world, American and European scales. In Europe, the first producer is Germany with 450 millions tons (FAO 2005).

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4.1.3 Breeding Objectives

Breeding purposes in plum concern both cultivar and rootstock. In cultivars, besides the selection for large and good-flavored fruits and for wide ranges of production time, the main objective relates to resistance to *Plum pox virus* (PPV), the causal agent of the sharka disease. PPV, a quarantine pathogen naturally transmitted by aphids, is among the most important *Prunus* diseases and is widely disseminated in European plum orchards, causing significant economic losses. For rootstock breeding, the remarkable variability of wild plum species is starting to be exploited to enlarge the narrow genetic bases of most cultivated plum species (Rom and Carlson 1987; Ramming and Cociu 1991; Dosba et al. 1994). Rootstock programs are being conducted that use Myrobalan plum (alone or crossed with another *Prunus* species) (Eremin 1978; Salesses et al. 1993, 1994) for its positive traits such as good vegetative propagation and adaptation to waterlogged soils (Okie 1987). Some accessions of this species also exhibit a high and wide-spectrum resistance to root-knot nematodes (RKN) *Meloidogyne* spp. (Esmenjaud et al. 1994, 1997) or a graft compatibility with most peach varieties (Salesses et al. 1994).

4.2 Selection for Resistance to PPV

In reaction to the spread of PPV across European borders, control programs have included the development of plum cultivars tolerant or resistant to PPV infection, and programs of strict eradication. Localization (Hoffman et al. 1997), concentration (Polak 1998) and systemic spread of the virus in the plant (Ferry et al. 2002) as well as spatial spread at the orchard scale (Dallot et al. 2003, 2004) has been investigated in plum and other stone fruit species.

4.2.1 Classical Breeding Approach

Development of resistance to PPV in plum has followed the classical approach of searching for natural resistance and incorporating this resistance into new varieties (Kegler et al. 1998). Quantitative resistance has been estimated in a high number of cultivated plums (Paprstein and Karesova 1998) and a qualitative factor such as a hypersensitive character (Hartmann 1998; Hartmann and Petruschke 2000) has also been detected in the European plum cv. Jojo.

Strategies aiming at combining both types of resistances in the hexaploid genome of *P. domestica* are being deployed. As an example, three European plum cultivars, 'Cacanska najbolja', 'Cacanska rana' and 'Cacanska lepotica' (also called 'Cacak Best', 'Cacak Early' and 'Cacak Beauty' respectively) are being used extensively for the introduction of tolerance and partial resistance to PPV (Hartmann 1998). A study by Decroocq et al. (2004) using 10 nuclear microsatellite markers (simple sequence repeat = SSRs) designed for apricot and four chloroplastic SSR markers from dicotyledonous angiosperms (Weising and Gardner 1999) has established that these Cacak accessions were full siblings and were also half siblings of Jojo. These results based on a total of 15 European plum cultivars also showed the cross transportability of the nuclear markers between two *Prunus* species belonging to the same *Prunus* subgenus (Prunophora) and established from both nuclear and chloroplastic markers the pedigree of all four cvs, which had always been previously a matter of discussion (Paunovic et al. 1978).

For a successful identification of the QTLs in the *Prunus* resistance sources, genetic studies need to be associated to the detection of candidate genes. Analogues of virus resistance genes were identified (Decroocq et al. 2005) in *P. davidiana*, a wild relative of peach, that co-localize with genomic regions linked to PPV in this source.

4.2.2 Genetically Engineered Plums

To control PPV spread in plants, attempts to develop genetic engineering technology can be regarded as an alternative approach to the conventional breeding techniques. For this purpose, Sanford and Johnston (1985) have proposed the pathogen-derived resistance as a new strategy to combat viral diseases. Subsequently many research teams have focussed their research program in the creation of transgenic plants resistant to virus infection. Scorza et al. (1994) have successfully engineered the full-length PPV CP gene in *Prunus domestica*. Results about the preliminary greenhouse testing showed that a transgenic clone designated as clone C-5 has been identified as resistant (Ravelonandro et al. 1997; Jacquet et al. 1998). The molecular mechanisms involved have been re-

ported as the post-transcriptional gene silencing or PTGS (Scorza et al. 2001). To verify the stability of PPV resistance in plums, transgenic clones were released in field conditions. For over five years under high inoculum pressure, and regardless of the PPV strains, D or M, the transgenic plum C5 remained healthy when compared to control clones expected to show clear PPV symptoms (Ravelonandro and Scorza 2004). Interestingly, cross hybridization of the transgenic clone C-5 with other plum species permitted to show that the virus transgene can be inherited in the progeny as a single gene trait (Ravelonandro et al. 2001).

4.3 Breeding Efforts for Rootstocks

In plum, no mapping results have yet been used in the specific objective of breeding varieties and available data mainly relate to the Myrobalan plum as a central species in rootstock programs. Breeding efforts have been devoted to the introgression of resistance to root-knot nematodes (RKN) *Meloidogyne* spp. from this latter species into rootstocks (Dirlewanger et al. 2004c; Esmenjaud 2004). Genome mapping and molecular breeding concern in priority interspecific crosses also involving, besides Myrobalan plum, the peach resistance sources Nemared (Ramming and Tanner 1983) and Shalil (Layne 1987). Major results have been obtained on the cross Myrobalan plum 'P.2175' x almond-peach Garfi × Nemared (= 'GN'). The objectives of this *Prunus* rootstock breeding program are to provide an efficient alternative to the use of highly toxic nematicides by developing a new generation of *Prunus* rootstocks bearing high resistance to RKN, using marker-assisted selection (MAS) for pyramiding *Prunus* resistance genes, and several additional characters such as adaptation to chlorosis and drought (from almond), tolerance to water logging (from plum) together with graft compatibility with peach (from peach) and good rooting ability (from plum) (Dirlewanger et al. 2004c; Esmenjaud 2004).

The complete characterization of one major resistance gene to RKN (*Ma*) from Myrobalan plum has been achieved and the molecular cloning of this gene is in progress. Recent advances in this work through the steps of high-resolution mapping, construction of a BAC library for chromosome landing, isolation of one BAC clone carrying the gene, detection of candidate genes, will be reported in this chapter. As an introduction to the molecular aspects of these breeding efforts detailed further for RKN resistance, we develop hereafter the basic knowledge on genetics of resistance in *Prunus* sources.

4.3.1 Genetics of RKN Resistance in *Prunus* Sources

Genetics of resistance to RKN has been studied in the Myrobalan plums P.2175 and P.2980 and in the peach sources Nemared, Shalil, Juseitou and Okinawa (Table 1).

Accessions P.2175 and P.2980 have been shown to carry one dominant allele (heterozygous) of a single resistance gene, designated *Ma1* and *Ma3*, respectively (Esmenjaud et al. 1996b; Rubio-Cabetas et al. 1998). Each of these *Ma* alleles confers a high and wide-spectrum resistance to *M. arenaria, M. incognita*, *M. javanica* and *M. floridensis* (Esmenjaud et al. 1997; Lecouls et al. 1997; Rubio-Cabetas et al. 1999; Handoo et al. 2004) and to the minor species *M. mayaguensis* (Rubio-Cabetas et al. 1999) which overcomes the resistance of the *Mi* tomato gene (Fargette et al. 1996). This *Ma* resistance was not overcome by any of the over-30 RKN species and isolates tested (Esmenjaud et al. 1994, 1997; Fernandez et al. 1994) and was not modified under conditions usually known as affecting plant defences to RKN such as high temperature and high inoculum pressure (Esmenjaud et al. 1996a). Within perennials, where the genetics of RKN resistance is poorly documented, the *Ma* gene from Myrobalan plum is the first genetic system fully characterized for resistance to a plant pest (Lecouls et al. 1997, 1999; Lecouls 2000; Claverie et al. 2004a, b; Lecouls et al. 2004).

Resistance in Nemared peach has been firstly studied in an F_2 population derived from self-pollination of an F_1 peach hybrid Lovell × Nemared by Lu et al. (2000) who proposed the *Mi* and *Mij* genes for resistance to *M. incognita* and both *M. incognita* and *M. javanica*, respectively. Resistance in Nemared has also been studied from interspecific crosses [P. 2175 × (Garfi × Nemared)] segregating both for *Ma* and Nemared resistance (Claverie et al. 2004a). Resistance from Shalil (the peach parent for the almond-peach GF.557) was established from the cross P. 2175 × GF.557 segregating both for *Ma1* and Shalil resistance (Claverie et al. 2004a). As those interspecific crosses involving Nemared and Shalil segregated

Table 1. Spectrum and genetics of resistance of main sources to root-knot nematodes used in *Prunus* rootstock breeding

Subgenus	Species	Resistance status to *M. arenaria* (MA)	*M. incognita* (MI)	*M. javanica* (MJ)	*M. floridensis* (MF)	Resistance gene and genotype	References
Prunophora							
	Myrobalan plum (*P. cerasifera*)					*Ma* gene controlling MA, MI, MJ and MF	
	P.2175	R[1]	R	R	R	(*Ma1 ma*)	Esmenjaud et al. 1994, 1996, 1997
	P.2980	R	R	R	R	(*Ma3 ma*)	Lecouls et al.1997
	P.2032[2]	S[1]	S	S	S	(*ma ma*)	Rubio-Cabetas et al. 1999
	P.2646[2]	S	S	S	S	(*ma ma*)	
	P.16.5[2]	S	S	S	S	(*ma ma*)	
Amygdalus							
	Peach (*P. persica*)						
	Nemared		R			*Mi* gene controlling MI and (or) *Mij* controlling MI and MJ	Lu et al. 2000
	Shalil						
	GF.557 = almond × Shalil peach					R_{Mia557} gene controlling MA and MI	Esmenjaud et al. 1994, 1997
	GF.557	R	R	S	S	($R_{Mia557}r_{Mia557}$)	Claverie et al. 2004a
	Nemared					R_{MiaNem} gene controlling MA and MI	Esmenjaud et al. 1997
	Nemaguard	R	R	R/S [3]	S	($R_{MiaNem}R_{MiaNem}$)	Claverie et al. 2004a
	Nemared	R	R	R/S	S	($R_{MiaNem}R_{MiaNem}$)	Dirlewanger et al. 2004a
	Juseitou		R	R		*Mia* gene controlling MI and *Mja* controlling MJ	Yamamoto et al. 2001 Yamamoto and Hayashi 2002
	Okinawa		R			*Mi* gene controlling MI race 1	Sharpe et al. 1969 Gillen and Bliss 2005

[1] R = resistant; S = susceptible

[2] Susceptible control accessions

[3] R/S: variable behavior in function of *M. javanica* isolates

identically for resistance to *M. incognita* and *M. arenaria*, a single gene controlling both RKN species was hypothesized and designated, respectively, R_{MiaNem} and R_{Mia557} in Nemared and GF.557 (Claverie et al. 2004a). Resistances in 'Juseitou' to *M. incognita* (gene *Mia*) and *M. javanica* (gene *Mja*) and in 'Okinawa' to *M. incognita* race 1 (gene *Mi*) have been studied in the F_2 populations Akame × Juseitou (Yamamoto et al. 2001; Yamamoto and Hayashi 2002) and Harrow Blood × Okinawa (Gillen and Bliss 2005).

4.3.2 Mapping of the RKN *Ma* Gene in Plum – Comparison with Peach RKN Genes

Molecular studies concerning *Ma* have been conducted to develop a local map of the gene and to locate it on the plum and reference *Prunus* maps. The data on comparative locations of RKN resistance genes from plum and peach have been firstly reported in Claverie et al. (2004a) and then confirmed in Dirlewanger et al. (2004a).

4.3.2.1 Local Map and Marker-Assisted Selection (MAS) for *Ma*

Random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) markers have been identified by bulked segregant analysis (BSA) (Michelmore et al. 1991) using intraspecific progenies involving P.2175 (*Ma1 ma*) and several susceptible parents (*ma ma*). Two reliable SCAR (sequence characterized amplified region) markers, $SCAL19_{690}$ (derived from a RAPD marker) and $SCAFLP2_{202}$ (derived from an AFLP marker), were shown to be linked in coupling phase to the dominant resistance alleles *Ma1* and *Ma3* (Lecouls et al. 1999, 2004). SCAL19 is located less than 1 cM from *Ma* and SCAFLP2 is cosegregating with *Ma*, as shown by the analysis of over 1,300 individuals belonging to diverse intra- and interspecific progenies (Claverie et al. 2004b).

4.3.2.2 Location of RKN Genes in the Prunophora Subgenus (Myrobalan and Japanese Plums)

In Myrobalan plum, three RFLP markers among 46 probes distributed all over the *Prunus* genome, revealed polymorphic fragments between the resistant and the susceptible bulks. All three RFLP markers lie on the linkage group G7 of the reference map (Joobeur et al. 1998) and cover 32 cM. This preliminary position of *Ma* on G7 was confirmed by the detection of a polymorphism or difference in amplification signal intensity between bulks for three SSR markers located on this group, pchgms6, UDP98-405, and CPPCT033. Genotyping the individuals of the couples of bulks completed by all other individuals previously characterized for *Ma* allowed to locate these markers on the same side of the gene at 2.3, 9.5 and 21.3 cM, respectively. These SSR markers are placed on the other side of the gene relative to the SCAR markers SCAL19 and SCAN12 (Claverie et al. 2004a) (Fig. 1).

Additionally, in the Japanese plum, a single dominant gene designated R_{jap} was hypothesized from a segregating progeny of 26 individuals between the RKN resistant accession J.222 (heterozygous) and the RKN susceptible accession J.13 (homozygous). The SCAR markers linked to *Ma* and all the SSRs available in the reference map for this G7 region were evaluated for their polymorphism in parents and all individuals of the progeny. On this small-sized cross, the markers pchgms6, CPPCT022 and SCAL19 cosegregated with the R_{jap} gene (Fig. 1), which shows that this gene lies on the G7 probably in the same position as *Ma* (Claverie et al. 2004a). In Prunophora, differences in allelism and polymorphism of genetic markers linked to resistance associated with co-location of the *Ma* and R_{jap} genes suggest the conservation of a resistance locus acquired before separation of the species Myrobalan and Japanese plums. It is likely that this location is conserved in cultivated and wild plum species including diploid to hexaploid species.

4.3.2.3 Comparative Location of Plum and Peach Genes – Consequences for MAS

All studies concerning peach mapping located the RKN resistance genes from this species in the linkage group G2 of the reference *Prunus* map T × E (Joobeur et al. 1998, 2000; Aranzana et al. 2003). The peach genes R_{Mia557} and R_{MiaNem}, carried by two *a priori* unrelated resistance sources, Shalil and Nemared respectively (Table 1), were colocalized in a subtelomeric position on the G2 (Claverie et al. 2004a; Arús et al. 2004). This location was different from the more centromeric position previously

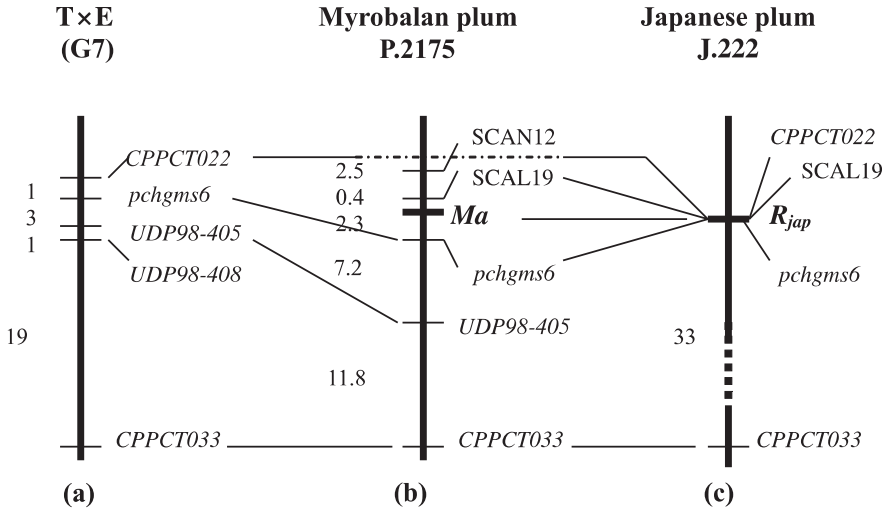

Fig. 1. Local maps of SSR (*in italics*) and SCAR (*normal letters*) markers linked to the *Ma* gene in the Myrobalan plum P.2175 (**b**) and to the R_{jap} gene in the Japanese plum J.222 (**c**) in comparison with SSR markers located on the linkage group G7 of the almond × peach reference *Prunus* map Texas × Earlygold (T×E) (**a**) (Aranzana et al. 2003). For the *Ma* gene, distances are expressed in cM using the Kosambi distance given by the MAPMAKER software version 3 (Lander et al. 1987) with a minimum LOD score of 3.0. For the *Rjap* gene, distances are expressed in recombination percentages

proposed by Lu et al. (1999) for the resistance gene *Mij* to *M. incognita* and *M. javanica* in Nemared near the SSR pchgms1 and the STS EAA/MCAT10. By contrast, R_{Mia557} and R_{MiaNem} were flanked by STS markers obtained by Yamamoto and Hayashi (2002) for the resistance gene *Mia* to *M. incognita* in the Japanese peach source Juseitou. Concordant results for the three independent sources, Shalil, Nemared and Juseitou, suggest that these peach RKN sources share at least one major gene for resistance to *M. incognita* located in this subtelomeric position.

The most beneficial and applied result is that *Ma* on the one hand and peach genes on the other hand are independent and can be pyramided into interspecific rootstock material. Construction of rootstock genotypes carrying *Ma* and peach genes by interspecific hybridization (e.g. Myrobalan plum × Amygdalus) is underway (Dirlewanger et al. 2004c; Esmenjaud 2004). These hybrids can thus cumulate favorable agronomic traits from both origins together with the complete-spectrum resistance controlled by the Myrobalan *Ma* gene and the more-restricted spectrum of Amygdalus genes. Indeed, the pyramiding of several genes in the same genotype may limit the risk of resistance breaking (Johnson 1983; Cook and Evans 1987; Roberts 1995) and thus extend the useful life of new rootstocks.

4.4 Construction of Maps for the 3-Way Interspecific Cross Myrobalan Plum × (Garfi × Nemared)

The mapping results reported here have been developed in Dirlewanger et al. (2004a). Inheritance and linkage studies were carried out with SSR markers in an F_1 progeny including 101 individuals of the cross between Myrobalan plum clone P.2175 and the almond-peach hybrid clone $(\text{Garfi} \times \text{Nemared})_{22}$ (= GN22). The *Ma* gene from P.2175 and the R_{MiaNem} gene from Nemared, are each heterozygous in the parents P.2175 and GN22, respectively. Two hundred and seventy seven *Prunus* SSRs were tested for their polymorphism. A genetic map was constructed for each parent according to the 'double pseudo-testcross' model of analysis (Fig. 2).

4.4.1 SCAR Analysis

$SCAL19_{690}$ and $SCAFLP2_{202}$, the two SCARs tightly linked to the *Ma* gene (Lecouls et al. 2004), were analyzed on the progeny. The five STS markers obtained

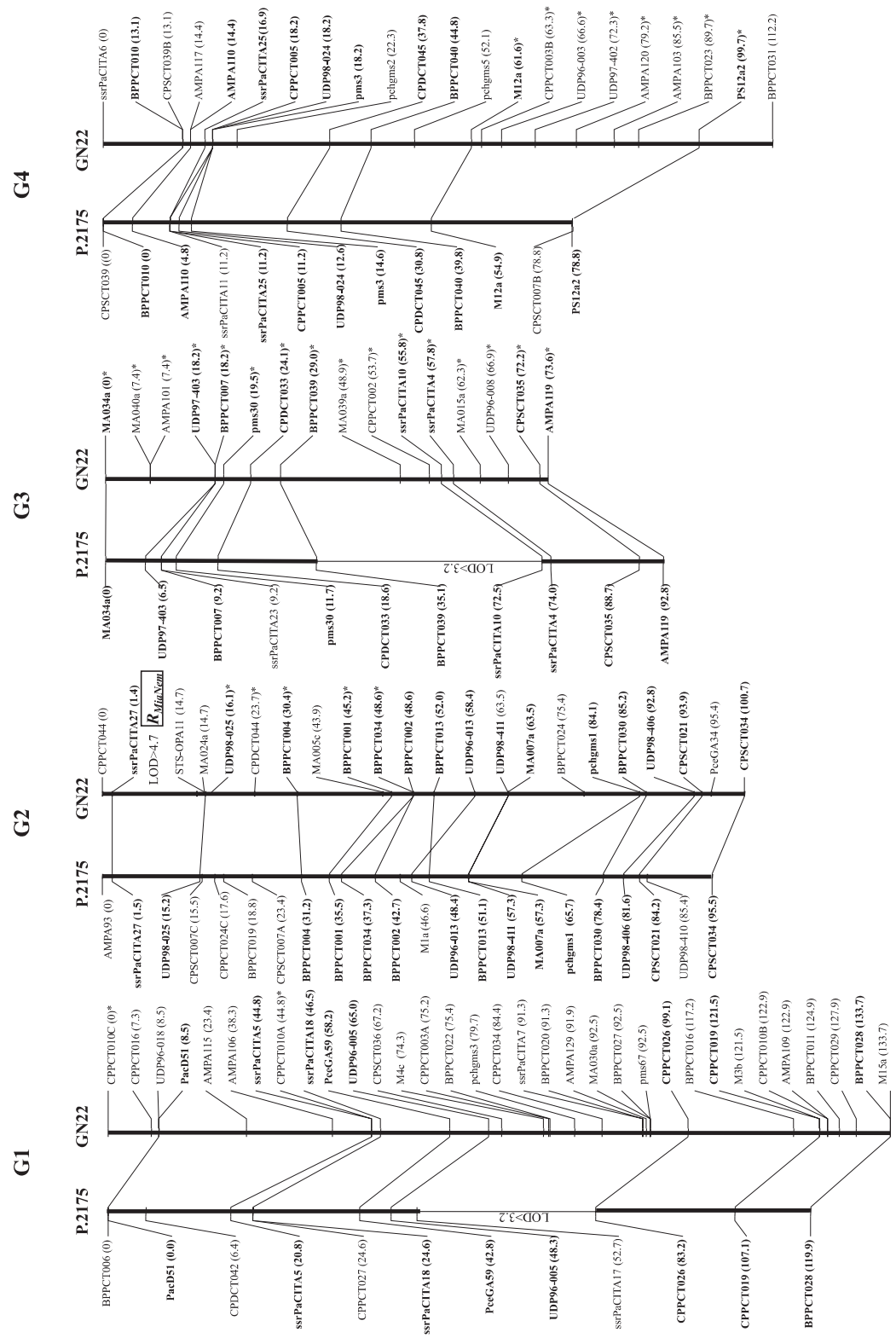

Fig. 2. Genetic maps obtained with the interspecific Myrobalan plum (P.2175) × almond-peach (GN22) F_1 progeny. Anchor-loci between the P.2175 and the GN22 maps (*in bold*) are connected by *lines*. Distorted loci ($P < 0.01$) are indicated by a *star* after the name

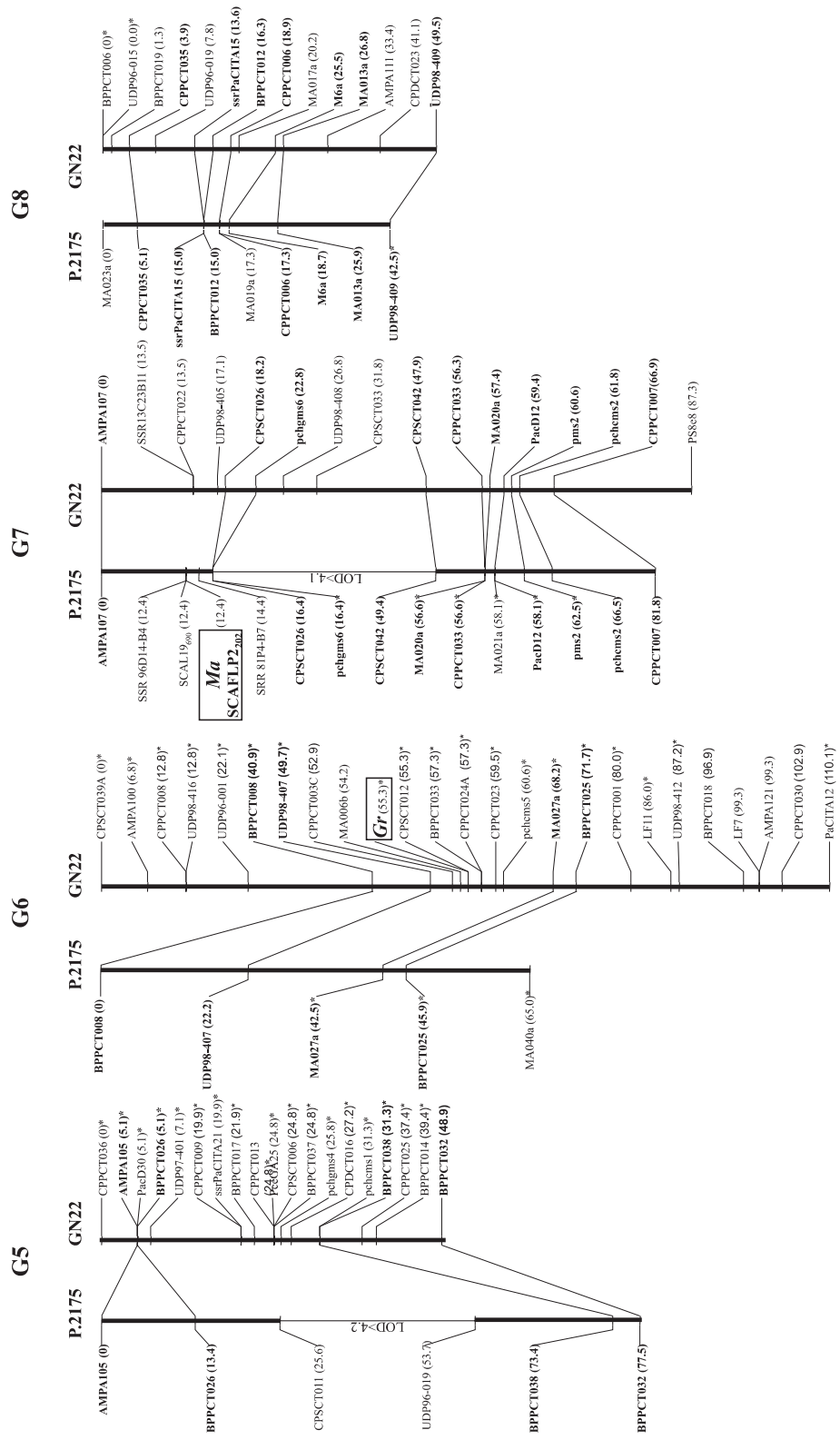

Fig. 2. (continued)

by Yamamoto and Hayashi (2002) linked to the resistance loci of the peach Juseitou, were also tested but only STS-OPA11 had a readable profile with a fragment of 481 bp segregating in the P.2175 × GN22 F_1 progeny.

4.4.2 SSR Analysis

Among the 277 SSRs originated from several *Prunus* species, 46 (16.6%) had complex profiles on acrylamide gels, 14 (5%) revealed no polymorphism, 104 (37.5%) revealed polymorphism in P.2175, 184 (66.4%) revealed polymorphism in GN22 and 84 (30.3%) were polymorphic in both parents. Thus the polymorphism detected in GN22 (66.4%) was much higher than in P.2175 (37.5%). The high degree of heterozygosity in GN22 results from its interspecific hybrid status. Most heterozygous SSRs in P.2175 were also heterozygous in GN22 (80.8%). These data confirm the high degree of microsatellite portability among *Prunus* previously reported by Cipriani et al. (1999) and Dirlewanger et al. (2002, 2004b). This high polymorphism between the Myrobalan plum P.2175 and GN22 has also been observed by Mnejja et al. (2004) between Japanese plum and peach (85%) or almond (78%), using 27 single-locus microsatellites. More SSRs deviated significantly from the expected ratio in GN22 (41.5%) than in P.2175 (10.6%); the interspecific status of GN22 may explain these results. In most cases, distorted segregations are more frequent in interspecific crosses than in intraspecific ones (Guo et al. 1991; Kianian and Quiros 1992) considering that mistakes between the coupling of homologous chromosomes during the metaphase 1 may occur in interspecific crosses. Among the 166 SSRs heterozygous in GN22, all those located on G3 and nearly all those located on G5 and G6, had a distorted segregation. In P.2175, distortions are located mainly in G6 and G7. Only the middle part of the G6 contained distorted segregating markers in both maps.

Many SSR markers (92) were already located on the T×E map (Aranzana et al. 2003), others were mapped on the peach P×F map (Dettori et al. 2001), on the apricot Stark Early Orange and Polonais maps (Lambert et al. 2004) or in the almond Ferragnès and Tuono maps (Joobeur et al. 2000). Here, 75 SSR markers were mapped for the first time.

4.4.3 Inheritance and Map Construction

4.4.3.1 Segregation of the *Ma*, R_{MiaNem} and *Gr* Genes

The 101 individuals from 2175 × GN22, tested to *M. floridensis* (Handoo et al. 2004) to evidence the *Ma* gene (Table 1), were shown to segregate into 40 resistant: 61 susceptible. This segregation deviated from the expected 1:1 ratio (P = 0.036). The 61 susceptible individuals were then processed for evaluation to *M. incognita*, in order to evidence the R_{MiaNem} resistance gene. Nevertheless, only a subset of both mapping populations could be evaluated, due to unsuccessful rooting of the cuttings. Within the 27 P.2175 × GN22 hybrids evaluated to *M. incognita*, 13 were resistant and 14 were susceptible thus fitting the expected 1.1 ratio. A high distorted segregation ratio (P = 0.00059) was observed for the color of the leaf, with 32 red-leaf and 66 green-leaf individuals.

4.4.3.2 *P.2175* Myrobalan Linkage Map

The P.2175 Myrobalan linkage map was constructed by analyzing the segregation of the *Ma* gene and 94 markers (92 SSRs, 2 SCARs) (Fig. 2). The P.2175 linkage map covered 524.8 cM with a LOD > 5.0 and 653.8 cM with a LOD > 3.2.

The *Ma* gene, already reported to cosegregate with the SCAR marker SCAFLP2 (Lecouls et al. 2004), cosegregated also with the SCAR $SCAL19_{690}$ and the SSR 96D14-B4. This SSR was identified within a BAC clone from the Nemared library (Georgi et al. 2002), containing $SCAFLP2_{202}$ (Lecouls, Personal Communication). The *Ma* gene and SSR 96D14-B4 segregated with the expected Mendelian 1:1 ratio; the two SCARs had distorted segregation (P = 0.037 each). They were located on P.2175 G7 at 12.4 cM from the top of the linkage group.

4.4.3.3 *GN22* Linkage Map and Evidence of Translocation

The map of the interspecific almond-peach GN22 parent from the P2175 × GN22 progeny was constructed by analyzing the segregation of the R_{MiaNem} gene, the *Gr* gene, and 166 markers (165 SSRs and 1 STS) (Fig. 2). With a LOD > 5.0, all markers were grouped into 7 linkage groups instead of the 8 expected. The

27 plants evaluated for resistance to *M. incognita* allowed the R_{MiaNem} gene to be mapped to linkage group 2 (G2) with a LOD>4.7. Six of the linkage groups, G1, G2, G3, G4, G5, G7, were homologous to those found in T×E with identical locus order and similar distances.

Thirty-eight markers formed a single group in the GN22 map and among them, 16 were already mapped in linkage group 6 and 11 in linkage group 8 in other maps [T×E (Aranzana et al. 2003), P×F (Dettori et al. 2001), J×F (Dirlewanger et al. 2006)]; 11 markers were not previously mapped. Ordering of this group (G6-G8) was difficult and linear order containing all loci could not be established. The map with the most markers included only 29 loci: 11 were already mapped in other maps on G6 and 11 on G8 (Fig. 2). The top of G6-G8 contained markers already mapped in the G6 but in the inverse order comparing to the T×E map, the bottom of G6-G8 contained markers already mapped in the G8 in exactly the same order as in T×E map. This pseudolinkage between G6 and G8 groups is a consequence of a reciprocal translocation between the chromosomes corresponding to G6 and G8. This was already reported in a 'Garfi' × 'Nemared' F_2 population (Jauregui et al. 2001). Reciprocal translocations are one of the most common structural chromosome rearrangements and have been detected in many species through the study of pollen viability and chromosome pairing during meiosis (Garber 1972).

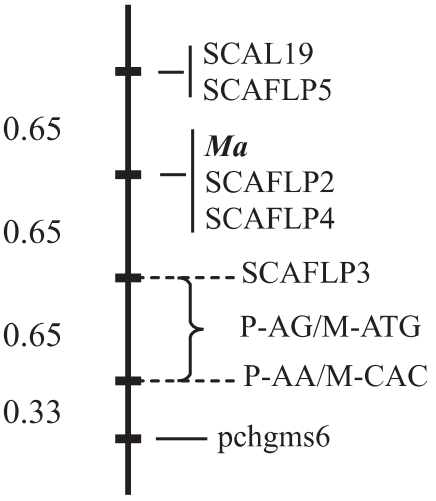

Fig. 3. Local map around *Ma* showing the position of AFLP and SCAR markers obtained from BSA. Distances expressed in recombination percentages have been evaluated from a population of approx. 300 individuals. P = *Pst*I; M = *Mse*I

With a LOD > 5.0, the GN22 map covered 716.0 cM, including the *Gr* gene located on G6 and 166 markers (165 SSRs and 1 STS). With a LOD > 4.7, the R_{MiaNem} gene controlling the nematode resistance from 'Nemared' was placed, as expected from location previously established by Claverie et al. (2004a), on G2 near the top of the group, between ssrPaCITA27 (13 cM) and the SCAR STSOPA11 that cosegregates with the SSR MAO24a (7.4 cM). The mean density of the map was 4.3 cM between markers.

4.4.4 Comparison of the *P.2175* and *GN22* Maps

Among the 73 SSRs markers polymophic in both parents and tested in the progeny, 68 were placed on both maps on homologous linkage groups. This shows a high level of colinearity between Myrobalan plum and the peach and almond genomes. This was already observed between apricot Stark Early Orange, Polonais and T×E (Lambert et al. 2004). These results reveal a strong homology of the genomes belonging to the *Prunophora* and *Amygdalus* sub-genera. By comparing all the *Prunus* maps sharing common markers, it is now possible to identify a set of single SSR loci covering all the genomes as it was proposed by Aranzana et al. (2003). Translocation events are now easily detected by using already mapped markers.

4.5 Strategy for Map-Based Cloning of the *Ma* Gene

Developing a positional cloning strategy for the *Ma* locus for resistance to RKN from the rootstock species *P. cerasifera* is a challenging task in particular because of the time and space required for producing and characterizing adequate populations. This project has been undergone because of the remarkable properties of the *Ma* gene which confers to this allogamous diploid plum, a complete-spectrum and a heat stable resistance to *Meloidogyne* spp. Conversely, the *Mi* gene from tomato, which is the RKN reference resistance (R) gene and the sole RKN R

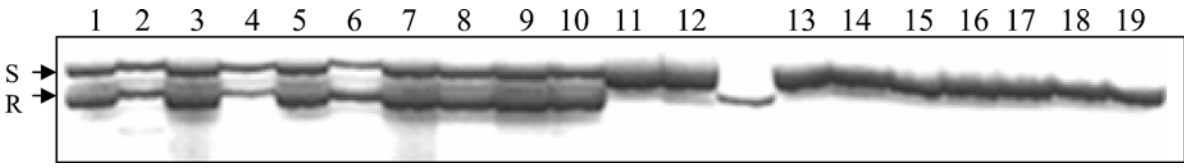

Fig. 4. Segregation of the SCAR marker SCAFLP4 in 19 individuals from different intraspecific progenies segregating for *Ma*. *Lanes 1–10*: *Ma* resistant individuals, heterozygous for the marker. *Lanes 11–19*: *Ma* susceptible individuals, homozygous for the marker. The *arrows* indicate the alleles in coupling with susceptibility (*S*) and resistance (*R*) in P.2175

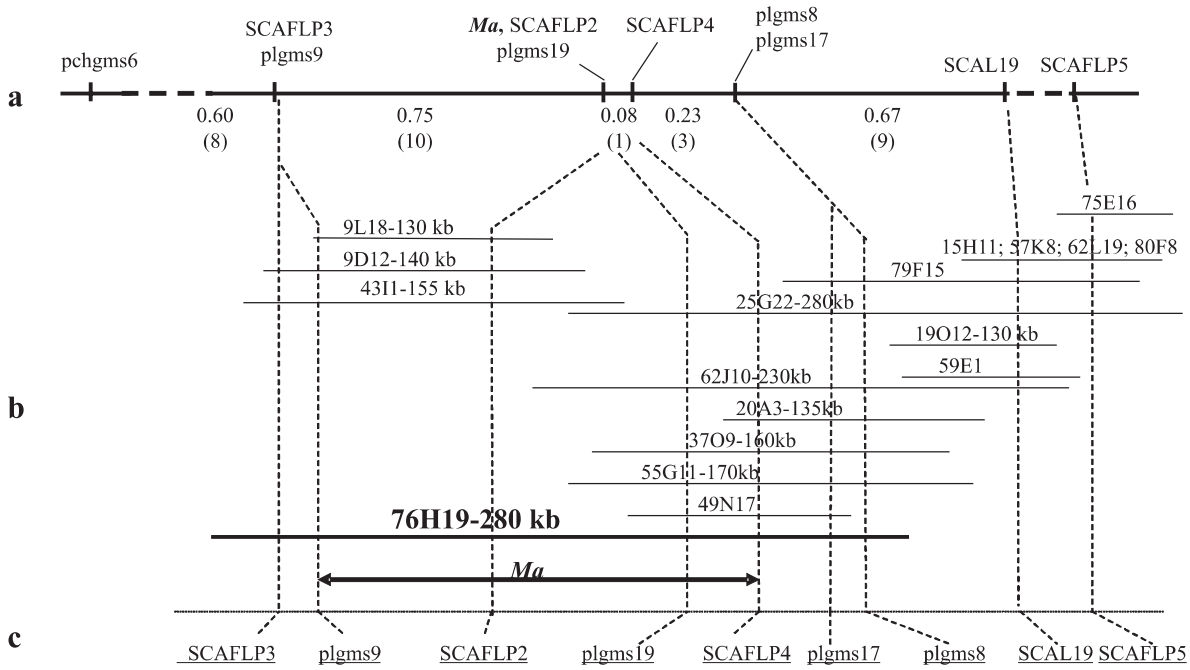

Fig. 5. Fine genetic mapping of *Ma* linked SSR (plgms) and SCAR (SCAFLP) markers (**a**) and physical mapping of positive BAC clones from the resistant contig (**b**). In (**a**), values between markers are recombination percentages (*upper row*) and numbers of recombinants among 1332 total individuals (between parenthesis, *lower row*). Amplification of the expected resistance allele of a marker from a BAC is represented by a cross between this BAC and the dotted vertical line joining the marker name (**c**). For some BAC clones, insert sizes are indicated after the BAC designation. BAC clones experimentally characterized by the same markers are grouped under the same representation. The *double arrow* indicates the interval containing the *Ma* locus (resistance allele)

gene cloned up-to-now, has a more restricted spectrum and a reduced efficiency at high temperature. Another favorable argument is that Myrobalan plum is a diploid species with a small and compact genome ($2n = 2x = 16$) estimated to be equivalent to the botanically closely related apricot species (*P. armeniaca*) (300 Mbp/1C; Arumuganathan and Earle 1991) i.e. with an average physical distance of about 300–400 kb per cM. The different steps of this strategy reported hereafter have been developed in Claverie et al. (2004b).

4.5.1 Detection of AFLP Markers by BSA, Development of PCR Markers and High-Resolution Mapping of the *Ma* Gene

Additional markers in the *Ma* region were obtained by BSA of 320 AFLP primer pairs combinations. Using a segregating population of 307 individuals, five AFLP markers tightly linked to the *Ma1* allele from P.2175 were obtained and mapped in the 2.3 cM interval spanning the gene, between the previously obtained

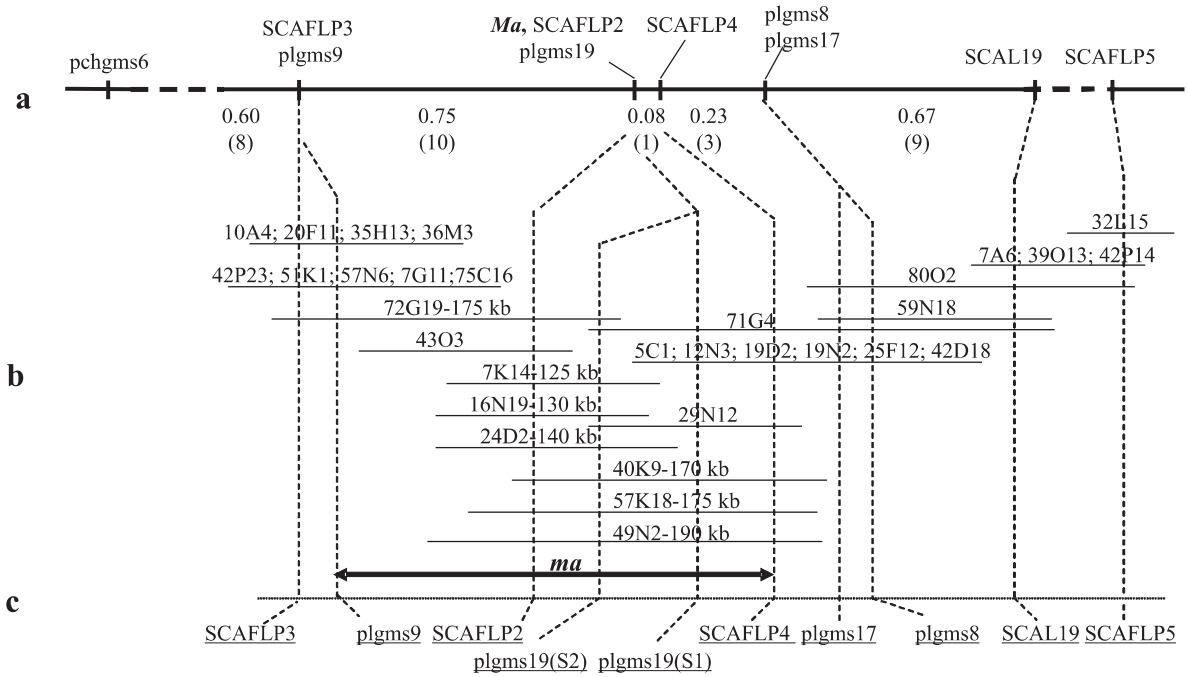

Fig. 6. Fine genetic mapping of *ma* linked SSR (plgms) and SCAR (SCAFLP) markers (**a**) and physical mapping of positive BAC clones from the susceptible contig (**b**). In (**a**), values between markers are recombination percentages (*upper row*) and numbers of recombinants among 1,332 total individuals (between parenthesis, *lower row*). Amplification of the expected susceptibility allele of a marker from a BAC is represented by a cross between this BAC and the *dotted vertical line* joining the marker name (**c**). For the SSR marker plgms19 two amplification products (differing in length by 4 base pairs) in coupling with susceptibility and genetically cosegregating were physically separated and designated as plgms19(S1) and plgms19(S2). For some BAC clones insert sizes are indicated after the BAC designation. BAC clones experimentally characterized by the same markers are grouped under the same representation. The *double arrow* indicates the interval containing the *Ma* locus (*ma* susceptibility allele)

markers SCAL19 and pchgms6 (Fig. 3). Three of these markers were sequenced and transformed into SCAR or CAPS (cleaved amplified polymorphism sequence) markers designated SCAFLP3, SCAFLP4 (Fig. 4) and SCAFLP5.

A total number of 1,332 individuals, from 21 crosses segregating for *Ma*, revealed 31 individuals recombining between the flanking markers SCAL19 and pchgms6 in the genetic interval of 2.3 cM encompassing the gene. These recombinant individuals were then genotyped with the markers SCAFLP2, SCAFLP3 and SCAFLP4 and RKN resistance tests allowed a finer location of the gene (Figs. 5a and 6a): *Ma* co-segregated with the SCAFLP2 marker and was separated from SCAFLP4 by a single recombination event.

4.5.2 BAC Library Construction

A total of 30,720 BAC clones distributed into four-size classes (sub-libraries) were organized into 384-well plates. Sub-library 1 consists of 9,513 clones with insert size ranging from 50 to 150 kb and an average of 120 kb, sub-libraries 2 and 3 grouped 19,200 clones with insert size ranging from 80 to 200 kb and an average of 150 kb. Sub-library 4 grouped 2,007 clones with insert size ranging from 110 to 350 kb and an average of 210 kb. Thus the average insert size of the whole library is estimated to be 145 kb with insert distribution ranging from 50 to 350 kb and the library has a 14–15× coverage of Myrobalan plum haploid genome. Considering that Myrobalan plum is highly heterozygous, this coverage must be expressed as a 7–8× coverage of the diploid genome.

4.5.3 Construction of Physical Contigs Spanning the *Ma* Region and Chromosome Landing

As the accession P.2175 is heterozygote and carries both R and S alleles of the *Ma* gene, R and S physical contigs were constructed by screening the BAC library

with the codominant co-segregating or tightly linked markers, SCAFLP2, SCAFLP3, SCAFLP4, SCAFLP5 and SCAL19 (Figs. 5a and 6a). The identified positive BAC clones were considered as belonging to either the resistant or the susceptible contigs based on their detection with either the resistant or the susceptible alleles of the codominant markers. Surprisingly the markers SCAFLP2 and SCAFLP4 only separated by 0.08 cM were detected together only in a single clone of the R contig and three clones of the S contig. Finally a single BAC clone ('BAC76H19') carried all together the resistant alleles of SCAFLP2, the flanking SCAFLP3 and SCAFLP4 markers and subsequently the *Ma* gene.

Thirteen random DNA sequences, from 224 to 827 bp long, were obtained from the 76H19 BAC subcloning and the sequencing of other-BAC ends that anchor to the gene region. Four of these sequences were shown to contain microsatellite repeats and served to generate four polymorphic SSR markers tightly linked to *Ma*. SSR amplifications were performed on recombinant individuals (and on parental material as controls) for a refined genetic mapping of the region surrounding *Ma*; the SSR plgms9 from the 9L18 T7 BAC end cosegregated with SCAFLP3, the SSR plgms19 cosegregated with *Ma* (and SCAFLP2) and the two others (plgms8 and plgms17) cosegregated and fell between SCAFLP4 and SCAL19. From amplification data in the BAC clones from the *Ma*-resistant and *Ma*-susceptible contigs, these newly developed markers were then placed on the resistant and susceptible physical maps (Figs. 5b and 6b).

4.6 Conclusion and Future Scope of Works

4.6.1 Resistance to Plum Pox Virus

Several genes are involved in the resistance to Plum pox virus in peach and apricot and identification of QTLs is in progress in both species (Guillet and Audergon 2001; Villanova et al. 2003; Decroocq et al. 2005). Because of the high synteny within *Prunus* and the close genetic relationships between them and in particular between apricot and plum, it is assumed that most genetic and mapping information acquired in peach or apricot about resistance to PPV will be easily transferable and thus exploitable in plum. In *P. davidiana* for example, identification of distinct genomic regions involved in resistance and their co-localization with virus resistance gene analogues (Decroocq et al. 2005) are the first steps towards marker-assisted selection of PPV resistance for peach from this wild peach species and might be useful later in plum species. The numerous SSR markers now characterized in plum (Decroocq et al. 2004; Dirlewanger et al. 2004a) are powerful tools in this way.

As a complement to conventional breeding, transgenic clone C5 is a promising source of high level PPV resistance transferable to progeny through cross-hybridization experiments (Ravelonandro and Scorza 2004). To alleviate the concern of consumers about genetically modified organisms (GMOs), research work is still necessary to evaluate the safe use of genetically modified fruits and the ability of such GMOs to contribute to a sustainable agriculture.

4.6.2 Genome Mapping

The *Ma* gene and 93 markers (2 SCARs, 91 SSRs) were placed on the P.2175 Myrobalan map covering 524.8 cM. In peach, the R_{MiaNem} gene, the *Gr* gene controlling the color of leaves, and 166 markers (1 SCAR, 165 SSRs) were mapped to seven linkage groups instead of the expected eight in *Prunus*. Markers belonging to groups 6 and 8 in previous maps formed a single group in the GN22 map and evidenced a reciprocal translocation, already reported in a Garfi × Nemared F_2, near the *Gr* gene. By separating markers from linkage groups 6 and 8 from the GN22 map, it was possible to compare the eight homologous linkage groups between the two maps using the 68 SSR markers heterozygous in both parents (anchor loci). All but one of these 68 anchor markers are in the same order in the Myrobalan plum map and in the almond-peach map, as expected from the high level of synteny within *Prunus*. The *Ma* and R_{MiaNem} genes confirmed their previous location in the Myrobalan linkage group 7 and in the GN22 linkage group 2, respectively. The SCAR markers ($SCAL19_{690}$, $SCAFLP2_{202}$) (Lecouls et al. 2004) cosegregated with *Ma* and 4 SSRs (SSR 96D14-B4, SSR 81P4-B7, SSR6, SSR12) were located in the same region.

All these data will be used in the *Prunus* rootstock breeding program aiming at developing a new generation of *Prunus* rootstocks bearing high resistance to RKN using MAS and several additional characters such as adaptation to chlorosis and drought (from almond), tolerance to waterlogging (from plum) together with graft compatibility with peach (from peach) and good rooting ability (from plum) (Esmenjaud 2004; Dirlewanger et al. 2004c). These data will also be available for the other rootstock programs relative to *Prunus* crops and particularly peach (Reighard 2002). The genetic linkage maps constructed from the interspecific F_1population issued from the cross P.2175 × GN22 will be used for the detection of QTLs involved in drought, waterlogging and chlorosis resistance. A subset of the progeny has already been evaluated for different ecophysiological parameters (predawn leaf water potential, conductance, transpiration, photosynthesis and growth parameters). A high variability of response was observed, especially for the water use efficiency, an essential condition for a breeding program (Kleinhentz et al. 2005), confirming that this material is promising for the selection of a new generation of *Prunus* rootstock associating the favorable characters of each species.

4.6.3 Towards Map-Based Cloning of *Ma*

The *Ma1* allele from the heterozygous parent P.2175 was accurately located using SSR markers available from *Prunus* maps. Applying an adapted BSA strategy resulted in three extra AFLP markers tightly flanking *Ma1* which were transformed into codominant SCAR markers. These markers, as well as the two closely linked markers obtained in previous studies, were used to build a high-resolution map, based on recombination events at the *Ma1* locus from segregating intra- and inter-specific crosses including more than 1,300 individuals. A BAC library of the parent P.2175 characterized by a large mean insert size (145 kb) and a 14–15 × haploid genome coverage was constructed. The markers tightly linked to the gene allowed the elaboration of the R and S contigs at the *Ma* locus. One 287 kb insert BAC carrying *Ma1* was detected in the R contig.

Because of the characteristics of *Ma* (i.e., complete RKN spectrum, heat stability, and no virulent isolate known), it may be of an outstanding interest to identify the gene and to study its structure, function and evolution. In this objective, a complete sequencing of the 280-kb insert has been performed. Bioinformatic sequence analysis revealed a cluster of three TIR-NBS-LRR (TNL) open reading frames (ORFs) lying between candidate ORFs from other multigenic families. New SSR markers directly derived from the BAC sequence reduced the physical interval encompassing *Ma* to a 70 kb region including a putative lectin/kinase receptor (LecRK) and the TNLs. Additional fine mapping, using 1,700 young Myrobalan plum segregating seedlings, still reduced this interval to 54 kb only containing the three TNLs as candidate resistance genes (Claverie 2004). The analysis of these sequences, in combination with a linkage disequilibrium study among Myrobalan plum accessions should allow to identify the best candidate to encode the *Ma* gene. Sequence analysis will also generate data about *Prunus* genome organization (genes, microsatellites, structure and distribution of repeated sequences) in this particular region that will be compared to the susceptible *Ma* region and extended via microsynteny to other *Prunus* or *Rosaceae* species. This study may also provide new information about the dynamics of the natural evolution of a resistance locus from a perennial, near-wild and self-incompatible plant (Salesses et al. 1993, 1994).

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5 Peach

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5.1 Introduction

5.1.1 Peach [*Prunus persica* (L.) Batsch]

In temperate regions, the family Rosaceae ranks third in economic importance. Its commercially valuable members include fruit producing (e.g., stone fruits, apples, brambles, and strawberries), nut-producing (almond), lumber producing (e.g., black cherry) and ornamental (e.g., roses, flowering cherry, quince, and pear) species. Rosaceae is the type family for the Rosales, the largest order in the Rosidae (Heywood 1978) or Calyciflorae (Benson 1979). It is traditionally divided into four well-defined subfamilies. The genus *Prunus*, within the subfamily Prunoideae, is characterized by species that produce drupes known as "stone fruits" where the seed is encased in a hard, lignified endocarp referred to as the "stone", and the edible portion is a juicy mesocarp. The agriculturally most important stone fruit species are *P. persica* (L.) Batsch (peach, nectarine), *P. domestica* L. (European or prune plum), *P. salicina* Lindl. (Japanese plum), *P. cerasus* L. (sour cherry), *P. avium* L. (sweet cherry), *P. armeniaca* L. (apricot) and almond (*P. amygdalus* Batsch) which is cultivated for its edible seed.

All commercial varieties of peach are *P. persica*, including nectarines that differ from peach in the absence of pubescence on the fruit surface. This character segregates as a simple trait presumably controlled by a single gene or a few closely linked genes.

5.1.2 Center of Origin and History of Dispersal

Peaches originated in China, with a cultivation history of over 4,000 years (Hesse 1975). Peach dispersal followed westward with human migration through trade routes and in the wake of conquering armies found it way to Greece. According to Pliny, peach was cultivated in Greece by 332 BC (Hedrick 1917). From Greece, peaches were further dispersed with expansion of the Roman Empire. The early writings of Pliny, Dioscorides, and Virgil exhibit references to peach and apricot (Hedrick 1917; Cullinan 1937). Peaches were brought to North and South America on the ships of the European explorers and settlers. Due to the fact that stone fruits have the seed encased in a hard, lignified structure (stone) obviates special storage conditions thus facilitating their dispersion over long distances. Peach seeds are viable for a year at room temperature and for several years if refrigerated (Scorza and Sherman 1996).

5.1.3 Peach production

Peach is a temperate fruit crop and is grown on all continents except Antarctica. Generally, commercial production lies between latitudes 30° and 45°. The major limiting factors for expansion of commercial production areas are extreme cold temperatures below −35 °C to −40 °C or insufficient length of cold temperature to satisfy dormancy. Table 1 lists the worldwide production, yield and harvest area for peaches and nectarines.

5.1.4 Breeding

Most major peach producing countries have active breeding programs. To develop a new peach cultivar usually takes 15–20 years and requires: 1) pollen collection from male parents; 2) individual hand emas-

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Table 1. Peach world cultivation statistics (FAOSTAT, http//faostat.fao.org)

Year	2000	2001	2002	2003	2004
Production (Mt)	13,317,455	14,005,372	14,712,287	15,355,170	15,561,206
Yield (Hg/Ha)	104,425	112,320	109,781	109,506	109,409
Area Harv. (Ha)	1,275,308	1,246,920	1,340,153	1,402,221	1,422,293

culation of flowers of female parents; 3) hand pollination; 4) collection of seed from fruit that developed from hybridization; 5) seed stratification and germination; 6) greenhouse or nursery culture of seedlings; 7) field planting of the seedlings; and 8) selection and testing of superior phenotypes. The juvenility period in peach is from 2 to 5 years (Sherman and Lyrene 1983). Maintenance, propagation, and selection of seedlings require a large investment of labor, equipment, materials and space, thus various time and space saving methods required for seedling evaluation include the use of high density fruiting nurseries, cultural manipulation, i.e., grafting seedlings onto mature rootstocks, girdling, growth regulator treatments, breeding with dwarf germplasm, and marker-assisted selection (Hansche and Beres 1980; Sherman and Lyrene 1983; Hansche 1990; Scorza 2003). The evaluation of superior seedling selections is a critical stage prior to cultivar release and requires the multiplication of elites on rootstock and evaluation of yield and horticultural characteristics including fruit quality under conditions simulating commercial production. This process generally requires replication in locations and/or years.

Although many commercial peach cultivars were developed from a restricted germplasm base and peach is predominantly self-fertilizing, they remain fairly heterozygous for many characters as evidenced from character segregation in progeny from self or out crosses with wild germplasm. In most cases the characteristics that are desirable for commercial cultivars, including large fruit size, high coloration of the fruit epidermis, and firmness of the flesh, are recessive (Bailey and French 1949). Therefore, integration of adaptive traits from germplasm requires several rounds of introgressive backcrossing to fix the new trait and regenerate the high quality value traits in the original cultivated parent.

Commercially grown peach cultivars represent only a small fraction of the genetic diversity of this species (Scorza et al. 1985; Mehlenbacher et al. 1990; Scorza and Okie 1990; Scorza and Sherman 1996). One of the major problems confronting the fruit breeding community is the loss of native germplasm through deforestation, urbanization, and lack of funds to support germplasm collection and maintenance in the centers of origin. Additionally, cultivation of high quality stone fruit cultivars displaces the lower quality native landraces that carry many of the locally important adaptive traits further accelerating the loss of genetic variability.

5.1.5 Breeding Goals

5.1.5.1 Disease and Pest Resistance

Peaches are susceptible to numerous pathogens and pests (Bailey and Hough 1975; Hesse 1975; USDA 1976; Mehlenbacher et al. 1990; Scorza and Okie 1990). Several book chapters and review articles have summarized the most important disease problems and have discussed breeding strategies and/or programs aimed at obtaining disease resistance (Bailey and Hough 1975; Hesse 1975; Okie et al. 1985; Layne and Sherman, 1986; Childers and Sherman 1988; Scorza 1991; Scorza and Sherman 1996). With the currently environmentally conscious public, chemical control of pests is coming under close scrutiny. As a result, many pesticides are no longer available to the grower, thus interest in natural resistance or engineered resistance has moved the forefront in breeding programs. This fact further underscores the importance of maintenance and study of natural germplasm resources since many native species carry resistance genes that could be introgressed into cultivated varieties.

Peach genotypes have been screened for resistance or tolerance to ring nematode (*Criconemella xenoplax*) (Okie et al. 1987), a primary factor in Peach Tree Short Life syndrome (PTSL), Cytospora canker caused by *Leucostoma* spp. (Scorza and Pusey 1984; Chang et al. 1989), and brown rot (*Monilinia fructicola*) (Gradziel and Wang 1993). These studies have revealed somewhat low but potentially useful levels

of disease resistance and in the case of tolerance to the ring nematode, rootstocks have been developed from initial isolates of tolerant material that ameliorate the effects of peach tree short life in the southeastern United States. Other studies examining the response of numerous peach and nectarine cultivars to *Stigmina carpophila, Monilinia laxa, Sphaerotheca pannosa, Tranzschelia pruni-spinosae, Taphrina deformans* and *Xanthomonas campestris* pv. *pruni* (Simeone 1985; Werner et al. 1986; Simeone and Corazza 1987; Scorza 1992), found most cultivars susceptible to these pathogens.

Other major pests of peach include fruit feeding insects which reduce fruit quality and marketability and insects which feed on vegetative parts of the tree causing reduced viability, performance and increased risk of fungal, bacterial and viral disease. Only a few cases of insect resistance in cultivated genotypes have been reported (Mehlenbacher et al. 1990; Scorza and Okie 1990).

Soil born pathogens also represent a major problem for peach tree cultivation. Nematodes of several different genera are major pathogens of peaches, these include: the dagger nematode (*Xiphenema* spp.) which is responsible for the spread of tomato ring spot virus, a serious pathogen in peach particularly in the US Mid-Atlantic States; root-knot nematodes (*Meloidogyne* spp.) which severely decrease the performance of the trees; the root lesion nematode (*Pratylenchus* spp.) which is associated with replant problems (Scorza and Okie 1990); and the ring nematode (*Criconemella xenoplax*) a primary factor in Peach Tree Short Life syndrome (Okie et al. 1987).

Plum Pox Virus Plum Pox Virus (PPV), also referred to as "Sharka" disease, is one of the most serious diseases of peach and other *Prunus* trees worldwide.

The "Sharka" disease of fruiting trees is caused by a potyvirus *Plum pox virus*. Like other potyviruses, its genome consists of a single RNA molecule/strain (680 to 900 nm length and 15 nm width) 9,800 nucleotides in length with a MW of 3.5×10^6 daltons. It encodes a VPg protein at the 5′ end and is poly adenylated at the 3′ end. According to the sequence the types of isolates can be divided into D, Dideron and M or Marcus serotypes (Laín et al. 1989; Maiss et al. 1989; Teycheney et al. 1989; Riechman et al. 1992; García et al. 1994; Candresse et al. 1998; Rosales et al. 1998).

The woody hosts for PPV are the *Prunus* species including; plums (*P. domestica*) and Japanese plum (*P. salicina*), the apricot (*P. armeniaca*) and peach (*P. persica*). Almonds (*P. dulcis*) can be infected by PPV but are asymptomatic. New PPV isolates that infect cherries (*P. avium* and *P. cerasus*) have also been described. Kalashyan et al. (1994) described a PPV-C in *P. cerasus* and Crescenzi et al. (1997) a PPV-C strain that infects most of the ornamental and wild *Prunus* species, some that are used as rootstocks for grafting trees such us *P. cerasifera, P. insititia, P. besseyi, P. tomentosa, P. spinosa*.

PPV produces symptoms on leaves and fruits. Symptoms vary according to the species, the isolate and the environmental conditions**.** The symptoms on leaves are chlorotic ring spots with necrosis. Symptoms on fruits appear before ripening, and appear as ring spots and deformations. The flesh appears brown and the pits show yellow ring spots. On plum species the affected fruits sometimes drop before reaching maturity.

As is typical with diseases caused by virusus, adequate procedures are presently not available to control the spreading of the virus on infected trees (Llácer and Cambra1998). Cross protection does not work for PPV strains. The spreading of the virus by aphids in a non-persistent manner makes the chemical control of aphids by spraying ineffective. In short term the control of the diseases relays on removing infected trees and planting virus free trees. In a long term the control will be the replacement of the susceptible varieties by resistant cultivars (Dosba et al. 1991).

Natural Resistance to Plum Pox Virus in *Prunus* Germplasm Resistance to pests and pathogens assumes particular importance when fruit quality is affected. Among virus diseases, Sharka disease is of particular concern as it is completely devastating to productivity and to fruit quality. Several laboratories in Europe examined *Prunus* germplasm for resistance to the virus. From this work, it was reported that a limited number of apricot varieties appear to have natural resistance to this disease including 'Goldrich', 'Stark Early Orange', 'Harlayne' 'Harcot' 'Stella' and 'Henderson' (Dosba et al. 1992; Karayiannis and Maniou 1994; Polak and Kominek 1995).

Evaluation of the susceptibility of plum and peach cultivars to Sharka disease has not resulted in the discovery of resistant cultivars like those in apricot. Introgression in a peach genetic background of the resistance available in a related wild species, *Prunus davidiana* and in some almond cultivars, is in progress through back cross progenies (Kervella et al. 1998; Foulongne et al. 2003; Martinez-Gomez et al., 2004).

Due to variable penetrance of the resistance character, to test a putatively resistant cultivar, one needs four years of monitoring after infection to assess the level of resistance or susceptibility. This slows the breeding process and makes finding new sources of resistance difficult. Therefore, it would be of major importance to develop efficient tools to screen for Sharka resistance, particularly where the resistance is recessive or only partial. These genes could then be pyramided to enhance or complement already existing resistant cultivars produced through conventional breeding or via transgenic approaches (see below). In woody plants, molecular tools can provide early information on the genetics of *Prunus* progenies and enable the use of marker-assisted selection (MAS) methods for more efficiently breeding resistant materials.

5.1.5.2
Environmental Stress Tolerance

Peaches are widely adapted throughout their range, and cultivars developed in one growing area are often utilized in many production regions. One of the most important breeding objectives is the development of varieties that perform well in the extremes of a species cultivation range. Thus, for example, in northern regions greater winter hardiness of both flower buds and whole trees is a major breeding consideration as it is the most important factor limiting production in mid-continent and northern climates (Bailey and Hough 1975; Hesse 1975; Mehlenbacher et al. 1990; Scorza and Okie 1990). Flower bud hardiness in peach has been shown to be a complex quantitative trait (Mowry 1964). Peach avoids low temperature injury through deep supercooling, a physical state that depresses the freezing point of cells. In *Prunus*, the degree of deep supercooling is related to cold hardiness of the xylem and flower buds. Cultivated species generally supercool to a lesser degree than hardy wild species (Quamme et al. 1982).

5.1.5.3
Growth Control

Control of tree architecture is of major concern in many fruit tree breeding programs. Genetic control of tree growth habit reduces the need for pruning and facilitates development of more productive, easily managed high-density production systems (Scorza 1984). In peach, several loci control tree size and canopy architecture producing compact (Mehlenbacher and Scorza 1986), spur-type (Scorza 1987), semidwarf (Fideghelli et al. 1979; Scorza 1984), columnar (Scorza et al. 1989, 2002), dwarf (Lammerts 1945; Monet and Salesses 1975; Hansche 1988;) and weeping (Monet et al. 1988) trees. Currently, identification and manipulation of genes controlling the columnar growth habit is underway through application of peach genomic resources and molecular marker mapping (Rajapakse et al. 1995; Scorza et al. 2002, Dr. Renate Horn, personal communication)

5.1.5.4
Fruit Characteristics

Ultimately, fruit quality drives the market for stone fruits. Breeding programs have produced very high quality fruits at maturity, however for storage and shipping of fruit to non-local markets, these varieties must be picked earlier than full maturity resulting in fruits of lesser flavor and aroma in the market place. This has led to a marked decrease consumption of peaches. In the 1960s the US average per capita consumption of peaches was 4.4 kg (Frecon 1988). In the past 20 years the consumption level has remained at 2.0 kg (Cristoso 2002). In comparison to other fruiting species in Rosaceae, (apples 16 kg/yr/capita) the reduced consumption of peaches is partly due to the marketing of immature fruit (Frecon 1988).

Increased firmness of ripe fruit is one of the major breeding targets in peach. Fruit firmness exhibits quantitative genetic control, however, major genes dramatically affecting fruit firmness were previously described including the stony-hard gene (Yoshida 1976), the slow-ripening genes (Ramming 1991). Several other peach fruit traits such as flesh color, melting flesh, soft melting flesh, freestone, low malic acid, and saucer shape are simply inherited. A more complete discussion of the inheritance of peach fruit quality traits can be found in Hesse (1975) and Scorza and Sherman (1996).

5.2 Construction of Genetic Maps

Although *Prunus* is an economically and biologically important genus, little was known about the genome structure and organization of its members up until the advent of DNA marker technologies. However, peach is considered the best genetically characterized species in the genus, and one of the best genetically characterized fruit trees (Mowrey et al. 1990). With the application of DNA marker technologies to the problem of developing genetic resources in trees,

peach has distinct advantages that make it suitable as a model species for structural, comparative and functional genomics. Peach has a relatively short juvenility period, 2–3 years compared to most other fruit tree species, such as, apple, pear, and citrus that have a juvenile phase ranging from 6–10 years (Sherman and Lyrene 1983). While some *Prunus* species such as cultivated plums and sour cherries are polyploid (Moore and Janick 1975), peach is a diploid with $n = 8$ (Jelenkovic and Harrington 1972) and has a comparatively small genome: 5.9×10^8 bp or 0.61 pg/diploid nucleus (Baird et al. 1994). This is equates to about 290 Mbp, about twice the value for *Arabidopsis thaliana* (Arumuganathan and Earle 1991). Finally, a peach transformation system has recently been reported (Perez-Clemente et al. 2005) indicating that peach transformation technologies are developing and these will be useful for facilitating functional genomic studies.

In addition to the importance of peach as a reference for Rosaceae genomics, the genetics of a large number of genes controlling fundamentally important traits has been described in peach. These include genes controlling flower development, fruit development, tree growth habit, dormancy, cold hardiness, disease and pest resistance. Extensive and detailed molecular genetic mapping efforts are being carried out worldwide, and many of these traits (both single gene and QTL) have been mapped. Thus, through the integrated study of genomics and genetics, peach promises to provide biological insight into many important pathways and genes associated with the growth and sustainability of fruiting trees.

5.2.1 Peach Genetics, a Brief History

The cultivated peach belongs to the Rosaceae family, subfamily Prunoideae, genus *Prunus* and subgenus *Amygdalus*. The peach karyotype consists of a clearly identifiable large submetacentric chromosome, and seven more chromosomes of smaller size, two of them acrocentric (Jelenkovic and Harrington 1972; Salesses and Mouras 1977). Although little is known about the chromosomal level location and organization of gene sequences in peach, recent results with fluorescence in situ hybridization (FISH) in the closely related almond (*P. dulcis*) have enabled detection of each chromosome individually based on chromosome length and the positions of the ribosomal DNA (rDNA) genes (Corredor et al. 2004). It is likely that peach chromosomal organization does not differ significantly from that of the other species of *Amygdalus* since crosses between peach and these closely related species are possible and produce fertile hybrids; including the species *P. ferganensis*, *P. mira*, *P. davidiana*, and *P. kansuensis*, and the cultivated almond. Crosses with species of other subgenera (*Prunophora* and *Cerasus*) such as apricot (*P. armeniaca*), Myrobalan plum (*P. cerasifera*), European plum (*P. domestica*), Japanese plum (*P. salicina*) or sour cherry (*P. cerasus*) are also possible, but fertile hybrids are only produced occasionally (Scorza and Sherman 1996).

A distinctive characteristic of peach is its self-compatible mating, unlike the majority of its congeneric species that exhibit various levels of gametophytic self-incompatibility. Selfing (Miller et al. 1989), plus important bottlenecks in its recent breeding history (Scorza et al. 1985), have resulted in a lower level of genetic variability of peach compared to the other *Prunus* crops (Byrne 1990). The high economic value of peach, its self-compatible nature that allows the development of F_2 progenies, and the possibility to shorten the juvenile period to 1–2 years after planting (Scorza and Sherman 1996) together suggest the peach can serve as an appropriate genetic and genomic reference species for *Prunus*.

A total of 42 morphological characters of simple Mendelian inheritance were discovered during the last century (Dirlewanger and Arús 2004), however until the recent development of molecular marker maps, only a few linkage relationships had been determined. Five linkage groups involving 11 major genes were reported by Monet et al. (1996).

5.2.2 Molecular Genetic Mapping in Peach

Chaparro et al. (1994) constructed the first molecular marker map in fruit trees consisting of 83 Random Amplified Polymorphic DNA (RAPD) markers, one isozyme and four morphological characters in a peach intraspecific F_2 progeny. Two more maps based on Restriction Fragment Length Polymorphism (RFLP) markers were published shortly there after; the first constructed in a peach $\times$ peach F_2 progeny (Rajapakse et al. 1995) and the second in a peach $\times$ almond F_2 progeny (Foolad et al. 1995). Later peach maps integrated dominant RAPDs

and Amplified Fragment Length Polymorphism (AFLP) markers with codominant (RFLPs) and morphological markers (Dirlewanger et al. 1998) or were constructed almost entirely with AFLPs (Lu et al. 1998). These maps were considered low level saturated maps having a low average marker density (4.5–8.5 cM/marker), and an excess of linkage groups over the eight expected based on karyotype analysis. These maps had large gaps without markers and many unlinked orphan markers (8–28%).

The first saturated linkage map, constructed exclusively with transferable markers (11 isozymes and 226 RFLPs, most of them detected with Rosaceae DNA probes) in a 'Texas' almond × 'Earlygold' peach F_2 population, was published by a European consortium (Joobeur et al. 1998). All markers were distributed into eight linkage groups with a total distance of 491 cM, representing an average density of 2.0 cM/marker, and maximal gap size of 12 cM. This map (abbreviated as the T×E map) was improved by the addition of 185 simple sequence repeat (SSR) markers, and 126 RFLPs most of them obtained with *Arabidopsis* DNA probes, and five sequence-tagged sites (Aranzana et al. 2003; Dirlewanger et al. 2004). Recently, 264 additional SSRs have been mapped to T×E using the "bin mapping" approach (Howad et al. 2005). From the 817 markers currently placed on the T×E map, 756 (92%) are based on known publicly available DNA sequences, with at least 198 (24%) of these sequences corresponding to a putative protein. Recent EST mapping has tentatively placed an additional 600 EST sequences on this map.

The *Prunus* scientific community has adopted the T×E map as the reference map for the genus. It provides a set of transferable markers that can be used as anchors for map construction in other progenies, a common linkage group terminology and marker order within each linkage group, and a highly polymorphic population that allows mapping markers that would not segregate in most peach intraspecific crosses. Table 2 presents a compilation of the inter- and intra-species peach maps that have been published. Those anchored on the *Prunus* general map are highlighted.

The network of maps interconnected with T×E reference map provides the density of markers necessary to saturate specific genomic regions of any progeny and to search genome wide for sufficient markers for quantitative trait loci (QTL) or other genetic analyses. Given that peach has a low level of intraspecific variation, a very dense "consensus" map with highly polymorphic markers well distributed in all genomic regions would insure that segregating markers are available in regions of interest in other peach crosses. To reach this goal, a supplementary effort will be required to increase the number of SSRs mapped in parallel with targeted strategies to fill regions with low SSR density (Wang et al. 2001, 2002; Georgi et al. 2002).

The existence of a single reference map has made it possible to locate the major genes and QTL that segregated in different populations (Table 3).

In total, 22 loci controlling simple characters were assigned to specific positions on the T×E map, 18 of these loci were mapped in intraspecific peach crosses and three that segregated in interspecific almond × peach crosses. For complex characters 28 QTLs for bloom and maturity time, fruit quality, tree architecture or disease resistance were also placed on the map (Abbott et al. 1998; Viruel et al. 1998; Dirlewanger et al. 1999; Etienne et al. 2002; Verde et al. 2002; Foulongne et al. 2003b).

With the current marker density, most simple characters are marked sufficiently for selection. Other strategies for gene tagging that do not require knowledge of the map position, such as bulked segregant analysis (Michelmore et al. 1991), have also been used successfully in peach (Chaparro et al. 1994; Warburton et al. 1996; Lu et al. 1998). In spite of this information being available, the use of markers for commercial breeding is still in its infancy. Marker-assisted selection is currently used in a rootstock breeding program to pyramid a root-knot nematode (*Meloidogyne* spp.) resistance gene coming from 'Nemared' peach (Lu et al. 1998; Yamamoto and Hayashi 2002; Arús et al. 2004) with another independent root-knot nematode resistance gene coming from Myrobalan plum (Claverie et al. 2004). However, selections using markers of other well-characterized genes affecting fruit characters (i.e. such as flesh color, skin pubescence, fruit shape or fruit sweetness) have not been reported. This is undoubtedly due to the fact that the variability of major traits of interest for the breeders (i.e. ripening time, fruit quality and other characters) is quantitatively inherited. There is published information on QTL characters in peach (Dirlewanger et al. 1999; Etienne et al. 2002), but a more detailed knowledge of the number, effects and map positions of the QTL affecting them is necessary before QTL associated markers can be routinely integrated in selection programs.

Table 2. Peach inter- and intra-specific maps

Population	Species	Type	Marker #	T×E Anchors	No. L.G.[1]	Total Map distance	References[2]
'Texas' 'Earlygold'	almond × peach	F_2	817	817	8	519 cM	Joobeur et al. 1998; Aranzana et al. 2003; Dirlewanger et al. 2004; Howad et al. 2005
NC174RL × 'Pillar'	peach	F_2	88	0	15	396 cM	Chaparro et al. 1994
'N J Pillar' × KV77119	peach	F_2	47	2	8	332 cM	Rajapakse et al. 1995
'Padre' × '54P455'	almond × peach	F_2	161	23	8	1,144 cM	Foolad et al. 1995; Bliss et al. 2002
'Ferjalou Jalousia®' × 'Fantasia'	peach	F_2	124	49	7	518 cM	Dirlewanger et al. 1998; Etienne et al. 2002
'Lovell' × 'Nemared'	peach	F_2	153	1	15	1,297 cM	Lu et al. 1998
'Garfi' × 'Nemared'	almond × peach	F_2	51	51	7*	474 cM	Jáuregui et al. 2001
IF7310828 × *P. ferganensis*	peach × *P. ferganensis*	BC_1	216	71	8	665 cM	Dettori et al. 2001; Verde et al. 2005
'Akame' × 'Juseitou'	peach	F_2	178	45	7*	571 cM	Yamamoto et al. 2001 and personal communication
'Summergrand' × P1908	peach × *P. davidiana*	F_2	153	57	8	874 cM	Foulongne et al. 2003a

[1] L.G. = linkage groups; *linkage groups 6 and 8 of these maps, were mapped as a single group due to the effects of a reciprocal translocation.

[2] When more than one reference is given, the data presented are either from the most recent publication or from the combination of the data from all publications.

Additional candidates for marker-assisted selection in peach are genes or QTLs that can be introgressed into peach from other wild or cultivated species, such as disease or pest resistances identified in *P. davidiana* (mildew, leaf curl, aphids, sharka) by Viruel et al. (1998) and Foulongne (2002). Introgression from wild species is facilitated with marker based whole genome selection approaches (Tanksley et al. 1989) that streamline the recovery of the genome of the cultivated species or elite genotype.

5.2.3 Comparative Mapping of Peach and Other *Prunus* Species

The transferable markers (RFLPs, SSRs and isozymes) mapped in the T×E population have been used for the construction of linkage maps in other *Prunus* species. Detailed comparisons can be made between this map and those of almond (Joobeur et al. 2000), apricot (Lambert et al. 2004), *P. davidiana* (Foulongne et al. 2003a), cherry (Dirlewanger et al. 2003) and *P. cerasifera* (E. Dirlewanger, INRA Bordeaux, 2004, personal communication). The order and distribution of the markers into the eight linkage groups was generally identical between species, suggesting a high degree of synteny. Occasional marker position discrepancies among species maps are attributed to the mapping of different duplicated loci detected by the same RFLP probe or SSR primer pair. An exception to the full collinearity observed within *Prunus* was reported by Jáuregui et al. (2001), who demonstrated the presence of a reciprocal translocation between linkage groups 6 and 8 in an F_2 progeny of 'Garfi' almond × 'Nemared' peach, and established the approximate position of the translocation breakpoint.

Taken together, these results strongly indicate that the group of *Prunus* species studied to date shares a nearly identical genome. Therefore, the information on gene sequence and position obtained in one *Prunus* species would be generally useful for the rest.

Table 3. Major genes and QTL placed on the *Prunus* reference map

Characters	L.G.[1]	Symbol[2]	Populations	References
Flesh color (white/yellow)	G1	*Y*	'Padre' × '54P455'	Warburton et al. (1996) Bliss et al. (2002)
Evergrowing	G1	*Evg*	'Empress op op dwarf' × PI442380	Wang et al. (2002)
Internode length	G1	QTL	(*P. ferganensis* × 'IF310828')BC1	Verde et al. (2002)
Powdery mildew resistance	G1	QTL	'Summergrand' × P1908	Foulongne et al. (2003b)
Flower color	G1	*B*	'Garfi' × 'Nemared'	Jauregui (1998)
Root-knot nematode resistance	G2		'P.2175' × 'GN22', 'Akame' × 'Juseitou'	Claverie et al. (2004), Yamamoto et al. (2001)
		Mi[3]	'Lowell' × 'Nemared', 'Garfi' × 'Nemared' 'Padre' × '54P455'	Lu et al. (1998), Bliss et al. (2002) Jáuregui (1998)
Ripening time, fruit skin color, soluble-solids content	G2	QTL	(*P. ferganensis* × 'IF310828')BC1	Verde et al. (2002)
Double flower	G2	*Dl*	'NC174RL' × 'PI'	Chaparro et al. (1994)
Broomy (or pillar) growth habit	G2	*Br*	Various progenies	Scorza et al. (2002)
Flesh color around the stone	G3	*Cs*	'Akame' × 'Jusetou'	Yamamoto et al. (2001)
Anther color (yellow/anthocyanic)	G3	*Ag*	'Texas' × 'Earlygold'	Joobeur (1998)
Leaf curl resistance	G3	QTL	'Summergrand' × P1908	Viruel et al. (1998)
Fruit weight, fruit diameter, glucose content	G3	QTL	'Suncrest' × 'Bailey'	Abbott et al. (1998)
Polycarpel	G3	*Pcp*	'Padre' × '54P455'	Bliss et al. (2002)
Flower color	G3	*Fc*	'Akame' × 'Jusetou'	Yamamoto et al. (2001)
Blooming time, ripening time, fruit development period	G4	QTL	'Ferjalou Jalousia®' × 'Fantasia'; (*P. ferganensis* × 'IF310828')BC1	Etienne et al. (2002) Verde et al. (2002)
Soluble-solids content, fructose, glucose	G4	QTL	'Ferjalou Jalousia®' × 'Fantasia'	Etienne et al. (2002)
	G4	*F*	(*P. ferganensis* × 'IF310828')BC1;	Verde et al. (2002), Dettori et al. (2001)
Flesh adhesion (clingstone/freestone)			'Akame' × 'Juseitou'	Yamamoto et al. (2001)
Flesh texture (melting/non-melting)	G4	*M*	'Dr. Davis' × 'Georgia Belle' and 'Georgia Belle'⊗	Peace et al. (2005)
	G5	*D*	'Ferjalou Jalousia®' × 'Fantasia'	Dirlewanger et al. (1998, 1999)
Non-acid fruit				Etienne et al. (2002)
Sucrose, malate, titrable acidity, pH, sucrose	G5	QTL	'Ferjalou Jalousia®' × 'Fantasia'	Etienne et al. (2002)
Skin hairiness (nectarine/peach)	G5	*G*	'Ferjalou Jalousia®' × 'Fantasia'; 'Padre' × '54P455'	Dirlewanger et al. (1998, 1999) Bliss et al. (2002)
Kernel taste (bitter/sweet)	G5	*Sk*	'Padre' × '54P455'	Bliss et al. (2002)
Ripening time, fruit skin color, soluble-solids content	G6	QTL	(*P. ferganensis* × 'IF310828')BC1	Verde et al. (2002)
Plant height (normal/dwarf)	G6	*Dw*	'Akame' × 'Juseitou'	Yamamoto et al. (2001)
Leaf shape (narrow/wide)	G6	*Nl*	'Akame' × 'Juseitou'	Yamamoto et al. (2001)
Male sterility	G6	*Ps*	'Ferjalou Jalousia®' × 'Fantasia'	Dirlewanger et al. (1998)
Powdery mildew resistance	G6	QTL	'Summergrand' × P1908	Foulongne et al. (2003b)
Leaf curl resistance	G6	QTL	'Summergrand' × P1908	Viruel et al. (1998)
Fruit shape (flat/round)	G6	*S**	'Ferjalou Jalousia®' × 'Fantasia'	Dirlewanger et al. (1998, 1999)

[1] L.G. = Linkage group; G6-G8 genes located close to the translocation breakpoint between these two linkage groups.
[2] QTL are included if they have been consistently found (at least in two independent measurements) in the indicated populations.
[3] One or two genes of nematode resistance with different notations and one QTL with have been described in this linkage group.

Table 3. (continued)

Characters	L.G.[1]	Symbol[2]	Populations	References
Leaf color (red/yellow)	G6–G8	*Gr*	'Garfi' × 'Nemared'; 'Akame' × 'Juseitou'	Jauregui (1998) Yamamoto et al. (2001)
Fruit skin color	G6–G8	*Sc*	'Akame' × 'Juseitou'	Yamamoto et al. (2001)
Leaf gland (reniform/globose/eglandular)	G7	*E*	(*P. ferganensis* × 'IF310828')BC1	Dettori et al. (2001)
Resistance to mildew	G7	QTL	(*P. ferganensis* × 'IF310828')BC1	Verde et al. (2002)
Powdery mildew resistance	G8	QTL	'Summergrand' × P1908	Foulongne et al. (2003b)
Quinase	G8	QTL	'Ferjalou Jalousia®' × 'Fantasia'	Etienne et al. (2002)

5.2.4 Comparative Mapping of Peach to *Arabidopsis*

In order to examine the evolution of the plant genome, it is extremely valuable to compare structural organization of relatively similar sized genomes of plants that have diverged over significant evolutionary time. Thus, identification of significantly conserved regions potentially identifies functional chromosomal units. The *Prunus* map and the *A. thaliana* genome sequence have been compared using a set of RFLP markers mapped in T×E obtained either with probes of different species (mainly *Prunus* and apple) that had a high level of sequence conservation with *Arabidopsis* (TBLASTX values lower than 10^{-15}) or with *Arabidopsis* probes that hybridized well to *Prunus* DNA (Dominguez et al. 2003). The position of 227 *Prunus* loci (map average density of 2.6 cM/marker) could be compared to that of 703 *Arabidopsis* homologous sequences. The criterion for declaring a syntenic region was that three or more homologous markers had to be located within 1% of the *Prunus* map distance (6 cM) and within a 1% of the *Arabidopsis* genome (1.2 Mb). In addition, blocks with gaps longer than 1% of either genome were rejected. With these stringent criteria it was possible to detect 37 syntenic regions, covering 23% and 17% of the *Prunus* and *Arabidopsis* genomes, respectively. The longest of these regions included 13 markers for a distance of 25 cM in linkage group 2 of *Prunus* and 16 homologous sequences spanning 5.4 Mb in chromosome 5 of *Arabidopsis*.

Similarly, higher resolution studies have not supported extensive preservation of localized genome structure between the two genomes. The sequence of peach bacterial artificial chromosomes (BACs) and BAC ends located in several locations in the peach genome was compared with that of *Arabidopsis*. (Georgi et al. 2003; Sook Jung, personal communication). Predicted genes in these sequences were homologous to genes scattered along the five chromosomes of *Arabidopsis*, with an approximate preservation limit of 2 genes. In summary, macro- and microsynteny results concur in detecting a fragmentary preservation between these two genomes putatively separated for more than 90 million years.

5.3 Genomics

5.3.1 Construction of the Peach Physical Map and its Use in Gene Discovery

5.3.1.1 Structural Genomics in Peach

Large-insert libraries and physical maps are important tools for map-based cloning of Mendelian loci (Arondel et al. 1992) and QTL (Frary et al. 2000). In peach BAC libraries were constructed for 'Nemared' rootstock and a haploid of 'Lovell'. The restriction enzymes used were *Hind*III and *Sau*3A1, respectively. The 'Nemared' library consists of approximately 40,000 clones with average inserts approximately 60 kb in size. The theoretical coverage of the genome is 8–10 fold but in practice it is approximately 4–5 fold. The haploid Lovell library consists of approximately 35,000 clones with an approximate average insert size around 80 kb yielding a theoretical twelve fold coverage of the genome.

Utilizing these BAC library resources the International Rosaceae Genome Consortium (IRGC) is constructing a complete physical map of the peach genome anchored on the general *Prunus* genetic

Table 4. Current summary data for the peach physical map

Number of clones fingerprinted	21,120
Number of clones used for map contig assembly	18,387
Number of singletons	7,194
Number of clones in contigs	11,193
Number of contigs	1,367
Size of contigs: >200 (chloroplast genome)	1
51–100 clones	1
26–50 clones	27
10–25 clones	3,478
3–9 clones	763
2 clones	228
Number of anchored contigs	149 (2,031 clones)
Physical length of contigs	210–230 Mb
Physical length of the anchored contigs	33 Mb

map (Joobeur et al. 1998) essentially following strategies utilized to develop the *Drosophila* physical map and others (Marra et al. 1999; Hoskins et al. 2000; Tao et al. 2001; Cone et al. 2002). The approach utilizes a combination of hybridizing mapped markers, BAC fingerprinting and in our case hybridizing expressed sequence tag (EST) sequences. With the current *Prunus* molecular marker map resources, 210 low-copy mapped RFLP markers, 4,000 peach fruit ESTs, 80 resistance gene analogs, 200 specific complimentary DNAs (cDNAs) and numerous specific AFLP markers have been hybridized to the BAC libraries. We completed BAC fingerprinting approximately 25,000 BACs (15,000 from the 'Nemared' library and 5,000 from the haploid 'Lovell' library from which approximately 15,000 have been used to construct an initial physical map (see map specifics in Table 4 and www.genome.clemson.edu/gdr/).

FPC (V4.7) (Soderlund et al. 2000) was used to construct an initial physical map of the peach genome following strategies employed to construct physical maps in other crops (Marra et al. 1999; Tao et al. 2001). Initially, the map was constructed at a cut-off from e^{-10} to e^{-12} and tolerance 5 to obtain all high confidence overlapping BAC inserts (contigs). These were then merged by testing end clones at cut-off values ranging e^{-8} – e^{11}. As there was a significant amount of hybridization data, merges were often achieved based on common hybridization of BACs in different contigs. However, if only BAC fingerprint data existed, we noted the merge points for further testing. Presently, the framework map is composed of ~1,000 contigs containing approximately 11,000 clones (see Table 4). Based on estimates of an average BAC insert size of 60 kb and an average of 60% degree of overlap in contigs, 80% or better of the peach genome should be high confidence contigs. Currently we are adding in orphan singleton BACs (approximately 7,000 not in contigs from initial map construction) and merging contigs at lower cutoff scores is underway to finalize the initial peach physical map. Preliminary estimates from trial merges of contigs suggests that the initial map will consist of 800–900 contigs with an average of 12 clones/contig upon completion of the analysis. Since the map includes marker hybridization data from the general *Prunus* genetic map, the developing physical map is directly anchored to the genetic map. From initial analysis of the integrated genetic/physical map, there is already evidence for duplication of some regions of the peach genome. The developing physical map is located at the *Prunus* genome website within the Genome Database for Rosaceae (GDR) at Clemson University www.genome.clemson.edu/gdr/. This database is under ongoing development (for details see below).

5.3.2 Functional Genomics

5.3.2.1 Peach EST Functional Genomics Database Development

With the support of the United States Department of Agriculture, the IRGC initiated a peach EST project with the central goal of developing the unique expressed gene set (unigene set) for peach. The cur-

rent efforts are centered on sequencing 30,000–40,000 cDNAs from libraries of developing fruit, shoot and seeds. Original expectations were that these would resolve into 3,000–4,000 unigenes, however, this number was obtained from the first 15,000 sequences finished. The data summary for the completed analysis of 23,000 cDNAs from developing peach fruit and almond seed libraries is available at the website www.genome.clemson.edu/gdr/. Sequencing of developing shoot and root cDNAs is in progress.

We have also begun mapping peach ESTs on the developing physical/genetic peach map and have determined that a significant portion of ESTs (11%) hybridized on our BAC libraries are placed directly on genetically mapped anchored contigs in the physical map. From the current 15,000 sequences, a peach/almond unigene set has been initiated. This unigene set consists of 3,842 putative unique genes.

5.3.2.2 Transcript Map

A set of 180 ESTs (11%) have been localized in 86 locations (involving 80 core markers) on the general *Prunus* genetic map by common hybridization with RFLP markers to BACs in the 'Nemared' library. This EST resource will provide candidate genes for marked regions of the *Prunus* maps containing traits of interest and will be available on-line through the *Prunus* genome database noted above. From the initial fruit unigene set, we have completed hybridizing in excess of 4,000 ESTs onto the 'Nemared' BAC library. From this set, 184 ESTs have been directly located on the general *Prunus* genome map through common hybridization of mapped molecular markers and ESTs. BACs have been identified in the 'Nemared' library for nearly 85% of these ESTs. Initial hybridizations of ~100 ESTs, that failed to detect BACs in the 'Nemared' library, on the haploid 'Lovell' BAC library have been 60% successful. Thus, upon completion of the physical map, virtually all unigene EST locations will be identified.

We are also mapping resistance gene analogues (RGAs) and resistance associated genes (RAGs). We have completed hybridizing over 80 different RGA/RAG genes. From these analyses, we have positioned on the general *Prunus* map/physical map approximately 40 RGAs and RAGs placing a number of these genes in regions known to contain resistance to powdery mildew, plum pox virus and parasitic nematodes (Lalli et al. 2005). This map serves as an initial starting point in the identification and marking of important disease resistance genes in peach and other *Prunus* species.

The structural and functional genomics databases of peach serve as tools for microsynteny analysis of regions of interest and for gene cloning investigations. With the integration of sequenced cDNA loci (EST loci), the physical map database immediately provides candidate genes located in the genetically marked intervals containing traits of interest. These associations provide the potential to greatly speed the process of gene discovery and characterization.

5.3.3 Comparative Physical Mapping of Peach and Other Model Genome Species

One of the most important contributions of DNA marker technology to fundamental studies in plant biology is the ability to rapidly compare genome organization in closely related as well as diverse species. Comparative mapping studies can identify highly conserved genome blocks, and regions of lesser conservation. Identification and molecular dissection of these evolutionarily conserved regions may uncover genetic associations that by virtue of their preservation, are implicated as important for plant development. In addition, comparative mapping information can serve as a starting point for initial mapping and gene cloning investigations in poorly characterized species.

The comparative genome sequence organization of plant genomes has not been examined as extensively as chromosomal mapping level studies, however, some reports suggest that within families, there is a significant preservation of gene repertoire and order among plants with quite different genome sizes (Dunford et al. 1995; Bennetzen et al. 1996; Chen et al. 1997; Kilian et al. 1997; Aramova et al. 1998). Initial comparative sequencing studies between *Arabidopsis* and rice have revealed some conservation of genomic structure in defined regions. The data suggests, however, that genes are being dispersed into and out of regions by mechanisms such as transposition, thus, obscuring microsynteny across great evolutionary distances (Van Dodeweerd et al. 1999). Future research is necessary to examine the degree of microsynteny within and among plant families.

As discussed in the genetic mapping section above, limited comparative mapping between peach and other model genome species was done utilizing molecular marker technologies (Dominguez et al. 2003). This lack of comparative data is also evident at

the high-resolution level, however, there are several reports suggesting that specific regions of the peach genome maintain a very limited microsynteny with the *Arabidopsis* genome (Georgi et al. 2002). These initial studies demonstrate that substantial genome rearrangements have occurred thus limiting the value of interfamily comparative genomics as a tool for gene discovery. However, within *Prunus*, the high level of genome preservation at the low-resolution scale suggests that utilization of the peach genome as an anchor for identification of important genes in other species is more promising. Initial high-resolution comparative studies of peach with plum and apricot suggest that the peach genome database will serve as an excellent source of candidate genes for traits in these species (D. Esmenjaud, INRA Antibes, France 2004, personal communication; M.Badenes, IVIA, Valencia Spain, 2004, personal communication).

5.4 Peach Tissue Culture and Transformation

Genetic transformation is a complementary method of stone fruit improvement that may be particularly useful to increase biotic and abiotic stress resistance and fruit quality (Scorza 1991, 2001; Scorza et al. 1995a; Srinivasan et al. 2004). Plant genetic transformation generally involves the transfer of DNA with the desired gene(s) into cells, and the regeneration of transgenic plants from the transformed cells through in vitro culture.

While genetic transformation is an important tool for peach improvement, a reliable and reproducible transformation and regeneration system from somatic tissue has yet to be developed. The following summarizes the reports of work in peach transformation and regeneration.

Although induction of somatic embryogenesis has been reported for peach, conversion of these somatic embryos into plants is far from routine (Scorza 2001). Raj Bhansali et al. (1990) induced somatic embryos from 1–3 mm long immature zygotic embryos of peaches and nectarines. Guohua and Yu (2002) produced embryogenic callus from immature cotyledons of four Chinese peach cultivars using a two-step process that induced up to 95% of the immature embryos to produce callus with up to eight somatic embryos per explant. Up to 75% of these somatic embryos produced shoots. Scorza et al. (1990a) produced somatic embryogenic cultures from immature (45–50 days post bloom) embryos. Following a 6-month culture period on the media of Hammerschlag et al. (1985) these cultures became growth regulator independent (habituated) and continually produced somatic embryos for up to four years. These embryogenic cultures only rarely germinated to produce viable shoots even when exposed to a number of treatments including cold treatment and various growth regulators.

Direct adventitious shoot regeneration without intervening somatic embryo production has been induced from callus derived from immature zygotic peach embryos (Hammerschlag et al. 1985). The use of immature zygotic explants limit source material availability to only a few months out of the year. Pooler and Scorza (1995) demonstrated adventitious shoot production from mature cotyledons of peach rootstock ('Nemaguard', 'Flordaguard', and 'Nemared') seeds that had been cold-stored at 4 °C for 1–3 years.

As with all peach regeneration systems developed to date successful regeneration is highly genotype dependent. Most of the preceding reports of regeneration from peach have focused on the use of zygotic tissues, and most from immature zygotic embryos. In contrast, Gentile et al. (2002) reported adventitious shoot regeneration from callus cultures of young leaves (1–2 mm long) from in vitro-grown peach shoots in a medium containing 9 µM BA and 0.54 µM NAA. Regeneration rates of 13–28% were obtained using three cultivars from diverse origins and two seedling selections. Most regeneration was obtained from leaf petioles.

Clearly, it is possible to regenerate peach plants in vitro. This has been achieved for the most part by using zygotic tissues. These explant sources have generally not been favored for tree fruit transformation because the ability to improve established cultivars is lost. Each seed-derived genotype is unique and not a clone of the parent. Transformation of zygotic tissues would be useful for providing unique and useful genes to breeding programs where they could be incorporated into new germplasm. Given the facts that the generation cycle for peach is approximately three years [a short cycle when compared with most tree fruit species (Sherman and Lyrene 1983)]; that most new peach cultivars are produced by breeding programs versus the selection of sports of established cultivars; and that peach varieties are continually replaced at a fairly rapid pace (10–12 years or less in some areas), the efficient transformation of peach

germplasm can be of great benefit to the genetic improvement of this species.

While the production of transgenic *Prunus* depends largely on the efficiency of regeneration of plants from transformed cells, the efficiency of transformation itself is also an important factor, one that takes on an even greater level of importance in the case of low regeneration rates. Several reviews have been published on transformation of *Prunus* species, including peach (Scorza and Hammerschlag 1992; Scorza et al. 1995a; Rugini and Gutierrez-Pesce 1999; Srinivasan and Scorza 1999, 2004). Transformation efficiency is affected by many factors including the method of transformation (e.g., *A. tumefaciens* or biolostics); transformation environment; and the antibiotic selection pressure. In most published reports, *A. tumefaciens* has been used to transfer the DNA plasmids carrying the gene(s) of interest to peach cells. Neomycin phosphotransferase (NPTII) has been used as the selectable marker, and in some cases, β-glucuronidase (GUS) or green fluorescent protein (GFP) as a visual marker of transformation (Pérez-Clemente et al. 2004; Padilla et al. 2006).

Although peach is infected by wild *A. tumefaciens* and crown gall disease is common in *Prunus* (Scorza and Sherman 1996), transformation efficiency of peach cells in vitro with disarmed *A. tumefaciens* appears to be relatively low (Padilla et al. 2006). Scorza et al. (1990b) reported the transformation of peach leaf segments, immature embryos, and long-term embryonic callus using *A. tumefaciens* strain A281 carrying plasmid pGA472 with the NPTII selectable marker. Transformation rates of 5% of immature embryos and up to 64% of leaf segments were observed. These explant sources did not undergo organogensis, thus no transgenic shoots were obtained from this work.

In addition to *A. tumefaciens*-based transformation particle bombardment (biolistics) has also been used to produce stably transformed embryogenic peach callus (Ye et al. 1994). Embryogenic callus derived from immature embryos was used as the starting material. No regeneration was obtained from the transformed embryogenic callus produced in this study. Transient expression tests of biolistic transformation of embryogenic callus, embryonic axes, cotyledons, and immature embryos demonstrated high levels of transformation efficiency. The ability to transform these explants was considered to be significant because regeneration from these tissues had been previously reported.

To date, there are only two reports of the development of transgenic peach plants. Smigocki and Hammerschlag (1991) regenerated transgenic peach plants from immataure zygotic embryos following transformation with a shooty mutant strain of *A. tumefaciens*, *tms*328::Tn5, which carried an octopine type Ti plasmid with a functional cytokinin gene and a mutated auxin gene. The use of this cytokinin-producing shooty-mutant strain of *A. tumefaciens* may have been responsible for the successful regeneration of transformed shoots and also for the altered growth habit of the transgenic trees (Hammerschlag et al. 1997). Pérez-Clemente et al. (2004) developed several transgenic peach plants by using zygotic embryo explants from stored seed. Efficiency of plant production was reported as 3.6 + 1.0%. In both reports of peach transformation few transgenic plants were produced and an efficient, reproducible transformation system remains to be developed.

Peach is not unique in the *Prunus* in its recalcitrance to transformation and regeneration. There are few reports of the successful production of transgenic *Prunus* species. Those species that have been transformed include apricot (*P. armeniaca*) (Laimer da Camara Machado et al. 1992), sweet cherry (*P. avium*) (Brasileiro et al. 1991), sweet × sour cherry (Dolgov and Firsov 1999), almond (*P. amygdalus*) (Miguel and Oliveira 1999) *P. avium* × *P. pseudocerasus* cv. Colt (Gutierrez-Pesce et al. 1998), *P. subhirtella autumno rosa* (da Camara Machado et al. 1995) and *P. domestica* (European or prune plum) (Mante et al. 1991; Padilla et al 2003). For most of these species there exists a single report of the development of only a few transgenic plants. Although the *P. domestica* system, uses mature seeds as the explant source and therefore is not a clonal system it has been used repeatedly to develop transgenic trees (Mante et al. 1991; Scorza et al. 1994, 1995b; Padilla et al. 2003) and presents what can be considered a reliable and routine system. It is such a system in terms of reliability and productivity that remains a goal for peach and one that will advance the utilization of gene transfer for peach improvement.

5.5 Future Directions

Significant progress has been made in recent years to understand the genome organization in peach and the other closely related species in Rosaceae. For

Prunus species, the genome organization is highly collinear and thus genetic resources developed in one key species will serve as a tool for identification, characterization and manipulation of important trait controlling genes in the other species. In this regard, genomic research in peach has significantly progressed toward the completion of a physical map/genetic map resource in peach with significant numbers of genes identified and mapped through EST and genomic sequencing efforts. This information is publicly available in the GDR. Recent reports utilizing these resources have demonstrated the importance of this database for identification and study of important fruit tree genes. Manipulation of these genes in peach awaits the development of a reliable transformation system for peach, however, recent reports (Perez-Clemente et al. 2005) suggest that this lies just around the corner and transformation in companion species such as *Prunus domestica* is routine.

Future work in peach will focus on the utilization of this gene information and marker systems for manipulation of important characters in the breeding schemes. The integration of the molecular genetic resources for peach with the traditional breeding programs promises to streamline the breeding process and provide new and improved varieties for the global market. Additionally, significant research efforts remain particularly in the characterization of many of the fundamental gene systems responsible for the unique and important life history traits of these fruit tree species, such as, endodormancy, cold hardiness, chilling requirements for flower bud break, growth habit and drupe fruit development. Other targets of research in peach should include the technologies of proteomics and metabolomics both areas that promise to provide much needed information the genetic control of important fruit quality characters as well as fundamental knowledge on the genetic basis of fruit tree physiology.

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6 Pear

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6.1 Introduction

Pears are among the oldest of the world's fruit crops. Cultivar development has been continuous since early days and now pears are grown in all temperate zones.

6.1.1 Origin and Early Development

Pear species belong to the genus *Pyrus*, the subfamily *Maloideae* (*Pomoideae*) in the family *Rosaceae*. There are 22 widely recognized primary species (Table 1), which are distributed to Europe, temperate Asia and mountainous areas of northern Africa (Bell et al. 1996). All species of *Pyrus* are intercrossable and there are no major incompatibility barriers to interspecific hybridization, in spite of the wide geographic distribution of this genus (Westwood and Bjornstad 1971). So, classification is often difficult, giving similar taxa designated as different species by some authorities. This genus is considered to originate in the mountainous area of western and southwestern China during the Tertiary periods (65–55 million years ago) and to spread into the east and west. Two sub-centers (Central Asia, and Eastern China) of diversity for the genus have been identified (Vavilov 1951). Dispersal is believed to have followed the mountain chains both east and west (Bell et al. 1996). Speciation probably involved geographic isolation and adaptation to colder and drier environments (Rubzov 1944). Kikuchi (1946) classified *Pyrus* species into three groups, small fruited species with two carpels, large fruited species with five carpels, and their hybrids with 3–4 carpels. Small fruited species, called as Asian pea pears, are used for ornamental purpose or rootstocks. Of large fruited species with five carpels, there are three major species, *P. communis* L. (pear or European pear), *P. bretschneideri* Rehd. or *P. ussuriensis* Maxim. (Chinese pear) and *P. pyrifolia* Nakai (Japanese pear: Nashi), which are commercially cultivated in temperate zone. *P. communis* is native to Europe, and is the main commercial species in Europe, North America, South America, Africa and Australia. *P. bretschneideri* is the main species in northern and central China. *P. pyrifolia* is the main species in Japan, southern and central China and Korea. *P. nivalis* Jacq., the snow pear is cultivated in Europe for making perry. *P. pashia* P. Don. is cultivated in northern India, Nepal and southern China. Asian pears are thought to have been domesticated in prehistoric times and to have been cultivated in China for at least 3,000 years (Lombard and Westwood 1987). European pears are thought to have been in Europe since as early as 1000 BC. Homer referred to a large orchard with pears in the Odyssey written in between 900 and 800 BC. The earliest written records of Japanese pears date back to the ancient manuscript of the Emperor Jito in AD 693 (Kajiura 1994).

6.1.2 Evolution of *Pyrus*

Maloideae includes the genus *Malus* (apple). The basic chromosome number of the *Maloideae* ($x = 17$) is high compared to other *Rosaceae* subfamilies ($x = 7$ to 9), indicating a polyploid origin. Classical biochemical studies on leaf phenolic compounds, isozyme studies and botanical data support the hypothesis of an allopolyploid origin (Chevreau et al. 1997). It has been suggested that the *Maloideae* arose as an amphidiploid of two primitive forms of *Rosaceae,* crossing a basic chromosome number of 8 and 9 (Sax 1931; Zielinski and Thompson 1967). These were possibly primitive members of the *Prunoideae* ($x = 8$) and *Spiraeodeae* ($x = 9$). A recent molecular study of the chloroplast gene *rbc*L suggests that *Spiraeodeae* is the maternal ancestor of *Maloideae* (Morgan et al. 1994). The majority of cultivated pears are functional diploids ($2n = 34$). A few polyploid (triploids and

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Table 1. *Pyrus* species. Adapted from Bell et al. (1996)

Species	Distribution
Asian pea pears	
P. calleryana Decne.	Central and South China
P. koehnei Schneid.	South China, Taiwan
P. fauriei Schneid.	Korea
P. dimorphophylla Makino	Japan
P. betulaefolia Bunge	North-East China
Asian large fruited pears	
P. pyrifolia Nakai	Japan, Korea, Central China
P. pashia P. Don.	Nepal, Pakistan, India, West China
P. hondoensis Nakai et Kikuchi	Japan
P. ussuriensis Maxim.	North-East China, Korea, Siberia
P. kawakamii Hayata	Taiwan, South-East China
West Asia	
P. amygdaliformis Vill.	Mediterranean Europe
P. elaeagrifolia Pall.	Turkey, Russia, South-East Europe
P. salicifolia Pall.	Iran, Russia
P. syriaca Boiss.	Lebanon, Israel, Iran
P. regelii Rehd.	Afghanistan, Russia
P. globra Boiss.	Iran
North Africa	
P. gharbiana Trab.	Morocco
P. longipes Coss. et Dur.	Algeria
P. mamorensis Trab.	Morocco
Europe	
P. communis L.	Europe, Turkey
P. nivalis Jacq.	Central, South, West Europe
P. cordata Desv.	South Europe

tetraploids) cultivars of *P. communis* and *P. bretschneideri* exist. Speciation in *Pyrus* has proceeded without a change in chromosome number (Zielinski and Thompson 1967). The genome size of *P. communis* has been estimated by using flow cytometry (Arumuganathan and Earle 1991). According to their report, DNA content of *P. communis* is a 1.03 pg/2C, compared to 0.54 pg/2C in peach and the genome size is approximately 496 Mbp/haploid nucleus.

6.1.3 Morphology and Growth Habitat

The flowers of *Pyrus* usually have 5 sepals, 5 petals, many stamens and 2 to 5 pistils. Asian pea pears (*P. calleryana, P. betulaefolia, P. dimorphophylla* etc.) have 2 pistils, while the major edible species (*P. communis, P. bretschneideri* and *P. pyrifolia*) have 5 pistils. The number of pistils equals the number of carpels. The *Pyrus* have mixed flower buds with leaf and flower initials, an ovary and 2–5 carpels. Carpels are united with each other at the receptacles and each locule has 2 ovules giving a rise to a maximum seed number of 10. The Pear fruits have a core with a fleshy pith and a cortex of flesh. The European pear (*P. communis*) combines a buttery juicy texture with good flavor and aroma. Asian pears are characterized by a crispy texture and unique flavor. Pears are usually grown as a compound genetic system, consisting of a fruiting scion grafted on a rootstock. The rootstock is used for the control of scion vigor and an adaptation to some environmental factors such as alkaline soil, flooding, drought cold hardiness and so on. In some cases of *P. communis* cultivation, trees consist of three components: the scion, the rootstock and an interstock. The

interstock is used where the scion and the rootstock will be incompatible, but will be united each other with the interstock.

6.1.4 Production and Economic Importance

Pear is the third important temperate fruit species after grape and apple, with a world production of 17.2 million metric tons (FAO: Food and Agriculture Organization of the United Nations 2003). Asia produced the most (11 million t), followed by Europe (3.1 million t), North and Central America (894,000), and South America (813,000). The European pear (*P. communis*) production is concentrated in five production area: Europe, North America, South America, South Africa and Oceania, while Asian pears (*P. bretschneideri* or *P. pyrifolia*) production is concentrated in Asia only. Different area produces different pear species. Among countries, China produced the most (9.4 million t), followed by United States (837,000 t), Italy (822,000 t), Spain (682,000 t), Argentina (560,000 t), South Korea (386,000 t), Germany (374,000 t), Japan (366,000 t), Turkey (360,000), and South Africa (320,000). Increased pear production in recent 10 years mainly reflects larger crops in China, and the production in China exceeds 50% of the world production. Production in China increased by 218% between 1990 and 1999. On the basis of the new plantations of pear trees, extension of world pear production is estimated (Segre 2002). A strong extension in pear growing is expected in South America and China. In many countries, pear production is concentrated in areas favorable to its cultivation which results in a good fruit quality. For example, in Italy, more than 70% of output of pears comes from the lowlands of Emilio Romagna and Veneto (Sansavini 1990) and in United States, production is concentrated in the states of California, Washington, and Oregon (Bell et al. 1996). The world yield average is stable but declining a little (Segre 2002). Yields in Asia and Europe are below the world average while other larger productive area is high. Pears figure prominently in international trade. Pear exports in 1998/1999 were 1.5 million metric tons increasing from 1 million metric tons in 1990 (Segre 2002). Main suppliers in Northern Hemisphere were Italy, Belgium, China and United States, and Argentina and Chile were leading exporters in Southern Hemisphere. Pears have many uses: fresh fruits, fruit juice, perry, syrup, cubes for fruit salads, canned products and dry fruits. But pears are grown mainly for the fresh market and for canning industry (Jackson 2003). About 80% of the total production is destined for fresh consumption.

6.1.5 Nutritional Composition

On the basis of 100 grams of the edible portion, European pears (*P. communis*) provide 54 calories of food energy (Standard Tables of Food Composition, released by Ministry of Education, Culture, Sports, Science and Technology, Japan 2003). They consist of 84.9% water, 0.3% protein, 0.1% fat, 14.4% carbohydrate, and 1.9% fiber. Of the major mineral nutrients, there are 140 mg of potassium, 5 mg of calcium, 4 mg of magnesium, 13 mg of phosphorus, 0.1 mg of iron. Of vitamin contents, there are 3 mg of ascorbic acid, and trace amounts of other vitamins. While Japanese pears (*P. pyrifolia*) provide 43 calories of food energy. They consist of 88.0% water, 0.3% protein, 0.1% fat, 11.3% carbohydrate, and 0.9% fiber. There are 140 mg of potassium, 2 mg of calcium, 5 mg of magnesium, 11 mg of phosphorus, and 3 mg of ascorbic acid. Asian pears are usually rich in water with less content of sugars and starch. Asian pears are characterized as a dietary or healthy fruit. The sugar content in fruit depends on the metabolism of unloaded sugars. Pears belong to the *Rosaceae*, in which the main translocating sugar is sorbitol. Sorbitol is converted into glucose, fructose, and sucrose in fruit. The composition of these four sugars plays a key role in sweetness of pear fruits. The differences in sugar composition within species are reported (Kajiura et al. 1979). According to their report, Japanese pears tend to have high sucrose content, on the other hand Chinese pears tend to have low content of sucrose, and European pears tend to have high fructose and starch contents.

6.1.6 Breeding Objective

6.1.6.1 Breeding of New Pear Varieties

In pear breeding programs, improvement of fruit quality is a main objective. A few reports on the inheritance of fruit characters have dealt with pears (Abe et al. 1993; Crane and Lewis 1949; Machida and

Kozaki 1975, 1976). It is very important, for increasing breeding efficiency, to elucidate the mode of the main characters which influences fruit quality. These characters are the fruit weight, flesh firmness, soluble solid content, organic acid content, ripening time and storage potential. However, the attributes that constitute good quality in one species may be different in others. This is the case with European pears and Asian pears. Attributes that constitute good quality among European pears are such as soft, buttery texture, but those among Asian pears are such as juicy, crisp, cracking flesh. The most distinctive characters of Asian pears are their maturation on the tree, no requirement of ripening treatment like off tree, and smooth round shape. Ideal fruit for Japanese market should be large (about 10 cm in diameter), regular and round (Kajiura 1994), although some old cultivars bear fruits with various types of shape, ranging from fusiform and pyriform to oblate. Asian pears are quite distinct from European pears. In European pears, fruit size is important and should exceed 7 cm in length and 6 cm in diameter (Bell et al. 1996). A pyriform shape is preferable. Some important commercial characters of pear breeding are given below.

Fruit Appearance Pears are mainly served as fresh marketing and must have an attractive appearance. The fruit color is the most important factor for the fruit appearance. There are wide variations in skin colors. Yellow, green and red pears attract Chinese consumers (Wei and Gao 2002). In Japan, yellow-green and brown russet pears are preferred. In European pears, the skin should be free of russet and should resist bruising from handling during harvest, grading, storing and ripening. The skin color should be golden yellow and bright with or without a red blush, although green or greenish-yellow type is also acceptable (Bell et al. 1996).

Disease Resistance Disease resistance has become a major concern in the development of new pear varieties. Pears are susceptible to a number of diseases, mostly caused by fungi. In European pears, resistance against fungal disease such as scab (*Venturia pirina*), powdery mildew (*Podosphaera leucotricha*), brown spot (*Stemphylium vesicarium*) and fire blight (*Erwinia amylovova*) is the important breeding objective. Especially, in North America as well as many regions of Europe, the fire blight is widespread in occurrence and devastating effect. In Europe, there is a reduction in pear growing area, mainly due to the fire blight (Deckers and Schoofs 2002). The prime objective of breeding programs in these regions is improved resistance to fire blight in these affected area. While in Asian pears, resistance against fungal disease such as scab (*Venturia nasicola*), rust (*Gymnosporangium asaticum)* and black spot (*Alternaria alternata* Japanese pear pathotype) is receiving attention. Rust (*Gymnosporangium*) and scab (*Venturia*) are differentiated into different species between European and Asian pears. Resistance to the black spot disease has been a major breeding objective in Japan and Korea.

Resistance to Insects Pear psylla, *Cacopsylla pyricola*, can be a limiting factor in European pear production. It is a native species that produces abundant honeydew, which allows a sooty fungus to grow on the fruit surface. The result can be severe tree injury. Codling moth (*Cydia pomonella* L.) is also single most important pest of pears. Resistance to Pear psylla and codling moth is a major breeding objective in Europe and North America.

Ripening Period The earlier harvesting often has a higher commercial value and the orchard scale can be increased owing to the dispersion of labor. The harvest season ranges from July to November in Japan. Early cultivars make growers more profit in Japan. So there is a trend for breeding earlier maturing cultivars in Japan. This is also the case with China. However, pear germplasm resources preserved at present are rich in mid season and late maturing cultivars while there are insufficient early maturing cultivars (Cao et al. 2000). Uniformity of maturity and uniform ripening are important in European pears.

Storage Quality For the world market, it would be useful to have new pear varieties with long storage ability, which would allow continuous marketing during the whole year. In Japan, before the development of the railway or road network growing regions were limited to the suburbs of big cities such as Tokyo and Osaka (Kajiura 1994). In addition, early-maturing cultivars were limited because of their short shelf lives. Even though refrigerating systems has been developed, late- or mid-maturing cultivars are predominant. So new pear varieties with long storage ability are needed especially for an international trade.

Growth Habit Most pears are upright and vigorous, although some variability occurs in breeding populations. But compact columnar habit is not observed in

pears, but found in apples. The recent trend in fruit production is oriented to dwarf and compact trees, which are easier to prune, chemical spray and harvest. A size-controlling rootstock can be a useful tool to produce trees of reduced height. However, a scion mutant with changes in growth habit is found. 'Conference Light', a mutant of 'Conference' shows a reduction of 20% in vegetative growth in comparison with standard 'Conference'. 'Abate Light' also shows a reduction of 40% in vegetative growth in comparison with standard 'Abate Fetel' (Deckers and Schoofs 2002).

Self-Compatibility Most pears show self-incompatibility and do not have a parthenocarpic ability. This self-incompatibility is not a preferable trait for growers because for good cross pollination and fruit set growers must plant cultivars together that are mutually compatible and that flower at the same time. In commercial production of pears, factors such as climate conditions at the time of bloom, effective periods of pollination, pollination methods are important and these need to be considered every year because pollination affects the stable pear production. In Asian countries such as Japan and Korea, artificial pollination is used for stable production and making perfect round shaped fruits. It would be very valuable if new pear varieties have the ability to self-pollinate. This major obstacle to achieve this objective is a shortage of germplasm resources with self-compatibility. A Japanese pear 'Osanijisseiki' and a Chinese pear 'Jinzhuili', self-comaptibility mutants of 'Nijisseiki' and 'Yali' respectively, are available for making new cultivars with self-fertile.

6.1.6.2 Breeding for New Pear Rootstocks

Pear cultivars are still almost exclusively propagated through budding or grafting onto rootstocks. Success in pear culture depends on the use of appropriate rootstocks. The development of improved rootstocks is an important phase of pear breeding. Currently, in vitro propagation of pear is easy, but the trees on their own roots often perform less well than those propagated on good rootstocks (Wertheim 2002). In European pear production, pear trees should not be too vigorous, as they must be suitable for high-density plantings. So the most important breeding objective in production of European pears is to develop rootstocks that induce smaller size and precocity. Clonal quince (*Cydonia oblonga* L.) rootstocks, which can induce different degrees of dwarfing relevant to high-density planting, are used in areas with not too cold winters. When pear is grafted on quince the tree may be reduced by 30 to 60% of the standard size. But, they are usually sensitive to fire blight and to lime-induced chlorosis with less iron absorption. Among quince rootstocks, 'Quince A' ('EMA'), 'B' ('EMB'), and 'C' ('EMC'), were released from East Malling, UK, have been the leading pear rootstocks planted in Europe and other regions. Of these three quince rootstocks, 'EMC' is dwarfing as a result of the heavy and precocious cropping that it induces. Recently, promising rootstocks are the selections 'QR 193-16' of the breeding program of East Malling, 'Pyrodwarf' from Germany, 'Pyriam' from France and Fox series from Italy (Deckers and Schoofs 2002). It may be better to develop dwarfing *Pyrus* rootstocks than to improve the quince rootstocks, because more grafting incompatibility is encountered between intergenic than intragenic grafts. However, the frequency developing dwarf *Pyrus* rootstocks is low and none of the present *Pyrus* clonal selections are easy to propagate. *P. communis* seedlings, especially of the main commercial cultivars 'D'Anjou', 'Winter Nelis' and 'Bartlett', are still widely used although trees on them can be too vigorous and they are susceptible to fire blight and pear decline. However, they have very good winter hardiness, good graft compatibility with scion cultivars and low susceptibility to lime induced iron chlorosis.

While in Asian pear production, a dwarf rootstock has not been developed. Japanese pear trees generally have their vigor controlled by being trained on a horizontal trellis and therefore developing a dwarf rootstock has not been a priority. Most widely used rootstocks are open-pollinated seedlings of semi wild *P. pyrifolia* and pea pear, *P. dimorphophylla* in Japan. Seedlings of *P. betulaefolia* strains originating in northern China should be used where stony pear (Yuzuhada: physiological disorder causing rough skin) is a problem. Strains of *P. calleryana* tolerant to water logging have been selected in Japan. The rootstocks used widely for Asian pears in China are seedlings of *P. bretschneideri, P. pyrifolia, P. betulaefolia* and *P. calleryana* (Wei and Gao 2002). Since rootstocks used for pear in Japan and China are all seedlings, which may be from interspecific or intraspecific hybridization and cannot provide uniform growth. This sometimes makes pear management complicated. In the future, uniform vegetative propagated rootstocks and methods for careful selections are needed.

6.2 Construction of Genetic Maps

Linkage maps and molecular markers would be useful in traditional crossbreeding programs for perennial crops such as fruit tree species. However, genetic studies in pear, as in many fruit trees, have been rare. There is little information on genetic linkage maps, and development of molecular markers on pears despite many researches on apple mapping and molecular markers. The long juvenile periods, the space necessary to manage large number of progenies and the high level of heterozygosity due to a gametophytic incompatibility have limited inheritance studies to a few morphological characters (Chevreau et al. 1997).

6.2.1 Development of Molecular Markers

6.2.1.1 Isozymes

The first report on the use of isozymes in pears was in 1980 by Santamour and Demuth to identify six ornamental cultivars of *P. calleryana* by peroxidase patterns. Peroxidase diversity has also been studied on several species of *Pyrus* (Menendez and Daley 1986) and on 172 cultivars of *P. pyrifolia* (Jang et al. 1991, 1992). Isozymes' variability of several enzymes in pollen was reported by Cerezo and Socias y Company (1989). However, these approaches are used for cultivar identification and in a desire to differentiate genetic sports. Chevreau et al. (1997) examined the inheritance and linkage of isozyme loci in *P. communis* varieties. They analyzed the polymorphisms of 11 enzymes (AAT: Aspartate aminitransferase, ENP: Endopeptidase, EST: esterase, LAP: Leucine aminopeptidase, PRX: Peroxidase, SOD: Superoxide dismutase, ADH: Alcohol dehydrogenase, DIA: Diaphorase, PGD: 6-Phosphogluconate dehydrogenase, PGI: Phosphoglucoisomerase, PGM: Phosphoglucomutase) in 11 progenies from controlled crosses. According to their report, 22 loci were identified and segregation was scored for 20 loci. Three pairs of duplicated loci forming intergenic hybrid bands were detected and these were found to correspond to equivalent duplicated genes in apple. They identified 49 active alleles and one null allele and revealed three linkage groups, which could all be related to existing groups on the apple map. Conservation of isozyme patterns, duplicated genes and linkage groups indicates a high degree of synteny between apple and pear. But no linkage map for pears was constructed based on the information of isozyme analysis.

6.2.1.2 DNA-Based Markers

RAPD RAPD has been widely used on pear genetic studies because RAPD has the advantages of being readily employed, requiring small amounts of genomic DNA. RAPD markers have been successfully used for identification and genetic relationships of pear. Oliveira et al. (1999) investigated molecular characterization and phenetic similarities between several cultivars of *P. communis* and *P. pyrifolia* and several wild species by RAPD markers. A total of 118 *Pyrus* spp. and cultivars native mainly to east Asia were analyzed by RAPD markers to evaluate genetic variation and relationships among the accessions (Teng et al. 2001, 2002). According to their reports, RAPD markers specific to species were identified, and the grouping of the species and cultivars by RAPD largely agrees with morphological taxonomy. RAPD markers have also been used to identify parentage (Banno et al. 2000). Banno et al. (1999) also identified an RAPD marker linked to the gene conferring susceptibility to black spot disease (*Alternaria alternata* Japanese pear pathotype).

AFLP AFLP technology is a powerful tool that combines DNA restriction and PCR amplification. AFLP has several advantages over the RAPD technique, like a higher number of loci analyzed and a higher reproducibility of banding patterns. Monte-Corvo et al. (2000) investigated the genetic relationships among 39 cultivars including 35 *P. communis* and 4 *P. pyrifolia* cultivars using AFLP and RAPD markers. They confirmed that AFLP markers were five times more efficient in detecting polymorphism per reaction. Although some differences can be noticed between the dendrograms resulting from AFLP and RAPD analyses, both techniques produced similar results. Yamamoto et al. (2002b) also made 184 and 115 polymorphic AFLP fragments using 40 primer combinations in the F_1 population originating from 'Bartlett' and 'Hosui', respectively. They reported that the average number of polymorphic fragments per primer combination was 4.6 in 'Bartelett' and 2.9 in 'Hosui'.

SSR SSRs are excellent sources of polymorphisms in eukaryotic genomes. The development of SSRs is labor intensive. However, SSRs have been very useful in

studying diversity in *Pyrus*. Yamamoto et al. (2002a) constructed a genome library enriched with (AG/TC) sequences from 'Hosui' Japanese pear using the magnetic beads method. They obtained 85 independent sequences containing 8–36 microsatellite repeats. Out of the 85 sequences, 59 contained complete (AG/TC) repeats. Thirteen primer pairs could successfully amplify the target fragments, and showed a high degree of polymorphisms in the Japanese pear. Kimura et al. (2002) identified 58 Asian pear accessions from six *Pyrus* species using these nine SSR markers with a total of 133 putative alleles. They obtained a phenogram based on the SSR genotypes, showing three major groups corresponding to the Japanese, Chinese and European groups. Moreover, nine apple SSRs were intergenetically applied to the characterization of 36 pear accessions (Yamamoto et al. 2001). All of the tested SSR primers derived from apple produced discrete amplified fragments in all pear species and accessions. The differences in fragment size are mostly due to the differences in repeat number. A total of 79 alleles were detected from seven SSR loci and thus pear and apple varieties could be differentiated. This data show that *Pyrus* family has a close genetic relationship with *Malus* family.

RFLP and Others RFLPs have been used to identify Japanese pears, including the parentage of 10 cultivars, with two minisatellite probes from human myoglobin DNA (Teramoto et al. 1994). Similar attempts have been made to distinguish *Pyrus* species with RFLPs of chloroplast DNA (Iketani et al. 1998; Katayama and Uematsu 2003). However, these markers were used for cultivar identification and investigating genetic relationships among *Pyrus* species. Itai et al. (1999) have identified RFLP markers linked to the locus that determine the rate of ethylene evolution in ripening fruit of the Japanese pear by using two probes of 1-aminocyclopropane-1-carboxylate (ACC) synthase genes in ethylene biosynthetic pathway. SCARs were developed from RAPDs to evaluate and identify *P. communis* and *P. pyrifolia* cultivars (Lee et al. 2004). ISSR markers also have been used for cultivar identification and taxonomic relationships in pears (Monte-Corvo et al. 2001). Another unique markers, copia-like retrotransposons have been identified in pears (Shi et al. 2002). They suggest that the transposition of retrotransposons take place during evolution leading to diversification. However, no data on the inheritance of these markers has yet been reported.

6.2.2 Constructing Linkage Maps

First linkage maps in *Pyrus* species were developed for 'Kinchaku' and 'Kosui' Japanese pears using RAPD markers (Iketani et al. 2001). Black spot and pear scab are the most severe diseases of Japanese pear. Only a few cultivars are susceptible to black spot, on the other hand most cultivars of Japanese pear are susceptible to pear scab. A survey of *P. pyrifolia* germplasm has identified 'Kinchaku' as the only cultivar having resistance. They used the pseudo-testcross method (Grattapaglia and Sederoff 1994) and constructed two separate maps from segregation data of 82 F_1 individuals. The reason for using the pseudo-testcross method is that it is very difficult to make F_2 or backcross populations because pears take long periods to progressing generation and don't have a self-pollination nature. The linkage map for 'Kinchaku' consisted of 120 loci in 18 linkage groups (LG) spanning 768 cM, while that for 'Kosui' contained 78 loci in 22 linkage groups extending over 508 cM. This is the first report of a linkage map of pear species. The resistance allele of pear scab (*Vn*) and the susceptibility allele of black spot were mapped in different linkage groups in 'Kinchaku'. However, in both maps the number of linkage groups do not converge into a basic chromosome number ($x = 17$). Therefore, the total map length is still not sufficient for covering the complete genome. The length of the apple genome was reported to be 1,200 cM or a little more (Conner et al. 1997). Pear has the same basic chromosome number as apple. In addition, the nuclear DNA content of pear species is estimated 3/4th or 4/5th of that of apple (Dickson et al. 1992). These two maps are estimated to cover at least about a half of the total pear genome (Iketani et al. 2001).

The second linkage maps were reported using 63 F_1 individuals obtained from an interspecific cross between the European pear 'Bartlett' and the Japanese pear 'Hosui' by Yamamoto et al. (2002b, 2004). They constructed maps based on AFLPs, SSRs (from pear, apple and *Prunus*), isozymes, and phenotypic traits (leaf color and S-genotype). The map of 'Bartlett' consisted of 256 loci including 178 AFLPs, 76 SSRs (32 pear, 39 apple, 5 *Prunus*), one isozyme and a self-incompatibility locus on 19 linkage groups over a total length of 1,020 cM (Fig. 1). The average distance between each pair of loci is 4.0 cM. The size of linkage groups ranges from 88 cM (LG 4) to 11 cM (LG 18). The segregation of many markers on LG 14 is largely

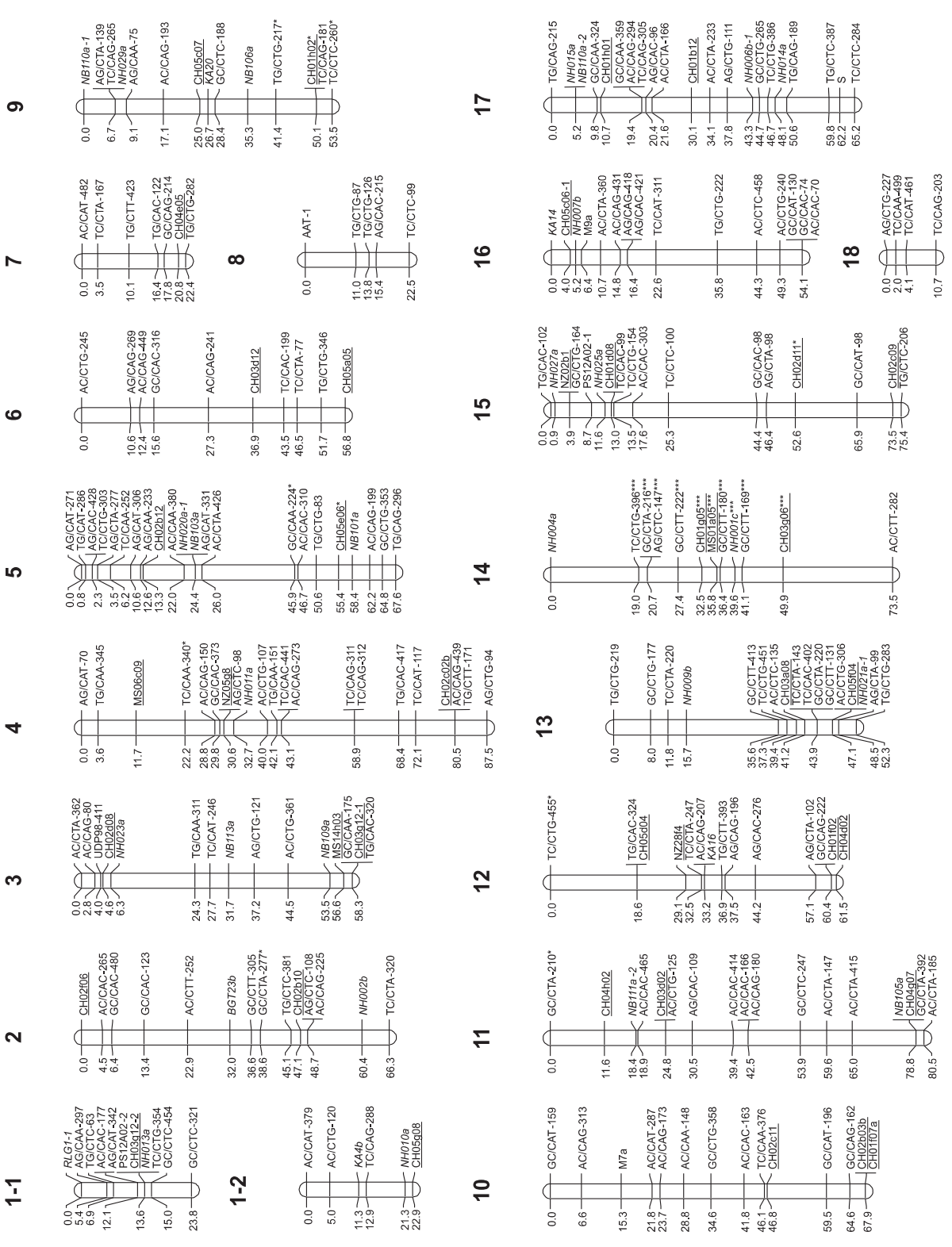

Fig. 1. A genetic linkage map of the European pear 'Bartlett' (Yamamoto et al. 2004). The designation of AFLP markers is based on primer combination and size. SSR loci from apple and pear are *underlined* and in *italics*, respectively. The self-incompatibility locus is denoted by *S*. *Asterisks* indicate distorted segregations of markers according to the chi-square test. Distortion at the 5%, 1% and 0.1% level are indicated as *, ** and ***, respectively

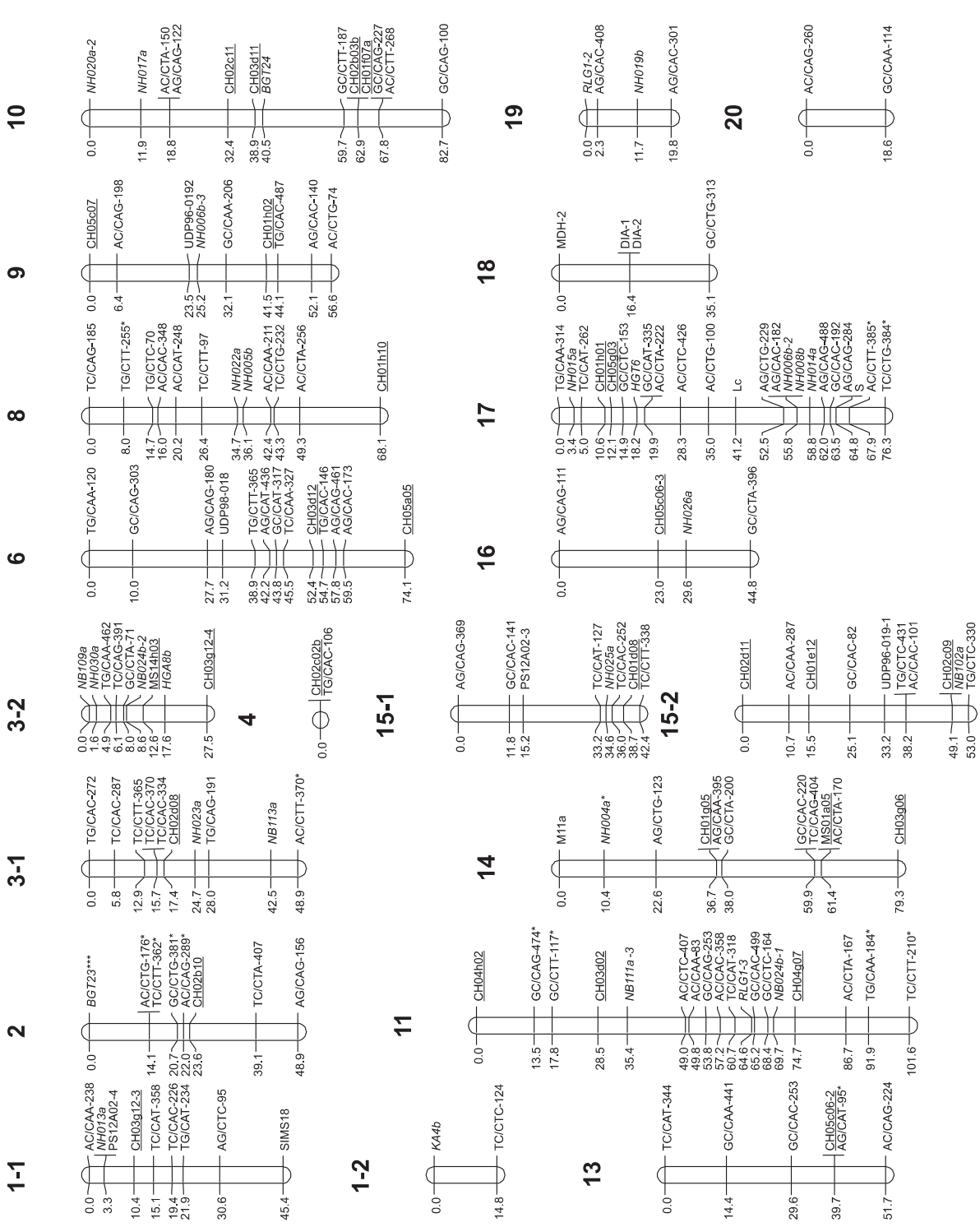

Fig. 2. A genetic linkage map of the Japanese pear 'Hosui' (Yamamoto et al, 2004). The *symbols* and *asterisks* are the same as Fig. 1. Young leaf color is denoted by *Lc*

distorted. The self-incompatibility locus (S-locus) is in the bottom of LG17. While the map of 'Hosui' contained 180 loci including 110 AFLPs, 64 SSRs (29 pear, 29 apple, 6 *Prunus*), two phenotypic traits and four other markers on 20 linkage groups encompassing a genetic distance of 995 cM (Fig. 2). Genetic linkage maps of these cultivars are aligned using 37 codominant markers that show segregating alleles in both cultivars (Yamamoto et al. 2002b, 2004). They found that of tested 80 SSRs obtained from apple, more than four-fifth could produce discrete PCR bands in pear. Similar findings were observed in European pears by another reaserch group (Pierantoni et al. 2004). Yamamoto et al. (2004) reported that 38 apple SSR markers showed 39 segregating loci on the linkage map of 'Bartlett', and that 27 SSRs produced 29 loci on that of 'Hosui'. Moreover, they considered synteny between pear and apple linkage maps. Total 36 SSRs originating from apple were mapped on the genetic linkage maps of 'Bartlett' and apple. Only two SSR loci were aligned to different linkage groups between pear and apple. Other 34 apple SSR loci were positioned in presumably homologous linkage groups of pear. All pear linkage groups were successfully aligned to the apple consensus map by at least one apple SSR, indicating that positions and linkages of SSR loci were well conserved between pear and apple. Their trials were the first major effort in comparing maps of apple and pear. Next, more SSRs and molecular markers for agronomically important characters could be developed to construct fine linkage maps resulting in useful for marker assisted selection.

Other maps were developed for two European pear cultivars 'Passe Crassane' and 'Harrow Sweet' using SSRs, MFLPs, AFLPs, RGAs and AFLP-RGAs markers in 99 F_1 individuals (Dondini et al. 2004). The existence of different levels of susceptibility to fire blight, one of the most terrible diseases, was distributed in European pear cultivars. This suggests that it is possible to identify quantitative trait loci (QTL) related to resistance in pear germplasm. 'Passe Crassane' is a susceptible cultivar and 'Hallow Sweet' is resistant. The 'Passe Crassane' map consists of 155 loci including 98 AFLPs, 37 SSrs, 6 MFLPs, 4 RGAs, and 10 AFLPs-RGA for a total length of 912 cM organized in 18 linkage groups. The average distance between each pair of loci is 5.8 cM. The size of each linkage group ranges from 7.0 to 92.9 cM. The 'Hallow Sweet' map consists of 156 loci including 101 AFLPs, 35 SSRs, 3 MFLPs, 3 RGAs and 14 AFLPs-RGA for a total length of 930 cM organized in 19 linkage groups. The sizes of these maps are comparable with the report by Yamamoto et al. (2002b).

6.3 Gene Mapping and QTL Detection

So far, there is only a report on the QTL analysis in *Pyrus* (Dondini et al. 2004). The only and the first QTL mapping involved the fire blight resistance in European pears. Fire blight continues to spread throughout western, central and southern Europe despite quarantine measures treated (Jock et al. 2002). The existence of different levels of susceptibility to fire blight is distributed in European pear cultivars. Fire blight resistance in pear is known as a quantitative trait (Dondini et al. 2002). Dondini et al. (2004) constructed two genetic linkage maps of the parental lines 'Passe Crassane' (susceptible) and 'Hallow Sweet' (resistant) using SSRs, MFLPs, AFLPs, RGAs and AFLP-RGAs markers and conducted QTL analysis of fire blight resistance. QTL analysis identified four regions of 'Hallow Sweet' associated with fire blight resistance, while no QTLs related to resistance were found in susceptible 'Passe Crassane'. This represents a first step of marker-assisted selection (MAS) approach in pear breeding programs designed to select new fire blight resistant genotypes. Moreover, the presence of each putative QTL of SSR markers makes it possible to transfer map information to different pear cross populations.

6.4 Marker-Assisted Breeding

A long juvenile period and high level of heterozygosity due to a strict gametophytic incompatibility have limited the parental combinations in pear breeding programs. Marker-assisted selection (MAS) is considered to be a powerful tool for increasing selection efficiency by identifying favorable genetic combinations in fruit trees as well as other crops. The major advantage of MAS is the ability to evaluate many traits at the seedling stage in fruit trees that have a long juvenile phase. Especially, MAS in pear breeding programs can be particularly important for traits that are difficult to evaluate. However, available markers for MAS are limited to some extent in *Pyrus*. Banno et al. (1999) tested 250 RAPD primers to screen

a pair of bulked DNA samples derived from open-pollinated progenies of Japanese pear 'Osa Nijisseiki' to identify markers linked to the susceptible *A* gene of black spot disease, caused by *Alternaria Alternata* Japanese pear pathotype. The CMNB41 primer generates a 2,350 bp fragment, which is present in the susceptible bulk, but not in the resistant one. This RAPD marker, CMNB41/2350, is at a distance of about 3.1 cM from the susceptible *A* gene. They found that the frequency of occurrence of the CMNB41/2350 marker was 96% in susceptible cultivars and progenies of 'Osa Nijisseiki' × 'Oharabeni'.

Ethylene production by cultivated Japanese pear fruits varies from 0.1 nl g^{-1} h^{-1} to 300 nl g^{-1} h^{-1} during fruit ripening, suggesting there are both climacteric and non-climacteric cultivars. Climacteric-type fruits exhibit a rapid increase in ethylene production and have a low storage potential, while non-climacteric fruits show no detectable ethylene production and fruit quality maintained for over a month in storage. Fruit storage potential is closely related to the maximum level of ethylene production in Japanese pear. Itai et al. (1999, 2003b) have cloned three ACC (1-aminocyclopropane-1-carboxylate) synthase genes (*PPACS1, 2, 3*), and studied their expression during fruit ripening. *PPACS1* was specifically expressed in cultivars of high ethylene production, while *PPACS2* was specifically expressed in cultivars of moderate ethylene production. Moreover, they have identified RFLP markers linked to the ethylene evolution rate of ripening fruit using RFLP analysis with two ACC synthase genes (*PPACS1* and *PPACS2*). RFLPs were designated as A (2.8 kb of *PPACS1*) linked to high levels of ethylene (> 10 nl g^{-1} h^{-1}) and B (0.8 kb of *PPACS2*), linked to moderate levels of ethylene (0.5 nl g^{-1} h^{-1}–10 nl g^{-1} h^{-1}), when the total DNA was digested by *Hin*dIII. These markers (A and B) are useful for the selection of Japanese pear cultivars with enhanced post-harvest keeping ability. These markers were converted to more convenient and easier PCR-based CAPS markers (Itai et al. 2003a). Furthermore, linkage analysis of these two markers were conducted in the F_2 populations derived from self-pollinated 'OT16', a F_1 of 'Osa Nijisseiki' (a self-compatible mutant of 'Nijisseiki') × 'Cili', which revealed that the recombination frequency between the two markers was 20.8±3.6%. F_2 populations in *Pyrus* have not been reported so far because of a strict gametophytic incompatibility. These are the first populations of self-pollinated F_2 in *Pyrus* species.

Most pear cultivars have been classified as self-incompatible. Therefore, the proposition of pollinizers inter-planted in the orchard is a requirement to get an economic crop from most of the cultivars (Sanzol and Herrreo 2002). The progression of our understanding of incompatibility in *Pyrus* has accelerated greatly since the mid-1990s. In *Pyrus*, gametophytic self-incompatibility is controlled by a single polymorphic gene locus, the S-locus. The S-locus harbors a multi-allelic gene, which encodes for S-RNase that blocks incompatible-tube growth through the style (Ushijima et al. 1998). In Japanese pear, cDNAs encoding S_1- to S_9-RNase have been isolated and sequenced (Sassa et al. 1997; Ishimizu et al. 1998; Takasaki et al. 2004). Based on the nucleotide sequences, Ishimizu et al. (1999) established a PCR-RFLP (S_1- to S_7-) system for S-genotype assignments in Japanese pear. Takasaki et al. (2004) modified this system and finally established the system for discriminating S_1- to S_9-allele in Japanese pear. Both S-alleleic constitution and cross-incompatibility groups have been for Japanese pear, although the situation contrasts with the scarce information available in European pear. In Asian countries, artificial pollination is often used for stable production, therefore knowing S-genotype of commercial cultivars is very important thing, in comparison with open-pollination in Europe. Recently, molecular techniques have started to be used for the identification of S-genotypes in European pears (Sanzol and Herreo 2002; Zuccherelli et al. 2002; Zisovich et al. 2004). Six *S*-allele (Sa- to Sh-) was identified using 10 cultivars by Zuccherelli et al. (2002), four S-allele (S1- to S4-) was identified using seven cultivars by Sanzol and Herreo (2002), and seven S-allele (Si- to So) was identified by Zisovich et al. (2004). Both the methods and the determination of S-gentypes will facilitate the stable production.

6.5 Future Scope of Works

Breeding pears is complicated by their long juvenile phase and complex genetic structure. Pears present a high level of heterozygosity, therefore a great deal of segregation must be taken into account for in breeding populations. Moreover, the lack of morphological markers in pears has been the obstacle to limit the improvement of selection techniques. However, approaches to the improvement of pears

by breeding have changed markedly in recent years, due to our expansion of knowledge and techniques on genes and gene function. This trend will be accelerated by the development of biochemical and molecular markers linked to important horticultural traits. Pear breeding will be entering a new era of knowledge acquisition with the start of large scale genomics programs. We expect pear genomics research programs to deliver a vast amount of data which will lead to a better understanding of this crop in terms of its relationship with the environment and its metabolic pathways. Finally, the increased knowledge provided by genomic studies will bring new tools to assist the creation of cultivars more adapted to the future request for stable pear production.

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7 Apricot

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7.1 Introduction

7.1.1 History of the Crop

Under the generic term apricot different species and one naturally occurring interspecific hybrid are usually included: *Prunus armeniaca* L., the common apricot; *P. armeniaca* var. *ansu* Komar, the Ansu apricot; *Prunus brigantina* Vill., the Briancon apricot or Alpine plum*; Prunus holosericea* Batal., the Tibetan apricot; *Prunus mandshurica* (Maxim.) Koehne, the Manchurian apricot; *Prunus mume* (Sieb.) Sieb. et Zucc., the Japanese apricot; *Prunus sibirica* L., the Siberian apricot, and *Prunus* × *dasycarpa* Ehrh, the black or purple apricot, a naturally occurring hybrid of *P. cerasifera* Ehrh. and

P. armeniaca L. (for reviews see Mehlenbacher et al. 1990; Layne et al. 1996; Faust et al. 1998). In addition, new interspecific hybrids have been recently obtained by artificial cross-pollination. Thus, "plumcot" is a putative hybrid between diploid plums (*Prunus salicina* Lindl.) and apricots (*P. armeniaca* L.) and "pluot" and "aprium" are complex hybrids considered to result from interspecific crosses of plums and apricots with subsequent backcrossing to plum (pluots) or to apricot (apriums) (Manganaris et al. 1999a; Ahmad et al. 2004). All apricot species are interfertile diploid species with eight pairs of chromosomes ($2n = 16$). In this review, we will pay attention to two cultivated species, *P. armeniaca*, the common or European apricot, and *P. mume*, the Japanese apricot.

Most cultivated apricots belong to the species *Prunus armeniaca* that originated in Central Asia where it has been cultivated for millennia and from where it was later disseminated both east and westward. Vavilov proposed three centers of origin: the Chinese Center (mountains of northeastern, central and western China), the Central Asian Center (mountains of Tien-Shan, Hindukush to Kashmir), and the Near-Eastern Center (mountains west of the Caspian Sea including the Caucasus and mountains of Georgia, Azerbaidjan, Armenia, Turkey and Northern Iran), the latter being a secondary center of diversification (Vavilov 1992). Bailey and Hough (1975) also suggested a North Chinese group (Siberian apricot and Manchurian apricot) and an East Chinese group (Ansu apricot).

According to Layne et al. (1996), the common apricot can be classified into six main ecogeographical groups: Central Asian, East Chinese, North Chinese, Dzhungar-Zailij, Irano-Caucasian, and European. Nevertheless, this classification is becoming complicated due to the introduction of new cultivars derived from crosses between genotypes of the different groups (Faust et al. 1998). The Central Asian group is the oldest and more diverse; most of the apricots belonging to this group are self-incompatible and show high chilling requirements. The Dzhungar-Zailij group includes mostly self-incompatible small-fruited cultivars. The Irano-Caucasian group includes mostly self-incompatible genotypes with low cold requirements from the Caucasian area, Iran, Iraq, North Africa and some cultivars from Southern Europe. The European group is the most recent and the least variable, comprising mainly self-compatible genotypes and includes the commercial cultivars of Europe, America, South Africa and Australia (for reviews see Mehlenbacher et al. 1990 and Faust et al. 1998).

Cultivation of *P. armeniaca* in China was practiced more than 3,000 years ago and spread through central Asia. Apricot culture was introduced in the Mediterranean region from Iran or Armenia around the first century BC (Zohary and Hopf 1993), although more recently new introductions were made from the Middle East, especially into Southern Europe (Faust et al. 1998). Thus, Spanish cultivars could be derived

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from North African genotypes brought by the Arabs (Hagen et al. 2002). As a result of trading and commerce, apricots were introduced into England and the United States (Virginia) in the 17th century (Ogawa and Southwick 1995). Later, apricot was introduced into California by the Spaniards in 18th century (for review see Faust et al. 1998).

The Japanese apricot (*Prunus mume* Sieb. et Zucc.) originated in Southeast China in warmer and more humid conditions than *P. armeniaca* (Mehlenbacher et al. 1990). It has been cultivated for over 3,000 years and wild forms can still be found in mountainous areas. In Japan, Japanese apricot has been planted as ornamental since ancient times. Later, ancient Japanese people found that Japanese apricot fruits have medicinal properties and the cultivation for fruit production started and spread across the country.

7.1.2 Botanical Description

P. armeniaca and *P. mume* are members of the family Rosaceae, in the genus *Prunus* L., subgenus *Prunophora* Focke and section *Armeniaca* (Lam.) Koch (Rehder 1940). Both *P. armeniaca* and *P. mume* are diploid ($2n = 16$). *P. armeniaca* has a small genome (5.9×10^8 bp) that is about twice the size of *Arabidopsis thaliana* and between that of two other important diploid species in *Prunus* with $n = 16$ such as peach (5.4×10^8 bp) and cherry (6.8×10^8 bp) (Arumuganathan and Earle 1991).

The common apricot grows in geographically diverse areas ranging from the cold winters of Siberia to the subtropical climate of North Africa and from the deserts of Central Asia to the humid areas of Japan and eastern China. However, commercial production areas are still very limited (Mehlenbacher et al. 1990). Some apricot cultivars are particularly prone to irregular productions that have been associated to the narrow adaptability range of the species. Indeed, while in other fruit tree species a few cultivars are grown all around the world, in apricot each cultivar is usually restricted to a particular geographical area with certain ecological conditions (Layne et al. 1996) and where apricot culture is most successful is in mild, Mediterranean climates. Rainfall and high humidity during the growing season, particularly at bloom or harvest, is a serious limitation due to fungal diseases, which can kill the flowers and shoots or rot the fruits (Ogawa and Southwick 1995).

Common apricots are small to medium sized spread trees capable of reaching 14 m in their native range. The one-year-old wood and spurs are thin, twiggy, and shorter lived than those of other *Prunus*. Leaves are elliptic to cordate and have serrate margins and long, red-purple petioles. Apricots produce perfect, perigynous, white to pinkish flowers borne singly or doubly at a node, with five sepals and petals and about 30 stamens, all of which emanate from the hypanthium or floral cup, and one pistil with a single carpel. In apricot, as in other *Prunus* species, two ovules are present in the flower although usually only one seed is produced (Rodrigo and Herrero 2002). Several cases of pollen sterility have been described and, although most commercial cultivars are self-fertile, self-incompatible cultivars exist (Schultz 1948). Floral buds are initiated in late spring or summer. The chilling required to initiate flowering (below 7 °C) ranges from 300 to 1,200 h. The heat requirement following chilling is very short, causing apricots to bloom early in most locations. The blooming period lasts one to two weeks in early spring. Dormant trees tolerate winter low temperatures, but early emergence from dormancy results in freeze injury to blossoms and even death of trees in some growing areas where late freezes occur. Thus, apricot is prone to frost injury due to the early bloom habit and, subsequently, the production area is limited by the danger of spring frost (Mehlenbacher et al. 1990; Layne et al. 1996).

The fruit of the apricot is a drupe consisting of a stony endocarp surrounding the seed, a fleshy mesocarp, and an exocarp (fruit skin). Fruits of the common apricot can be freestone or clingstone with round to oval shape and glabrous to pubescent fruit skin. Fruit flesh can be sweet or sour and flesh color is mostly orange, but a few white-fleshed cultivars exist (Layne et al. 1996). Fruits are climacteric and require 3–6 months for development, depending on cultivar (Jackson and Coombe 1966). Ripe fruits are soft to touch and highly susceptible to decay-causing organisms (Ogawa and Southwick 1995).

The Japanese apricot is a deciduous tree of large stature, occasionally reaching almost 10 meters in height. The type of petals is variable depending on the cultivar. Petals are either single or multiple, white, pink or red. Flowers consist of one pistil including two ovules and more than 50 stamens. Some cultivars show male-sterility or self-incompatibility. Fruit size is also variable from 5 to 50 g depending on cultivars. The fruits are clingstone and smaller than European

Table 1. FAO: Apricot production (t) (%), area (ha) (%), yield (t/ha). Average data from 2000 to 2004. (Faostat 2005)

Country	Production (%)	Area (%)	Yield
World	2,595,871 (100)	395,250 (100)	6.57
Turkey	448,800 (17)	63,665 (16)	7.05
Iran	278,664 (11)	30,929 (8)	9.01
Italy	182,570 (7)	16,044 (4)	11.48
France	138,548 (5)	15,431 (4)	9.01
Spain	132,893 (5)	22,630 (6)	5.87
Pakistan	130,113 (5)	12,985 (3)	10.02
Morocco	101,200 (4)	13,056 (3)	7.74
Syria	89,340 (3)	12,549 (3)	7.11
Ukraine	84,862 (3)	10,740 (3)	8.01
USA	82,274 (3)	7,645 (2)	10.76
China	81,873 (3)	17,680 (4)	4.65
Greece	72,389 (3)	4,700 (1)	15.40
Russia	70,400 (3)	19,400 (5)	3.63
Egypt	69,714 (3)	5,620 (1)	12.41
Algeria	67,562 (3)	25,378 (6)	2.66
South Africa	64,322 (2)	5,960 (2)	10.79

apricots. Since the fruits are sour, the entire production is utilized in some processed form (pickles, concentrate, liquor, or juice). With the development of fruit processing techniques, some cultivars with high quality for pickles have been selected (Horiuchi et al. 1996).

7.1.3 Economic Importance

The common apricot is an edible fruit mainly cultivated in Mediterranean climates. Apricot production is widely distributed and apricots are produced commercially in 60 countries on about 395,000 ha. Total world production has reached about 2.6 million tons although a few countries (Turkey, Iran, Italy, France, Pakistan and Spain) account for over than 50% of that production (FAOstat 2005). Yields average more than 6 t/ha, ranging from just 2 to over 15 t/ha in some European countries (Table 1).

Traditionally, apricot has been one of the few temperate fruit trees not affected by overproduction and often premium prices are reported for both fresh and processed fruits. However, this crop is challenged by a number of problems: yields are subjected to yearly fluctuation mainly due to frosts and low adaptation of many cultivars, several pests and diseases threaten the crop, and the spread of yield-efficient but tasteless cultivars often is causing consumer disaffection (Bassi 1999).

The variability within apricot species is large. However, only one or two major cultivars lead most of the production in each production area. This is partly responsible for large fluctuations in yield and makes apricots vulnerable to adverse environmental conditions, diseases and pests (Mehlenbacher et al. 1990). Thus, more than 80% of the world production is based on less than 30 cultivars. This situation is changing in Mediterranean countries where, due to the problems associated with sharka (caused by the Plum Pox Virus, PPV), new cultivars from North America and European breeding programs are being introduced (Badenes et al. 2003).

In some regions there is an enormous amount of diversity because trees have been commonly grown from seed for many centuries. Production from seedling orchards is still important in countries such as Turkey, Iran, Iraq, Afghanistan, Pakistan or Syria, whereas in other countries most of the production relies on a few clonally propagated cultivars well adapted to local conditions (Table 2).

Apricots are T- or chip-budded onto rootstocks usually during summer or fall, although June budding is practiced occasionally. Apricot seedlings are the most popular rootstock worldwide. Other root-

Table 2. Leading apricot cultivars in the world. (Mehlenbacher et al. 1990; Ogawa and Southwick 1995; Bassi 1999)

Country	Cultivar
Algeria	Canino, Amor Leuch
Australia	Hunter, Moonpark, Story, Trevatt, Pannach, Watkins
Canada	Goldcot, Goldrich, Harcot, Harglow, Hargrand, Harlayne, Harogem, Veecot, Velvaglo, Vivagold
China	Bak-Ta-Sin, Caoxing, Chu-In-Sin, Dahongxing, GulotiLochak, Hongjing zhen, Huax-iandjiexing, Hvang-Sin, Isko-Dari, Liganmeix-ing, Nan zhoudajiexing, Konak Doraz, Kzil Kumet, Luotao xhuang, Maj-Ho-Sin, Manti-Rujuk, Shi-Sin, Shoyinhouz, Tulaki,
France	Bergeron, Canino, Earlyblush, Fantasme, Goldrich, Hatif Colomer, Helena du Roussillon, Ivresse, Luizet, Malice, Modesto, Orange Red, Polonais, Rouge de Roussillon, Rouge de Fournes, Tirynthos, Tomcot
Greece	Bebeco, Tirynthos, Luizet
Hungary	Bergeron, Ceglédi Bìborkajszi, Ceglédi Orìas, Gönci Magyar Kajszi, Magyar Kajszi (Hungarian Best), Mandula Kajszi
Italy	Baracca, Bella di Imola, Boccuccia, Cafona, Canino, Ceccona, Fracasso, Goldrich, Monaco Bello, Palummella, Portici, Reale di Imola, San Castrese, Tirynthos, Vitillo
Iran	Tabarza, Tokbam, Damavand, Malayer, Lasgherdi
Morocco	Canino, Amor Leuch
New Zealand	CluthaGold, Sundrop, Valleygold
Pakistan	Shakarpara
Portugal	Bulida, Canino
Romania	Callatis, Comandor, Excelsior, Favorit, Litoral, Mamaia, Olimp, Neptun, Saturn
South Africa	Bulida, Empress, Imperial, Lady Sun, Palsteyn, Peeka, Royal, Soldonné, Super Gold
Spain	Bulida, Canino, Galta Rocha, Mauricio, Moniqui, Palabras, Paviot, Pepitos, Real Fino
Syria	Ajamy-Hamoy, Balladi Falik-Huby, Balladi Khashabi, Balladi Maourdi, Canino, Hamani, Hamoy, Klaby, Malty, Shahmy, Sindyany, Shakrbara, Tadmory, Wazary
Turkey	Aprikoz, Cataloglu, Cologlu, Darende, Hacihaliloglu, Hasanbey, Kabaasi, Sekerpare, Soganci, Tokaloglu, Yegen
Ukraine	Krasnoschekii, Krasnoshchekii Pozdnii, Krasnyi Partizan, Nikitskii
USA	Blenheim (Royal), Castelbrite, Katy, Modesto, Patterson, Tilton

stocks include peach seedlings, Myrobalan (*Prunus cerasifera*) cuttings or seedlings and *Prunus insititia* rooted suckers (for a review see Crossa-Raynaud and Audergon 1987).

There are many different uses of apricots. It is enjoyed as fresh fruit, but a large portion of the worldwide production is preserved primarily by drying (Faust et al. 1998). All fruits for the fresh market are hand-harvested. Mechanical trunk shakers and catching frames used for processed fruits may increase trunk injuries and incidence of canker diseases. Apricots are also utilized as canned, dried, frozen, and baby food. Other products include wine, brandy, jam, and nectar. Ground apricot pits are used to clean jet engines, and the kernel oil is used for soaps and perfume (Ogawa and Southwick 1995). In some Asian regions, apricots used for edible seed and for seed oil are more important than apricots grown for fruit (Bailey and Hough 1975; Layne et al. 1996). Seeds of Central Asian and Mediterranean apricots are generally "sweet" and, thus, the seeds can be used as a substitute for almonds, or crushed for almond-like cooking oil. Mature fruits for drying purposes are usually held for further ripening, treated with sulfur dioxide, and placed on wooden trays in the sun (Ogawa and Southwick 1995).

Apricot quality consists of a balance of sugar and acidity as well as a strong apricot aroma. Central Asian and Irano-Caucasian cultivars are lower in acidity than European and Japanese cultivars (Mehlenbacher et al. 1990). Fresh fruits have and edible portion of 94% and are an excellent source of Vitamin A (carotene) and Vitamin C (ascorbic acid) (Wills et al. 1983) (Table 3).

The cultivation of Japanese apricot for fruit production is limited to Japan, China and Korea and some other Asian countries. The annual fruit

Table 3. Nutrient composition of apricot fruit. (Wills et al. 1983)

Component	Unit	Range
Water	g/100g	85.3–85.6
Protein	g/100g	0.7–0.9
Dietary fiber	g/100g	2.0–3.0
Energy	kJ/100g	141–167
Sugars	g/100g	
Sucrose		4.4–5.1
Glucose		1.1–2.7
Fructose		0.3–0.5
Titratable Acidity	Meq H+/100g	17.5–33.7
Organic Acids	Mg/100g	1.5–2.6
Maleic		440–770
Citric		660–2130
Ascorbic		7–16
Carotene	Mg/100g	153–617
Thiamin	Mg/100g	0.02–0.03
Riboflavin	Mg/100g	0.03–0.04
Niacin	Mg/100g	1.1–1.4
Potassium	mg/100g	320–350
Sodium	mg/100g	1–3
Calcium	mg/100g	15–16
Magnesium	mg/100g	9
Iron	mg/100g	0.3
Zinc	mg/100g	0.1–0.2

production in Japan is approximately 100,000 tons. The fruits are not consumed fresh but rather processed in different ways to make them palatable. Most of them are processed and consumed as pickles ("Ume-boshi"). Japanese apricot fruits have a higher content of organic acids such as citric acid and malic acid than other fruits. The pickled fruits have been reported to aid the digestive system, increase saliva, and even act as a cure for a hangover. The flesh of the fruits produces an extract known as "bainiku-ekisu". This by-product is the grated, condensed flesh of the fruit. Recent studies (Chuda et al. 1999; Utsunomiya et al. 2002) have reported that "bainiku-ekisu" includes a bioactive substance, known as mumefral, produced during the fruit processing, which improves human blood fluidity. Other uses of Japanese apricot include traditional medicinal purposes and juices (Yoshida 1994). More than 300 cultivars have been described being 'Nanko', 'Shirokaga' and 'Ryukyo Koume' the most popular. The flowers of the Japanese apricot are revered for their beauty and mume trees have been increasingly used as ornamentals (Faust et al. 1998) and several cultivars such as 'Kankobai', 'Kobai', and 'Koume' are grown as early blooming, small landscape trees.

7.1.4 Breeding Objectives

The main objective of any fruit tree breeding program is to develop new cultivars with the best quality and in the most economical way possible. There is a wealth of diversity in common apricot germplasm, but cultivar improvement is slowed by the high degree of heterozygosity within the species. Although most of the production in many countries still comes from chance seedlings and local cultivars (Bassi 1999), the main cultivars of many of the producing countries belong to the European group that shows a narrow genetic base (Mehlenbacher et al. 1990). The main factors limiting the expansion of apricot growing areas include a lack of agronomic and adapted varieties, a limited market value of most native cultivars and a poor adaptability of cultivars out of their native area (Badenes et al. 1998). Thus, although apricot breeding objectives differ depending on the country and on the main use of the product (dry, fresh or canned), some selection criteria are common to most apricot breeding programs (Bailey and Hough 1975; Mehlenbacher et al. 1990; Layne et al. 1996; Lespinasse and Bakry 1999):

- One of the main objectives in most apricot breeding projects is climatic adaptation. Most apricot cultivars are highly specific in their ecological requirements. Consequently, commercial production is limited to some locations, where usually one or two cultivars account for most of the production. Therefore, there is a need to evaluate apricot cultivars in each production area to look for high and regular production. Depending on the location, this adaptation involves breeding for late blooming to avoid frost damages, for early blooming in frost free areas to develop early maturing cultivars or for greater midwinter cold hardiness in colder areas. Local fruit tree adaptation is expressed in terms of productivity and regularity of production, and directly related to specific environmental conditions.

- Fruit quality. Some of the most important characteristics for the fresh market are large size (more than 60 g), attractive appearance (a bright blush over bright orange or cream), freestone, firm flesh, resistance to skin cracking and uniform ripening. For canning apricots, good orange skin and flesh colors are preferred as well as uniform medium size, regular shape, good texture, high sugar content, small pit and a good balance of acid and sugar. For drying purposes, high soluble solids are needed.
- Introduction of self-compatibility in some self-incompatible interesting cultivars. Although most apricot cultivars are self-compatible, self-incompatibility is present in some interesting cultivars and cultivars used as parents in breeding programs.
- Disease resistance: important diseases include apricot chlorotic leaf roll (Mediterranean countries), bacterial canker caused by *Pseudomonas* spp., *Xanthomonas pruni* (America and South hemisphere), *Monilia*, *Gneumonia* (Eastern European countries). A special case is the menace of sharka, caused by the plum pox virus (PPV) that is causing important damages in most Mediterranean countries. There is no treatment to cure virus-infected trees, and once a tree is infected it serves as a source of infection for other trees. In countries where the level of infection is low and infected trees are restricted to limited areas, eradication has allowed to maintain a low level of infection and, more rarely, to eliminate the disease. However, eradication has proven insufficient in most countries because newly planted healthy trees become infected in a short time. Breeding programs for resistance/tolerance to sharka have been initiated in France (Audergon 1995), Greece (Karayiannis and Mainou 1999), Italy (Bassi et al. 1995) and Spain (Egea et al. 1999; Badenes et al. 2003) and they are yielding some interesting results (Egea et al. 2005).

Regarding Japanese apricot, its most notable biological characteristic is self-incompatibility although self-compatible cultivars are occasionally found. Some cultivars show also male sterility. Self-sterility limits fruit set in many locations and may force growers to plant unprofitable pollinating cultivars. Other important breeding objectives include in order of importance: big fruit size (more than 30 g), tolerance to gumming in fruits by which some fruits often lose their commercial value, late flowering which avoids frost damage, early ripening, and resistance or tolerance to scab and bacterial canker (H. Yaegaki, personal communication).

7.1.5 Classical Breeding Achievements

A high parent-offspring correlation was detected for fruit size and flesh firmness, two of the most important traits related to fruit quality, in the progeny analysis of some apricot crosses (Lapins et al. 1957). However, very little information is available in apricot about simple associations between morphological traits and fruit quality. Perez-Gonzales (1992) reported a wide range of variability among accessions representing apricot germplasm from Central Mexico for 20 morphological and phenological variables, especially for those factors associated with yield efficiency. Fruit weight was correlated with morphological traits such as tree growth habit, apical and basal diameter of fruiting spurs, and bud and leaf size. On the other hand, Badenes et al. (1998) showed a narrow range of variation among 55 cultivars from Spain, France, Italy, Greece, Tunisia, and USA for 18 morphological, phenological and fruit quality traits using principal component analysis. The only correlation observed between morphology and phenology was blossom and budbreak season with internode length. These results confirm that cultivars of the European group, the youngest in origin and the source of most of the commercial cultivars are difficult to sub-group morphologically and have a narrow genetic base (Bailey and Hough 1975; Layne et al. 1996).

In spite of its lower variability most of the progress in common apricot breeding has been carried out through hybridization and selection within the European group. However, a vast amount of mostly unexplored genetic variability is available within the other groups. A limited amount of published information of Irano-Caucasian cultivars comes from Armenia, Iran, Turkey, and North Africa. Soviet researchers published extensively on Central Asian cultivars and their hybrids. Information from Pakistan, Afghanistan, and China is very limited, although these areas are known to be rich sources of genetic diversity. Thus, information on genetic variability in apricot is primarily from collections where the European group is overrepresented lacking the enormous amount of variability

present in other groups (for review see Mehlenbacher et al. 1990 and Layne et al. 1996).

Most of the leading cultivars in the world come from local cultivars well adapted to one or very few areas (Bassi 1999). A number of apricot cultivars have been selected for several interesting traits such as disease resistance, climatic adaptation, fruit quality and tree growth habit (Mehlenbacher et al. 1990). Frost resistance has been also searched in apricots from wild species such as *Prunus sibirica* or *Prunus mandshurica* (Dosba 2003) and from native cultivars in Turkey (Akca and Sen 1999).

Released cultivars have been mainly obtained from open pollination, selfing, and, more recently, from controlled crosses (Layne et al. 1996). A number of apricot cultivars from controlled pollinations have been introduced in Argentina, Australia, Canada, Czechia, France, Hungary, Romania, Russia, South Africa, Spain, and the USA. While in most of these countries the production is still based on local cultivars, in others, like Canada and Romania, most of the production comes from cultivars developed from breeding programs specifically tailored for those regions (Bassi 1999).

The determination of the inheritance of a few traits of interest as self-compatibility (Burgos et al. 1997) or male sterility (Burgos and Ledbetter 1994) and to the identification of sources of tolerance/resistance to sharka (reviewed in Martinez-Gomez et al. 2000) have taken place in recent years. The genetic control of sharka resistance is still not very well known since contradictory reports have been published. Thus, the results of Dosba et al. (1991), Moustafa et al. (2001) and Vilanova et al. (2003a) suggested a two-loci control of the trait whereas the results of Dicenta et al. (2000) fit with a monogenic control and Guillet-Bellanger and Audergon (2001) suggest a control of the resistance by at least three loci. Further studies involving a larger number of individuals are needed to clarify the genetics of sharka resistance.

Regarding Japanese apricot, most main commercial cultivars resulted from natural selection carried out within the original areas of production. Because of the best quality for "Ume-boshi" processing, 'Nanko' is the most important cultivar so far. 'Nanko' was selected in Wakayama prefecture where more than half of total Japanese apricot fruits in Japan are produced.

Conventional breeding is still today the most important method of obtaining new European and Japanese apricot cultivars. As with other fruit tree species, this method is time-consuming and laborious; hence, the development of molecular markers linked to important biological character is absolutely required.

7.2 Construction of Genetic Maps

Genetic maps can be a useful tool to locate traits of interest to perform marker-assisted selection. Although advances in the construction of linkage maps in *Prunus* have been mainly obtained in peach, in the last few years, several genetic maps have been published in apricot:

- Hurtado et al. (2002a) developed two apricot maps composed of RAPD, AFLP, RFLP and SSR markers with 81 F_1 individuals from the cross 'Goldrich' × 'Valenciano'. A total of 132 markers (33 RAPDs, 82 AFLPs, 4 RFLPs, 13 SSRs) were placed into eight linkage groups in the 'Goldrich' map defining 511 cM of total map distance with an average distance between adjacent markers of 3.9 cM. A total of 80 markers (19 RAPDs, 48 AFLPs, 4 RFLPs, 9 SSRs) were placed into seven linkage groups on the 'Valenciano' map defining 467.2 cM of total map distance with an average interval of 5.8 cM between adjacent markers.
- Vilanova et al. (2003a) developed a map composed of AFLPs and SSRs from an F_2 population of 76 individuals from self-pollination of 'Lito' (an F_1 individual of 'Stark Early Orange' × 'Tyrinthos'). A total of 209 molecular markers (180 AFLPs and 29 SSRs) were assigned to 11 linkage groups covering 602 cM of total map distance with an average distance between adjacent markers is 3.84 cM.
- Lambert et al. (2004) used RFLP and SSR markers, previously mapped, in an F_2 progeny of the interspecific cross almond cv Texas × peach cv Earlygold (Joobeur et al. 1998; Aranzana et al. 2002) to develop two maps using 142 F_1 hybrids from a cross between the apricot cultivars 'Polonais' and 'Stark Early Orange' (Fig. 1); a total of 141 markers were placed on the map of 'Stark Early Orange' with a total length of 669 cM and 110 markers on the 'Polonais' map with a total length of 538 cM. Most markers present in each linkage group were aligned with those of the almond cv Texas × peach cv EarlyGold F_2 progeny map that is considered as a saturated

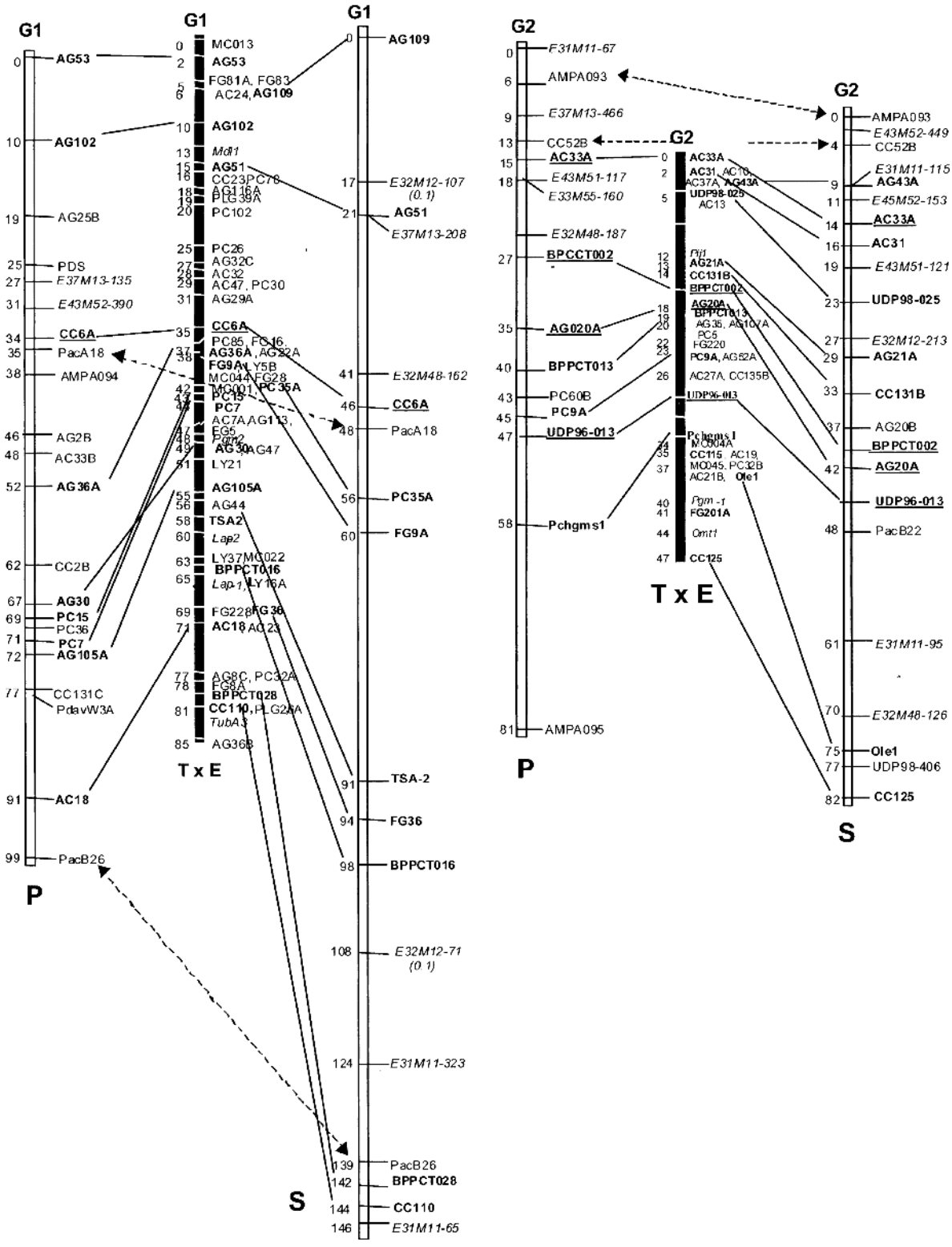

Fig. 1. Genetic maps obtained with the Polonais (P) × Stark Early Orange (S) (P×S) progeny compared to that of Texas × Earlygold (T×E) almond × peach. (Reproduced by permission of Lambert et al., Theoretical and Applied Genetics 108:1120–1130)

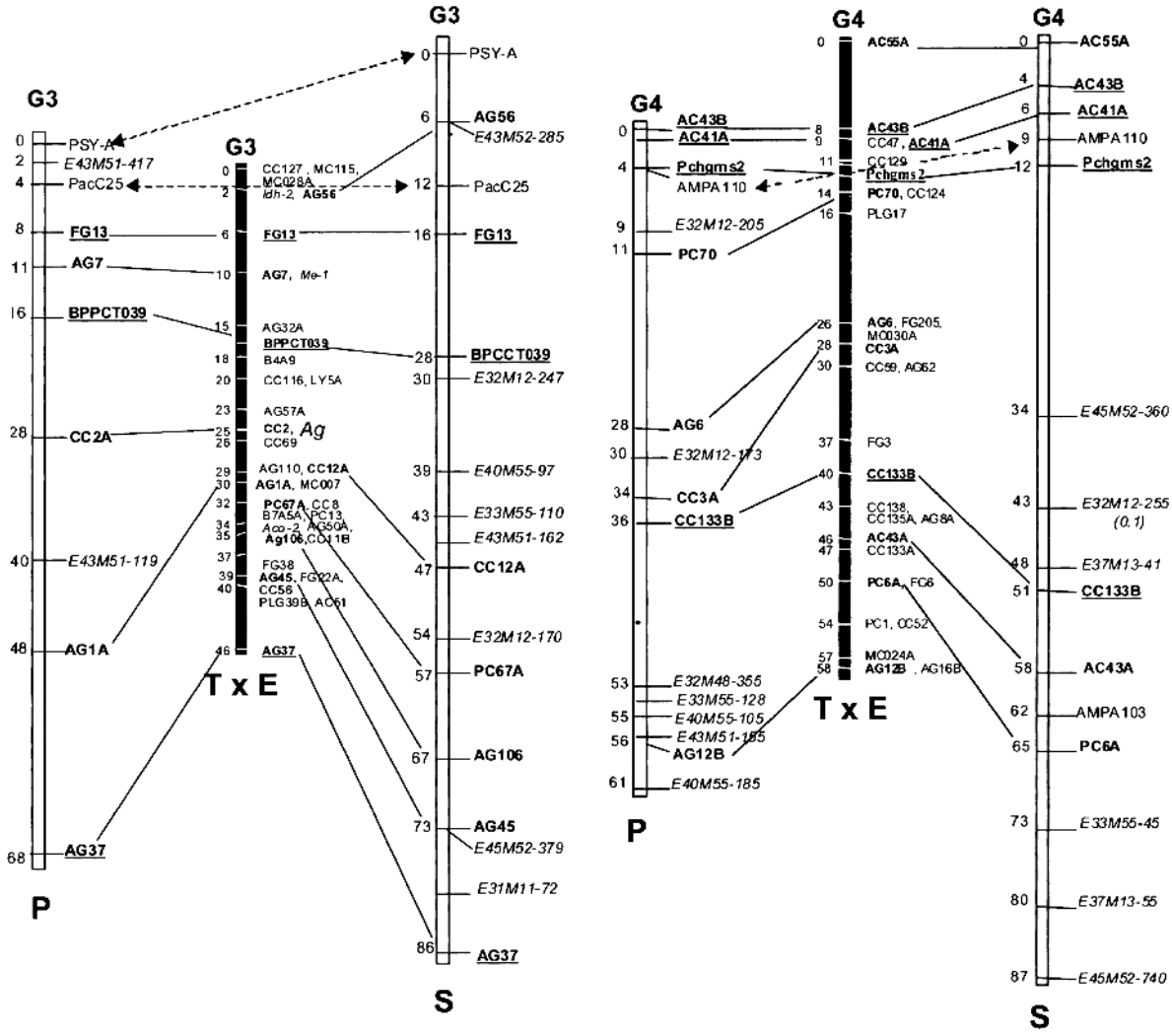

Fig. 1. (continued)

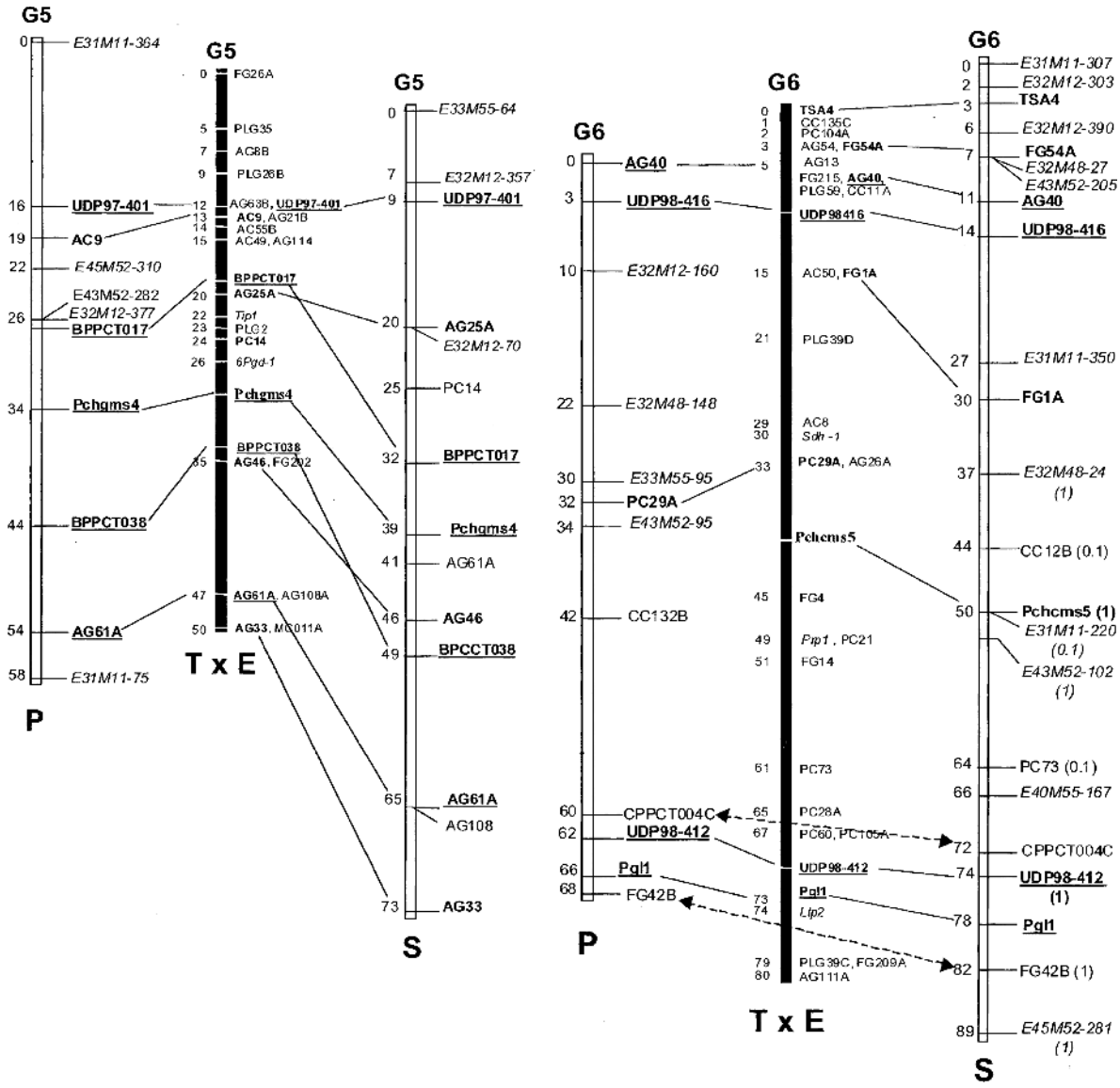

Fig. 1. (continued)

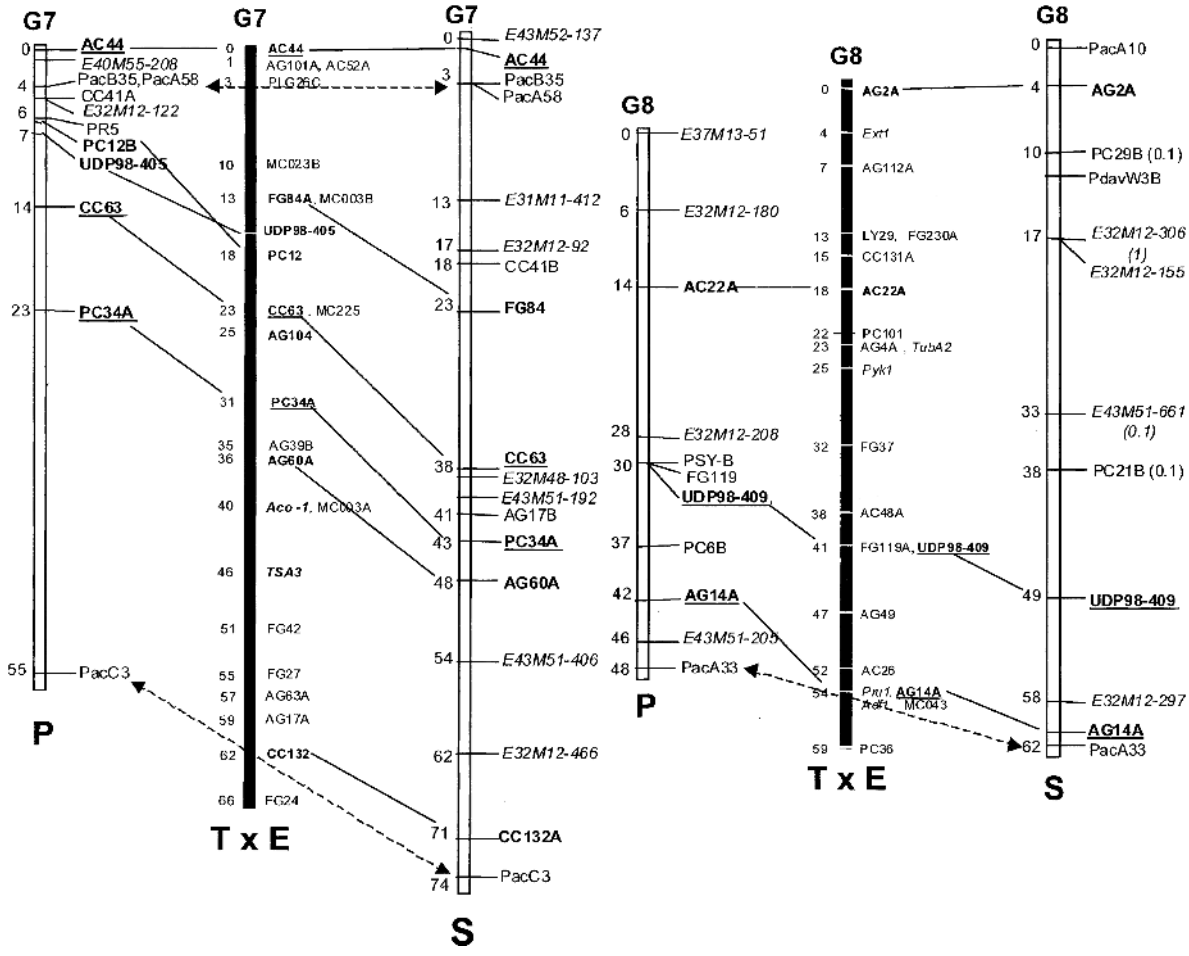

Fig. 1. (continued)

map (Joobeur et al. 1998; Aranzana et al. 2002). The results show a high degree of colinearity between the apricot and the peach and almond genomes suggesting a strong homology among *Prunus* genomes

Regarding Japanese apricot, although no reports of classical mapping efforts have been published to date, attempts to generate a first genetic map have been initiated recently (H. Yaegaki, personal communication).

7.3 Marker-Assisted Breeding:

7.3.1 Germplasm Screening

The information available on morphological apricot descriptors includes mainly varieties from the European group. They are based on a wide range of characteristics, such as tree vigor and growth habit, leaf size and shape, productivity, disease resistance or fruit quality (Crossa-Raynaud 1969; Brooks and Olmo 1972; Couranjou 1977, Fideghelli and Monastra 1977; Guerriero and Watkins 1984; Perez-Gonzales 1992; Badenes et al. 1998).

More recently, as in other fruit tree species (Wünsch and Hormaza 2002), different molecular markers have been used to fingerprint apricot cultivars and for genetic diversity studies. Molecular characterization of apricot cultivars was initially carried out using isozymes. Thus, Byrne and Littleton (1989a) studied isozymes on 69 accessions including European, Central Asian, North Chinese apricots and their hybrids, and found polymorphism at three of the seven examined enzymes; a few cultivars were uniquely identified. Battistini and Sansavini (1991) found four enzyme systems showing enzymatic polymorphism and could separate the 50 cultivars studied into 16 groups on the basis of the zymograms observed. Badenes et al. (1996) used 10 enzymatic systems, six of which were polymorphic, and were able to separate 94 apricot accessions in three geographical groups: North American, Irano-Caucasian and European. Likewise, Manganaris et al. (1999b) studied the enzyme variability among 17 apricot cultivars and 56 genotypes from intraspecific crosses using 20 enzyme systems, 15 of which were polymorphic. These studies with isozymes were not able to address the genetic diversity of apricot on a larger germplasm basis because of a lack of representative accessions from more diverse origins (Badenes et al. 1996; Manganaris et al. 1999b) or a lack of informative markers (Byrne and Littleton 1989a). Identification of interespecific hybrids between diploid plums and apricots has been also reported using isozymes. Thus, a useful marker was found for identifying plum × apricot hybrids among six enzymes (Byrne and Littleton 1989b) and 14 plum and 12 apricot specific alleles were found as useful markers for identifying plumcot, pluot and aprium hybrids (Manganaris et al. 1999b).

Due to the low polymorphism obtained with isozymes, in the last two decades efforts have been dedicated to obtain more efficient cultivar identification and diversity studies with the use of DNA-based molecular markers. Shimada et al. (1994) studied the genetic relationships among 54 Japanese apricot cultivars with 95 RAPD primers and classified them in seven groups that reflected their origin: 1) Taiwan mume, 2) Ko-ume, 3) Chuu-ume, 4) Ou-ume with white flower, 5) Ou-ume with pink flower, 6) Anzu-ume or Bungo-ume, and 7) Sumomo-ume. RAPDs were also applied to determine the parentage of Japanese apricot cultivars (Ozaki et al. 1995). Later, Takeda et al. (1998) investigated the relationships between 33 common apricot cultivars and two related species (*P. sibirica* L. and *P. brigantina* Vill.) with 18 RAPD primers, clustering the genotypes into two main groups, cultivars originated in the East (eastern China and Japan) and cultivars from the West (Europe, Central Asia and Western China). Mariniello et al. (2002) could identify 19 out of 25 cultivars analyzed with 44 RAPD primers.

RFLPs have also been used in apricot for fingerprinting and diversity studies. Thus, 45 different phenotypes from 52 apricot (*Prunus armeniaca* L.) cultivars (de Vicente et al. 1998) were identified using 31 selected probes developed in almond. The similarity matrix obtained from the molecular data was used to construct a dendrogram that separated the Spanish apricot cultivars from those from Europe and North America.

AFLPs have also been used for fingerprinting and diversity studies in apricot and the results obtained agree with the known historical movement of apricot cultivation. Hurtado et al. (2002b) examined 16 cultivars with six primer sets obtaining 231 polymorphic markers that allowed to distinguish all the cultivars studied. Similarly, Hagen et al. (2002) studied 47 apricot cultivars with five *Eco*RI-*Mse*I AFLP primer combinations revealing 379 polymorphic markers show-

ing a gradient of decreasing genetic diversity of varieties from the former USSR to Southern Europe. Panaud et al. (2002) studied 19 genotypes of a Saharian oasis with seven primer combinations producing a total of 197 amplification bands, of which 97 were polymorphic allowing the identification of all the genotypes studied. Similarly, Ricciardi et al. (2002) studied five apricot cultivars and 34 local Apulian ecotypes with four primer combinations resulting in 267 polymorphic bands from a total of 409 amplification fragments allowing the identification of all the genotypes. Geuna et al. (2003) used five primer combinations to unequivocally fingerprint 118 accessions resulting in 165 polymorphic fragments. Regarding Japanese apricot, recently 14 cultivars from China and Japan have been characterized with AFLPs with 12 primer combinations producing a total of 470 amplification bands, of which 284 were polymorphic allowing the identification of the genotypes studied and their grouping according to the known origin (Fang et al. 2005).

More recently, microsatellites have been used for genotype identification and variability studies in apricot. In a first step, primer pairs developed in other *Prunus*, mainly peach, were used. Thus, Hormaza (2002) identified 48 apricot genotypes with 20 primer pairs from peach grouping the cultivars according to their geographical origin and/or known pedigree information. Similar results were obtained by Zhebentyayeva et al. (2003) with 74 cultivars and 12 primer pairs, Romero et al. (2003) with 40 cultivars and 11 pairs of primers and Sánchez-Pérez et al. (2005) with 25 genotypes and 14 primer pairs. Ahmad et al. (2004) used 25 SSRs developed in cherry and three in peach to fingerprint seven apricot, one plumcot and six pluot cultivars confirming the transferability of SSRs among *Prunus* species. SSRs have also been used for identification of *P. mume* genotypes. Thus, Gao et al. (2004) reported the identification of 24 genotypes from diverse geographical areas with 14 SSRs derived from different *Prunus* species (nine from peach, five from sweet cherry and one from sour cherry). More recently, SSRs have also been specifically isolated in apricot. Thus, Lopes et al. (2002) and Messina et al. (2004) reported the isolation from genomic libraries of 21 and 99 SSRs, respectively, whereas Decroocq et al. (2003) isolated 10 EST SSRs from a leaf apricot cDNA library and Hagen et al. (2004) developed 24 new loci (13 from genomic libraries, eight from fruit EST libraries and three from a leaf cDNA library).

7.3.2 Marker-Assisted Selection and Gene Identification

The best example of the development of molecular markers linked to a trait of interest in Japanese apricot is self-incompatibility. Conventional assessment of self-incompatibility, as determined by pollination and pollen tube growth tests, requires several years after the tree reaches the flowering age. Recent identification of pistil and pollen-S determinants, namely S-RNase and SFB, respectively, enabled to develop molecular marker for S-haplotypes. Tao et al. (2002a) and Yaegaki et al. (2001) cloned cDNAs encoding S-RNases and established molecular typing system for S-haplotypes using the S-RNase sequence information. Cloning of cDNAs encoding pollen-S candidates, SFBs, led to a firm determination of S-haplotypes because the use of molecular markers for both pistil and pollen determinants became available (Yamane et al. 2003). This is very useful especially when S-RNase genes from different S-haplotypes gave the same PCR and RFLP bands. In addition, Tao et al. (2000, 2002b) and Yamane et al. (2003) revealed unique PCR or hybridization bands derived from S-RNase or SFB, linked to a mutated S-haplotype conferring self-compatibility in Japanese apricot. Regarding common apricot, the self-incompatibility trait has been mapped on linkage group G6 using an F_2 population derived from the self-pollination of an F_1 individual ('Lito') originated from a cross between 'Stark Early Orange' and 'Tyrinthos' (Vilanova et al. 2003a) and, more recently, the putative genes controlling gametophytic self-incompatibility have also been identified (Romero et al. 2004). Moreover, several research groups have determined apricot S alleles by PCR analysis (Halasz et al. 2005; Qi et al. 2005; Vilanova et al., 2005).

Another important trait for breeding purposes is resistance to sharka. Hurtado et al. (2002a) mapped the sharka resistance trait in linkage group 2 using an F_1 population derived from the cross between 'Goldrich' and 'Valenciano' whereas Vilanova et al. (2003a) mapped the trait in the G_1 linkage group using an F_2 population derived from the self-pollination of an F_1 individual ('Lito') originated from a cross between 'Stark Early Orange' and 'Tyrinthos'. The conservation of plant disease resistance genes has allowed the screening of apricot to isolate resistance gene analogs (RGAs) to find markers associated with resistance genes (Dondini et al. 2004; Soriano et al.

2005); one putative marker only present in sharka resistant genotypes has been recently reported (Dondini et al. 2004).

Regarding the study of specific genes, ripening-related genes are being widely studied in most *Prunus* species, including apricot, where fruit is the product of interest. Thus, a full length ACC-oxidase cDNA has been isolated from a cDNA library made from ripe apricot fruits based on sequence conservation among ACC-oxidases (Mbéguié-A-Mbéguié et al. 1999), a polyphenol oxidase expressed in leaves and mature fruits and turned off during fruit ripening has also been isolated from an immature green fruit cDNA library (Chevalier et al. 1999) and two expansins have been isolated from a ripe apricot fruit cDNA library (Mbéguié-A-Mbéguié et al. 2002). Expressions of ACC synthase and ACC oxidase have also been studied in Japanese apricot (Mita et al. 1999). As other fruit tree species, apricots contain some allergenic compounds and the most important is a protein that belongs to the family of lipid transfer proteins (LTP) (Pastorello et al. 2000) which is highly similar to peach and almond LTPs (Conti et al. 2001).

7.4 Future Scope of Works

Conventional apricot breeding has been successful for the development of new cultivars. New approaches with biotechnological tools offer the possibility of speeding up the development of new cultivars with improved characteristics. Promising results have been obtained in the development of molecular markers, fingerprinting and diversity studies although QTL analysis and gene identification, marker development and marker-assisted selection for important agronomic traits are strongly required to facilitate apricot breeding programs. Although genetic maps are a great advance to locate genes and QTLs, even in saturated maps, genetic markers are still too far in base pairs from genes. Physical maps can bridge the gap between markers and genes. Advances in the development of a physical map in peach (as a model species for the Rosaceae) will be very useful for apricot in the near future (Jung et al. 2004) due to the synteny observed among *Prunus* species. Similarly, the construction of BAC libraries in apricot can help in that direction (Vilanova et al. 2003b). Thus, any attempts for marker conversion such as EST analysis, saturated map construction and QTL detection (probably increasing the number of individuals in the progenies and improving the evaluation of phenotypic traits) or the application of gene analogs will undoubtedly open the door to clone and transfer genes of interest.

However, as in most *Prunus* tree species, the lack of an efficient transformation system hinders studies on the gain and loss of function in transgenic experiments. Plant regeneration from somatic seeds of adult trees is necessary to preserve genetic integrity of apricot cultivars but there are just limited reports of regeneration of transgenic apricot plants (Burgos and Alburquerque 2003). An efficient transformation system would allow the introduction of tolerance/resistance to sharka in adult material following the approaches used with by Da Câmara Machado et al. (1992) who regenerated from cotyledons transgenic plants with the PPV coat protein (PPV-CP).

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8 Strawberry

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8.1 Introduction

The genome composition of the octoploid ($2n = 8x = 56$), cultivated strawberry, *Fragaria* ×*ananassa*, is among the most complex of any crop species. The most recently proposed genome composition model for the octoploid *Fragaria* species, AAA′A′BBB′B′ (Bringhurst 1990), implies the presence of up to four distinct subgenomes (A, A′, B, and B′). The genomic complexity of the octoploid species has prompted attention to diploid relatives, such as *Fragaria vesca* ($2n = 2x = 14$), as model systems for strawberry genetics and genomics (Davis and Yu 1997; Sargent et al. 2004). Previous reviews have summarized the history of strawberry breeding and genetics (Darrow 1966; Galletta and Maas 1990; Hancock 1999) and initial developments in strawberry biotechnology (Hokanson and Maas 2001). This review updates the state of progress in the genetic and genomic characterization and manipulation of the cultivated strawberry and its respective diploid model system.

8.1.1 Origin of the Cultivated Strawberry

The genus *Fragaria* is comprised of 23 species variously distributed throughout the northern hemisphere and also extending southward along the western coast of South America and to Hawaii. Historically, several *Fragaria* species and novel hybrids have been brought into cultivation in various parts of the world, including *F. chiloensis* in South America, and *F. moschata* and *F. vesca* in Europe (Darrow 1966; Hancock 1999). However, the current economic significance of all other *Fragaria* species combined is insignificant compared to that of *F.* ×*ananassa*. Therefore, use of the term "cultivated strawberry" in this review will refer specifically to *F.* ×*ananassa* unless otherwise qualified.

The origin of the cultivated strawberry traces to the 1700s, when representatives of the octoploids *F. chiloensis* and *F. virginiana* – previously brought to Europe from South and North America, respectively – were grown in proximity in European horticultural gardens. Cross-pollination produced hybrids that were quickly recognized for their unique and desirable combinations of morphological and fruit characteristics, and were brought into cultivation and breeding (Hancock 1999). This recent origin makes *F.* ×*ananassa* one of the youngest of contemporary crop species. The cultivated species' immediate octoploid progenitors, *F. chiloensis* and *F. virginiana*, may have arisen from a common octoploid ancestor (Potter et al. 2000); however, no lineage has been established connecting the octoploids to any lower ploidy level. Several diploid species have been suggested as possible ancestors of the octoploid species, including *F. vesca*, *F. iinumae*, *F. daltoniana*, and others (Hancock 1999). The need for a comprehensive phylogenetic treatment of *Fragaria* that identifies the diploid ancestors of the cultivated species is becoming increasingly acute, as researchers begin to develop *F. vesca* and perhaps other diploid species as model systems for strawberry genetics and genomics.

8.1.2 Systematics and Phylogenetics

The strawberry genus, *Fragaria,* belongs to the family Rosaceae, subfamily Rosoideae. *Fragaria* has been represented in two molecular phylogenetic studies of the Rosaceae family (Morgan et al. 1994; Eriksson et al. 1998), but these broad studies included only one or two *Fragaria* species, respectively, and provided no insight into species relationships within *Fra-*

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garia. The monophyly of *Fragaria* is considered to be well-supported (Potter et al. 2000), but species relationships within *Fragaria* have not been adequately delineated. Molecular phylogenetic resolution within *Fragaria* has been limited, in part, by the low levels of variability detected in the nuclear ITS (internal transcribed spacer of rDNA) and chloroplast DNA (cpDNA) sequences that have been used for phylogenetic analysis (Harrison et al. 1997; Potter et al. 2000).

The Harrison and Potter studies provide an informative foundation for further, more detailed investigations of *Fragaria* phylogenetics. Significantly, the Potter study draws attention to *F. vesca*, *F. nubicola*, and *F. orientalis*, as possible progenitors to the octoploids. However, neither study discerned the reticulate phylogenetic history expected for the octoploids, or for hexaploid *F. moschata*. Of course, uniparentally inherited cpDNA sequence alone cannot provide evidence of phylogenetic reticulation. However, study of the biparentally inherited, nuclear ITS sequence has also failed to provide evidence of reticulate evolution in the *Fragaria* octoploids. Although ITS has been widely used for phylogenetic analysis at the specific and generic level in angiosperms, concerted evolution can homogenize ITS regions in allopolyploids, potentially erasing the contribution(s) of all but one diploid progenitor (Wendel et al. 1995). Perhaps this has been the case for the ITS region in *Fragaria* octoploids.

8.1.3 Karyotype

The basic chromosome number in *Fragaria* is $x = 7$ (Ichijima 1926). The recognized *Fragaria* species comprise a polyploid series, including twelve diploid ($2n = 2x = 14$) species, four tetraploids ($2n = 4x = 28$), one hexaploid ($2n = 6x = 42$: *F. moschata*), and four octoploids ($2n = 8x = 56$). Synthetic octoploids have been constructed via controlled, interspecific hybridizations accompanied by chromosome doubling, in an effort to broaden the octoploid gene pool available to strawberry breeders (Evans 1977; Bors 2000). The decaploid level has been obtained in controlled crosses accompanied by chromosome number manipulation (Scott 1951; Ahmadi and Bringhurst 1992). Decaploids referred to as *Fragaria* ×*vescana* were derived from crossing *F.* ×*ananassa* ($2n = 56$) with tetraploid forms of *F. vesca* var. *semperflorens* ($2n = 28$) followed by backcrossing to *F.* ×*ananassa* (Bauer 1993). Other decaploids have been derived from crosses involving octoploid cultivars and diploids *F. vesca* or *F. nilgerrensis* (Mochizuki et al. 2002). Many other natural or synthetic hybrids of various even and odd ploidy levels have been described (Darrow 1966; Bringhurst and Gill 1970; Staudt 1999; Staudt et al. 2003), reflecting the broad potential for interspecific hybridization both within and between ploidy levels in *Fragaria*.

Chromosomes are quite small in all *Fragaria* species, and only minor variation in chromosome morphology has been described (Ichijima 1926; Senanayake and Bringhurst 1967; Iwatsubo and Naruhashi 1989, 1991). Satellites have been observed on one chromosome pair in five diploid species: *F. vesca*, *F. iinumae*, *F. nipponica*, *F. nubicola*, and *F. daltoniana* (Iwatsubo and Naruhashi 1989, 1991), and chromosome morphology, *per se*, has provided no basis for differentiating the subgenomes in the octoploids.

8.1.4 The Strawberry Plant

A detailed description of strawberry morphology and physiology is provided in Darrow (1966 – Chap. 18). Strawberries are perennial, herbaceous, low-growing plants. Strawberries are capable of vegetative propagation via the production of runners (stolons), which are trailing, above-ground stems that can take root at their nodes to establish new, clonal daughter plants. A runnerless mutant form is known in *F. vesca*. Strawberry leaves are generally trifoliate; however, pentafoliate leaves occur in the diploid species *F. pentaphylla*, and are sometimes seen in other species as well.

The fleshy red strawberry "fruit" is actually the expanded receptacle of the strawberry flower. The true fruit of strawberry are the seed-like achenes borne on the surface of the receptacle. Each achene is derived from an individual, monocarpelate pistil, and if successfully fertilized contains a single seed. Strawberry flowers typically have five white petals, exceptions including higher petal number in some Asian species (Staudt 1989, 2003, 2005) and pink flowers in certain novelty varieties (e.g., 'Pink Panda'). Typically, strawberry is a short day plant, flowering in response to short day lengths and low temperature (Battey et al. 1998). However, day neutral forms have been identified in octoploid *F. virginiana* and diploid *F. vesca* (Ahmadi et al. 1990; Brown and Wareing 1965; Sakin et al. 1997). The day neutral (everbearing), or "*Sem-*

perflorens" form of *Fragaria vesca* ssp. *vesca* is of European origin, and is often termed the 'Alpine' form.

Sex determination in strawberry varies among species (Hancock 1999). Contemporary cultivars of *F.* ×*ananassa* are hermaphroditic; however, gynodioecy and/or trioecy have been reported in the octoploid species *F. chiloensis* and *F. virginiana* (Ahmadi and Bringhurst 1991; Staudt 1989; Ashman 2003), and in tetraploid *F. orientalis* and hexaploid *F. moschata* (Staudt 1989). A genetic model was proposed for trioecy in the octoploids involving a sex determination locus with three alleles (F, H, and M) (Ahmadi and Bringhurst 1991). According to this model, females are heterogametic (F/H or F/M), hermaphrodites may be heterogametic (H/M) or homogametic (H/H), and males are homogametic (M/M). The diploid species are reportedly all hermaphroditic, except for *F. vesca* ssp. *bracteata*, in which gynodioecy also occurs (Ahmadi and Bringhurst 1991). Gametophytic self-incompatibility occurs in the diploid species *F. viridis*, *F. nubicola*, *F. mandshurica*, *F. nipponica*, *F. yezoensis*, *F. gracilis* and *F. pentaphylla*, while diploids *F. vesca*, *F. iinumae*, *F. nilgerrensis*, and *F. daltoniana* are self-compatible (Staudt 1989; Hancock 1999). Self-incompatibility is not known to occur in the octoploid species.

8.1.5 Breeding

Detailed accounts of the history of strawberry domestication and breeding are to be found in Darrow (1966) and Hancock (1999). Before obtaining the first genotypes of *F.* ×*ananassa* in the 1760s, *F. vesca* and *F. moschata* (Hautbois) were cultivated in Europe. *F. viridis* was also cultivated but was less important than the former two species. In 1764, Duchesne identified clearly the parentage of the modern, cultivated strawberry. This strawberry appeared as a vigorous, perfect hermaphrodite displaying fruit a little smaller than the Chilean and with pineapple aroma. Duchene suspected a cross between the Scarlet strawberry (*F. virginiana*) as pollen source, and the Frutillar (*F. chiloensis*) (Darrow 1966). At the same period, this new species was also reported in England and in Holland.

The first breeding work on modern strawberries was conducted in the middle of the 1800s, mainly in England and in America. In the 1900s and particularly after World War II, breeding programs appeared in public institutions. In 1961, the protection of new plant varieties by an intellectual property right (International Convention for the Protection of New Varieties of Plants in Paris, http://www.upov.int/index.html), allowed private companies to develop their own breeding programs. During the twentieth century, the efficiency of strawberry production and the fruit quality were drastically improved with the development of superior production environments and with the breeding of cultivars specifically adapted to these superior environments. In this context, the breeders have to integrate the research in production physiology and cultural practices in order to optimize their selection strategies.

The methodology of strawberry breeding mainly involves pedigree selection, since strawberry is highly heterozygous as observed in other polyploid species. Large genetic variability among strawberry progenies is the major factor for the selection of desirable characters. Traditionally, the best cultivars are crossed and from their progenies, the best genotypes are selected. The succession of crosses between the best genotypes and the selection in the progenies constitute recurrent breeding associated to pedigree selection in which the choice of the parents and the choice of the best combination are critical.

Selection for a new cultivar starts with the cross, and ends with the release of the new cultivar, which takes about 8–10 years. Usually, the process of the breeding program is as follows. The first cycle is obtained with controlled crosses among selected parents chosen for their phenotype values in the considered location. Since main characters of interest are quantitative ones, the genetic gain is achieved with the choice of the best genotypes phenotypically selected for their desirable traits, then making numerous crosses to promote the best combination of alleles. Selfing to fix character is rarely used since inbreeding depression is observed. However, selfing can reveal genetic potential of some genotypes to be used further as parents. The first year of evaluation is performed on the basis of seedling performance. Approximately, 1–3% of the genotypes are kept and further evaluations are performed on plots of runner plants. After a few years of screening on the desirable characters, the selected genotypes are evaluated in multi-location trials under commercial conditions. Controlled tests are required to analyse some characters in the breeding program, i.e. disease resistance tests or simulation of fruit conservation test.

Although some breeding objectives vary according to the area of cultivation, traditional main breeding objectives are the following (Rosati 1993): a production of relative large berry size in order to limit the cost of harvest, a firmer fruit with regular shape and long shelf life, which is easy to harvest, an increase in the total yield, an improvement in fruit appearance (color, shape, brightness), and disease resistances. When cultivars have to be adapted to specific regions or to specific markets, specific objectives are included in the breeding programs such as developing production for processing, time of ripening (very early or very late ripening). Breeding for good taste and flavor is also an important objective to fit the quality market needs. The evolution of cultural practices leads also to new objectives such as resistance to powdery mildew which is more important in greenhouse production.

8.1.6 Nutritional Composition

Strawberries present many specific nutritional characteristics known to have health benefits. They are particularly rich in vitamin C (60 mg per 100 g fresh fruit corresponding to 75% of the daily need), richer than oranges, and contain a high amount of potassium (180 mg per 100 g of fresh fruit). Besides these essential nutrients, strawberries contain a high content of ellagic acid more commonly found in the form of water-soluble ellagitannins. This phenolic compound is known as a naturally occurring dietary antimutagen and anticarcinogen (Maas and Galetta 1991; Clifford and Scalbert 2000). In vitro, strawberry appears to exert a weaker antioxidant activity as compared to other berries. They are rich in pelargonidin-3-glucoside, the major strawberry anthocyanin, and ascorbic acid, both of which are weak antioxidants (Törrönen and Määttä 2002). However interesting results have been reported on beneficial effects of strawberries in experimental animals (e.g., Joseph et al. 1999).

8.1.7 Economic Importance

Globally, 214,118 Ha of strawberries were cultivated in 2004, representing a worldwide production of 3,113,840 Mt (FAOSTAT data 2004). A large part of the cultivated area is located in Europe (63.3% of the total area), followed by Asia (14.8%) and North and Central America (13.8%). However, Europe and North and Central America have a comparable production level with 1,164,650 Mt and 1,022,521 Mt. The USA is the world's leading strawberry producer with 840,000 Mt. Spain ranks second (285,600 Mt) followed by the Republic of Korea, Japan, Mexico, Italy, the Russian Federation, Turkey, Poland and Germany. During the last ten years, some countries like Turkey, Morocco and Egypt have strongly increased their production. Germany (109,824 Mt) and France (93,591 Mt) are importing a large quantity of strawberries whereas Spain and the USA are exporting a large part of their production (212,327 and 94,666 Mt respectively).

8.2 Genetic Characterization

8.2.1 Genome Composition

The first model of octoploid *Fragaria* genome composition – AABBBBCC – was proposed by Federova (1946). Cytological (meiotic pairing) evidence also provided the basis for Bringhurst's initial, partially differentiated AAA′A′BBBB genome composition model for the octoploid strawberry species (Senanayake and Bringhurst 1967). Subsequent genetic evidence, notably the absence of any indication of polysomic inheritance patterns, prompted the proposal of the prevailing, fully differentiated AAA′A′BBB′B′ model (Bringhurst 1990). This last cytological formula implies that the genome of *F.* ×*ananassa* is highly diploidized. A recent study using CAPS (cleaved amplified polymorphic sequence) markers detected only disomic inheritance in *F.* ×*ananassa* (Kunihisa et al. 2005). The diploidization of the wild octoploid strawberry *F. virginiana*, one of the parents of the cultivated species, was also suggested by studying SSR (single sequence repeat) markers (Ashley et al. 2003). However, a final conclusion regarding the diploidized status of the octoploïd genome requires the analyses of markers spread over the whole genome, as will be further detailed in the linkage mapping section.

8.2.2 Genome Size

C-value determinations based on flow-cytometric measurement were reported for one representative of *F. vesca* (1 C = 164 Mb), and for two *F.* ×*ananassa* cultivars (1 C = 562 Mb and 1 C = 571 Mb) (Akiyama et al. 2001), where 1 C is the DNA content of an unreplicated haploid nucleus. In this study, *Arabidopsis thaliana* was used as a standard, and it was assigned a C-value of 125 Mb based on the length of the *Arabidopsis* genome sequence (Arabidopsis Genome Initiative 2000). However, when Bennett et al. (2003) measured the *A. thaliana* C value via flow cytometry (in comparison to a *Caenorhabditis elegans* standard), they obtained a value of 157 Mb, which is about 25% larger than the 125 Mb value determined on the basis of genome sequencing. Using the *A. thaliana* flow-cytometric C-value of 157 Mb as an appropriate standard for flow-cytometric analysis, the 164 Mb C-value reported for *F. vesca* (Akiyama et al. 2001) should be proportionately corrected upward by 25% to 206 Mb, and the *F.* ×*ananassa* values of 562 Mb and 571 Mb should be corrected to 708 Mb and 720 Mb, respectively. Notably, if the two corrected octoploid C values are divided by four to obtain an average C-value for the "basic" (x = 7) subgenome size in *F.* ×*ananassa*, the resulting values of 177 Mb and 180 Mb, respectively, are less than the corrected 206 Mb size of the *F. vesca* genome.

In a similar study using *A. thaliana* as reference (1C = 157 Mb), the diploid (*F. vesca*), hexaploid (*F. moschata*) and octoploid (*F.* ×*ananassa*) genotypes displayed genome sizes of 264 Mb, 731 Mb and 884 Mb, respectively (Denoyes-Rothan, unpublished results). These values, which are corrected in accord with the reference of Bennett et al. (2003), are slightly higher than the corrected values of Akiyama et al. (2001). In both the Akiyama and Denoyes-Rothan studies, the evident diminution of the size of the octoploid genome relative to the diploid one is similar (12% and 16% less than the size expected if the octoploid genome was four times the size of the diploid one). This discrepancy prompts speculation that the genomes originally contributed to the octoploid species by their diploid ancestors were not of uniform size – some being smaller than the 206 Mb size of *F. vesca*. Alternately, the octoploid subgenomes may have undergone some size reduction since the origin of the octoploid species. This smaller size could be due to events which followed the origination of a polyploid such as loss of DNA segments (reviewed in Osborn et al. 2003). It is evident that a comprehensive survey of C-values in the diploid and polyploid species is needed to provide a basis for future investigations of genome evolution in *Fragaria*.

8.2.3 Gene Nomenclature

This review provides a useful opportunity to consider the status of gene nomenclature in strawberry. No uniform nomenclatural guidelines have been established for strawberry, and very few gene names have been assigned. Nevertheless, some conflicts and inconsistencies have occurred, drawing attention to the need for development of a uniform gene nomenclature system for strawberry.

The first use of gene names in strawberry was the assignment of the symbols *s*, *c*, and *r* to the monogenic recessive traits, respectively, of perpetual flowering, yellow/white fruit color, and non-runnering, in *F. vesca* (Brown and Wareing 1965). In this instance, the single-letter gene symbols correspond to the dominant, wild type forms of the respective traits: seasonal flowering (*S*), colored fruit (*C*), and runnering (*R*). Subsequently, Guttridge (1973) employed the gene symbol *j* with reference to the perpetual flowering trait in *F. vesca* f. *semperflorens* cv 'Baron Solemacher', one of the two everbearing varieties previously studied by Brown and Wareing (1965). Although not explicitly stated, the symbol *j* evidently referenced the dominant, wild type "June bearing" (*J*) or seasonal bearing form. More recently, Albani et al. (2004) introduced the symbol *SFL* (*SEASONAL FLOWERING LOCUS*) in relation to the locus governing seasonal (*SFL*) versus perpetual (*sfl*) flowering in *F. vesca* f. *semperflorens*. Again, although not explicitly stated by the authors (Albani et al. 2004), the chosen gene symbol referenced the dominant, wild type form of the trait (seasonal flowering). Thus, three different gene symbols, *s*, *j*, and *sfl*, have already been introduced for what is probably a single locus conferring the mutant form, perpetual flowering. This example emphasizes the need for the establishment of a genetic nomenclature committee for strawberry at the earliest available opportunity. This committee should be charged with establishing guidelines for gene nomenclature in strawberry, and for resolving existing nomenclatural conflicts. Of even greater benefit would be the adop-

tion of a common nomenclatural system for all of the species within the Rosaceae family.

8.2.4 Morphological Markers

Few monogenic morphological markers have been identified in strawberry, in large part because of the genetic and genomic complexity of the octoploid cultivated species. At the diploid level, a few simply inherited traits have been described in the classical literature (reviewed by Brown and Wareing 1965), but few genes have been named. Other than the *c*, *s*, and *r* loci described in the previous paragraph, the only other named morphological marker at the diploid level is the *arb (arborea)* locus conferring a long stemmed phenotype (Guttridge 1973). At the octoploid level, very few simply inherited traits have been described (Scott and Lawrence 1975; Galetta and Maas 1990) or gene symbols assigned. A series of monogenic, dominant determinants (*Rpf* genes) of resistance to red stele disease (causal organism *Phytophthora fragariae* var. *fragariae*) have been described by Van de Weg (1997), and linkages to molecular markers have been identified for three of these (Haymes et al. 1997; Hokanson and Maas 2001). Another dominant gene, *Rca*2, conferring resistance to *Colletotrichum acutatum* (Denoyes-Rothan et al. 2005) has been described. In contrast, quantitative variation has been assessed in a plethora of traits and studies in *F.* ×*ananassa* (reviewed in Galletta and Maas 1990). Despite their major significance to strawberry breeding, quantitative genetic studies that do not include a molecular or mapping component fall outside the scope of this review.

8.2.5 Isozymes and Molecular Markers

Hokanson and Maas (2001) carefully summarized applications of isozymes and the initial phase of molecular marker (RAPD, RFLP, AFLP and first SSRs) development in *Fragaria*. The investigations of PGI (phosphoglucoisomerase) isozymes by Bringhurst and coworkers provided the first evidence that a single gene could be represented by four distinct loci, all of which could be expressed, in the octoploid strawberry (Arulsekar et al. 1981). RFLP (restriction fragment length polymorphism) markers using probes developed from *Prunus* were used for mapping in the octoploid strawberry (Viruel et al. 2002). Among the 123 probes tested, 27 (22%) revealed polymorphism whereas 60–75% revealed polymorphism in *Prunus*, indicating a low overall level of variability in strawberry in the conditions of the study. RFLP markers, which potentially reveal homologous loci, could be very useful in the study of synteny between species of the Rosaceae family.

AFLP (amplified fragment length polymorphism) markers were utilized exclusively in construction of the first published octoploid map (Lerceteau-Köhler et al. 2003). RAPD (randomly amplified polymorphic DNA) markers were employed in construction of the first *Fragaria* linkage map, in *F. vesca* (Davis and Yu 1997). A remarkable aspect of this mapping study was the development of a method of identifying codominant RAPD markers by heteroduplex analysis (Davis et al. 1995) that resulted in placement of 11 codominant RAPD markers on the map. Nevertheless, concern about the comparatively poor transferability of RAPD markers between mapping populations (Sargent et al. 2004), a concern that also applies to AFLP and other anonymous marker types, has prompted intensive attention to the development of sequence-specific, PCR-based markers such as SSR markers for strawberry.

The high cost of SSR marker development via genomic library screening – the source of the initial wave of *Fragaria* SSRs (Nourse et al. 2002; Ashley et al. 2003; James et al. 2003; Sargent et al. 2003; Cipriani and Testolin 2004; Hadonou et al. 2004; Lewers et al. 2005; Monfort et al. 2006) – has been drastically reduced by the advent of EST (expressed sequence tag) database mining as a means of discovering SSR loci within the rapidly growing body of publicly available cDNA and genomic sequences for strawberry (Lewers et al. 2005; Sargent et al. 2006). Lewers et al. (2005) showed that SSRs developed from the genomic library were only slightly superior to GenBank-derived SSRs in their ability to detect polymorphisms. An anticipated advantage of SSRs was their portability at the intra or inter-generic levels. In Lewers et al. (2005), most of SSRs from various species of *Fragaria* amplified within the genus *Fragaria*. Differential patterns of SRR marker transferability from octoploid to various diploid *Fragaria* species are reported in Davis et al. (2006), who also review the general issues relevant to SSR marker transferability within *Fragaria*. Preliminary studies on transferability between genera of the same Rosoideae tribe such as

Rosa showed that about 30–50% of the microsatellites amplified. However, this transference was very low between genera from different tribes such as between *Fragaria* and *Prunus* (Denoyes-Rothan, unpublished results).

The CAPS technique (Konieczny and Ausubel 1993) has also been explored as a gene-based marker technology for strawberry (Kunihisa et al. 2003, 2005). In this method, PCR primers located in exon sequences flanking one or more introns are used to amplify intron-containing sequences, and the amplification products are subjected to restriction digestion with the goal of detecting sequence polymorphisms that are not readily detectable as intron length polymorphisms. This promising method offers the opportunity to conveniently map genes that do not contain SSRs.

8.3 Linkage Mapping

The first instances of genetic linkage to be described in *Fragaria* each involve an isozyme marker and a single gene morphological trait in *F. vesca*. In a cross between Alpine *F. vesca* cultivars 'Yellow Wonder' and 'Baron Solemacher', the yellow fruit color trait (*c* locus) was linked to the SDH (shikimate dehydrogenase) isozyme locus, with a recombination frequency of 1.1% (Williamson et al. 1995). Analysis of several segregating populations derived from crosses between non-runnering, 'Alpine' cultivars and wild type runnering plants detected a linkage (~18% recombination frequency) between the non-runnering (*r*) locus and the PGI-2 (phosphoglucoseisomerase 2) isozyme locus (Yu and Davis 1995).

The first *Fragaria* linkage map was also constructed in *F. vesca* (Davis and Yu 1997). This map defined the expected number of seven linkage groups, covered a total map length of 445 cM, and consisted of 80 markers of which 75 were RAPD markers. The remaining five markers were the SDH and PGI-2 isozyme markers, which anchored linkage groups I and II, respectively, the *r* locus, the *Adh* (alcohol dehydrogenase) gene detected molecularly as an intron length polymorphism, and the *c* locus, which was not segregating in the cross but was added to the map based upon its known close linkage to the SDH isozyme locus (Williamson et al. 1995).

A subsequent diploid linkage map (Sargent et al. 2004), based on the interspecific cross *F. vesca* × *F. nubicola* (Fv × Fn), consisted of 78 markers and spanned a map distance of 448 cM, nearly identical to the 445 cM length of the initial *F. vesca* map (Davis and Yu 1997). The Fv × Fn map marked the beginning of a shift toward use of SSR markers for mapping in strawberry, and contained 68 SSR markers out of a total of 78 markers mapped. The development of this map is ongoing, and has been expanded to a 182 marker version by the addition of new microsatellite loci (Sargent et al. 2006).

The first reported instance of linkage in the octoploid strawberry was that of Haymes et al. (1997), who used bulked segregant analysis (Michelmore et al. 1991) to identify seven RAPD markers linked to the R*pf1* gene for resistance to *Phytophthora fragariae* (red stele) resistance in *F.* ×*ananassa*. Two SCAR (sequence characterized amplified region) markers closely linked in coupling phase to the R*pf1* gene were subsequently developed and found to be widely associated with resistance in a survey of 133 European and North American cultivars and breeding selections (Haymes et al. 2000).

The first published octoploid map was that of Lerceteau-Köhler et al. (2003) for *F.* ×*ananassa*. This mapping study employed a two-way pseudo-testcross strategy, combined with a single dose restriction fragment (SDFR) analysis applied to 789 AFLP markers. Due to the difficulty of accurately detecting repulsion phase linkage in cases of polyploids with polysomic inheritance, as described in Wu et al. (1992) and detailed recently in Qu and Hancock (2001), a two-step mapping procedure was applied (Grivet et al. 1996; Fregene et al. 1997). In a first step, markers linked in coupling phase were mapped into cosegregation groups and in a second step the data matrix was inverted to test the repulsion phase between markers allowing the definition of linkage groups. Separate female and male maps were constructed, and had total map lengths of 1,604 cM and 1,496 cM, respectively. The female map consisted of 235 markers assigned to 30 linkage groups, or an average of 7.8 markers per group (Fig. 1). The male map consisted of 280 markers assigned to 28 linkage groups, or 10 markers per group. Dividing the total map lengths by the number of linkage groups yields average linkage group lengths of 53.5 cM and 53.4 cM for the female and male maps, respectively, which is slightly less than the average linkage group lengths of 63.6 cM

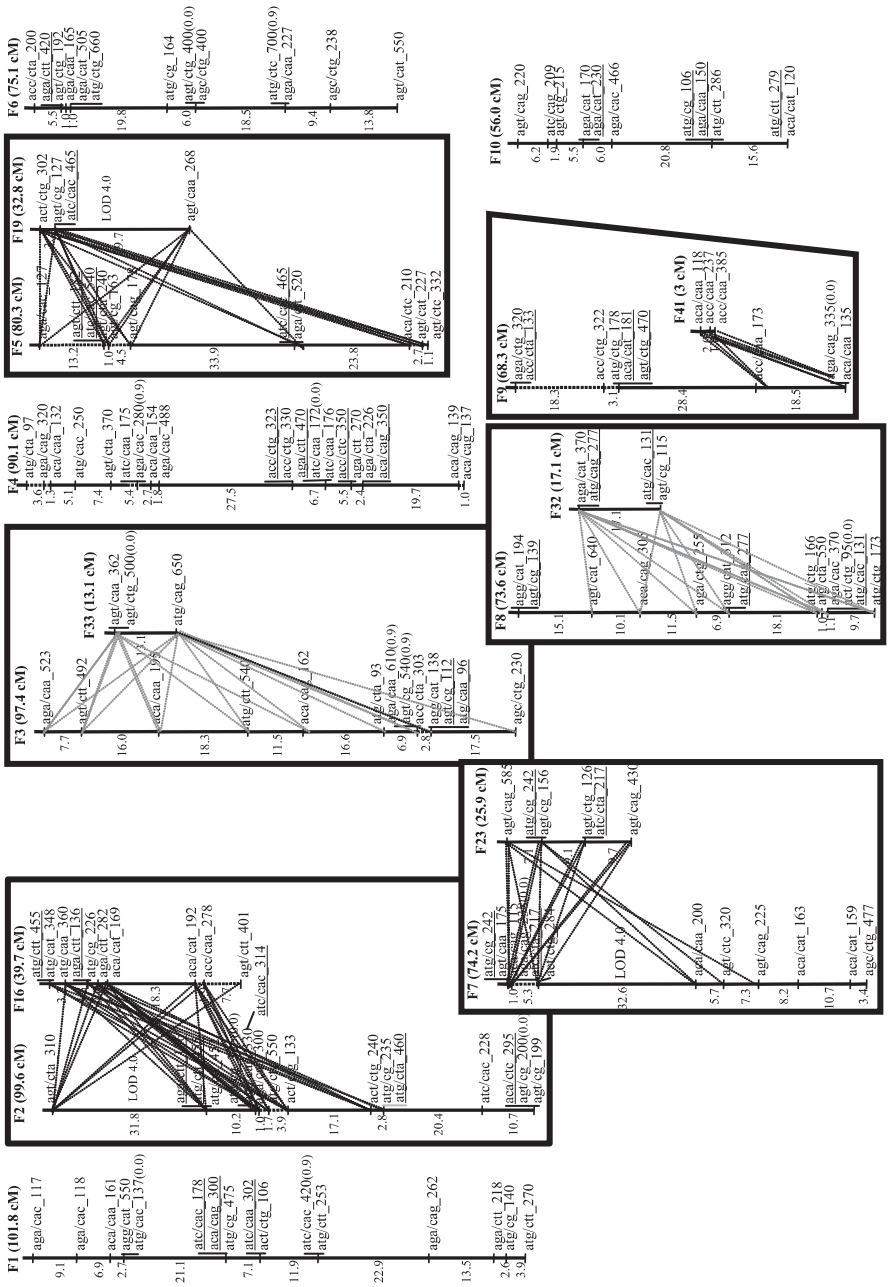

Fig. 1. Linkage map of the female parent (Capitola) (Lerceteau-Köhler et al. 2003). Linkage groups were numbered in order of descending size. Uncertain marker orders in the co-segregation groups (alternate orders not ruled out data LOD = 1) are represented by a *dotted line*. The 3:1 segregating markers are *underlined* and not included in the count of groups with only two markers. Linkage in the repulsion phase between two markers is represented by a *dashed line* (LOD = 3.0). *Boxes* represent linkage groups in the repulsion phase. The genetic distance, in centimorgans, of each marker to its closest neighbouring marker is given in *parentheses*

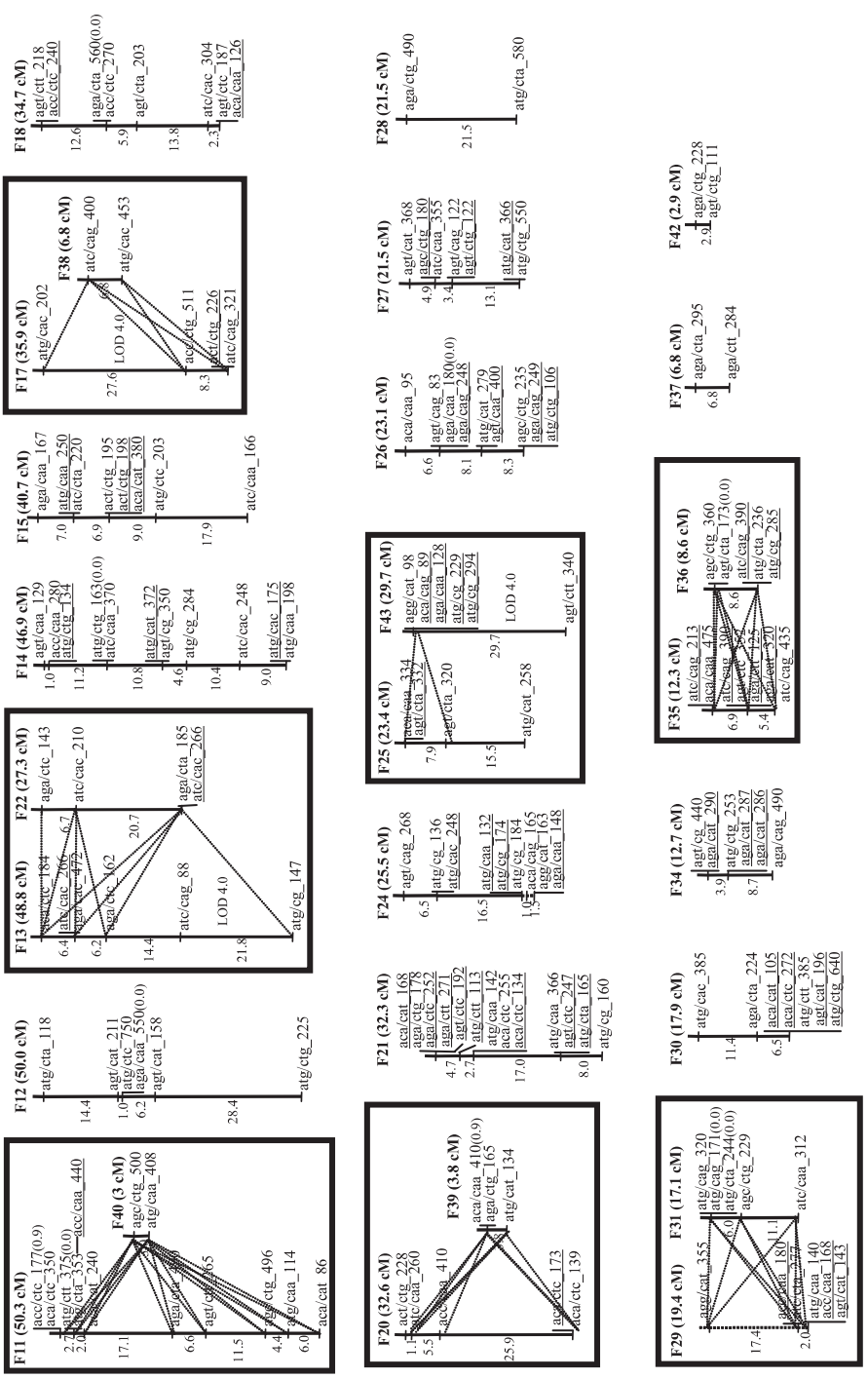

Fig. 1. (continued)

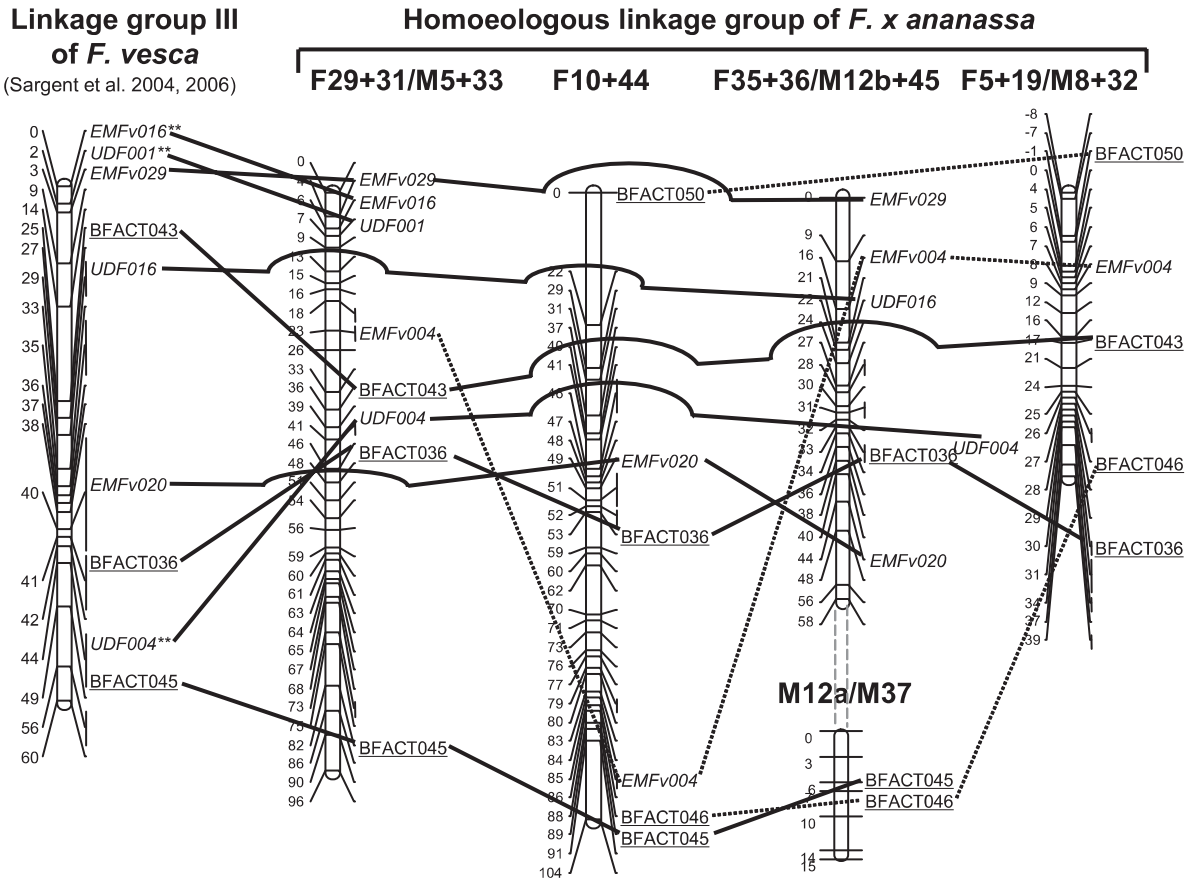

Fig. 2. Comparison of Fragaria vesca and *F.* ×*ananassa* linkage groups (LG). The genetic distances are expressed in map distances (cM) according to Kosambi. Only microsatellites (SSRs) involved in comparisons of linkage groups are indicated (in *underlined*, SSR from *F. x ananassa* and in *italic* SSR from F. vesca). The other markers (SSRs or AFLP) are indicated by *dashes*. Connections between microsatellites located on the four homoeologues LG of *F.* ×*ananassa* and on their homologue LG in *F. vesca* are indicated by *continuous lines*. Connections between microsatellites located exclusively on the four homoeologues LG of *F.* ×*ananassa* are indicated by *dotted lines*

and 64.0 cM calculated, respectively, for the *F. vesca* (Davis and Yu 1997) and *F. vesca* × *F. nubicola* (Sargent et al. 2004) diploid maps described above, both of which had about 11 markers per linkage group. However, since the octoploid map is not fully saturated, the previous figures might be biased and a direct comparison between the diploid and the octoploid maps might be problematic. The analysis of repulsion phase showed that most of the groups were in coupling/repulsion phase reflecting a disomic behavior. However, the presence of some large groups displaying only single dose markers in coupling phase suggested that the entire genome might not be completely disomic.

Recently, a comparison between the octoploid (Lerceteau-Köhler et al. 2003) and the diploid (Sargent et al. 2004, 2006) maps was initiated using microsatellites (Denoyes-Rothan, unpublished results). The first step was the construction of an integrated map in *F.* ×*ananassa*. The pattern of conserved linkages between *F.* ×*ananassa* and *F. vesca* allows the assignment of linkage groups of *F.* ×*ananassa* as potentially homoeologous and homologous to one linkage group of *F. vesca* (Fig. 2).

8.4 Gene Mapping

In the diploid species, *F. vesca*, gene mapping has initially focused on fruit and flowering aspects. A candidate gene mapping approach undertaken by Deng and Davis (2001) discovered an association between the *c* (fruit color) locus and the flavanone 3-hydroxylase (*F3H*) gene in *F. vesca*. Molecular markers linked to the *F. vesca* seasonal flowering locus were identified by Albani et al. (2004). Initially, three ISSR (inter simple sequence repeat) markers (Cekic et al. (2001) linked to the seasonal flowering locus were identified, and were then converted to sequence-specific SCAR markers (Albani et al. 2004). SCAR2 cosegregated with the seasonal flowering locus, which was in turn flanked by SCAR1 (3.0 cM distance) and SCAR3 (1.7 cM distance). The identification of these and other markers linked to the flowering locus provides a starting point for positional cloning of this important locus.

In *F.* ×*ananassa*, gene tagging has so far focused on disease resistances. As previously mentioned, RAPD markers and their derived SCAR markers linked to the R*pf1* gene for resistance to *Phytophthora fragariae* (red stele) resistance have been identified in *F.* ×*ananassa* (Haymes et al. 1997, 2000). The development by Haymes of RAPD markers closely linked to the R*pf3* and R*pf3* red stele resistance genes has also been reported (Hokanson and Maas 2001). A bulked segregant analysis (BSA) was recently used to identify molecular markers linked to the *Rca2* gene conferring resistance to *Colletotrichum acutatum* pathogenicity group 2, which causes anthracnose in the octoploid strawberry *F.* ×*ananassa* (Lerceteau-Köhler et al. 2005). Among the four AFLP markers linked to the resistance gene, two were converted into SCAR markers (STS-Rca2_417 and STS-Rca2_240) and were located at 0.6 cM and 2.8 cM from the resistance gene respectively. Studying the presence of the STS-Rca2_417 marker in 43 cultivars of *F.* ×*ananassa* showed that 81.4% of the resistant/susceptible genotypes were correctly predicted. All these developed SCARs constitute new tools for indirect selection criteria of disease resistance genotypes in strawberry breeding programs (Fig. 3).

A complementary approach to the identification and mapping of resistance genes is the use of degenerate PCR primers targeted to conserved sites in the NBS (nucleotide binding site) domain of many plant resistance genes to isolate resistance gene analogs (RGAs) (Leister et al. 1996; Kanazin et al. 1996; Yu et al. 1996). Martinez Zamora et al. (2004) have reported the isolation of RGAs from cultivated and wild strawberries. Seven distinct families of RGAs were described.

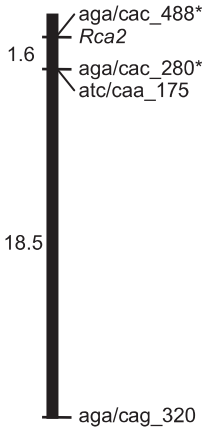

Fig. 3. A genetic map of the chromosome region containing the *Rca2*, *Colletotrichum acutatum*, pathogenicity group 2, resistance gene (Lerceteau-Köhler et al. 2005). The map is based on 62 F_1-individuals from the 'Capitola' × 'Pajaro' cross. AFLP markers labeled with an *asterisk* (∗) were successfully converted into SCAR markers

8.5 QTL Detection

Only a few QTL (quantitative trait locus) studies have been published to date in strawberry. New approaches such as genetic association have been initiated for studying the relationship between the underlying genotype and the observed phenotype. These studies have their extension in the pedigree approach (Van de Weg et al. 2005). The first QTLs published on strawberry concerned fruit quality (Lerceteau-Köhler et al. 2004) and were detected using a segregating population of 213 individuals of a cross between Capitola and CF1116, two genotypes with many contrasting fruit quality traits. A total of 34 traits involved in fruit quality were evaluated, including developmental and fruit aspect related traits, texture related traits, fruit acidity, sugar and ascorbate concentrations. Amino acid concentrations were quantified using one-dimensional proton NMR spectroscopy. Most of the traits except the amino acid concentrations

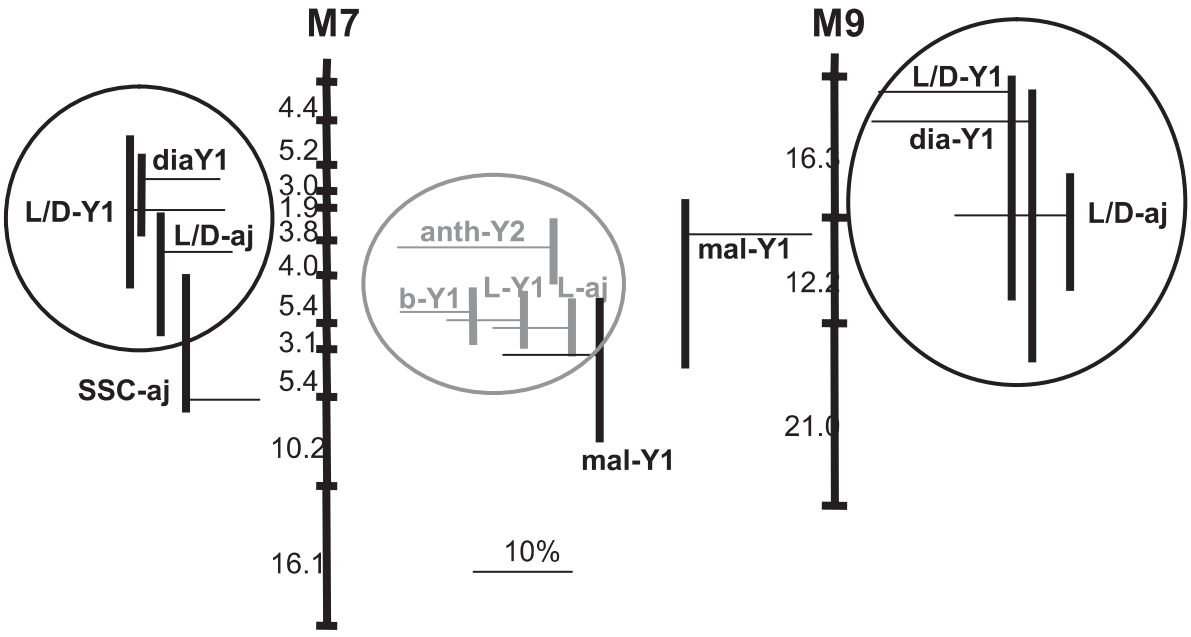

Fig. 4. QTL clusters for fruit quality traits (*mal*, malate; *anth*, anthocyanin; *L/D*, length/diameter ratio; *dia*, diameter; *L* and *a*, external skin color parameters; *SSC*, soluble-solids content) detected on the CF1116 linkage map (Lerceteau-Köhler et al. 2004). Analyses were conducted on two years data (*1* and *2*), and on year-adjusted data. The linkage groups M7 and M9 are in the coupling/repulsion phase. *Horizontal bar* represents the percentage of phenotypic variation explained by a QTL. *Vertical bar* represents the one-LOD support confidence interval

were evaluated during two successive years. A total of 22 significant QTLs were detected by simple interval mapping (LOD > 3.0) in year 1, four on the female map and 18 on the male map, whereas 17 were detected in year 2, ten on the female map and seven on the male map. Only two QTLs could be detected both years. When removing the year effect, 22 QTLs were observed, eight on the female and 14 on the male map. The percentages of phenotypic variance explained by each QTL ranged from 6.5% to 16.0%. An example of QTL cluster of fruit quality-related traits is given in Fig. 4.

QTLs associated with *C. acutatum* and *P. cactorum* resistances were also detected in the same population (Denoyes-Rothan et al. 2004). One hundred eighty five progeny were inoculated with *C. acutatum* by dipping cold stored plants obtained from vegetative multiplication in a conidial suspension adjusted to 2.10^6 conidia per ml. Cold stored plants of the all progeny were also inoculated separately by *P. cactorum* by inserting an agar disk containing mycelium into the crown. Each inoculation was conducted twice, named experiments 1 and 2. For resistance to *C. acutatum*-pathogenicity group 1, five QTLs with LOD scores ranging from 2.0 to 2.8 and spread over three female and two male groups were mapped. The individual QTL effects (R^2) ranged from 5.8 to 12.2%. No QTLs common to experiments 1 and 2 were detected. For resistance to *P. cactorum*, five QTLs with LOD scores ranging from 2.0 to 2.6 and spread over two female and three male groups were mapped. The individual QTL effects (R^2) ranged from 6.5 to 10.2%. Two QTLs, one on female group (F19) and one on male group (M2a), were detected in both experiments. No QTL for *P. cactorum* resistance overlapped the QTLs for *C. acutatum* resistance.

In both fruit quality and disease resistance QTL studies, different putative QTLs were found depending on the technique, the year or the experiment. These differences could be attributed to an environment variation or to a lack of accuracy in the notation. Therefore, before using QTLs in breeding programs, it is necessary to know in which conditions the QTL is expressed. Since there was no QTL for the different studied resistances (*C. acutatum*-pathogenicity groups 1 and 2, and *P. cactorum* resistances), a pyramidal strategy as suggested by Hospital and Charcosset (1997) should be considered for constructing a durable resistance to both pathogens in a breeding scheme.

8.6 Marker-Assisted Selection

The identification of molecular markers linked to QTLs, as well as to qualitative trait loci, enhances the opportunity for use of marker-assisted selection (MAS) in strawberry. Luby and Shaw (2001) have specified criteria that can be used to assess whether MAS will make economic sense in fruit breeding programs. Among these are the requirements for inexpensive marker technologies and for markers that have highly robust marker-locus association. The ultimate test will be whether MAS can provide an economical and substantially improved probability of selecting superior individuals as compared with the best conventional breeding and evaluation practices (Luby and Shaw 2001). Already at least one commercial strawberry breeder is utilizing molecular markers for MAS. RAPD-derived SCAR markers are being used at Driscoll Strawberry Associates in California to screen 1,500 – 30,000 seedlings per year for markers associated with day-neutrality and resistance to *Colletotrichum acutatum* (T. Sjulin, personal communication).

8.7 Development of Genomics Resources

As of July 2005, approximately 7,000 strawberry genomic and cDNA sequence entries were listed in GenBank. By April 12, 2006, this number exceeded 20,000. Although these numbers are small in comparison to the GenBank entry lists for many crop species, they represent a dramatic uptrend over the prior 2–3 year period, before which the number of GenBank entries was well under 1,000. Many thousands of additional strawberry EST sequences are in the bioinformatics pipeline. DNA microarray technology has been employed by only one laboratory, resulting in the identification of genes involved in fruit ripening and flavor, including genes *SAAT* (strawberry alcohol acetyltransferase) (Aharoni et al. 2000), and *FaNES1* (*F.* ×*ananassa* Nerolidol Synthase1) and related genes (Aharoni et al. 2004).

Initiation of positional cloning efforts has been hampered by the general unavailability of high molecular weight genomic libraries for strawberry. BAC (bacterial artificial chromosome) and fosmid libraries have been constructed from *F. vesca* genomic DNA at the University of Reading and the University of New Hampshire, respectively, have yet to be described in peer reviewed publication. However, initial sequencing of genomic fosmid clones from *F. vesca* (Davis, unpublished results) suggests that gene density in *F. vesca* is about 1 gene per 6 kb.

8.8 Conclusion and Future Prospects

Despite its genomic complexity, the small size of the basic *Fragaria* genome makes the strawberry a favorable subject for genomics resource development. The next few years should bring rapid progress in strawberry genomics in several areas. These areas include linkage mapping, positional cloning, functional genomics, and possibly the complete sequencing of a basic strawberry genome. Much of the genomics and mapping data being generated for strawberry and other rosaceous crops is being coordinately assembled and disseminated through the Genome Database for Rosaceae (GDR). Details are available at the site http://www.mainlab.clemson.edu/gdr/.

Second generation linkage maps at the diploid and octoploid levels will be constructed using sequence-specified, transferable markers such as gene-based SSRs, CAPS, and other PCR-based marker technologies. Establishment of robust associations, and ideally identities, between gene-based markers or candidate genes and quantitative or qualitative trait loci will promote the wider evaluation, and potentially adoption, of MAS methods by strawberry breeders. The enhanced transferability of gene-based markers, as compared with the anonymous RAPD and AFLP markers used for construction of the first generation diploid and octoploid maps, respectively, will greatly facilitate map comparison between *Fragaria* species within and between ploidy levels. Mapping with gene-based, codominant markers in the octoploid will enhance the opportunity to detect polysomic inheritance, if it exists at all, in the octoploids.

The high gene density evident from preliminary genomic sequence samples in strawberry favors efforts to positionally clone genes known only by phenotype, particularly in the diploid model system. However, for such efforts to move forward, routine methods for constructing BAC libraries with inserts in the ~150 kb range are needed. The primary obstacle here is the isolation in large quantities of high qual-

ity, high molecular weight DNA, which has proven to be a particular problem in strawberry. Efforts to overcome this obstacle are in progress.

EST resource development for strawberry still lags far behind that of many other fruit crops, including other rosaceous species such as apple and peach. The advent of new technologies for economical, high-throughput, short-read sequencing of individual cDNA molecules in the absence of cloning, such as that recently introduced by the private company 454 Life Sciences, promises to open up an enormous opportunity to expand the strawberry EST database, and to extend the opportunity for strawberry microarray analysis beyond the narrow private sector in which it currently resides.

Finally, the prospect of obtaining the complete sequence of a basic strawberry genome looms inevitably in the minds of strawberry genomicists. The less-than 200 Mb size of the basic ($x = 7$) strawberry genome makes it by far the smallest genome of any rosaceous crop species. An inbred line of the diploid model species, *Fragaria vesca*, almost certainly a genome contributor to the octoploids, would be an excellent subject for complete genome sequencing.

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9 Raspberry

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9.1 Introduction

Rubus is one of the most diverse genera in the plant kingdom, comprising over 400 species (Bailey 1949) subdivided into 12 subgenera (Jennings 1988). Ploidy levels range from diploid to 14-ploid (Nybom 1985). Members of the genus can be difficult to classify into distinct species for a number of reasons including hybridization between species and apomixes (Robertson 1974). The domesticated subgenera contain the raspberries, blackberries, arctic fruits and flowering raspberries, all of which have been utilised in breeding programs. The most important raspberries are the European red raspberry, *R. idaeus* L. subsp. *idaeus*, the North American red raspberry *R. idaeus* subsp. *strigosus* Michx and the black raspberry (*R. occidentalis* L.). Rubus subgen. Idaeobatus is distributed principally in Asia but also East and South Africa, Europe and North America. In contrast, subgen. Eubatus is mainly distributed in South America, Europe and North America (Jennings 1988). The members of subgenus Idaeobatus sp. are distinguished by the ability of their mature fruits to separate from the receptacle. The subgenus is particularly well represented in the northern hemisphere.

The place of origin of raspberry has been postulated to be the Ide mountains of Turkey. Jennings (1988) and Roach (1985) have given extensive accounts of early domestication. Records were found in 4th century writings of Palladius, a Roman agriculturist, and seeds have been discovered at Roman forts in Britain; hence, the Romans probably spread cultivation throughout Europe. The British popularized and improved raspberries throughout the middle-ages, and exported the plants to New York by 1771.

Rubus species are prostrate to erect, generally thorny shrubs producing renewal shoots from the ground (called canes). They are perennials only because each bush consists of biennial canes, which overlap in age. Leaves are compound with 3–5 leaflets, the middle one being the largest; margins serrate to irregularly toothed.

Small (0.5–1.5 cm), white to pink flowers are initiated in the second year of planting. The gynoecium consists of 60–80 ovaries, each of which develops into a druplet. There are 60–90 stamens. Raspberries produce copious amount of nectar and attract bees. The flowers of *Rubus* are structurally rather similar to those of strawberries, with five sepals, five petals, a very short hypanthium, many stamens, and an apocarpous gynoecium of many carpels on a cone-like receptacle. Raspberries are an aggregate fruit, composed of individual drupelets, held together by almost invisible hairs. In *Rubus* each carpel will develop into a small drupelet, with the mesocarp becoming fleshy and the endorcarp becoming hard and forming a tiny pit that encloses a single seed. Each drupelet usually has a single seed, though a few have two. Fruiting begins in the second year of planting and can continue for more than 15 years if properly managed. Fruit development occurs rapidly, taking only 30–36 days for most raspberry cultivars.

Canes grow one year and fruit the next, but there are also primocane varieties which fruit in the first year. The biennial growth cycle of raspberry stems begins when a bud from below soil level develops and elongation of the internodes carries the growing point, protected by leaf scales, to the soil surface. At the surface, leaves expand to form a tight rosette around the growing point. Elongation of the shoot starts in spring and continues until autumn, by which time the shoot will have attained a height of 2 to 3 m. In red raspberries (*R. idaeus* L.), shortening days and falling temperatures in late summer cause shoot elongation to cease and dormancy to set in. This is a gradual process extending over several weeks and once a stage of complete dormancy is reached it is not readily reversible. Black raspberries (*R. occidentalis* L.) or purple raspberries (hybrids between red and black raspberries) and most blackberries differ

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from red raspberries both in time when dormancy begins and intensity of dormancy attained. In these fruits, growth continues well into autumn. The initiation of flower buds usually starts at the same time as the canes begin to acquire dormancy. In the spring of the second year, vegetative primocanes become fruiting canes. The fruit is composed of a large number of one-seeded druplets set together on a small conical core (Jennings 1988).

The traditional method of culture harvests fruit annually from each plant, although both non-fruiting vegetative canes (primocanes) and fruiting canes (fructocanes) are present. This main season summer-fruiting crop is usually supported on a post-and-wire system designed to carry the weight of fruits and to protect canes from excessive damage due to wind, harvesting and cultivation. Primocanes are produced in numbers excessive to requirements for cropping in the following season so many must be removed by pruning in winter and early spring to reduce inter-cane competition and create an open crop canopy for efficient light capture. Old dead fruiting canes must also be removed by pruning after harvest. Such pruning operations remove sources of fungal inoculum from the plantation and are important for the long-term health of the crop.

Primocanes and fruiting canes are in close proximity resulting in a complex plant-architecture that provides spatial and temporal continuity for pests and pathogens to colonise a range of habitats (Willmer et al. 1996). The complex nature of the plant architecture also creates a barrier of foliage that impedes spray penetration of plant protection chemicals, thus requiring specialised chemical application equipment (Gordon and Williamson 1988). Healthy plantations are expected to crop productively for more than 10 years, but this is only possible if the planting stocks and soils are free from persistent viral, bacterial and fungal diseases and certain pests, hence the importance of quarantine arrangements and certification schemes to protect the propagation industry and fruit production (Jones 1991; Smith 2003).

In a mature plantation the raspberry roots spread completely across the inter-row space. Young canes ('suckers') developing from root buds (Hudson 1959) in the inter-row space must be removed, to prevent competition of these suckers for light, water and nutrients with the crop. Uncontrolled suckers also represent a reservoir for pests and pathogens. Cultivation of the inter-row space is another alternative way to remove suckers and weeds, but repeated cultivation by machinery leads to loss of soil structure and soil erosion on slopes where raspberries are often grown. Effective weed management by residual herbicides, or cultivation, is essential to remove weeds as alternative hosts for nematodes that are vectors for many viruses (Murant 1981; Harrison and Murant 1996) and to reduce humidity around the base of plants where several pathogens thrive and sporulate at high humidity.

There has been increased interest in the sale of raspberry fruits harvested from 'organic production' – farming based on methods relying entirely on crop rotation and avoidance of pesticide application except certain substances currently permitted by the national regulatory authority for organic farming. However, with woody perennial crops the difficulties of maintaining healthy productive plantations over many years are profound and it is too early to judge the overall success of these ventures in *Rubus* cane fruits.

Increasing popularity of autumn-fruiting raspberries, in which late season fruit is harvested from berries forming on the upper nodes of primocanes, has extended the production season and the period of attack of some foliar and cane pests. Some very early spring fruits with high value can also be obtained from the remaining lower nodes of these over-wintered primocane-fruiting types. Primocane-fruiting raspberries tend to be grown in the warmer areas of Europe where the temperature in autumn is relatively high and there is little risk of early autumn frosts.

Interest has also been shown in extended-season production under glass or under plastic structures in northern European countries, e.g. Belgium (Meesters and Pitsioudis 1993; Verlinden 1995) and the UK (Barry 1995) and now in the Mediterranean fringe, e.g. Spain and Greece, and this trend will affect their pest and disease status. To satisfy these production systems, long primocanes grown in northern regions, such as Scotland, are lifted, chilled and stored for long periods for planting in late spring for late summer harvest under plastic. The concept of extended-season-production would mean that by careful manipulation of plant dormancy cycle and flower initiation it should be possible to produce fresh raspberries in Europe for sale in almost all months.

The genomic number of *Rubus* is seven and species representing all ploidies from diploid to duodecaploid are found in nature. The range in size is from 1 – 4 µm (Jennings 1988). The genome has been estimated to be 275 Mbp.

Self-incompatibility systems occur in some *Rosaceous* species and it is common among many of the diploid *Rubus* species (Keep 1968). In contrast all polyploidy species are self-compatible as are the domesticated forms of the diploid raspberries.

Raspberries are grown in many parts of the world with production estimated at 385,000 Mt (http://faostat.fao.org), Europe is estimated to produce around 316,000 Mt. Cane fruit production, mainly red raspberry (*Rubus idaeus* L.), is an important high-value horticultural industry in many European countries because it provides employment directly in agriculture, and indirectly in food processing and confectionary. Most raspberry production is concentrated in the northern and central European countries, although there is an increasing interest in growing cane fruits in southern Europe e.g. in Greece, Italy, Portugal and Spain. In many production areas, the fruit is grown for the fresh market, but in central Europe e.g. Poland, Hungary and Serbia, a high proportion of the crop is destined for processing. In the UK there has been a major movement away from processing towards fresh fruit production under protected cultivation for the high-value fresh market. Commercial blackberries are also grown, mainly in east Europe, and arctic raspberries (*R. arcticus* L.) are produced commercially on a small-scale in Finland (Koponen et al. 2000).

Fruit has become important in the human diet due to increased consumers awareness of healthy eating practices. In 2003, the global fresh fruit market was valued at £7.6 bn at current prices, having increased by just 3.9% since 1999. The fresh fruit sector accounts for 38.1% of the overall market and is gaining share due to continuing trend towards convenience food. Banana account the largest segment of the fruit sector with 22.5% of the market in 2003. In term of soft fruit, strawberries remain the UK's best selling soft fruit, but other fruit such as raspberry, are gaining popularities because the increasing all year round availability. Raspberries have always been attractive as fresh dessert fruits or for processing from frozen berries into conserves, purees and juices. It is interesting to see that raspberries were first used in Europe for medicinal purposes (Jennings 1988), but there is now heightened interest focused on these foods as major sources of antioxidants, such as anthocyanins, catechins, flavonols, flavones and ascorbic acid, compounds that protect against a wide variety of human diseases, particularly cardiovascular disease and epithelial (but not hormone-related) cancers (Deighton et al. 2000; Stewart et al. 2001; Moyer et al. 2002). As a result, the consumption of these berries is expected to increase substantially in the near future as their value in the daily diet is publicised. A concerted effort by the public health authorities in Finland, for example, has promoted the consumption of small berry fruits to their populations (Puska et al. 1990) and in 2002, a similar initiative was launched in Scotland (Berry Scotland Project www.berryscotland.com) though success here has yet be demonstrated.

Five parent cultivars dominate the ancestry of red raspberry; 'Lloyd George' and 'Pynes Royal' entirely derived from the European sub-species and 'Preussen', 'Cuthbert' and 'Newburgh' derived from both European and North American subspecies. Domestication has resulted in a reduction of both morphological and genetic diversity in red raspberry (Haskell 1960; Jennings 1988) with modern cultivars being genetically similar (Dale et al. 1993; Graham and McNicol 1995). This is of concern as a lack of genetic diversity can lead to inbreeding depression and susceptibility to external stresses. Extensive genetic diversity has been found in wild raspberry germplasm offering scope for expanding the genetic base of cultivated raspberries (Graham et al. 1997b; Marshall et al. 2001; Graham et al. 2003).

The objectives of breeding programs vary from region to region, but certain traits are always considered important. Breeding for high yields of easily harvested, quality fruit remains the priority in any commercial breeding program (www.fruitgateway.co.uk). The incorporation of novel resistance/tolerance to pests and diseases is regarded as essential for the development of cultivars suitable for culture under integrated pest (crop) management (IPM (ICM)) systems (Gordon et al. 2002a, b). The selection of resistant or tolerant cultivars is essential for reduced pest and disease pressure on the raspberry plantation. Careful thought however must be given to the management of reducing chemical applications as these may result in previously well-controlled pests or diseases becoming an unexpected problem in their own right. Additionally specification of cultivars, for example by UK supermarkets where the cultivars Glen Ample and Tulameen have been selected because of their high fruit quality, has lead to increased pesticide use because these cultivars are aphid susceptible (S.C. Gordon personal communication) again challenging the concepts of IPM and highlighting the

often conflicting demands breeders face. Most raspberries are produced by small enterprises, frequently lacking the resources to fund adequate support from technical advisory services to manage this complex crop in a low input system. However, a survey in New England, USA in the early 1990s showed that farmers generally knew more about IPM than did consumers, wholesalers and food processors (Hollingsworth et al. 1993). Similarly, when driven by legislation and adequate state support, cane fruit IPM systems can be vibrant and generate considerable local and international interest e.g. Whatcom County (Nootsack) IPM raspberry program in Washington State, USA (MacConnell et al. 2002). Resistance breeding is becoming increasingly urgent due to the withdrawal of and undesirability of remaining chemical control measures. Fruit quality though, determines the ultimate success of a cultivar and these objectives may prove to be conflicting. Initial market acceptance of most fruits is based on color and appearance, as other factors are not evaluated until later when the product is consumed. Usually, the consumer associated eye appeal with quality. For an extensive review of fruit quality parameters in plant breeding see Sistrunk and Moore (1983). There is pressure from the supermarkets and some consumers to develop organic sources of many crops including raspberry. There appears to be no large-scale organic production of cane fruit in Western Europe, except for a few isolated producers. However, several large-scale producers are adopting the 'biodynamic' production system in central Europe. Many growers who have tried organic production in Western Europe in the past have failed due to the lack of control of perennial weeds within the crop and to infestation by raspberry beetle. Although derris (rotenone) sprays applied to the green fruit will give some protection against raspberry beetle, experimental and commercial experience suggests that the level of control is inadequate to satisfy the demands of the consumers. In trials in the early 1970s, comparing the efficacy of different insecticides, derris was considerably less effective than the then standard, malathion (Taylor 1971). Safety concerns of some organically approved products are being raised. Some producers, particularly in Scandinavia are keen to develop organic or very low-input production to exploit the demand. The geographical isolation of plantations coupled with low winter temperatures result in low pest burdens in these areas. Organic production will greatly benefit from cultivars with high levels of resistance or tolerance to the major pests and diseases. The large number of characteristics in any breeding program coupled with long generation times and problems with inbreeding depression have prompted the move towards marker-assisted breeding in red raspberry. For a review of breeding objectives and breeding techniques see Daubeny 1996.

Raspberry breeders have successfully produced cultivars that vary in growth habit, pest and disease resistance, spinelessness, fruit quality and primocane varieties.

9.2 Construction of Genetic Linkage Maps

Breeding methods used in raspberry have changed very little over the last 40 years or so. Little novel germplasm has made its way into commercial cultivars. However, with the narrowing genetic base coupled with the increasing demands from consumers, new breeding methods are required to meet demands. The speed and precision of breeding can be improved by the deployment of molecular tools for germplasm assessment and the development of genetic linkage maps. Such genetic linkage maps can facilitate the development of diagnostic markers for polygenic traits and the identification of genes controlling complex phenotypes. Understanding the genetic control of commercially and nutritionally important traits and the linkage of these characteristics to molecular markers on chromosomes is the future of plant breeding. Red raspberry (*Rubus idaeus*) is a good species the application of such techniques, being diploid ($2n = 2x = 14$) with a very small genome (275 Mbp). Indeed, the haploid genome size of raspberry is only twice the size of *Arabidopsis*, making it highly amenable to complete physical map construction, thereby providing a platform for map-based gene cloning and comparative mapping with other members of the Rosaceae (Dirlewanger et al. 2004). The availability of abundant genetic variation in natural and experimental populations and adaptation to a range of diverse habitats (Graham et al. 1997b; Marshall et al. 2001; Graham et al. 2003) offers researchers a rich source of variation in morphology, anatomy, physiology, phenology and response to a range of biotic and abiotic stress. The ability to vegetatively propagate individual plants provides opportunities to capture genetic variation over generations and replicate

individual genotypes to partition and quantify environmental and genetic components of variation of genetic linkage maps. These are necessary to develop diagnostic markers for polygenic traits and, in the future, possibly identify the genes behind the traits. The Rosaceae is an economically important family of perennial fruit bearing crops that includes members of the following genera: *Malus* (apple), *Pyrus* (pear), *Rubus* (raspberry, blackberry), *Fragaria* (strawberry) and *Prunus* (stone fruits). In addition, the family also includes a number of important ornamental plants such as roses, flowering cherry, crab apple and quince. Molecular marker applications have been reviewed in *Rubus* (Antonius-Klemola 1999) and in the small fruits (Hokanson 2001). Linkage maps have been generated in other woody species (Ritter et al. 1990; Grattapaglia and Sederoff 1994; Bradshaw et al. 1994; Bradshaw and Stattler 1995) and in the small (soft) fruit crops a few maps exist. In the diploid strawberry (*Fragaria vesca*) and diploid blueberry (*Vaccinium* spp.) 445 cM and 950 cM or 1,288 cM long linkage maps based on RAPD markers have been constructed (Rowland and Levi 1994; Davis and Yu 1997; Qu and Hancock 1997). Maps of other *Rosaceous* crops include *Prunus* maps (Dirlewanger et al. 1997, 1998; Joobeur et al. 1998, Ballester 2000; Joobeur et al. 2000; Dettori et al. 2001; Aranzana et al. 2003), apple (Hemmat et al. 1994; Maliepaard et al. 1998; Liebhard et al. 2003). Resources are being developed in strawberry to enhance maps based on RAPD markers (Sargent et al. 2003; Graham 2005). The first genetic linkage of raspberry has recently been constructed (Graham et al. 2004b). This 789 cM genetic linkage map was constructed utilising a cross between the phenotypically diverse European red raspberry cultivar Glen Moy and the North American cultivar Latham. SSR markers were developed from both genomic and cDNA libraries from Glen Moy. These SSRs, together with AFLP markers, were utilised to create a linkage map. An enhanced with further SSR and EST-SSR and gene markers has recently been completed (Graham et al. 2006).

9.3 Gene Mapping

Mapping in raspberry is at an early stage. Preliminary work is underway to map genes underlying a number of commercially important traits. Gene *H* in raspberry has recently been mapped to Group 2 of the raspberry map (Graham et al. 2006). Raspberry breeders in general have limited resources and rarely include a primary screen for fungal diseases. It has been reported that some disease resistances are associated with distinctive morphological traits, most notable cane pubescence (fine hairs). Pubescence is determined by gene *H* (genotype *HH* or *Hh*), the recessive allele of which gives glabrous canes (genotype *hh*). Gene *H* is rarely homozygous because it is linked with a lethal recessive gene (Jennings 1988). Raspberry cultivars and selections with fine hairs (pubescent canes) are more resistant to cane botrytis (*Botrytis cinerea*), cane blight (*Leptosphaeria coniothyrium*) and spur blight (*Didymella applanata*) than non-hairy ones (Knight and Keep 1958; Jennings and Brydon 1989) but more susceptible to cane spot (*Elsinoe veneta*), powdery mildew (*Sphaerotheca macularis*) and yellow rust (*Phragmidium rubi-idaei*) (Keep 1968, 1976; Anthony et al 1986; Jennings and McGregor 1988; Williamson and Jennings 1992). How Gene *H* has the large increase or decrease in disease resistance has not been determined. It has been suggested that it is due to linkage with major resistance genes or minor gene complexes that independently contribute to the resistance or susceptibilities of the six diseases affected. An alternative explanation is that the gene itself is responsible through pleiotrophic effects on each of the resistances (Williamson and Jennings 1992). This gene has now been mapped and further mapping of the disease resistance genes is underway (Graham et al. 2006). Other work underway is aimed at identifying the gene(s) responsible for resistance to raspberry root rot (Graham and Smith 2002). Two regions on two linkage groups have been identified and further research aimed at confirming these in a second population through glasshouse and field trials is underway (Graham, Smith and Tierney unpublished data). Efforts to map aphid resistance by anchoring data marker data from appropriate segregating populations to the published raspberry maps are underway (Sargent, Knight Personal Communication).

9.4 Analysis of Quantitative Trait Loci

Preliminary quantitative trait loci (QTL) mapping has been carried out in raspberry using the recently developed genetic linkage map (Graham et al. 2004b, 2006). Morphological data based on the segregation of cane spininess, and root sucker density and diameter were quantified in two different environments. Breed-

ing for spinelessness is a major concern for breeders and there are several major genes that confer this trait (Jennings and Ingram 1983; Jennings 1988). The mapping parents differ for spine morphology with Glen Moy having a spine-free phenotype (being homozygous for gene *s* (Jennings 1988), whereas Latham is a densely spiny cultivar, the genetics of which has not been determined. The progeny generated from the cross were all spiny, though the extent of spines varied continuously from a very sparsely spiny cane to the densely spiny phenotype of the Latham parent. From the phenotypic data it was proposed that two or more genes are involved. This was supported by the mapping data where a number of markers were identified, linked to the spiny phenotypes. These markers mapped onto linkage group 2, and there appeared to be two linked regions within this group accounting for 98% of the variation.

Large differences exist in the extent of root sucker production in cultivated raspberries. Control measures based on the chemical burning of early canes produced from suckers are required in commercial plantations to optimise fruit yield (Jennings personal communication). Roots of red raspberry have adventitious buds, which develop on most roots. The number, density and distance from the mother plant of the root suckers varies between genotypes. Only a proportion of the buds normally develop into suckers. Knight and Keep (1960) have shown that the ability to produce suckers in red raspberry is determined by the recessive gene sk_1 or by the complementary genes sk_2 and sk_3. Interestingly, and probably not surprisingly, the measurements of density and spread map to the same linkage group (group 8), with an overlap in the location of the QTLs for the two traits (Graham et al. 2004b).

A number of QTLs for fruit quality parameters have been identified on the raspberry maps and some candidate genes which underlie these traits have been identified. For example a QTL for fruit size has been located (Graham unpublished data) with a vacuolar $^{H+}$-ATPase (Martinoia et al. 2000).

9.5 Marker-Assisted Breeding

A number of DNA-based marker systems have been developed for use in raspberry (Antonius-Klemola 1999; Hokanson 2001; Graham et al. 2002a). Genetic markers have been used to widely to examine genetic variation within and between *Rubus* spp. An M13 bacteriophage probe has been used to examine different *Rubus* spp. and a number of red raspberries (Nybom et al. 1990). A minisatellite probe was used by Kraft et al. (1996) to demonstrate that fingerprints of out-crossing species vary considerably compared to vegetative and apomictic clones. Chloroplast DNA sequence probes were used by Waugh et al. (1990) and Howarth et al. (1997) to examine genotypic and taxonomic relatedness in raspberry. Ribosomal DNA ITS region has been used to construct a phylogenetic tree with representatives from 20 species (Alice and Campbell 1999). RAPD markers have been widely used to examine the relatedness of raspberry cultivars and species (Graham and McNicol 1995; Graham et al.1997b; Coyner 2000).

Marker-assisted selection is developing into a powerful tool for plant breeding, through its ability to select plants with the desired trait(s) accurately and at an early stage of growth. Rather than screening for a particular phenotype (trait), a breeder can screen for a marker tightly linked to the gene of interest that is identified through the construction of a linkage map in a population segregating for that trait. Alternatively, bulked segregant analysis can be used to identify markers linked to a particular trait, the position of which can then be determined on a linkage map (Graham and Smith 2002).

9.6 Map-Based Cloning

Map-based cloning has yet to be carried out in raspberry. Genetic engineering technologies, if they become widely acceptable to customers, could allow high quality cultivars to be transformed with genes conferring resistance to a range of pests and diseases (Watt et al. 1999), thus offering the prospect of reduced pesticide application. Recent research in strawberry has demonstrated that introduction of the Cowpea protease trypsin inhibitor (CpTi) gene resulted in promising levels of control in glasshouse feeding trials and field trials against larvae of *Otiorhynchus sulcatus* (Graham et al. 1997a, 2002b). Use of gene transfer technologies to improve resistance to mites, insects and nematodes would be especially valuable because of the toxicity of acaricides, insecticides and nematicides, many of which are likely to be withdrawn from use in minor crops in the future. Fruit quality and other stress resistance genes would be

valuable. However, it is vitally important that these genetically engineered crops are not toxic or pose a serious allergenic risk to humans, do not harm beneficial organisms (e.g., natural enemies of pests, crop pollinators, soil micro-organisms) or affect the wider environment. Large-scale 'risk assessments' of genetically engineered crops such as the Farm-Scale Evaluation of oil seed rape, sugar beet and maize are currently being undertaken in the UK to ensure that, on release, they are environmentally benign.

9.7 Advanced Works

Advanced work for red raspberry is still at an early stage. A search of the NCBI nucleotide database for *Rubus* retrieved only 1,744 sequences a large number of which were actually viral sequences. In comparison, a similar search for the genus *Prunus*, also a member of the Rosaceae family, yielded 325,773 sequences of which 76,619 originated from *Prunus persica* (peach).

The number of raspberry sequences is, however, very likely to increase rapidly as efforts are under way to generate EST libraries from different tissues and developmental stages. At the Scottish Crop Research Institute, cDNA libraries have been generated from leaves (approximately 6,500 clones), canes (approximately 8,000 clones) and roots (approximately 7,300 clones) and further libraries will be constructed from fruit and shoots in the near future (Graham, Smith and Tierney unpublished data). Bacterial colony filters derived from the above libraries have been subjected to hybridization screening to identify simple sequence repeats (SSR) markers and will be partially sequenced.

A further project aims at the characterization of bud dormancy in woody perennial plants on a molecular level and generated in total 5,300 ESTs from endodormant (true dormancy) and paradormant (apical dominance) raspberry meristematic bud tissue (Mazzitelli et al. unpublished data). PCR-products from these cloned cDNA fragments have been spotted onto glass slides and are currently being used in microarray experiments to identify genes that show differential expression. At present, approximately 380 clones exhibit up or down regulation during the endodormancy – paradormany transition.

Large insert genomic libraries (BACS) are invaluable tools and a source of genomic DNA for physical mapping, positional cloning and as a scaffold for whole genome sequencing. *Rubus idaeus* is an ideal candidate for BAC library construction, since it is diploid ($2n = 2x = 14$) and has a very small genome (275 Mbp). Indeed, the genome size of raspberry is only twice that of the model plant *Arabidopsis*, making it highly amenable to complete physical map construction, and thereby providing a platform for map-based gene cloning and comparative mapping with other members of the Rosaceae.

One of the most challenging steps required for the construction of plant large-insert genomic libraries is the isolation of high molecular weight DNA (HMW-DNA), either in the form of embedded protoplasts or nuclei. Raspberry and other soft-fruit species have, however, proven recalcitrant to standard genomic DNA extractions as they contain very high levels of carbohydrates, particularly polysaccharides, and polyphenolic compounds. They require heavily modified methods for ordinary genomic DNA isolations (Woodhead et al. 1998) and the utilization of activated charcoal in tissue culture to prevent growth inhibition due to excess polyphenolics released into the medium (Millan-Mendoza and Graham 1999). To prepare HMW-DNA suitable for the construction of BAC libraries we have developed a novel nuclei isolation procedure (Hein et al. 2005). The method is based on a modified buffer system including 4% (w/v) PVP-10 described by Peterson et al. (2000) and utilizes a combination of nylon filters and Percoll™ gradients to purify nuclei extracts prior to embedding in agarose plugs. The isolated HMW-DNA is of high quality and has been used for the construction of the first publicly available red raspberry BAC library from the European red raspberry cultivar Glen Moy, which has also been utilised as a parent for the first reported genetic linkage map of *R. idaeus* based on a cross with the phenotypically diverse North American cultivar Latham (Graham et al. 2004b). Currently, the library comprises over 15,000 clones with an average insert size of approximately 130 kb (6–7 genome equivalent). Hybridization screening of the BAC library with chloroplast (*rbcL*) and mitochondrial (*nad1*) coded genes revealed that contamination of the genomic library with chloroplast and mitochondrial clones was very low (>1%) (Hein et al. 2004a).

Future work will focus on anchoring the physical map to the genetic map, which will enable alignment of the maps and the identification of genomic

regions harbouring genes controlling important phenotypes. An integrated physical/genetic map will also allow the extent of synteny or collinearity of the *Rubus* genome with other members of the Rosaceae to be determined.

The availability of a detailed genetic linkage map, together with a deep coverage bacterial artificial chromosome library, will be of great value in the identification of the genetic factors that underpin a wide range of commercial characteristics such as appearance, genetic resistance, texture and sensory (taste and aroma) attributes of fruit. The establishment of gene-phenotype relationships will allow gene-based selection in breeding and the functional assignment of genes for commercially important traits.

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10 Blueberry

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10.1 Introduction

Blueberries are members of the *Ericaceae* or Heath family, genus *Vaccinium*, subgenus *Cyanococcus*. Genus *Vaccinium* consists of blueberries, cranberries, lingonberries and many related wild species. The genus is very diverse, containing about 400 species, mostly found in the tropics at high elevation, but also in temperate and boreal regions. *Vaccinium* is widely distributed plant genus and exhibits a high level of morphological diversity. Fruits from several *Vaccinium* species are collected from the wild for food. *Vaccinium* species in section *Cyanococcus* are used to develop blueberry cultivars which are grown for their edible fruit. Most are shrubs like the blueberries; however diverse range of growth forms from epiphytes to trees exists.

Blueberry is an important small fruit crop and the most recent major fruit crop to be cultivated, having being domesticated during the twentieth century. They are high value crop which can thrive on acidic imperfectly drained sandy soils, once considered worthless for agricultural crop production. North America is the major producer of blueberries. The total area devoted to growing commercial blueberries in North America is approximately 74,000 ha. Blueberries are one of the richest sources of antioxidants of all fresh fruits and vegetables (Prior et al. 1998). In addition, fresh blueberries are fair source of vitamin C (Matzner 1967). Blueberries are produced commercially in 16 countries worldwide. Worldwide average yields have increased almost by 50% in the last 10 years to just over 4,000 lbs/acre. Acreage has also increased by 37%, causing production to double in the last 10 years. Major countries of blueberry production are USA and Canada, together account for the major blueberry production. In addition to these, other countries including Poland, The Netherlands, France, Italy, Mexico, New Zealand and Lithuania also produce blueberries.

10.1.1 Cytology

According to Longley (1927), the genus *Vaccinium* has a basic chromosome number of 12. Blueberry exists at three ploidy levels: 2× ($2n = 24$), 4× ($2n = 48$) and 6× ($2n = 72$). Diploid population ($2n = 24$) includes species: *V. myrtilloides* Michx, *V. corymbosum* L., *V. pallidum* Ait., *V. darrowi* Camp, *V. elliottii* Chapm, *V. tenellum* Ait. *V. boreale*. Important tetraploids are *V. angustifolium* Ait, *V. corymbosum*, *V. hirsutum* Buckley, *V. simulatum* Small, *V. myrsinites* Lam. These tetraploids may have resulted from crosses between members of the same species, resulting in autotetraploids, or between members of different species as allotetraploids. Species *V. corymbosum* L. and *V. australe* Small, are natural tetraploids. The third important group of species is represented by the hexaploid ($2n = 72$) population of which *V. ashei* Reade, and *V. constablaei* Gray, are members. There are seven diploid, six tetraploid and two hexaploid species (Rowland and Hammerschlag 2005). Since no fundamental sterility barrier exists between homoploid *Vaccinium* species, many polyploids have arisen naturally (Coville 1927; Newcomber 1941). These polyploids particularly tetraploids ($2n = 48$), are thought to be responsible for the wide range of adaptation of the genus (Newcomber 1941). Ahokas (1972) concluded that the diploid Vaccinum genome ($x = 12$) is actually "homoeologous tetraploidy" (secondary polyploidy), based on Hall and Galletta's findings (1971).

Three species are of major economic importance: (1) The highbush blueberry, *Vaccinium corymbosum* (2) The lowbush blueberry *Vaccinium angustifolium* and (3) the rabbiteye blueberry, *V. ashei*. Most of the worldwide blueberry production comes from the highbush blueberry.

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10.1.2 Commercial Blueberries

Commercially grown blueberries can be divided into five major groups.

(1) Highbush Blueberry: In Highbush types (4×) *Vaccinium corymbosum* makes up much of the genetic material of the northern highbush. It is a very variable deciduous shrub that is typically 4 to 6 ft. tall. It is most widely planted blueberries, popular with home gardeners throughout the northern US and southern Canada. There are over 100 named varieties of northern highbush blueberries.
(2) The wild or lowbush blueberries of North America: The lowbush types (2× and 4×) include managed wild populations of *V. angustifolium*, *V. myrtilloides, V. boreale* and improved lowbush cultivars. In North America most are *Vaccinium angustifolium* and are known as sweet lowbush blueberry. The lowbush is not commercially planted, but thousands of acres of natural stands are pruned, sprayed and harvested. It is a dwarf, woody, usually deciduous shrub that is found growing in wide range of areas. The term lowbush applies to those species that are less than 3 ft.
(3) Rabbiteye: The Rabbiteyes (6×) are all wild selections and hybrid cultivars of *V. ashei*.
(4) Southern Highbush: Southern highbush blueberries (4×) cover hybrids that may contain genetic material from two, three and sometimes four *Vaccinium* species. These are predominantly highbush *V. corymbosum* germplasm but which have the low-chilling species *V. darrowi* in their parentage, as well as *V. angustifolium* and in some cases *V. ashei* and *V. tenellum* (Lyrene 1990; Ballington et al. 1991). Southern highbush were specifically hybridized for superior fruit, soil adaptability, heat tolerance, and low winter chilling.
(5) Halfhigh Blueberries: Halfhigh blueberry (4×) is a term given to a group of blueberries that do not exceed about 3 ft. at maturity, but most have the bushy, woody habit of highbush cultivars. These are species hybrid or backcross derivatives of lowbush-highbush hybrids, usually involving *Vaccinium angustifolium and V. corymbosum* parentage. The half high category refers to bushes intermediate in height between high bush and low bush (Galletta and Ballington 1996; Hokanson 2001; Rowland and Hammerschlag 2005).

10.1.3 Breeding Objectives

10.1.3.1 Horticultural Attributes

The early objectives in blueberry breeding included large berry size, light blue color, small scar, firmness of fruit, good dessert quality and productivity. Each of these objectives has been realized, but all are not yet combined in a single variety. The trait that receive emphasis in selection vary with the location and type of blueberry, but generally include plant vigor, disease resistance, desirable plant architecture, easy of clonal propagation, large fruit size, good flavor, light blue fruit color, long storage life, season of ripening, and consistent high yields. Other objectives of breeding work involve the development of cultivars with greater winter hardiness, drought resistance and adaptation to mechanical harvesting.

10.1.3.2 Biotic Stress Resistance

Insects The control of blueberry insects is one of the most important phases of blueberry culture. Some of these insects seriously reduce the productivity of the bush, while others impair the quality of berries lowering their value. Blueberries are subject to attack from many different insects. Blueberry maggot is the major fruit pest of blueberry. The adult is a small fly which lays an egg under the skin of the developing fruit. The tiny larva feeds within the fruit. Cranberry fruitworm, plum curculio, blueberry bud mite are other important insects. Mites are tiny pests (<1/100th inch) inhabit the leaf and flower buds, feeding on them before they emerge. The other problematic insects to blueberries are: blueberry blossom weevil, blueberry leafminer, blueberry stem borer, cherry fruitworm, cranberry fruitworm, cranberry rootworms and grubs, scale insects, sharp nosed leafhopper, blueberry crown girdler, black vine borer, and cranberry rootworm.

Diseases Blueberries can be attacked by a host of fungi, bacteria, and viruses. Most of the diseases vary in severity and economic importance from one blueberry growing region to another. Lowbush blueberries, highbush blueberries, and rabbiteye blueberries have similar types of diseases, but the disease that is most important in one type may be minor in the other. Mummy berry is probably the most

Table 1. Important diseases and pests of blueberry

	Causal Agents
Diseases	
Fusicoccum canker	*Fusicoccum putrefaciens*
Anthracnose	*Colletotrichum gloeosporioides*
Botrytis blight	*Botrytis cinerea*
Mummy berry	*Monilinia vaccinii-corymbosi*
Red leaf rose bloom	*Exobasidium vaccinii*
Blueberry stunt	Mycoplasma-like organism
Blueberry Shoestring	Blueberry Shoestring Virus
Stem blight	*Botryosphaeria dothidea*
Stem canker	*Botryosphaeria corticis*
Phomopsis twig blight and canker	*Phomopsis vaccinii*
Alternaria fruit rot	*Alternaria* spp.
Insects	
Blueberry maggot	*Rhagoletis mendax* Curran
Sharp-nosed leafhopper	*Scaphytopius magdalensis* Provancher
Blueberry aphid	*Illinoia pepperi* MacGillivray
Cranberry fruitworm	*Acrobasis vaccinii* Riley
Cherry fruitworm	*Grapholita packardi* Zeller
Plum curculio	*Conotrachelus nenuphar* Herbst
Blueberry bud mite	*Acalitus Vaccinii* Keifer

widespread threat to blueberry in almost all countries. It is characterized by the formation of dried-out mummified fruit at harvest. The disease kills leaves, shoots, and flowers and then produces the spores on these dead tissues that infect the fruit later. It may reduce yields by up to 10% in severe infestations of some main commercial areas. Other common diseases of blueberry are blueberry stunt, blueberry shoestring, leaf mottle, scorch and red ringspot viruses, stem blight, stem canker, botrytis, anthracnose, phomopsis twig blight, canker, alternaria and fruit rot, fusicoccum canker and red leaf rose bloom (Galletta and Ballington 1996; Rowland and Hammerschlag 2005).

Viruses cause several diseases in blueberry. Two viruses of importance in North America are Blueberry scorch and red ringspot viruses. Virus diseases are the most difficult to control since infection may occur several months, or possibly years, before symptoms are seen, and the only effective control usually involves removing infected bushes. Fruit and foliar diseases are controlled with a combination of proper cultivar selection, cultural practices, and fungicides. Stem and root diseases are more difficult to control. Disease-free planting stock, promotion of good plant growth, removal and destruction of infected plant parts, and the selection of well drained ground all help reduce the incidence and severity of root and stem diseases. Table 1 lists important diseases and pests of blueberry (Galletta 1975; Luby et al 1991; Galletta and Ballington 1996).

10.1.4 Blueberry Breeding

There has been a great breeding effort in the highbush blueberry than any other *Vaccinium* species (Draper and Scott 1971). The breeding of highbush blueberries began in about 1900 while rabbiteye breeding began in about 1940. Of the three types of blueberries, lowbush blueberries have benefited the least from cultivar development, and most lowbush blueberries still come from native plants. Breeders have released several blueberry cultivars comprised of diverse species and from widely different geographical areas (Ballington 1990; Lyrene 1990).

Three themes have been evident in the breeding of both highbush and rabbiteye cultivars: recurrent selection (Lyrene 1988), the proven-cross method,

and interspecific hybridization (Lyrene and Ballington 1986; Lyrene and Perry 1988). Interspecific hybridization continues to be the keystone to the success of the cultivated blueberry improvement program. The genus *Vaccinium* has many species. Within section *Cyanococcus*, interspecific crosses are easy to make and interspecific hybrids are usually vigorous and fertile if the two species involved have the same chromosome number. Coville (1937) undertook interspecific hybridization for blueberry breeding. Most of the native species of blueberry could be hybridized with the cultivated types and provide unique genes. (Draper 1977; Draper et al. 1982). Crosses of diploid × tetraploid, diploid × hexaploid, and tetraploid × hexaploid species give varying result, depending on the species involved. Many breeders utilized various species for blueberry improvement. (Moore 1965; Sharp and Sherman 1971; Ballington 1990; Lyrene 1990). Wild *V. corymbosums* selections have been used for many years for developing blueberry cultivars.

The first varieties to be introduced were hybrids of *V. corymbosum* and *V. australe*. Modern cultivars were derived from the hybrids of *Vaccinium angustifolium* and *Vaccinium corymbosum* and from the hexaploid hybrids of *V. ashei* and *V. constablaei*. (Ballington 1980). There has been an emphasis in several breeding programs in the past to develop highbush blueberry varieties (*V. corymbosum*, 4×) with low chilling requirements, suitable for growing in the southern United States because they are early ripening. Crosses between high-chilling northern highbush cultivars and the low-chilling southern evergreen diploid, *V. darrowi* Camp, have been important in the development of low-chilling highbush cultivars (Sharpe and Darrow 1959; Sharp and Sherman 1971). Crosses between *V. ashei* and *V. constablaei* have resulted in the release of two rabbiteye hybrid cultivars (Ballington 2001). However traditional breeding approaches for blueberry are labor-intensive due to heterozygosity, polyploidy, and length of evaluation trails. Hybridization between certain species has been difficult to achieve due to chromosome number difference and the inability to easily induce polyploidy. Attempts to transfer genes between tetraploid highbush cultivars and hexaploid rabbiteye cultivars have not been highly successful.

10.2 Application of Marker Technologies

10.2.1 Protein Markers

Isozymes have been used in genome analysis of higher plants both to determine phylogenetic and evolutionary relationships and in genetic linkage analysis. Hill and Vander Kloet (1983) used isozyme markers for genetic studies in blueberry and reported limited variation in four enzyme systems. Among four *Vaccinium* sections Vorsa et al. (1988) studied diploid, tetraploid and hexaploid *Cyanococcus* species for isozyme polymorphisms using 12 enzyme systems. Further Kreb and Hancock (1989) used isozyme markers to investigate the mode of inheritance in tetraploid *V. corymbosum* and reported that it has tetrasomic inheritance in the four enzyme systems analyzed. Bruederle et al. (1991) extended isozyme analysis of 20 loci to the investigation of population genetic structure among diploid blueberry species *V. elliotii*, *V. myrtilloids*, and *V. tenellum*. They found that the diploid species exhibit high level of variation within populations as expected for highly self-sterile, outcrossing crops in taxa. Hokanson and Hancock (1998) examined levels of allozymic diversity in native Michigan populations of diploid *V. myrtilloids* and the tetraploid *V. angustifolium* and *V. corymbosum*. The number of polymorphic loci is very limited within a gene pool, and polymorphism is low. DNA markers were favored for most purposes.

10.2.2 Molecular Markers

Several types of DNA markers are now available for use in genetic mapping. PCR-based markers such as random amplified polymorphic DNA (RAPD), arbitrary primer-PCR (AP-PCR), inter-simple sequence repeat (ISSR), expressed sequence tag (EST), cleaved amplified polymorphic sequences (CAPS) and microsatellites have also been used mainly for the analysis of plant genomes. With respect to molecular genetic research in blueberry molecular markers have been developed for DNA fingerprinting, analysis of genetic relationship and mapping. Several cDNA and genomics clones have been isolated and an EST database has been made publicly available. The type of markers currently available includes isozyme, RFLP, RAPD, ISSR, EST-PCR, CAPS and microsatellite markers.

Molecular markers have been identified that are useful for DNA fingerprinting of representative selections and cultivars of three major commercial grown types of blueberries: the highbush, lowbush and rabbiteye types. Haghighi and Hancock (1992), performed RFLP analysis on various genotypes representing the blueberry species *V. corymbosum*, *V. angustifolium*, *V. darrowi* and *V. ashei*, using chloroplast specific and mitochondria specific probes. In this study, high polymorphism was observed in mitochondrial genome while no polymorphism was detected in chloroplast genome. Aruna et al. (1993) and Levi et al. (1993) reported successful amplification of RAPD markers from blueberry DNA. Aruna et al. (1993, 1995) reported good results from DNA of native selections and improved cultivars of rabbiteye blueberry, *V. ashei*. In this study the extent of genetic relatedness among 19 cultivars of rabbiteye blueberry, 15 improved cultivars and the four original selections from the wild were used. Their analysis was consistent with phylogenetic data provided for rabbiteye blueberries. Levi et al. (1993) described an RAPD protocol from several different woody plants including blueberry, cherry, peach, pear and apple.

Levi and Rowland (1997) used RAPD and SSR-anchored primers for the usefulness for amplifying blueberry DNA. These markers were used to differentiate and evaluate genetic relationship among 15 highbush (*V. corymbosum*) or highbush hybrid cultivars, two rabbiteye (*V. ashei*) cultivars and one southern lowbush (*V. darrowi*) selection from the wild. The *V. ashei* cultivars and *V. darrowi* selection grouped out separately from the *V. corymbosum* cultivars. The study indicated that RAPD and SSR-anchored primers are useful for identifying blueberry cultivars or selection. Burgher et al. (1998) screened 26 wild lowbush (*V. angustifolium*) clones, including six named cultivars and 12 selections. Clustering of genotypes correlated fairly well with geographic origin of clones. Further Bugher et al. (2002) used RAPD analysis with low bush blueberry selections and native accessions that has been collected from various geographic regions in Atlantic Canada and Maine. This analysis successfully distinguished all the clones. Arce-Johnson et al. (2002) reported using two RAPD primers to distinguish five highbush Chilean cultivars. Rowland (2003a) reported development of EST-PCR markers for fingerprinting and genetic relationship studies in blueberry. The polymorphic EST-PCR and CAPS marker developed in this study distinguished all the genotypes indicating that these markers should have general utility for DNA fingerprinting and examination of genetic diversity in blueberry. Further, EST-PCR primers were tested for their ability to amplify fragments in related *Ericaceae*, cranberry and rhododendron (Rowland et al. 2003b).

Microsatellite or SSRs have recently become important genetic markers in plant genome research. The development of SSR in plants is accelerating, and SSR loci are now being incorporated into established genetic maps of the major plant species. SSRs are particularly attractive for distinguishing between cultivars because the level of polymorphism detected at SSRs loci is higher than that detected with any other molecular marker assay. Boches (2005) reported microsatellite markers for *Vaccinium* from EST and genomic libraries. SSR markers were derived from two Expressed Sequence Tags (EST) libraries and from microsatellite enriched genomic library constructed from *V. corymbosum* cultivar Blue crop DNA. Recently, Boches et al. (2006) used 20 EST-SSR and eight genomic microsatellite loci to determine genetic diversity in 69 *Vaccinium corymbosum* L. accessions consisting of 13 wild accessions and 56 cultivars (one half-high, 18 southern highbush and 37 northern highbush).

10.3 Genetic Linkage Mapping

Different types of markers have been used for generating the linkage maps. PCR-based markers are being used extensively for the construction of linkage maps. Within the *Ericaceae* molecular linkage maps have been developed only for blueberry (Rowland and Levi 1994; Qu and Hancock 1997). Initial RAPD based genetic linkage maps have been developed for three diploid and one tetraploid blueberry populations. Rowland and Levi (1994) reported the construction of an initial genetic linkage map for diploid blueberry using a population resulting from a test cross between the F_1 interspecific hybrid US 388 (*V. darrowi* × *V. elliottii*) and another *V. darrowi* clone US 799. The map comprises 70 RAPD markers mapped to 12 linkage groups in agreement with the basic blueberry chromosome number and cover a total genetic distance of over 950 cM, with a range of 3–30 cM between adjacent markers. Qu and Hancock (1995) have used RAPD markers to establish a tetrasomic mode of inheritance in interspecific hybrids of diploid *V. darrowi* and tetraploid *V. corymbosum*.

Vorsa and Rowland (1997) reported RAPD for the estimation of 2*n* megagametophyte heterozygosity in a diploid blueberry *Vaccinium darrowi* Camp. Qu and Hancock (1997) reported construction of an RAPD based genetic linkage map of tetraploid blueberry population that should be segregating for high fruit quality, heat tolerance and cold tolerance. The population resulted from a cross of US75 (a tetraploid hybrid of a diploid *V. darrowi* selection Fla 4B and tetraploid *V. corymbosum* 'Bluecrop') and another *V. corymbosum* 'Bluetta'. A total of 140 RAPD markers unique to Fla 4B that segregated 1:1 in the tetraploid population were mapped into 29 linkage groups. Rowland et al. (1999) constructed RAPD-based genetic linkage map using diploid blueberry populations shown to be segregating for both chilling requirements and cold hardiness. The population resulted from test crosses between F_1 interspecific hybrids, *V. darrowi* × diploid *V. corymbosum*, and another *V. darrowi* clone and another diploid *V. corymbosum* clone. Recently a few EST-PCR markers have been added to these maps; the map of the *V. corymbosum* test cross currently comprises approximately 90 RAPD and EST-PCR markers and the map of the *V. darrowi* test cross comprises approximately 70 RAPD and EST-PCR markers (Rowland et al. 2003c; Rowland and Hammerschlag 2005).

One of the unique contributions of genetic mapping is the possibility of detection of genomic regions controlling quantitative traits. Most of the agronomically and economically important traits are controlled by a relatively large number of loci. Such loci are called as quantitative trait loci (QTL). A preliminary QTL analysis using current genetic linkage map and cold hardiness data for the *V. corymbosum* test cross population have identified one putative QTL associated with cold hardiness that explains ~20% of the genotypic variance (Rowland et al. 2003c). With further saturation, these maps and segregating populations should allow researchers to map genes and QTLs controlling the important traits.

Muthalif and Rowland (1994a, b) studied changes in protein levels associated with low temperature exposure in floral buds of blueberry cultivars with different levels of cold hardiness. Characterization of cold-responsive proteins revealed them to be members of a family of proteins known as dehydrins. Levi et al. (1999) reported isolation of 2.0 kb dehydrin cDNA which encodes the 60 kDa dehydrin. The sequence of 2 kb cDNA was further used to design primers to amplify alleles of two dehydrin-related genes from the cold sensitive and cold tolerant parent plants. Panta et al. (2004) reported mapping of dehydrin related gene to linkage group 12 of the current genetic linkage map of blueberry. Dhanraj et al. (2004, 2005) reported that family of dehydrins of 65, 60 and 14 kDa accumulates in floral buds during winter, and the levels of these proteins correlate with cold tolerance. A cDNA clone from blueberry floral bud RNA that encodes the 14 kDa dehydrin was identified and sequenced.

Genetic mapping provides a direct means of investigating the number of genes influencing a trait, the location of these genes along the chromosomes, and the affects of the variation in doses of these genes. The most successful applications will be in those species with well developed molecular marker maps. A large number of monogenic and polygenic loci for various traits have been identified in a number of plants, which are currently being exploited by breeders and molecular biologists together for marker-assisted selection. Tagging of useful genes like the ones responsible for conferring resistance to plant pathogen, insect, drought tolerance and a variety of other important developmental pathway genes, is a major target. Identification of the marker loci that are linked to the trait of interest is followed by the utilization of linkage association in genetic improvement program. Once linkage between a trait and a marker locus is established, it is possible to use the information in the selection of the breeding lines. The availability of more molecular marker based maps in blueberry would facilitate the localization of genes controlling traits such as fruit quality, fruit size, plant vigor, disease resistance and various environmental tolerances.

10.4 In Vitro Culture and Genetic Engineering

Most blueberry genotypes can be cloned in large numbers with great rapidity from small amount of starting by means of in vitro shoot culture (Nickerson 1978; Cohen and Elliott 1979). Shoot tip propagation of blueberry was initiated by Layrene (1978), who reported successful in vitro propagation of rabbiteye blueberry seedling. Since then, several reviews (Smagula and Lyrene 1984; George et al. 1987; Zimmerman 1980, 1991; Galletta and Ballington 1996) have summarized the in vitro technology for blueberry propagation. The first studies on shoot tip propagation of lowbush blueberry (*V. angustifolium*) were conducted by Frett and Smagula (1983). Further Smagula

and Litten (1989) and Litten et al. (1992) studied mycorrhizial inoculation of lowbush blueberry as an aid to micro-propagation. The earliest studies on shoot tip propagation of highbush blueberry (*V. corymbosum*) date back to 1979-1980 (Cohen and Elliott 1979; Cohen 1980; Zimmerman and Broome 1980).

Wolfe et al. (1983) conducted studies to compare various media and to determine the optimum medium for micropropagating highbush blueberry. Young and Cameron (1985a, b) studied influence of growth regulators, nitrogen form and effect of light on micropropagation of rabitteye blueberries. Grout and read (1986) studies the influence of the stock plant propagation method on propagation and rooting of halfhigh blueberry 'Northblue'. Rooting and establishment of in vitro blueberry plantlets in the presence of mycorrhizal fungi was studied by Lareau (1985). Chandler and Draper (1986) studied the effect of zeatin and 2iP on shoot proliferation of highbush blueberry clones. Grout et al (1986) conducted studies on the influence of stock plant propagation method, tissue culture and leaf-bud propagation of 'Northblue' blueberry. Long-term effects of in vitro propagation of 'Northblue' halfhigh blueberry under greenhouse and field conditions have been reported (El-Shiekh et al. 1996). Noè and Echher (1994) and Noè (1998) studied the influence of irradiance on the in vitro growth of highbush blueberry. Growth vigour and yielding of highbush blueberry from semi-woody cuttings and in vitro was studied by Smolarz and Chiebowska (1997). Isutsa et al. (1994) conducted investigations to identify environmental conditions that would accelerate rooting and acclimatization and improve survival of ex vitro blueberry microcuttings. Gonzalez et al. (2000) initiated studies to develop a uniform method of micropropagation using nodal segment from mature field-grown highbush blueberry plants. Further Cao et al. (2003) investigated the effect of sucrose concentration in the propagation medium on shoot proliferation and on gene delivery into highbush blueberry shoots.

The first success with organogenesis from highbush blueberry (Billings et al. 1988) occurred from leaf explants of in vitro propagated shoots. Rowland and Ogden (1992, 1993) investigated zeatin riboside (ZR) for highbush blueberry regeneration from leaf explant. Hruskoci and Read (1993) studied the in vitro shoot shoot regeneration from internode segment and internode-derived callus in blueberry. Cao and Hammerschlag (2000) reported improved shoot organogenesis from leaf explant of highbush blueberry. Cao et al. (2002) also reported that growth regulator pre-treatments enhance shoot organogenesis from leaf explants of 'bluecrop'.

As a tool in cultivar breeding, in vitro chromosome doubling with colchicines is feasible with blueberry (Lyrene and Perry 1982). Lyrene and Perry (1982) reported that a combination of colchicine facilitates chromosome doubling in blueberry. Different methods have been used to induce tetraploids in *V. darrowi*, *V. elliottii* and *V. darrowi* × *V. elliottii* hybrids (Perry and Lyrene, 1984), 8× plants from 4× *V. corymbosum* clones (Goldy and Lyrene 1984) and 6× plants from triploid (*V. coryombosum* (4×) × *V. elliotii* (2×)) hybrid (Dweikat and Lyrene 1989). The use of 2*n* gametes to obtain elevated polyploids is also possible with blueberry, and is more efficient than colchicine doubling in many situations.

Transformation is a powerful approach to introduce genes of interest and accelerate the breeding process for many fruit crops. Transformation is particularly suited to blueberry since it has a polyploidy genome and is asexually propagated. To date, regeneration has been reported for only a few commercial blueberry cultivars (Billings et al. 1988; Callow et al. 1989; Rowland and Ogden 1992, 1993; Hruskosi and Read 1993). Cao et al. (1998) studied several factors that influenced the efficiency of *Agrobacterium*-mediated transfer of an intron containing β-glucuronidase (GUS) gene into leaf cells of several commercially important blueberry cultivars and into callus derived from these cells during the early stages of transformation. There is one report of transformation but has not been confirmed by Southern analysis (Graham et al. 1996).

Hancock et al. (1990) conducted transformation studies with the highbush, sierra with *Agrobacterium tumefaciens*. They investigated the effect of concentration of *A. tumifaciens*, length of co-cultivation and antibiotic treatments on transformation. Rowland and Ogden (1993) initiated transformation studies with *A. tumifaciens* strain C58C1/pGA482. Graham et al. (1996) reported transformation of half high North country using disarmed *A. tumifaciens* strain LBA4404 containing a binary vector with an intron containing GUS marker gene (Vancanneyt et al. 1990). Cao et al. (1998) conducted an in-depth study on factors that influence the early stages of transformation. They used 10 highbush blueberry cultivars and disarmed *Agrobacterium* strain LBA4404. Recently, Song and Sink (2004) described an efficient shoot regen-

eration method and results of transient transformation studies that led to *A. tumefaciens*-mediated stable transformation of four selected highbush blueberry cultivars.

10.5 Future Scope of Works

The increasing use of biotechnology in blueberry research, in fields such as diverse as linkage mapping, gene cloning, functional genomics, tissue culture and genetic transformation has increased ability to manipulate species for the advantage in breeding programs. There is still much work needed to provide a better understanding of gene regulation and phenotypic expression, generation of high-density genetic maps, and transformation system.

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11 Almond

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11.1 Introduction

The almond [*P. dulcis* (Mill.) D.A. Webb; syn. *P. amygdalus* Batsch] is a species of genus *Prunus* and subgenus *Amygdalus* (Rosaceae, subfamily Prunoideae) that is commercially grown worldwide. The cultivated almond is thought to have originated in the arid mountainous regions of Central Asia (Grasselly 1976a). Several wild species are also found growing in these mountainous areas from Tian Shan mountain in western China through the mountainous areas and deserts of Kurdistan, Turkestan, Afghanistan and into Iran and Iraq (Grasselly 1976b; Kester and Gradziel 1996). The *Prunus* species *P. fenzliana* (Fritsch) Lipsky, *P. bucharica* Korschinsky and *P. kuramica* Korschinsky (of the Section Euamygdalus) from these regions are described as the wild species most closely related to almond (Grasselly 1976b; Browick and Zohary 1996), and may be the ancestral species of the modern cultivated almond (Kester et al. 1991). Ladizinsky (1999), however, identified only *P. fenzliana* as the wild ancestor of almond. *P. webbii* (Spach) Vieh, which is thought to have originated on the Balkan peninsula, is also described as closely related to almond (Grasselly 1976a, b; Browick and Zohary 1996). The evolution and distribution of almonds, both in cultivation and in the associated semi wild state, has been divided into three stages: Asiatic, Mediterranean, and Californian, corresponding to the geographical areas where is grown (Fig. 1) (Grasselly 1976a; Kester et al. 1991; Kester and Gradziel 1996).

The fruit of almond, as with other *Prunus* species, is a drupe where the mature, stony endocarp together with the seed forms a propagation unit comparable to a botanical seed surrounded by its protective testa. The almond is the earliest deciduous fruit and nut tree to bloom in spring due to its low winter chilling requirements and quick growth response to warm temperatures. The almond growth cycle is adapted to a Mediterranean type climate (Kester et al. 1991; Kester and Gradziel 1996). Almond is a predominantly self-incompatible species. This self-incompatibility is gametophytic and it is controlled by a single *locus* with multiple codominant alleles (Socias i Company and Felipe 1988; Dicenta and García 1993a). Since self-compatible almond cultivars were reported in Puglia region in Italy, self-compatibility has become one of the main objectives for almond breeding programs in Europe and the USA (Grasselly et al. 1981; Vargas et al. 1984; Socias i Company and Felipe 1988; Dicenta and García 1993a; Gradziel and Kester 1998). Cultivated almond is among the most polymorphic of all cultivated fruit and nut species (Hauagge et al. 1987a; Byrne 1990; Kester et al. 1991; Socias i Company and Felipe 1992; Bartolozzi et al. 1998; Martínez-Gómez et al. 2003a). Sixteen ($2n = 2x = 16$) small, but distinguishable (Corredor et al. 2004), chromosomes and a small diploid genome of approximately 300 Mbp (Baird et al. 1994) also characterize this species.

Horticulturally, almonds are classified as a nut in which the edible seed (the kernel) is the commercial product. Almond kernels are concentrated energy sources because of their high lipid content. The oil is primarily unsaturated, composes mostly oleic and linoleic fatty acids (García-López et al. 1996). The kernel also contains considerable proteins, minerals, and some vitamins (Kester et al. 1991; Kester and Gradziel 1996). However, native almond species predominantly have bitter kernels because of high levels of the glucoside amygdalin (Grasselly 1976b; Kester et al. 1991).

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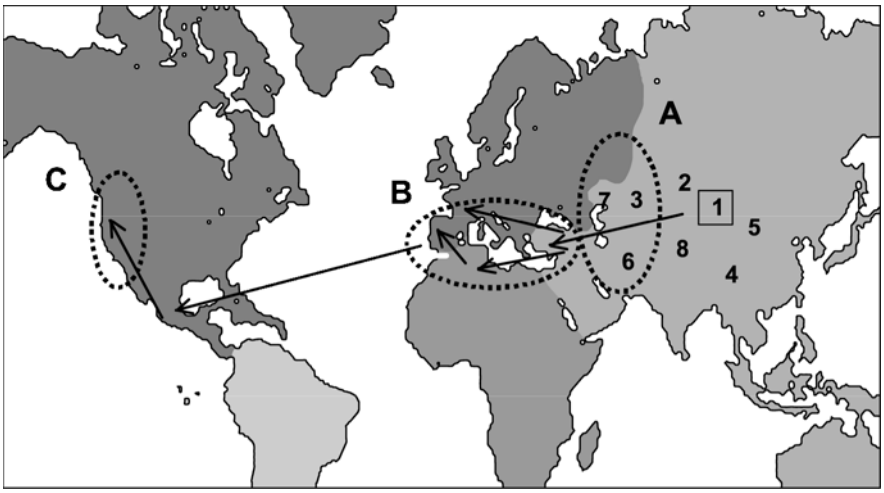

Fig. 1. Map of world showing the origin for almond [*Prunus dulcis* (*1*)] and different relative *Prunus* species [*P. bucharica* (*2*), *P. fenzliana* (*3*), *P. davidiana* (*4*), *P. persica* (*5*), *P. scoparia* (*6*), *P. webbii* (*7*), and *P. argentea* (*8*)], the dissemination routes for the cultivated almond [→], and the three main areas for diversification and cultivation of almonds [Asiatic (*A*), Mediterranean (*B*), and Californian (*C*)]

The principal almond-producing area of the world is the central valley of California with around 50% of the world production. In 2003, worldwide annual almond production exceeded 1679 thousand metric tons, including 741 thousand metric tons in California. The second major almond-producing area includes the European countries bordering the Mediterranean Sea, including Spain (the second leading country after the United States with 197 thousand metric tons in 2003), Italy (91 thousand metric tons) and Greece (40 thousand metric tons). Finally, emergent areas exist in central and southwestern Asia including Syria (139 thousand metric tons in 2003), Iran (109 thousand metric tons) and Turkey (38 thousand metric tons) (Fig. 2) (FAO 2004).

The three basic objectives of almond improvement are to increase yield (self-compatibility, late flowering, flower density, and productivity), to improve quality (maturity date, kernel bitterness), and to decrease production costs (pest and disease resistance, drought resistance) (Socias i Company 1998). The efficiency of breeding programs depends on the information available on the transmission of those traits to be improved. There has been a considerable progress in the study of inheritance of agronomic traits in almond. In this species most of the important agronomical characteristics are quantitative. These quantitatively inherited characters constitute the bulk of the variability selected during the breeding process (Kester and Asay 1975; Grasselly and Crossa-Raynaud 1980; Dicenta et al. 1993a, b; Socias i Company 1998). Late flowering allows the avoidance of the spring frosts in colder areas and has been an objective of early almond breeding programs (Kester 1965; Vargas et al. 1984; Dicenta et al. 1993a; Socias i Company et al. 1999). Genetic studies have demonstrated a positive response to selection for this trait (Kester et al. 1973; Dicenta et al. 1993a). Flowering density and productivity are also two important traits, which have been studied by Kester and Asay (1975), Grasselly and Crossa-Raynaud (1980), Vargas et al. (1984) and Dicenta et al. (1993a). Few studies have been performed regarding the time of maturity (Kester and Asay 1975; Dicenta et al. 1993b). On the other hand, other important agronomic traits in almond seem to be controlled by major genes, including kernel bitterness or self-compatibility. There are many studies regarding the transmission of the kernel traits (see Kester et al. 1977; Vargas et al. 1984; Dicenta et al. 1993b). In addition, kernel bitterness has been characterized as a monogenic trait, the bitter genotype being recessive (Heppner 1923, 1926; Dicenta and García 1993b; Vargas et al. 2001). Finally, self-compatibility was studied by different authors who have determined its monogenic nature with a multi-allelic *S* series, and identified the S_f allele as the responsible for self-compatibility (Socias i Company and Felipe 1988; Dicenta and García 1993a; Ortega and Dicenta 2003). Self-compatibility is expressed within the styles of flowers and results in the successful growth to fertilization of self-pollen tubes (Bošković et al. 1997, 2003).

The absence of extensive crossing barriers among the different *Prunus* species in the initial hybridization and the subsequent backcrosses, demonstrates a direct accessibility of this rich germplasm to almond breeding (Browicz and Zohary 1996; Gradziel et al. 2001a; Martínez-Gómez et al. 2003b). The encouraging performance of interspecific hybrids and back-

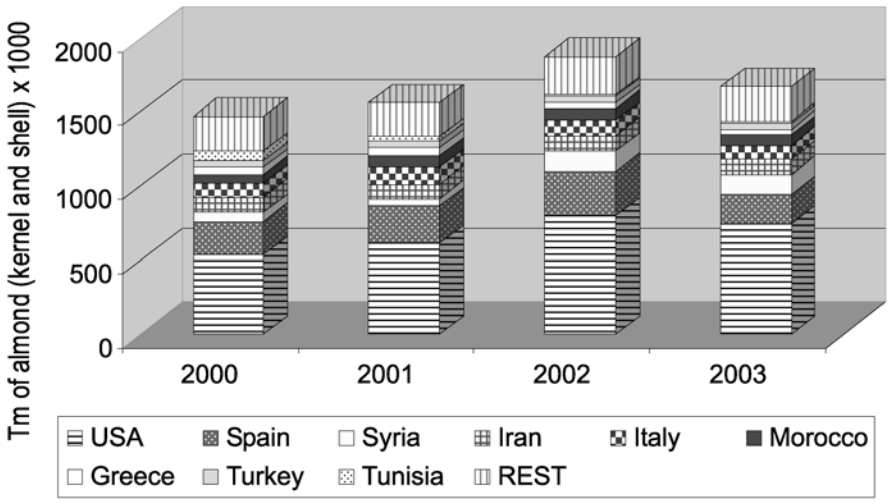

Fig. 2. World almond production according with FAO (2004)

crosses to date, support continuing opportunities for transferring useful traits, including self-compatibility, resistance to important pests and diseases, improvement of seed oil quality, tolerance to aberrant environments, and modified tree architecture and bearing habit (Gradziel et al. 2001a). The direct utilization of these related almond species as a rootstock, mainly under non-irrigated native conditions, has been reported by several authors (Grasselly 1975; Denisov 1988). Interspecific crosses have also been used as peach and plum rootstocks (Kester and Hansen 1966; Felipe 1975). Related species have also been reported as having potential in almond breeding to improve the quality of kernels and as sources of self-compatibility (Kester and Gradziel 1996; Gradziel and Kester 1998; Gradziel et al. 2001a). However, a major impediment to the full utilization of this rich germplasm is the tedious selection process emphasizing the need for accurate molecular markers allowing efficient and rapid selection tools (Martínez-Gómez et al. 2003b, c).

11.2 Variability Analysis with Molecular Markers

Traditionally, the identification and characterization of almond cultivars has been based on morphological traits. However, such traits are not always available for analysis, are affected by changing environmental conditions and may only be visible in adult materials and so requiring a long time for their analysis. Molecular markers have offered a solution to many of these problems allowing a fast, accurate, highly discriminative and environmentally stable test that has been used for variability analysis, pedigree determinations or cultivar identification (Wünsch and Hormaza 2002; Martínez-Gómez et al. 2003b; Sánchez-Pérez et al. 2004a). Moreover, some markers, such as isozymes, restriction fragment length polymorphisms (RFLPs), simple sequence repeats (SSRs) and other markers derived from the knowledge of specific genome sequences; allow the comparison of variability among homologous regions of the same or different species.

Isozymes were the first molecular markers used because of their environmental stability, their codominant expression, and their good reproducibility (Arulsekar et al. 1986; Hauagge et al. 1987a, b; Cerezo et al. 1989; Foolad et al. 1995; Vezvaei et al. 1995; Sathe et al. 2001). Isozyme studies have detected high levels of variability in almond and allowed the individual identification of most genotypes studied. A comparative study of isozyme variability in *Prunus* (Byrne 1990) showed that almond and Japanese plum, both with a strong self-incompatibility system were more variable than apricot and peach that have different degrees of self-compatibility. Nevertheless, their utilization is limited by the small number of loci that can be analyzed with conventional enzyme staining methods, as well as a low variation at most loci. On the other hand, RFLPs are codominant and can detect a virtually unlimited number of markers, thus providing an efficient method for discovering linkages between markers and for constructing genetic maps. RFLPs also proved to be useful for variability analysis and cultivar identification in almond

(Viruel 1995). However, RFLP analysis has important limitations: it is laborious and time-consuming and it often involves the use of radioisotopes. The recent utilization of PCR-based markers has increased the opportunities for mapping and tagging a wide range of traits. RAPDs, based on the PCR amplification of random locations in the genome, typically use arbitrary primers. A single oligonucleotide is utilized for this random amplification of genomic DNA. Unlike RFLPs, RAPDs can be obtained with a simple method, but have some disadvantages when compared to isoenzymes and RFLPs: they are dominant markers and have a variable degree of repeatability which limit their utilization for cultivar identification and map construction. RAPD techniques have been used in almond for the study of germplasm variability (Bartolozzi et al. 1998; Martins et al. 2003).

SSR (or microsatellite) markers, also based on the PCR technique, are currently becoming the markers of choice for genetic fingerprinting studies for a wide range of plants. Because of their high polymorphism, abundance, and codominant inheritance, they are well suited for the assessment of genetic variability within crop species, and of the genetic relationships among closely related species (Gupta et al. 1996; Powell et al. 1996). In the case of *Prunus*, SSR markers covering the almost whole genome have been obtained in different species including peach, apricot, Japanese plum and cherry almost (Cipriani et al. 1999; Downey and Iezzoni 2000; Sosinski et al. 2000; Testolin et al. 2000; Cantini et al. 2001; Aranzana et al. 2002, 2003; Dirlewanger et al. 2002; Georgi et al. 2002; Wang et al. 2002; Yamamoto et al. 2002; Clarke and Tobutt 2003; Decroocq et al. 2003; Schueler et al. 2003; Hagen et al. 2004; Messina et al. 2004; Mnejja et al. 2004). Recently, the first set of almond SSRs has been published (Testolin et al. 2004). They have been successfully used for the molecular characterization and identification of almond cultivars (Martínez-Gómez et al. 2003a; Testolin et al. 2004) and related *Prunus* species (Martínez-Gómez et al. 2003c). Electrophoresis in polyacrilamide gels with radioactive and silver staining was the first method used in the analysis of the PCR amplified fragments of DNA obtained from the SSR markers. Electrophoresis in Metaphor® agarose gels was an alternative method to polyacrilamide gels due to its easier application (Morgante et al. 2003). More recently, new methods for PCR amplified DNA have been developed including the utilization of automated sequencers. While the use of Metaphor® agarose gels appears less useful for genotype characterization that the other two methods, this method may be the most convenient in mapping of populations involving alleles separated more than 5 bp due to its lower cost and easier routine application (R. Sánchez-Pérez et al. 2006) (Fig. 3). The comparative analysis of the variability of five *Prunus* species with 125 SSRs has detected that the most polymorphic species was almond, followed by Japanese plum, apricot, cherry and peach (M. Mnejja and P. Arús, unpublished results). This is the same order of variability that was found with isozymes by Byrne (1990), which confirms almond as a species with a very high level of polymorphism.

11.3 Construction of Genetic Linkage Maps

Linkage analysis was first performed in almond using isozyme genes (Arús et al. 1994a; Vezvaei et al. 1995), but the low number of isozymes that can be analyzed with conventional enzyme staining methods in a given population precluded the use of these markers for the construction of genetic maps. The development of RFLPs at the beginning of the 1980s provided a virtually unlimited source of high quality markers located all over the genome, making map construction with markers a feasible endeavor for most animal and plant species. The first map for almond was constructed by Viruel et al. (1995) based almost entirely on these markers (120 RFLPs and 7 isoenzymes) for the F_1 progeny between 'Ferragnès' and 'Tuono' (the F×T map). This map detected the eight expected linkage groups and spanned approximately 400 cM. Another map constructed by Foolad et al (1995) with an F_2 population of the interspecific cross between a peach selection (54P455) and the almond cultivar 'Padre' (P×5), had a similar marker composition (101 RFLPs and 6 isozymes). This map was longer than that of Viruel et al. (1995), with a total length of about 800 cM.

As a result of a European project (see Arús et al. 1994b), a saturated linkage map of for *Prunus* was obtained in an almond (cv. 'Texas', syn. 'Mission') × peach (cv. 'Earlygold') F_2 progeny (Joobeur et al. 1998) including 246 markers (235 RFLPs and 11 isozymes). All markers studied mapped in the eight linkage groups found, with a total distance of 491 cM. Given that this map (the T×E map), considered as the

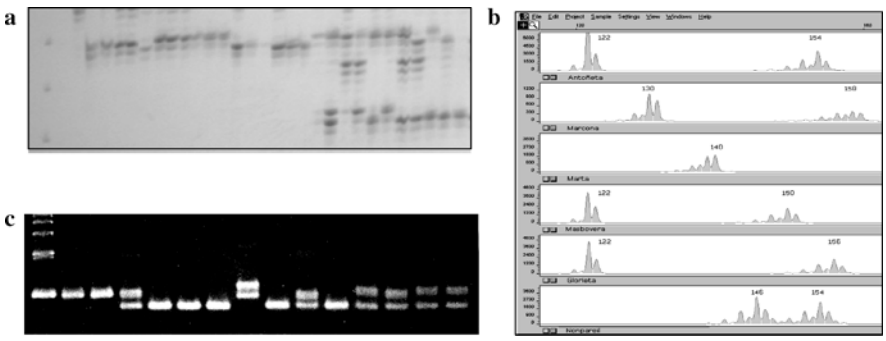

Fig. 3. Analysis of DNA polymorphisms of SSR markers in several almond cultivars using polyacrylamide electrophoresis gels (*a*), Metaphor® agarose electrophoresis gels (*b*) and automated capillary sequencers (*c*)

Prunus reference map, had many markers in common (67) with the 'Ferragnès' × 'Tuono' (F×T) map, it was possible to compare them, having the same distribution of makers among linkage groups and a complete colinearity of markers within each linkage group. For that reason the terminology for linkage groups coined for the F×T almond map was adopted for the T×E reference *Prunus* map. The T×E map has been progressively improved (Aranzana et al. 2003) with the addition of more markers of good quality, such as additional RFLPs and simple-sequence repeats (SSRs). The current version (Dirlewanger et al. 2004a) includes 562 markers (361 RFLPs, 185 SSRs, 11 isozymes and 5 STSs), which cover a total distance of 519 cM with high density (average density 0.92 cM/marker and largest gap of 7 cM).

The development of markers that could be obtained with simpler methods than RFLPs, such as RAPDs and SSRs (particularly given the latter's high quality) fostered the improvement of other maps, and more saturated versions were produced such as the F×T map (Joobeur et al. 2000) with 174 markers and the P×5 ['Padre' (almond) × 54P455 (peach)] map (Bliss et al. 2002) with 161 markers including six morphological genes and eight resistance-gene analog sequences.

Two more maps were constructed after T×E and used the information of this map to elaborate framework maps with a low number of markers selected from it that covered the whole genome at distances of 10–25 cM. The first of these maps was obtained in the F_1 progeny of the cross between two almond cultivars 'Felisia' (syn. D-3-5) and 'Bertina' which allowed a study of the map position of genes involved in self-incompatibility (Ballester et al. 1998, 2001), shell hardness (Arús et al. 1999) and blooming time (Ballester et al. 2001). The second map was based on an F_2 progeny between 'Garfi' almond and 'Nemared' peach (Jáuregui et al. 2001), which located genes involved in nematode resistance, and flower color (Jáuregui 1998).

The similar order of molecular markers observed in different *Prunus* maps when compared to the *Prunus* reference map, suggests a high level of synteny within the genus (Aranzana et al. 2003; Dirlewanger et al. 2004a, b; Lambert et al. 2004). This homology among the genomes of *Prunus* species is in agreement with the low level of breeding barriers to interspecific gene introgression and supports the opportunity for successful gene transfer between closely related species (Gradziel et al. 2001a; Martínez-Gómez et al. 2003b). In addition, the synteny among *Prunus* genomes offers important opportunities to transfer and compare genetic information from linkage maps generated in different species of this genus.

The *Prunus* reference map has been compared with the *Arabidopsis* sequence, finding 23 syntenic blocks between them, which covered 23% of the *Prunus* map distance and 16% of the *Arabidopsis* genome (Dominguez et al. 2003). Microsynteny studies have found also a fractional conservation between these two distant taxons (Georgi et al. 2003) and indicate that the sequence of *Arabidopsis* can be employed to a limited extent for gene or marker search in *Prunus*.

11.4 Major Gene and QTL Mapping, and Gene Cloning

The usual approach for the analysis of marker-trait association is the use of mapping populations segregating for the agronomic characters of interest. The analysis of cosegregation among markers and characters allows establishment of the map position of major genes and QTLs responsible for their expres-

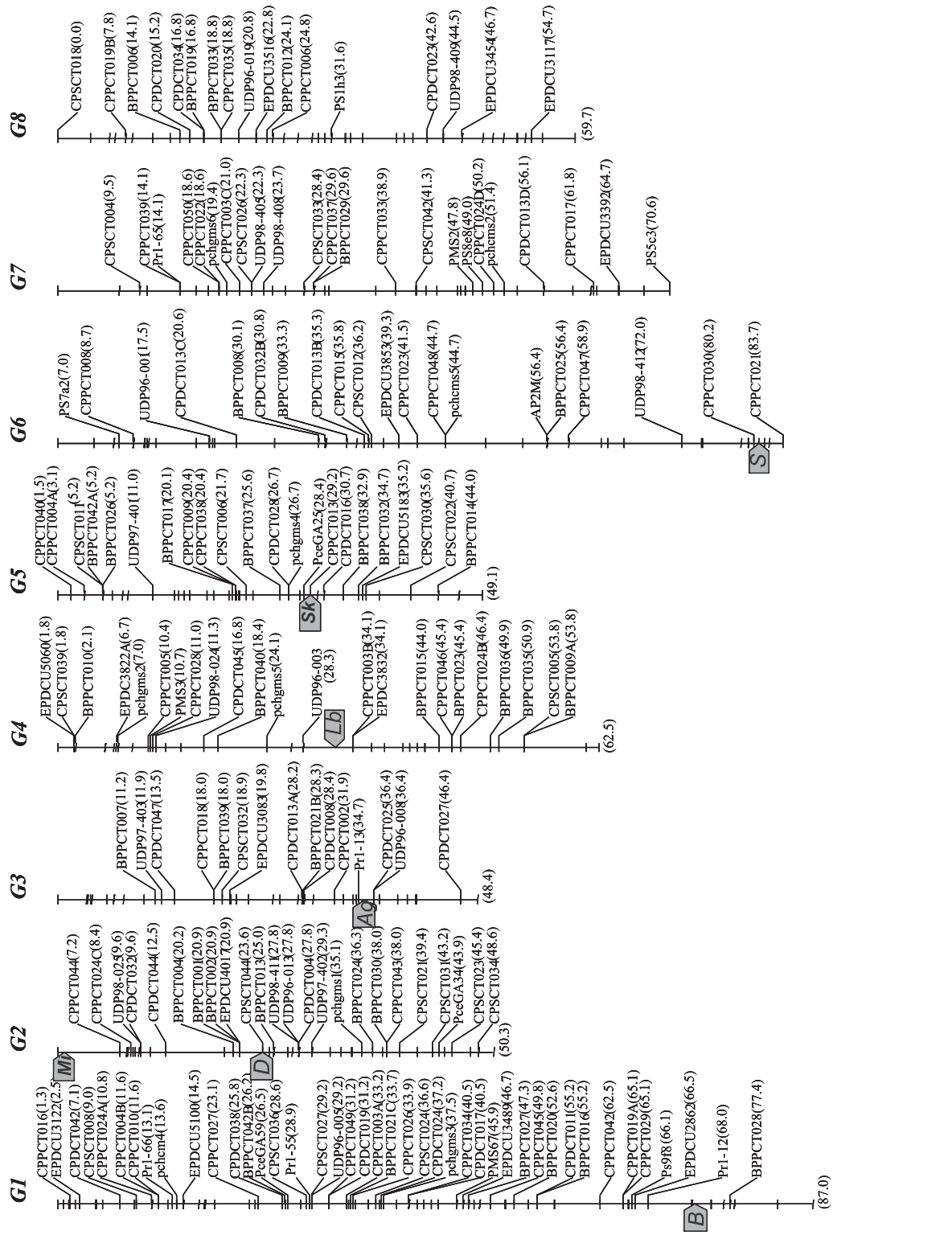

Fig. 4. Map of the 'Texas' (almond) × 'Earlygold' (peach) F2 population obtained only with the SSR markers of the map of Dirlewanger et al. (2004a) and with the approximate location of flower color (*B*), nematode resistance (*Mi*), shell hardness (*D*), anther color (*Ag*), blooming time (*Lb*), kernel taste (*Sk*), and self-incompatibility (*S*) genes

Table 1. Markers associated to main agronomic traits in almond

Trait	Symbol	Linkage group	Populations	Marker	Reference
Flower color	*B*	G1	'Garfi' (almond) × 'Nemared' (peach)	RFLP	Jáuregui 1998
Shell hardness	*D*	G2	'Ferragnés' (almond) × 'Tuono' (almond)	RFLP	Arús et al. 1999
Nematode resistance	*Mi*	G2	'Garfi' (almond) × 'Nemared' (peach)	RFLP	Jáuregui 1998
Nematode resistance	*Mi*	G2	'Padre' (almond) × '54P455' (peach)	RFLP	Bliss et al. 2002
Anther color	*Ag*	G3	'Texas' (almond) × 'Earlygold' (peach)	RFLP	Joobeur 1998
Blooming time	*Lb*	G4	'D.3.5' (almond) × 'Bertina' (almond)	RAPD	Ballester et al. 2001
Kernel taste	*Sk*	G5	'Padre' (almond) × '54P455' (peach)	RFLP	Bliss et al. 2002
Kernel taste	*Sk*	G5	'Texas' (almond) × 'Earlygold' (peach)	RFLP	Joobeur 1998
Self-incompatibility	*S*	G6	'D.3.5' (almond) × 'Bertina' (almond)	RAPD	Ballester et al. 2001
Self-compatibility	*S*	G6	'Ferragnés' (almond) × 'Tuono' (almond)	RFLP	Ballester et al. 1998
Self-compatibility	*S*	G6	'Ferragnés' (almond) × 'Tuono' (almond)	RFLP	Arús et al. 1999
Self-compatibility	*S*	G6	'Padre' (almond) × '54P455' (peach)	RFLP	Bliss et al. 2002

sion (Arús and Moreno-González 1993). Some of the linkage maps developed in almond include markers associated with several traits of horticultural value. With the previously reported high level of synteny between the genome of *Prunus* crops, and the existence of a reference map, a considerable number of genes studied in different populations of almond have been integrated in a single map. The approximate position of these genes is providing in Fig. 4 and their description in Table 1. The important characters and QTLs that are presently being mapped in almond include flower color (*B*) in the linkage group 1 (G1) (Jáuregui 1998), nematode resistance (*Mi*) (G2) (Jáuregui 1998; Bliss et al. 2002), shell hardness (*D*) (G2) (Arús et al. 1999), anther color (*Ag*) (G3) (Joobeur 1998), blooming time (*Lb*) (G4) (Ballester et al. 2001), kernel taste (*Sk*) (G5) (Joobeur 1998; Bliss et al. 2002), and self-incompatibility (*S*) (G6) (Ballester et al. 1998, 2001; Arús et al. 1999; Bliss et al. 2002). Although in some cases the location of these genes has been established in low-density maps, their position can be further defined by using the information provided by the network of maps available for *Prunus* (Dirlewanger et al. 2004a). *Prunus* genome synteny should also facilitate the successful transfer of sets of markers and coding sequence among species (Aranzana et al. 2003; Decrocq et al. 2003; Dirlewanger et al. 2004a, b). Candidate gene approaches have also proven to be useful for finding associations between genes involved in relevant metabolic pathways and the major genes or QTLs as have been reported in peach (Etienne et al. 2002).

Bulked segregant analysis (BSA), where two pooled DNA samples are formed from plant sources that have similar genetic backgrounds but differ in one particular trait, is another powerful approach for the analysis of molecular marker-horticultural trait association. A strategy combining different markers with bulked segregant analysis was used to identify markers linked to loci of specific fruit characters in peach × almond crosses (Warburton et al. 1996). In addition, Ballester et al. (2001) using this methodology identified three RAPD markers associated with self-incompatibility and a gene conferring delayed blooming in almond.

Although gene cloning studies in almond are very scarce, the first gene sequence reported in *Prunus* was that of extensin obtained from almond developing seeds (García-Mas et al. 1992), to which followed some other genes abundantly expressed during seed development (García-Mas et al. 1995, 1996). The genes involved in the self-incompatibility trait have also been characterized. Ushijima et al. (1998) cloned the cDNAs encoding *S*-RNases from almond after studying the primary structure and the sequence diversity of the *S*-RNases in other related *Rosaceae* species. These studies have been completed by other research groups studying other *S* alleles (Channuntapipat et al. 2001; Ma and Oliveira 2001; Certal et al. 2002). In addition, Ushijima et al. (2001) cloned and characterized the cDNAs encoding *S*-RNases in an almond cultivar 'Jeffries' which is a somaclonal mutant of 'Nonpareil' (*Sc* and *Sd* self-incompatibility alleles) and has a dysfunctional *S* allele haplotype

both in pistil and pollen. Results indicated that at least two mutations had occurred to generate this mutant, the deletion of the *Sc* allele haplotype and the duplication of the *Sd* allele haplotype. On the other hand, Suelves and Puigdomenech (1998) identified and sequenced a gene highly expressed in the floral organs of almond and coding for the cyanogenic enzyme (R)-(+)-mandelonitrile lyase. However, the study of the mRNA levels during seed maturation and floral development in fruit and floral samples indicated a lack of correlation between these characteristics and levels of mandelonitrile lyase mRNA and the level of kernel bitterness of almond cultivars classified as homozygous or heterozygous for the sweet trait or homozygous for the bitter trait. In addition, Vezvaei et al. (2004) developed a strategy for the discovery the glucosyltransferase gene responsible for producing bitter kernel in almond using degenerate primers based on consensus regions of glucosyl-transferase genes for other plants.

11.5 Marker-Assisted Breeding

Developing new cultivars is a long and tedious process in almond, involving the generation of large population of seedlings from which the best genotypes are selected. Whereas the capacity of breeders to generate big populations from crosses is less limited, the management, study and selection of these seedlings remain the main limiting factors in the generation of new releases (Kester et al. 1991; Kester and Gradziel 1996; Socias i Company 1998). Marker-assisted selection (MAS) is emerging as a very promising strategy for increasing selection gains (Arús and Moreno-González 1993; Luby and Shaw 2001). Knowledge provided by advances in molecular genetics promise faster and more efficient approaches to cultivar improvement. Early selection utilizing molecular markers allows accurate screening of seedlings several years before the mature plant traits can be evaluated in the field, makes possible the accumulation of different genes/QTLs for horticultural traits of interest, and shortens the number of generations to recover the desired genotype particularly after a cross with an exotic genotype or wild species (Arús and Moreno-González 1993; Baird et al. 1996; Dirlewanger et al. 2004a). Selection by molecular markers is particularly useful in fruit, nut, and other tree crops with a long juvenile period, when the expression of the gene is recessive or the evaluation of the character is difficult, as with resistance to biotic or abiotic stresses (Luby and Shaw 2001; Scorza 2001; Testolin 2003). If sufficient mapping information is known, MAS can dramatically shorten the number of generations required to "eliminate" the undesired genes of the donor in backcrossing programs. Selection of marker loci linked to major genes can be sometimes more efficient than direct selection for the target gene (Arús and Moreno-González 1993; Baird et al. 1996).

A very promising application of MAS is the manipulation of self-incompatibility in almond. Almond self-incompatibility alleles (*S*-alleles) were initially identified in the field through controlled crosses with a series of known *S*-genotypes (Kester and Gradziel 1996; Certal et al. 2002). Molecular methods have been developed in two areas: identification of stylar *S*-RNases by electrophoresis in vertical polyacrilamide gels (Bošković et al. 1997, 2003), and the amplification of specific *S*-alleles using appropriately designed primers for PCR and electrophoresis in horizontal agarose gels (Tamura et al. 2000; Channuntapipat et al. 2003; López et al. 2004). This latter technique is being routinely used for the identification of cross-incompatibility groupings for current almond cultivars and for efficiently breeding self-compatibility into new cultivars (Gradziel et al. 2001b; Ortega and Dicenta 2003) allowing earlier and more accurate selection of the most common self-incompatibility or self-compatibility alleles. More recently, a multiplex-PCR strategy has been developed for the unequivocal identification of self-incompatibility and self-compatibility alleles. This multiplex PCR opens the possibility to identify new *S*-alleles using different sets of primers (Sánchez-Pérez et al. 2004b) (Fig. 5).

In a recent study to determine the genetic basis of mechanisms involved in almond drought tolerance, several genes that were strongly expressed in response to dehydration of almond have been identified. A differential expression technique based on cDNA-AFLP (amplified fragment length polymorphism derived technique for RNA fingerprinting) has been used to identify transcripts that accumulated in mature embryos and in in-vitro-cultured plantlets subjected to desiccation or abscisic acid treatment. This study showed that the levels of expression of the identified genes in leaves of young trees of eight almond cultivars differing in drought

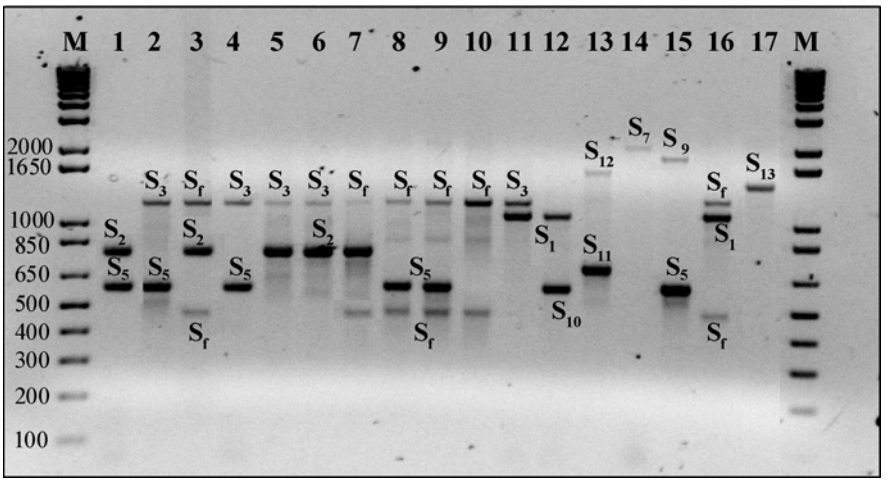

Fig. 5. Agarose 1.5% gel showing amplified *S*-alleles (self-compatibility and self-incompatibility) in 17 almond cultivars and breeding lines using PCR specific primers AS1II (Tamura et al. 2000), CEBASf (Sánchez-Pérez et al. 2004b) and AmyC5R (Tamura et al. 2000)

tolerance provided valuable information for breeding drought resistance in almond (Campalans et al. 2001).

11.6 Advanced Works and Future Scope

Apart from the molecular markers described (isoenzymes, RFLPs, RAPDs, and SSRs), other markers being used in the development of marker associated traits in almond and other *Prunus*, are those based on single point mutations and those obtained from either cDNA sequences (expressed sequences tags, ESTs) or databases (cloned gene analogs, CGAs) (Van Nocker et al. 2002; Testolin 2003, Jung et al. 2004). The large-scale single-pass sequencing of ESTs can give a more global picture of the genes involved in the development and function of organs and tissues. A recent collection of ESTs from peach and almond based on cDNA libraries has been released to public databases, and more than 3,800 putative unigenes have been detected (http://www.mainlab.clemson.edu/gdr/) (Main et al. 2004). This work is complementary to the other works regarding EST development in *Prunus* performed by different research groups in other European countries (Grimplet et al. 2004; Pozzi et al. 2004). Lazzari et al. (2004) also presented a collection of 6,817 ESTs prepared from four cDNA libraries obtained from mesocarps of peach as part of the work of the Italian National Consortium for Peach Genomics (http://www.itb.cnr.it/ESTree). In almond, a study of expressed transcripts during pistil development has selected and partially sequenced over 1,000 clones from a cDNA library. Analysis of these ESTs using the National Center for Biotechnology Information (NCBI) databases indicated significant similarity to protein coding sequences in the database. The EST analysis has provided a preliminary picture of the numerous almond genes potentially involved in pistil development and provides an extensive reservoir for future gene cloning and genetic mapping in almond (Jiang and Ma 2003). As part of a worldwide collaboration effort to increase and enrich the genomics resources in different *Prunus* species, the fabrication of different *Prunus* microarray using unigene sets as probes is being initiated. A group of nearly 4,600 unique ESTs derived from peach mesocarp and developing almond seeds have been sequenced to analyze the expression profile of the unigene set during fruit development and the identification of additional genes involved in this process (McCord et al. 2004). The development of microarrays has also been described in peach for the study of fruit quality by Trainotti et al. (2003, 2004) including the development of markers associated to these important horticultural characteristics.

A recent strategy for the location of new markers in an established genetic linkage map is the "selective" or "bin" mapping approach. This technique allows mapping with the use of a subset of plants of a population from which a map is already available (Vision et al. 2000). The plants of this subset are selected to maximize the information on linkage, so that their joint genotype for any marker identifies a small as possible unique genome fragment (a bin). The advantage of this strategy is that it allows mapping with less time and cost and is adequate for simplifying the construction of high-density maps or for the addi-

tion of large numbers of markers (such as SSRs or EST-derived markers) to a previous map. Recently, Howad et al. (2005) have incorporated 151 SSRs to the *Prunus* reference map using only six individuals from the T×E ('Texas' × 'Earlygold') *Prunus* reference population. The use of this set of six individuals, promises to be a useful resource for *Prunus* geneticists in the future.

Twin seeds (multiple embryos within the same seed coat) occur spontaneously in several almond cultivars including the Californian 'Nonpareil' and 'Mission' (Kester and Gradziel 1996). Seedlings from the same twin peach seed are frequently viable and show similar growth habits, though occasionally one of the seedlings show weak growth and develops poorly. Some of these low-vigor plants have been shown to be haploids from which true-breeding dihaploids can be generated (Gulcan 1975) for genetic studies, hybrid rootstock production, and transformation and regeneration studies. In addition, some of the low-vigor twin almond seedlings were found to be aneuploids (Martínez-Gómez and Gradziel 2003) and thus, have value for developing near isogenic lines (NIL). A collection of these haploid/aneuploid NILs has been presented as an interesting germplasm to aid in genetic (locating genes, selective transfer of particular chromosomes) and molecular (isolation and sequencing of genes, genetic transformation, etc.) studies for the development of new strategies of markers linkage to agronomic traits in almond (Sánchez-Pérez et al. 2004c).

In conclusion, the typical long generation time, along with the extensive space requirements and other limitations to generating the required large segregating almond progeny populations, have frustrated the development and testing of new almond cultivars. These same limitations, however, make molecular-based strategies that improve breeding efficiency particularly valuable to tree crops. Because they are vegetatively propagated, most *Prunus* tree crops such as almond have a unique advantage over other agronomic crops since desirable, unique gene/genomic combinations can be 'captured' and disseminated by clonal propagation. Future research needs include the comparative mapping between the most important genera of fruit crops and the numerous wild species. Almond species include a large number of intercompatible species which provide an enormous gene pool available for breeding. Little use has been made of this variability because the slowness of classical breeding methods. However, genomic methodologies, including the development of quick gene sequencing and cloning tools, may make it possible to rapidly discover and incorporate genes of interest from this exotic material. Additional advantages encouraging the utilization of new technologies to almond tree crop improvement include a small genome size, high levels of synteny between genomes, and a well-established international network of cooperation among researchers.

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12 Pistachio

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12.1 Introduction

12.1.1 History of the Crop

Pistachio (*Pistacia vera* L.), a deciduous, dioecious, and wind-pollinated tree species, is a member of the family Anacardiaceae. This family includes other known species such as cashew (*Anacardium occidentale* L.), mango (*Mangifera indica* L.), ambarella (*Spondias dulcis* Forst.), purple mombin (*Spondias purpurea* L.), poison ivy and poison oak (*Toxicodendron* spp.), pepper tree (*Schinus* spp.), or sumac (*Rhus* spp.). Although pistachio is widely cultivated in Mediterranean countries, its probable origin is central and southwestern Asia. The most complete surveys of the current range of wild pistachio trees were made by Whitehouse (1957), who traveled to southwestern Asia in the late 1920s, and by Zohary (1952, 1973). These two authors note that *P. vera* grows wild in the low mountains and foothills of the semi-desert region of south-central Asia. The range extends from northeast Iran and northern Afghanistan to western Tien-Shan and the Karatau mountains through Turkmenistan, Uzbekistan, Tajikistan, Kazakhstan and Kyrgyzstan (Kayimov et al. 2001). East of the Karatau range, in the central Tien-Shan range, wild pistachio trees exist in only a few, separate, small areas, and the species also grows in Baluchistan (western Pakistan) (Anwar and Rabbani 2001). Historical records, however, tell of pistachio trees growing in places where none exists today, and the present distribution has been influenced by exploitation of pistachio trees by local human populations, who used them as a source of fuel and heavy pasturing of cattle, preventing natural renewal (Whitehouse 1957).

The presence of pistachio nuts in archeological excavations provides evidence that pistachio has long been associated with human activities, although these reports do not always indicate if the nuts found are from *P. vera* or from closely related *Pistacia* species such as *P. palaestina* or *P. atlantica* (Hormaza et al. 1994a). Pistachio cultivation is very ancient and probably started in areas close to wild pistachio stands, likely from seedlings obtained from the best wild trees (Whitehouse 1957). Remnants of true pistachio nuts dated from the sixth millennium BC have been found, east of the Zagros mountains, in Shortughai, Afghanistan (Wilcox 1991) and in Yahya in the Soghum valley of southeastern Iran (Prickett 1986), two places that were situated close to wild pistachio stands. From its presumed center of origin, pistachio cultivation was extended within the ancient Persian Empire from where it gradually expanded westward. In fact, according to Joret (1976), the name pistachio seems to derive from the word pista-pistak in the ancient Persian language Avestan. In Assyria, about the tenth century BC, the Queen of Sheba monopolized the limited crop of nuts for her exclusive use and that of her guests (Whitehouse 1957). Pistachio trees were also planted in the gardens of the king Merodach-Baladan of Babylon around the eighth century BC (Brothwell and Brothwell 1969). Pistachio nuts, called botnim in Hebrew, are mentioned in the Bible (Genesis 43:11) as precious gifts carried from Canaan to Egypt by the sons of Jacob (Zohary 1982). In the second century BC, Nicander found pistachios in Susa, a village in southwestern Iran close to the current border with Iraq (Joret 1976). In the first century BC, Poseidonius finds cultivated pistachios in Syria which misled Greek and Roman writers to consider Syria as the site of origin for pistachio (Joret 1976), a misconception that persisted until recent times (Zohary 1973). Pliny wrote in his *Natural History* that pistachio was introduced into Italy from Syria by the Roman consul in Syria, Lucio Vitello, at the end of the reign of the emperor Tiberius early in the first century AD (Bonifacio 1942). From Italy it was introduced into Spain by Flavius Pompeius, and, probably at that

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time, to other Mediterranean regions of Southern Europe, North Africa, and the Middle East (Lemaistre 1959). Pistachio cultivation was also extended eastward from its center of origin and it was reported in China around the tenth century AD (Lemaistre 1959). More recently, its culture has begun in Australia and in California.

12.1.2 Botanical Description

Pistachio (*Pistacia vera* L.) is a diploid ($2n = 30$) (Zohary 1952; Ila et al. 2003) member of the Anacardiaceae. This virtually cosmopolitan family in the Sapindales/Rutales (Wannan and Quinn 1991) comprises about 70 genera and over 600 species (Mitchell and Mori 1987). The most widely accepted classification divides the family into five tribes: Anacardieae, Rhoeae, Semecarpeae, Spondiadeae, and Dobineae (Mitchell and Mori 1987; Wannan and Quinn 1991), with *Pistacia* belonging to the tribe Rhoeae. Zohary (1952) considers the genus to comprise 11 species divided into four sections (*Lentiscella*, *Eu Lentiscus*, *Butmela* and *Eu Terebinthus*, where *P. vera* is included), although some authors recognize as many as 15 species (Whitehouse 1957). Except the North American species *P. texana* and *P. mexicana*, *Pistacia* species are distributed mainly within the Mediterranean region, Western and Central Asia and the Middle East.

The pistachio is a small to medium sized, bushy, deciduous tree which grows slowly to a height of about 6–9 meters with a single or several trunks. Leaves are compound-pinnate, hairy when young and glabrous when old, with three to five oval leaflets. Pistachio is dioecious and both the staminate and pistillate inflorescences are panicles formed in the axils of the previous year's growth, consisting of up to several hundred individual flowers (Crane and Iwakiri 1981). Both types of flowers are apetalous and wind is the pollinating agent. *P. vera* shows perfect dioecy since mature pistillate flowers have no trace of stamens and mature staminate flowers lack any evidence of female structures (Wannan and Quinn 1991). However, stamen and carpel primordia are initiated in both male and female flowers, but the development of organs of the opposite sex becomes arrested at the primordial stage (Hormaza and Polito 1996). Mature pistillate flowers consist of two to five tepals and a pistil with three stigmas. The commercial pistachios are known as nuts, but the pistachio fruit is indeed a drupe with a fleshy exocarp and mesocarp (hull) and a hard, bony, dehiscent endocarp (shell) that splits longitudinally along its suture beginning at the apex when the fruit has ripened. The pistachio nuts of commerce comprise the endocarp (shell) and the edible kernel, which has a papery seed coat, which color ranges from yellowish to green. Although the fleshy hull loosens at maturity, it has to be removed from the nut in processing either by hand or mechanically.

12.1.3 Economic Importance

Although some species other than *P. vera* produce seeds that are eaten or used for oil and soap production by local populations in their native ranges (Hepper 1992), *P. vera* is the only commercially important species in the genus *Pistacia* (Whitehouse 1957). Among the nut tree crops, pistachio tree ranks sixth in world production behind almond, walnut, cashew, hazelnut and chestnut (Mehlenbacher 2003). Total pistachio world production has reached about 650,000 tons (2002–2004 average) and the main world producer is Iran with more than 300,000 tons followed by Turkey, USA and Syria (Faostat 2004). Pistachio nuts are an excellent source of vegetable protein, with a high arginine and unsaturated fat content, mainly monounsaturated, as well as phytosterols. Pistachios also contain appreciable levels of copper, magnesium, phosphorus and calcium as well as many vitamins such as vitamin E and folic acid (Favier et al. 1995).

Most pistachio cultivation is based on clonally propagated scion cultivars grafted onto seedling rootstocks of the same species, or of other *Pistacia* species or hybrids. Different rootstocks are used in the different growing areas, thus, *P. atlantica*, *P. integerrima* and hybrids *P. atlantica* × *P. integerrima* are the main rootstocks in California, *P. vera* seedlings are used in Turkey, while *P. mutica*, *P. khinjuk* as well as seedlings of *P. vera* are the main rootstocks used in Iran. In areas where wild forms exist, wild *P. vera* individuals are occasionally grafted in situ with selected genotypes (Zohary and Hopf 1988). The few cultivars of *P. vera* described, probably less than 100 worldwide, are thought to be derived from only a few primitive varieties (Maggs 1973) resulting in a high degree of genetic vulnerability. These cultivars are relatively few generations removed from their wild ancestors and usually only a few cultivars are grown in a given pistachio producing area. The main cultivars grown in Iran are Ohady, Kaleh ghochi, Ahmad

Aghai, Badami Zarand, Rezaii and Pust piazi (Esmailpour 2001); in Turkey, Uzun, Kirmizi, Halebi, Siirt, Beyazben, Sultani, Değirmi and Keten Gömleği (Ak and Açar 2001); and in Syria, Ashoury, Red Oleimy and White Batoury (Hadj-Hassan 2001). The California pistachio industry relies almost exclusively on two seedling selections: 'Kerman', a nut-producing female cultivar, and 'Peters', a male cultivar used as a pollinizer.

The pistachio is a xerophitic tree that requires long and hot summers and moderately low winter temperatures. These conditions are met in Iran, Turkey and California but some pistachio producing countries from the Mediterranean basin like Syria and Tunisia usually require varieties with lower chilling requirements. Similarly, in regions with spring frosts or high summer humidity and rainfall, the pistachio trees may suffer during blooming and can be affected by diseases not found in dry areas (Crane 1984). Pistachios are adapted to a variety of soils, but commercial production is best on well-drained, deep, light, sandy loams with high lime content. Although pistachio can thrive in arid conditions, yields in non-irrigated conditions are much lower than in irrigated conditions such as California. Several diseases and pests can affect pistachios (Holtz 2002) although their incidence is variable depending on the cultivar and environmental conditions.

12.1.4 Breeding Objectives

The main tasks carried out in the different existing pistachio collections include characterization and evaluation trials (Caruso et al. 1998). Only recently, a few breeding programs have started in different places, like California (Parfitt et al. 1995; Chao et al. 1998, 2003), Spain (Vargas et al. 1987, 1993, 2002; Batlle et al. 2001) and Turkey (Atli and Kaska 2002). As in other tree-crop species, traditional breeding progress has been slow and many of the selections are still in experimental collections.

Selection and breeding in male pistachio trees is directed towards obtaining pollinators that produce a large amount of viable pollen, with an overlap in blooming with the female cultivar of interest (Martinez-Palle and Herrero 1994). Most of the breeding effort has been focused on the fruiting related problems of female pistachios. The main traits of interest in current pistachio breeding programs include:

- Increasing the percentage of split shells. Split shells make pistachio nuts more attractive to consumers since the kernels can be extracted with the fingers avoiding cracking. The percentage of split shells depends on the cultivar and on environmental conditions (Crane 1984).
- Reduction in the number of blank or unfilled nuts. Blanks are fruits without kernels resulting mainly from embryo abortion and subsequent fruit development (Crane 1973). The extent to which blanks occur varies upon cultivar and rootstock and year to year (Crane 1984). It has been observed that, in blank nuts, the shell does not split, indicating the involvement of the seed in shell dehiscence.
- Increased and regular yields trying to diminish alternate bearing. Alternate bearing is a common problem in most pistachio cultivars, resulting in a heavy crop one year followed by little or none the following year. This problem is observed in other fruit crops and, in pistachio, it seems to be the result of the premature abscission of inflorescence buds during a heavy crop year (Crane and Nelson 1971) although the physiological causes are still not clear (Roussos et al. 2003).
- Vegetative propagation. Pistachio rootstocks are produced from seed. The genetic variation found in seedling rootstocks results in a great variability in the performance of the grafted cultivar affecting diverse traits such as fungal resistance, shell splitting, blank nut production or yield. Consequently, a greater uniformity is desirable and it could be obtained by using vegetative propagated rootstocks (Crane 1984). Important advances have taken place in micropropagation (Parfitt and Almehdi 1994) and micrografting (Onay et al. 2004a). Recently, clonal propagation from leaf cuttings of the rootstock UCB-1 (hybrid between *P. atlantica* and *P. integerrima*) widely used in California has been also reported (Almehdi et al. 2002).
- Increase in nut size, quality and appearance. As in other crops, the improvement of quality traits are becoming important breeding objectives towards a greater acceptance of the product by the consumer and to reduce the number of undesirable fruits.
- Later flowering. Late flowering in regions with late frosts is a desirable breeding and selection trait to avoid flower damage that would affect production and to avoid spring fungal infections.
- Resistance to both aerial and soil fungal diseases. Several fungal diseases affect pistachio but their impact is highly variable depending on the cultivar

and the environmental conditions. The most important include: panicle and shoot blight (caused by *Botryosphaeria dothidea*), botrytis blossom and shoot blight (caused by *Botrytis cinerea*), alternaria late blight (caused by *Alternaria alternata*), Verticillium wilt (caused by *Verticillium dahliae*), Armillaria root rot (caused by *Armillaria mellea*), Schizophyllum wood decay (caused by *Schizophyllum commune*), Sclerotinia shoot blight (caused by *Sclerotinia sclerotiorum*), Phomopsis shoot blight (caused by *Phomopsis spp*.) and Septoria leaf and fruit spot (caused by *Septoria spp*.) (Michailides et al. 1995; Holtz 2002).
- Other interesting traits in rootstock breeding include resistance to nematodes and salinity.

Although a few exceptions have been described in various *Pistacia* species (Ozbek and Ayfer 1958; Crane 1974; Kafkas et al. 2000) dioecy is the norm in pistachio. Dioecy represents an inconvenience for pistachio breeding because pistachio seedlings need between five to eight years to reach reproductive maturity and both sexes are phenotipically indistinguishable at the seedling stage (Hormaza et al. 1994b). However, molecular methods (see below) can facilitate breeding and selection by enabling screening for gender at the seedling stage, thereby simplifying the breeding of male and female plants for different objectives, with savings of time and economic resources.

12.1.5 Classical Breeding Achievements

Breeding and introducing new pistachio varieties is of great interest for the pistachio industry in different areas of the world. The use of a single variety, like 'Kerman' in the US, makes pistachio production very vulnerable to new diseases, and limits the possibility of extending the ripening season. Additionally, due to the little breeding efforts made until recently, most of the varieties used today have low levels of the desired characteristics like percentage of split nuts, number of blanks or exhibit an extreme alternate bearing. This situation limits production and, therefore, breeding towards the improvement of these characteristics can greatly improve yields. Due to the relatively recent initiation of pistachio breeding programs and to the long time required to achieve results in fruit tree species, the progress obtained in this direction following classical selection breeding approaches is slow. The most advanced pistachio breeding programs are now evaluating advanced selections from breeding crosses. This is the case of the program initiated at the University of California-Davis in the USA (Parfitt et al. 1995; Chao et al. 1998, 2003), at the IRTA Mas Bové in Spain (Vargas et al. 1987, 1993, 2002; Batlle et al. 2001) and at the Pistachio Research Institute in Gaziantep in Turkey (Mehlenbacher 2003). In other countries like Iran, Turkey, Israel or Australia, current pistachio genetic improvement involves evaluating cultivars, local seedling populations and species (Mehlenbacher 2003). In Turkey, monoecious *P. atlantica* genotypes are being investigated to determine the mechanism and inheritance of sex determination in the species (Kafkas 2002). Regarding disease resistance, the impact of *Verticillium* wilt on the susceptible species *P. atlantica* and *P. terebinthus* is now minimized by using *P. integerrima* hybrids, resistant to this fungus, as rootstock in infected soils (Morgan et al. 1992). Also, heritable resistance to *Alternaria* (Chao et al. 2001) and to *Botryosphaeria* (Parfitt et al 2003) has been identified in pistachio progenies.

12.2 Marker-Assisted Breeding

No genetic maps have been released so far in pistachio, although some advances have been made in the use of molecular tools for germplasm screening and breeding for specific traits. Results on genetic transformation of pistachio are also not yet available, although efficient somatic embryogenesis protocols have been reported (Onay et al. 1995, 1996, 2000, 2004b).

12.2.1 Germplasm Screening

Several studies have been conducted in pistachio concerning intra- and inter-specific genetic relationships, patterns of inheritance, or breeding histories. As in other fruit tree species, identification of pistachio cultivars has been traditionally carried out through pomological, morphological and horticultural traits (Zohary 1952; Grundwag and Weker 1976; Lin et al. 1984), and the consensus on those traits has allowed the release of descriptors for pistachio (IPGRI 1997).

More recently, as in other fruit tree species (Wünsch and Hormaza 2002), different molecular markers have been used to fingerprint pistachio cultivars

and to perform genetic diversity studies. Molecular characterization of *Pistacia* cultivars and species was initially carried out using isozymes (Loukas and Pontikis 1979; Dollo 1993; Barone et al. 1993, 1996; Rovira et al. 1995; Vargas et al. 1995). However, insufficient isozyme polymorphism among closely related cultivars limits their usefulness for fingerprinting and genetic diversity studies. In the last two decades efforts have been dedicated to obtain a more objective identification of genotypes with the use of DNA-based molecular markers. Initial work on molecular identification of pistachio using DNA markers was carried out by Hormaza et al. (1994a) and Dollo et al. (1995). They examined 15 pistachio cultivars with 33 RAPD primers and selected 14 primers that produced 143 amplification fragments, 37 of them being polymorphic. UPGMA cluster analysis grouped the cultivars according to their geographical origin distinguishing two major clusters, one comprising cultivars originated in the Mediterranean countries and the other from Iran and the Caspian Sea. Those studies were continued later (Hormaza et al. 1998), increasing both the number of genotypes closer to the pistachio center of origin and the number of RAPD primers. The results obtained with 29 genotypes and 37 primers agreed with earlier observations since most of the new genotypes fell into the Iranian-Caspian cluster. RAPD markers have also been used more recently to study the diversity of local pistachio germplasm in Turkmenistan (Barazani et al. 2003).

Recently, a first set of microsatellite markers has been developed in pistachio (Ahmad et al. 2003). In this work, a genomic library enriched for dinucleotide and trinucleotide repeats from the cultivar 'Kerman', was used to identify 14 SSRs that resulted in 46 putative alleles in a set of 17 pistachio cultivars (six from Syria, eight from Iran and two from Turkey). These microsatellites have been initially used by Ahmad et al. (2003) to identify the set of cultivars studied, analyze their genetic similarity and to establish a true to type assay based in the DNA extraction from pistachio kernels and shells. Twelve of those markers, together with 104 polymorphic markers produced by eight primer combinations following the Sequence-Related Amplified Polymorphism (SRAP) technique, have also been recently used to identify four commercial pistachio rootstocks (*P. atlantica* cv. 'Standard Atlantica', *P. integerrima* cv. 'Pioneer Gold', and the *P. atlantica* × *P. integerrima* hybrids 'PGII' and 'UCB-1') detecting variation in the UCB-1 rootstock (Ahmad et al. 2005).

At the interespecific level, molecular DNA markers have been used in *Pistacia* to analyze the phylogenetic and similarity relationships among the species of the genus. Thus, Parfitt and Badenes (1997) determined the phylogenetic relations among 10 *Pistacia* species using PCR-RFLP chloroplast DNA analysis. This analysis led to the classification of the *Pistacia* species into two main groups, *Lentiscus* and *Terebinthus,* with all the species of the former group being evergreen with paripinnate leaves, and the species in *Terebinthus* group, including *P. vera*, being deciduous with imparipinnate leaves. This work confirmed the morphological observations of Zohary (1952) that *P. vera* and *P. khinjuk* are the most primitive *Pistacia* species and thus confirming Central Asia, the natural area of this species, as the origin of diversity of the genus. The interspecific relationships described by Parfitt and Badenes (1997) using chloroplast DNA were later confirmed by Kafkas and Perl-Treves (2001) using RAPD nuclear DNA markers. Additionally, Kafkas and Perl-Treves (2001) were able to separate *P. vera* from *P. khinjuk* and established species-specific RAPD markers for the identification of unknown *Pistacia* germplasm. In a subsequent study, Kafkas and Perl-Treves (2002) analyzed the interspecific relations of nine *Pistacia* species using RAPD markers, and included two species (*P. palaestina* and *P. eurycarpa*) that had not been analyzed before. In this study, the species analyzed grouped in two clusters, one comprising single-trunked trees, including *P. vera*, and a second group comprising shrubs or small trees including *P. lentiscus* and *P. terebinthus,* and differing from the classification made by Parfitt and Badenes (1997). However, other works with RAPDs and AFLPs (Katsiotis et al. 2003; Golan-Goldhirsh et al. 2004) grouped the species in agreement to the initial classification, with one group containing evergreen species and including *P. lentiscus*, and a second group containing deciduous species and including *P. vera* and *P. terebinthus*. RAPDs have also been used to study the genetic diversity of *P. lentiscus* populations in Southern Spain and Northern Africa (Werner et al. 2002). The identification of RAPD markers specific to *P. lentiscus* or *P. terebinthus*, has also allowed to identify hybrid genotypes of *Pistacia* × *saportae* Burnat (*P. lentiscus* × *P. terebinthus*), a *P. vera* rootstock, using RAPD marker profiles (Werner et al. 2001). Recently, a set of microsatellite markers developed in mango by Viruel et al. (2005) have also been used to analyze the genetic relationships among *Pistacia* species and among several pistachio cultivars (Viruel and Hormaza, un-

published data). The study of the transferability of SSR markers between the two genera, showed that 44% of the SSRs developed in mango are conserved in the four *Pistacia* species studied (*P. vera*, *P. atlantica*, *P. therebinthus* and *P. lentiscus*) and, thus, they can be added to the set of microsatellite markers available for studies in *Pistacia* species.

12.2.2 Marker-Assisted Selection

The use of molecular markers linked to sex determination in *Pistacia* is one recent application of marker-assisted selection in this species. Pistachio, as well as other species of the genus, is dioecious, and is characterized by a long juvenile period needing five to eight years to reach maturity. Since morphological markers do not allow distinguishing female from male plants prior to flowering, determination of the plant gender at an early vegetative stage would greatly facilitate breeding, selection and management of this species. The first molecular marker linked to sex determination in *P. vera* was identified by Hormaza et al. (1994b) using bulked segregant analysis (BSA). To identify this marker, the DNA of seven male and seven female pistachio trees from two different crosses (Lassen × Peters and Kerman × Peters) were bulked and screened for polymorphisms with 700 RAPD primers. One RAPD marker ($OPO08_{945}$) was found to be present in female genotypes and absent in male genotypes and, thus, linked to the gene(s) controlling sex determination in *P. vera*. Subsequently, this marker has proved to be useful for sex identification in a large number of genotypes (Yakubov et al. 2005). A similar approach was followed by Kafkas et al. (2001) to identify markers linked to sex determination in the wild *Pistacia* species *P. eurycarpa* and *P. atlantica*, used as *P. vera* rootstocks. In this work, two markers linked to *P. eurycarpa* sex determination (one present in the male bulked DNA and the other in the female bulked DNA), and one in *P. atlantica* (present in the female bulked DNA) were found from the screening of 472 RAPD primers. In both works (Hormaza et al. 1994b; Kafkas et al. 2001), hybridization signals of the identified sex-related RAPD markers were found in repetitive sequences and a low frequency of sex related polymorphisms was observed. These results suggest that sex determination in *Pistacia* species must be restricted to a small region of the genome comprising one or few genes, surrounded by repetitive sequences.

12.3 Future Scope of Works

Fruit tree breeding is hampered by factors that make progress slower and more expensive than other crops. The large generation time of fruit trees makes traditional breeding including crossing, evaluation and selection a lengthy process. Additionally, a great amount of space and resources are needed to maintain living trees. Thus, the size of progenies and breeding programs and, consequently, the variability that can be screened is usually limited. Molecular markers tagging single gene traits or quantitative loci allow early seedling screening, reducing space and resources. Therefore, although crossings can only be carried out at maturity, fruit tree breeding can still greatly benefit from molecular genetics. Transformation can also help to make rapid advance in fruit breeding. The introduction of desired characters in elite germplasm eliminates the long time needed to introduce traits of interest by recurrent crossings. Additionally fruit trees are vegetatively propagated and, therefore, the improvement, once it is introduced, can be maintained through clonal propagation. The drawbacks of transformation are that regeneration protocols are still not available for most fruit tree species and, that the evaluation of transgenic fruit trees is still expensive and time consuming (Scorza 2001).

In pistachio, important advances have been obtained using classical breeding and selection approaches. Thus, germplasm has been characterized using phenotypical descriptors and molecular markers, breeding programs based on controlled crosses of selected genotypes are currently underway in different countries, information of heritability of some traits is available and advances in propagation and regeneration have been reported. However, the integration of molecular tools with conventional methods will be a qualitative advance in pistachio breeding programs. Initial molecular works in pistachio have initiated by molecular marker studies to identify germplasm and to study the genetic variability available. Advances have also been made in the identification of molecular markers linked to traits of interest such as sex determination; these markers allow the rapid and early screening of a large number of seedlings. However, for a widespread use of molecular breeding in pistachio, saturated genetic maps should also be built in this species and other genes of interest should be tagged to carry out marker-assisted selection and map-based

cloning. Additionally the availability of genetic maps will allow QTL identification and genome selection. Therefore, strong interdisciplinary breeding programs combined with appropriate networks that put together conventional breeding and molecular techniques are highly needed in pistachio to make a qualitative advance similar to that currently occurring in other fruit tree species such as those of the Rosaceae.

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13 Olive

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13.1 Introduction

13.1.1 Brief History of the Crop

The tree species olive (*Olea europaea* L.) is among the most ancient of crops of the Mediterranean region (Zohary and Spiegel-Roy 1975) (Figs. 1 and 2). In the archaeological records, woods of cultivated olive from Eastern Spain and Southern France have been dated up to the Neolithic age (Terral 2000). Before its domestication, wild olive was endemic across the Mediterranean region, but particularly in the Middle East. Wild olive grows abundantly in thick forest, and is believed to be indigenous to the Mediterranean Basin (Green 2002). The domestication process is thought to have involved the selection of trees of large fruit size and/or high oil content, and their vegetative propagation, either directly planted via cuttings or grafted onto indigenous oleasters. There is evidence for contemporaneous starting of olive domestication at both ends of the Mediterranean. In the Near East it occurred in the Early Bronze Age (second half of the 5th millennium BCE), as has been demonstrated both by the discovery of olive oil presses and by the presence of pollen grains, stones and wood remains (Zohary and Spiegel-Roy 1975; Liphschitz et al. 1991); while analysis of archaeological charcoal and olive stones have dated domestication to the end of the Bronze Age in the north-western Mediterranean area (Terral 2000; Terral et al. 2004). From the 6th century BC, cultivated olive spread throughout the Mediterranean, reaching Tunisia and Sicily, and later Northern Italy. With the European settlement of America after the XV century, olives arrived in the New World, but only in recent times has its cultivation extended significantly beyond the Mediterranean area. Today, it is grown commercially in Australia, South America (Argentina and Chile) and South Africa.

13.1.2 Botanical Description

Olive belongs to the *Oleaceae* family, sub-family *Oleideae*. The family includes about 30 genera (Johnson 1957), accounting ornamental shrub species such as jasmine (*Jasminum fruticans* L.), lilac (*Syringa vulgaris* L.) and forsythia (*Forsythia* × *intermedia* Zabel); and tree species, such as ash (*Fraxinus excelsior* L. and *F. angustifolia* Vahl.), privet (*Ligustrum vulgare*

Fig. 1. An ancient olive tree (*Olea europaea* L.)

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L.) and phyllirea (*Phyllirea angustifolia* L., *P. media* L. and *P. latifolia* L.). The genus *Olea*, sub-family *Oleideae,* includes two sub-genera*: Olea* and *Paniculatae.* The former is divided in two sections: *Olea*, which contain only *O. europaea* (including both cultivated and wild forms), and *Ligustroides*. According to recent revisions of *O. europaea* taxonomy (Green and Wickens 1989; Green 2002), this species is divided into six sub-species, based on morphology and geographical distribution:

1) subsp. *europaea*, with the two botanical varieties *europaea* (cultivated olive) and *sylvestris* (wild olive), widely distributed throughout the Mediterranean Basin;
2) subsp. *cuspidata,* distributed from SE Asia to SW China, as well as from the Arabian peninsula through East and South Africa;
3) subsp. *laperrinei*, restricted to the Sahara region;
4) subsp. *maroccana*, restricted to Morocco;
5) subsp. *cerasiformis*, restricted to the island of Madeira;
6) subsp. *guanchica*, restricted to the Canary Islands.

Wild olive fruits are smaller in size and have lower mesocarp oil content than do cultivars (Terral and Arnold-Simard 1996). Populations of wild olive are restricted to a few isolated areas of native Mediterranean forest, where pollen/stones may be wind/bird-distributed (Lumaret et al. 2004). Molecular analysis, using both nuclear and cytoplasmic markers, has shown that the eastern and western Mediterranean populations are strongly differentiated from one another (Besnard et al. 2001a, 2001b, 2002b; Lumaret et al. 2004). On the contrary, cultivated olives do not show such geographical structure, even though their variability is quite high. It has been repeatedly shown evidence for the multilocal selection of most cultivars (Besnard et al. 2001b; Rotondi et al. 2003), empirically undertaken by olive growers from naturally cross-bred genotypes. At least 1,275 cultivars have been described (Bartolini et al. 1998), but many other local varieties and ecotypes contribute to the richness of the olive germplasm. Few cultivars are dispersed over a widespread area; rather, the majority is highly localized.

Fig. 2. Ripening olive fruits of cultivar Frantoio

The olive is a long-living evergreen tree, which can attain a mature height of up to 15 m and a spread of 9 m; its life span is typically more than 500 years, but trees older than 2,000 years have been recorded. Mature leaves are elliptic and characteristically gray-green in color, as a result of the presence of star-hairs. Flowers are wind pollinated, and although most cultivars are self-incompatible, some are self-compatible. The flowers are generally hermaphroditic, but certain cultivars are male-sterile (Besnard et al. 2000), while others are purely staminate. The fruit is a drupe, with a thick, fleshy oil-accumulating mesocarp. When pulped, the mesocarp is made up of oil (22%), water (50%), proteins (1.6%), carbohydrates (19.1%), cellulose (5.8%) and minerals (1.5%).

Green olives destined for canning are usually harvested when the fruit is completely developed and the skin color starts to change from green to reddish, while olives used either as a source of oil, or for processing as black olives, are picked later in the ripening process, when oil accumulation is completed and the skin has become black. The characteristic compound oleuropein, which confers a strong bitter taste to the fruit, makes the fruit unpalatable, so that pretreatment is necessary before table consumption.

Olive trees grow in semi-arid to temperate climates, on almost any well-drained soil with a pH below 8.5, and are reasonably tolerant of mild soil salinity. They show cold winter hardiness, tolerating temperatures as low as −12 °C. Even though olive has the ability to initiate vegetative shoots from the base of the trunk, productivity may be compromised for several years following episodes of severe cold-induced die-back.

Significant pests and diseases include the olive-specific pathogens *Spilocaea oleagina* Cast., causing olive leaf spot, and the olive fruit fly (*Bactrocera oleae*). Major non-specific pathogens cause *Verticil-*

lium wilt (*Verticillium dahliae* Kleb.) and olive knot (*Pseudomonas syringae* subsp. s*avastanoi*). *B. oleae* directly attacks the fruit mesocarp, and can have serious consequences on production, by inducing early fruit fall or causing total disruption of the pulp. Plant propagation is generally by cutting or grafting onto seedling rootstocks. Cultivars are mostly diploid ($2n = 2x = 46$) (Falistocco and Tosti 1996; Minelli et al. 2000), but tetraploid plants have been reported (Rugini et al. 1996). The DNA content is 2.2 pg per 1C nucleus (Rugini et al. 1996), equivalent to a genome size of 2.2 Gbp (De la Rosa et al. 2003).

13.1.3 Economic Importance

Olive is one of the most important crops as a source of oil, and for table consumption. Olive oil has favorable nutritional properties, and as a result, its consumption, traditionally restricted to the Mediterranean area (77% of the world production area), is increasing worldwide (mainly United States, Canada, Australia and Japan). Some varieties are cultivated specifically for table consumption, but the majority is used for oil extraction.

Virgin olive oil is mechanically extracted from pressed or centrifuged pulped fruit. In the commonest process (the continuous extraction system), two centrifugations generate three fractions: oil, pomace and vegetable water. Olive production is concentrated in Southern Europe, mainly Spain and Italy, followed by Greece, Portugal and France, which together account for about the 85% of world production. Turkey, Syria, Lybia, Morocco, Algeria and Tunisia are also important producers. Over the last ten years olive cultivation has extended around the world, from South Africa to Latin America (Argentina and Chile), California, New Zealand and Australia and, since the late 1990s, there has been a strongly rising production trend in these countries; nevertheless, the major producers remain in Europe. Olive oil production in Europe in 2003 was 2.3 Mt, while competitor oil-producing crops such as rapeseed and sunflower generated, respectively, 4.2 Mt and 4.9 Mt (data from FAOSTAT database).

Olive oil is a relatively expensive vegetable oil due to its high cultivation costs and limited production. Fruit production can start 3–5 years after planting, if properly cultivated, but generally optimal yields are not attained before trees are 10 years old. Mean production per tree (15–50 kg fruit) and per unit area (about 2 t/ha) are low in comparison with other oil crops, and extractability rarely exceed 24% of fresh weight (depending on variety, agro-climatic conditions and extraction method). Yield is unpredictable from year to year, but the source of much of this variation remains unclear. Oil accumulates in the fruit mesocarp, and to a lesser extent, also in the seed (Harwood and Sanchez 2000).

Virgin olive oil is overwhelmingly made up of triglycerides (98–99%), along with a small proportion of other compounds. The dominant triglyceride fatty acid species are the mono-unsaturated acids oleic (18:1) (57–78%), palmitic (16:0), and stearic (18:0), and the poly-unsaturated acids linoleic (18:2) (7–19%) and linolenic (18:3) (0.6–0.8%) (Salas et al. 2000). The minor compounds (alcohols, polyphenolic compounds, chlorophyll, carotenoids, sterols, tocopherols and flavonoids) contribute to the organoleptic qualities, taste, flavor, and nutritional value (Servili and Montedoro 2002; Garcia-Gonzalez et al. 2004), which may distinguish olive oils originating from different production regions. Recent studies have shown that olives contain antioxidants in abundance (up to 16 g/kg), represented by acteosides, hydroxytyrosol, tyrosol and phenilpropionic acids. Olive oil, especially extra virgin, contains smaller amounts of hydroxytyrosol and tyrosol, but also contains secoiridoids and lignans, as well as other compounds deemed to be anticancer agents (e.g. squalene and terpenoids) (Fabiani et al. 2002; Owen et al. 2004).

The European Union has developed a PDO (Protected Designation of Origin) assignation to olive oils with important regional traditional origins. Oil quality is strongly cultivar-dependent, but is also affected by agro-climatic factors and agronomic practices.

Various categories of olive oil have been defined (Reg. CEE 1513/01):

- *virgin oil:* oil produced by mechanical or other physical means under conditions (e.g. temperature) that do not lead to any chemical alteration in the oil, and which has not undergone any treatment other than washing, decantation, centrifugation and filtration. Within this category is included the 'extra virgin olive oil': virgin olive oil which has a free acidity, expressed as oleic acid, of not more than 0.8 grams per 100 grams, and the other character-

istics of which correspond to those fixed for this category in this standard;
- *refined oil:* oil obtained from virgin oil by refining methods which do not lead to an alteration in the initial glyceric structure;
- *olive oil*: oil consisting of a blend of refined and virgin olive oil;
- *olive-pomace oil*: oil obtained by treating olive pomace with solvents, to the exclusion of oil obtained by re-esterification processes and of any mixture with oils of other kinds.

Two types of adulteration have been identified: blending of virgin olive oils with olive oils of lower grade, and mixing olive oils with other vegetable oils. Mislabeling of olive oils is of considerable concern, as this results in the product not being of the claimed grade (Lai et al. 1994; Yoke et al. 1994; Spangenberg and Ogrinc 2001). The International Olive Oil Council (1993) and the Codex Alimentarius Commission (1993) have therefore produced standards for virgin, refined and olive-pomace oils. Instruments such as Protected Designation of Origin (PDO), Protected Geographical Indication (PGI) and Traditional Specialty Guaranteed (TSG) are all important for quality control in this context.

13.1.4 Breeding Objectives

Primary goals in olive breeding are directed towards overcoming current limiting factors for production. These include: shortening the juvenile stage; increasing fruit number and size; increasing oil content and quality (fatty acid composition, polyphenol content, etc.); stabilising yield; dwarfing, and other manipulations of tree architecture to facilitate mechanical pruning and harvesting; improving resistance to pests (in particular olive fruit fly, *Bactrocera oleae*) and diseases (leaf peacock spot, caused by *Spilocaea oleagina*; Verticillium wilt, *Verticillium dahliae*; and olive knot, *Pseudomonas savastanoi*). Other important objectives relate to improvement in cold tolerance (to allow cultivation in more northerly areas) and to the promotion of self-fertility (to reduce reliance on pollinators). Tree architecture and vigour are particularly important because the height of the tree prevents mechanical harvesting and pruning, thereby increasing the costs of cultivation. Although the olive is generally considered to be a drought-tolerant species, its productivity is strongly reduced under drought conditions, and thus there is interest in the possibility of tolerant cultivars, as well as those that can thrive on saline and heavy soils. Rootstock selection is focused on the ability to control scion vigour, and to improve the level of resistance to biotic and abiotic stresses.

13.1.5 Breeding Achievements

In spite of its economic importance to all Mediterranean countries, there has been little directed olive breeding to date, despite the pressing need to improve productivity and agronomic performance. Most selection programs have so far relied on clonal selection, on the assumption that in a long-living plant such as olive, natural mutations generating any positive alteration in a trait of agronomic interest, can be maintained by vegetative propagation (Rallo 1995; Belaj et al. 2004). Exploration of phenotypic variability in agronomic characters has led to the identification of valuable clones within numerous olive cultivars (Suárez et al. 1990; Lavee et al. 1995; Bartolini et al. 2002; Grati-Kammoun et al. 2002). However, in spite of the significant efforts made towards clonal selection, very few clones have outstanding performance (Loussert and Berrichi 1995; Tous et al. 1998). Similarly, induced mutagenesis has not been encouraging, and so far has succeeded in producing only a compact mutant of the cv. Ascolana Tenera (Roselli and Donini 1982). The evaluation of minor local cultivars, present in every cultivation area, has recently been exploited to identify individuals highly adaptive to extreme environmental conditions (Pannelli et al. 2003; Rotondi et al. 2003). Clonal rootstocks with high rooting ability have been identified from crossbred populations (Baldoni and Fontanazza 1990), and other selected rootstocks have shown ability to control scion vigor and resistance to frost injury (Pannelli et al. 2002). The use of the cvs. Souri, Muhasan and Barnea as rootstocks under dry conditions, after 10 years from planting, did not show any significant effect on tree vigor, shape and fruit production (Lavee and Schachtel 1999).

Experiments of genetic transformation are in progress with the aim to select disease resistant cultivars or to introduce key genes involved in important metabolic pathways (Rugini et al. 2000; Rugini and Baldoni 2004).

The long generation time has severely hindered both classical breeding and genetic studies (De la Rosa et al. 2003). It is possible to greatly reduce the length of the juvenile phase by using forcing protocols, but the evaluation of the agronomic performance of mature plants still requires at least five years of experimentation (Santos Antunes et al. 1999). Furthermore, the genetic control of the major traits is unknown (De la Rosa et al. 2003). Vigor, leaf size and fruit shape seem controlled by major genes showing dominance (Bellini 1993), while the inheritance of other characters, such as fruit size, flowering intensity, fruit set, ripening time and yield remains uncertain (Bellini 1993; Parlati et al. 1994). Very few cultivars have been emerged from formal breeding programs.

A new cultivar (Maalot) resistant to *Spilocea oleagina* has been selected from the selfed F_1 progeny of a semi resistant seedling probably of Chemlali (Lavee et al. 1999). From seedling populations obtained by unknown parents two other cultivars were selected: 'Barnea', with vigorous and upright growth, and 'Kadesh', as a table olive (Lavee 1978; Lavee et al. 1986).

Three new olive cultivars (Arno, Tevere and Basento) were released from the progeny of the cross 'Picholine × Manzanilla' (Bellini et al. 2002) and their performance is still under evaluation.

The University of Adelaide has recently established a selection program utilizing the plant olives locally reproduced from cultivars previously introduced in Australia and well adapted to that environment. The aim of the project is the identification of new improved olive cultivars showing superior morphological and oil characteristics (Sedgley 2000).

13.2 Construction of Genetic Maps

The first linkage map of the olive genome was based on RAPD (random amplified polymorphic DNA) and AFLP (amplified fragment length polymorphism) dominant markers, along with a small number of codominant RFLPs (restriction fragment length polymorphisms) and SSRs (simple sequence repeats) (De la Rosa et al. 2003). The mapping population consisted of a progeny derived from two highly heterozygous cultivars, Leccino and Dolce Agogia. The Leccino map covered 2,765 cM and comprised 249 markers, falling into 22 major and 17 minor linkage groups (the latter each involving less than four markers). The Dolce Agogia map was of similar length (2,445 cM) and comprised 236 markers arranged in 27 major and three minor linkage groups. Mean inter-marker distances were similar in both maps (13.2 cM in Leccino and 11.9 cM in Dolce Agogia). AFLP and RAPD markers were homogeneously distributed across all of the linkage groups. Based on the olive genomic size, estimated around 3,000 cM (Wu et al. 2004), the Leccino × Dolce Agogia map is thought to have covered about 80% of the genome. A second linkage map was constructed by Wu et al. (2004), based on RAPDs, SCARs and SSRs exploiting the progeny of a cross between the cultivars Frantoio and Kalamata. The greater use of codominant markers allowed the integration of the two parental maps to generate 15 linkage groups, covering 101 loci and 879 cM with a mean inter-marker distance of 10.2 cM.

In situ hybridization using tandem repeated sequences has allowed most of the olive chromosomes to be distinguished, and has also revealed structural heterozygosity in three chromosome pairs (Minelli et al. 2000).

At present, no further olive genome mapping data are available, and as yet, no QTL have been detected, neither is there any detailed analysis on genome organization.

13.3 Gene Mapping

Mapping of gene sequences has concentrated on orthologous genes characterized in other species (Table 1). Particular attention has focused on genes encoding key enzymes involved in fatty acid biosynthesis, modification, triacylglycerol synthesis and storage. These include enoyl-ACP reductase (*ear*), stearoyl-ACP desaturase, omega 6 plastidial desaturase (*fad6*), omega 3 plastidial desaturase (*fad7*), cytochrome b5 (*cyt b5*), omega 6 cytoplasmic desaturase (*fad2*), omega 3 cytoplasmic desaturase (*fad3*), acyl-CoA:diacylglycerol acyltranferase (DGAT) and oleosin (Hatzopoulos et al. 2002). The temporal and transient expression of stearoyl-ACP desaturase (a key enzyme for the conversion of 18:0 stearic acid to 18:1 oleic acid, the main component of olive oil) has been studied during fruit development (Haralampidis et al. 1998). Expression of a cDNA encoding an ω-3 fatty acid desaturase

Table 1. Genes and expressed sequences identified in olive

GenBank Accession Number	Gene encoding for	Authors (Year of publication)	Length (bp)
AJ536118	Partial putative copia retrotransposon RNaseH gene and gene encoding retrotranscriptase	Natali L, Giordani T, Maestrini P, Cavallini A (2005)	1,164
AJ536119	Partial putative copia retrotransposon RNaseH gene and putative LTR	Natali L, Giordani T, Maestrini P, Cavallini A (2005)	499
AJ536120	Partial putative gypsy retrotransposon RNase gene, and genes encoding retrotranscriptase and integrase	Natali L, Giordani T, Maestrini P, Cavallini A (2005)	1,930
AY772187	Fatty acid desaturase 6 (fad6)	Moressis A, Banilas G, Hatzopoulos P (2005)	1,597 (complete cds)
AJ810085	Beta-1,3-glucanase (glu-4 gene)	Caliente R, Barea J, Azcon C, Ferrol N (2004)	1,032
AJ810086	Beta-1,3-glucanase (glu-5 gene)	Caliente R, Barea J, Azcon C, Ferrol N (2004)	643 (partial cds)
AY445635	Acyl-CoA:diacylglycerol acyltransferase 1 (DGAT1)	Giannoulia K, Hatzopoulos P (2004)	1,836 (complete cds)
AY738639	Phenylalanine ammonia-lyase (PAL) gene	Tosti N, Baldoni L (2004)	713 (partial cds)
AY788899	Actin	Tosti N, Baldoni L (2004)	427 (partial cds)
AY083161	Oleosin	Giannoulia K, Haralampidis K, Milioni D, Hatzopoulos P (2002)	792 (complete cds)
AY083162	Beta-glucosidase (bglc)	Gazis F, Hatzopoulos P (2002)	1,902 (complete cds)
AY083163	Fatty acid desaturase 2 (fad2)	Nikoloudakis N, Hatzopoulos P (2002)	1,452 (complete cds)
AY083164	Enoyl ACP reductase (ear)	Poghosyan Z, Hatzopoulos P (2002)	1,674 (complete cds)
AJ428575	Cu/Zn super-oxide dismutase	Butteroni C, Afferni C, Tinghino R et al. (2002)	459
AY095446	Photosystem II protein D1 (PSBA)	Muleo R, Proietti C, Paolucci I et al. (2002)	942 (partial cds)
AF492010	Monosaccharide transporter (MST)	Oliveira JM, Geros HV, Tavares RM (2002)	726 (partial cds)
AF479171	26S ribosomal RNA gene	Soltis DE, Senters A, Kim S et al. (2002)	1,603 (partial sequence)
AF428256	Acyl carrier protein (ACP)	Guerrero CM, Valpuesta V, Baldoni L (2001)	763
AJ416434	Ty1-copia-like retrotransposon, Toe21 gene	Stergiou G, Katsiotis A (2001)	264
L49289	18S ribosomal RNA gene	Johnson LA, Soltis DE, Soltis PS (2001)	1,729 (partial sequence)
AF426829	Cu/Zn-superoxide dismutase	Corpus FJ, Barroso JB, Romero-Puertas MC et al. (2001)	312 (partial sequence)
AY040811	trnT-trnL intergenic chloroplast spacer	Baldoni L, Guerrero CM, Abbott AG et al. (2001)	658
AF384051	Expansin	Ferrante A, Hunter DA, Reid MS (2001)	486 (partial cds)
AF384050	Anthocyanidin synthase	Ferrante A, Hunter DA, Reid MS (2001)	789 (partial cds)
AF384049	Chalcone synthase	Ferrante A, Hunter DA, Reid MS (2001)	571 (partial cds)
AY059387	Putative cullin protein	Butowt R, Rodriguez-Garcia MI (2001)	2,637
AF429429/AF429430	Polyubiquitin OUB1 and OUB2	Butowt R, Rodriguez-Garcia MI (2001)	1,184/1,666
AF427107	Manganese superoxide dismutase	Corpus FJ, Barroso JB, Romero-Puertas MC et al. (2001)	435 (partial cds)
AY036055	Hexose transporter pGlT	Butowt R, Rodriguez-Garcia MI (2001)	2,039

Table 1. (continued)

GenBank Accession Number	Gene encoding for	Authors (Year of publication)	Length (bp)
AF130163	NADH dehydrogenase subunit F (ndhF)	Olmstead RG, Kim KJ, Jansen RK, Wagstaff SJ (2000)	2,217
AF288707	Cytochrome c oxidase subunit I (cox1) mitochondrial gene	Zilhao IT, Tenreiro RP, Fevereiro PS (2000)	447 (partial cds)
AF225275	Ribosomal protein S16 (rps16) chloroplast gene	Wallander E, Albert VA (2000)	853 (partial intron)
AF191342	Copper/zinc superoxide dismutase (SOD1)	Alche JD, Castro AJ, Rodriguez-Garcia MI (1999)	276 (partial cds)
AB025343	Lupeol synthase	Shibuya M, Zhang H, Endo A et al. (1999)	2,546
AB025344	Cycloartenol synthase	Shibuya M, Zhang H, Endo A et al. (1999)	1,983 (partial cds)
AJ236163	ATP synthase beta subunit	Albach DC, Soltis PS, Soltis DE, Olmstead G (1998)	1,493
Z70240/Z70241	Cytochrome oxidase; subunit 3, cox3 gene	Perrotta G, Cavallotti A, Quagliariello C (1997)	1,817/1,818
AJ001766	Chloroplast ribulose 1,5-bisphosphate carboxylase large subunit (rbcL) gene	Oxelman B, Backlund M, Bremer B (1997)	1,402 (partial cds)
AJ001369/AJ001370	Cytochrome b5 genes 1 and 2	Martsinkovskaya AI, Poghosyan ZP, Haralampidis K et al. (1997)	688/752
AF027288	NADH dehydrogenase (ndhF) chloroplast gene	Oxelman B, Backlund M, Bremer B (1997)	2,193 (partial cds)
U58141	Stearoyl-ACP desaturase	Baldoni L, Georgi LL, Abbott AG (1996)	1,493
AF511041	Retrotransposon	Muleo R, Intrieri MC (2002)	484 (partial sequence)
AY095446	PSBA gene	Muleo R, Proietti C, Paolucci I et al. (2002)	942 (partial cds)

has been studied in leaves, anthers and embryos (Poghosyan et al. 1999), and two cytochrome b_5 genes and their spatial and temporal patterns of expression have been characterized during flower and fruit development (Martsinkovskaya et al. 1999). The differential expression of other genes such as diacylglycerol acyltransferase (DGAT) and oleate desaturase has been evaluated in various tissues (Giannoulia et al. 2000; Banilas et al. 2005). Finally, a candidate stearoyl-ACP desaturase was mapped on linkage group 4 of cv. Leccino (De la Rosa et al. 2003).

13.4 Marker-Assisted Breeding

The very preliminary works performed on olive genomics are far before producing effective results toward the selection of new cultivars by the use of molecular tools.

For that reason and considering the lack of knowledge on the real useful variability already present in the cultivated and wild olive germplasm, attention has been focused in the last ten years mainly on the evaluation of such germplasm. The large number of cultivars and wild populations, in fact, positions olive as a crop species with a very extensive germplasm. The geographic distribution of variability within the *Olea* genus and the genetic relationships among the different species have been studied using various molecular methods, including cpDNA profiles (Lumaret et al. 2000; Baldoni et al. 2002), AFLPs (Angiolillo et al. 1999; Baldoni et al. 2000), and rDNA and mtDNA polymorphisms (Besnard and Bervillé 2002; Besnard et al. 2002a, 2002b). The wild relatives of cultivated olive (oleasters) have been widely analysed using RFLP markers derived from mitochondrial, chloroplast and nuclear DNA, which, in addition to allozyme markers, provide evidence for the survival of indigenous oleaster populations, particularly in the western Mediterranean (Lumaret and Ouazzani 2001; Lumaret et al. 2004). Within wild populations, a clear distinction between the eastern and western Mediterranean has been noted (Besnard and Bervillé 2000; Besnard et al. 2002b; Bronzini de Caraffa et al. 2002).

Internal transcribed spacer 1 (ITS-1) sequences, RAPD and inter-SSR (ISSR) markers have been deployed to evaluate the colonization history of *O. europaea* (Hess et al. 2000). Some *Olea europaea* retroelements have also been identified (Hernandez et al. 2001) and their copy number has been estimated (Stergiou et al. 2002).

The development of SCAR markers has been attempted from RAPDs (Hernandez et al. 2001), and one such has been reported by Mekuria et al. (2001) to be linked to tolerance to leaf peacock spot.

DNA fingerprinting is a powerful aid for the identification of olive oil provenance, since it can be used to generate a profile specific for any given plant genotype. Over the last decade, molecular markers have been widely applied also to characterize and identify olive cultivars. These analyses have utilised RAPDs (Fabbri et al. 1995; Belaj et al. 1999; Mekuria et al. 1999; Barranco et al. 2000; Gemas et al. 2000; Belaj et al. 2001; Besnard et al. 2001c; Belaj et al. 2002; Guerin et al. 2002), AFLPs (Angiolillo et al. 1999; Rotondi et al. 2003; Owen et al. 2005; Montemurro et al. 2005), ISSRs (Hess et al. 2000; Pasqualone et al. 2001; Vargas and Kadereit 2001) and SSRs (Rallo et al. 2000; Sefc et al. 2000; Carriero et al. 2002; Cipriani et al. 2002; Bandelj et al. 2004). The same methods have also been applied to trace the geographic origin of batches of olive oil (Muzzalupo and Perri 2002; Busconi et al. 2003; Breton et al. 2004; Pasqualone et al. 2004; Testolin and Lain 2005).

Single Nucleotide Polymorphisms (SNPs) are currently under development (Reale et al. 2006) in order to clearly distinguish inter-cultivar variability and characterize the clonal variants.

13.5 Future Scope of Works

Projects are currently under development in order to address gaps in genetic mapping and molecular breeding in olive. Three main areas of interest can be resumed:

1) completing the research on the evaluation, characterization and utilization of the available genetic resources, both on cultivated varieties and wild relatives;
2) continuing the project on genomic, functional and physical mapping;

3) establishing new breeding programs and completing those in progress by the extended application of marker-assisted selection.

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14 Citrus Fruits

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14.1 Introduction

14.1.1 Background

Citrus, belonging to the family Rutaceae, is one of the world's most important fruit crops with a total world production of 105 million metric tons. It is commercially grown in the tropical and subtropical regions around the world, primarily between the latitudes of 40°N to 40°S. The literature record of citrus domestication and cultivation history dates back to 2100 BC (Webber 1967; Scora 1988). It is considered to have originated from the Malay Archipelago and Southeast Asia, occurring from Northern India to China and in the South through Malaysia, the East Indies and the Philippines. Movement of citrus worldwide was achieved by distant explorers, traders, and church missionaries. More recent evidence suggests that Yunnan Province in the Southwest China may be the center of origin due to the diversity of species found there (Gmitter and Hu 1990). The network of rivers in this area could have provided an on-route dispersal to the south (Sauer 1993). Much has been written on the evolution of modern citrus cultivars and its broad diversity (Swingle and Reece 1967). Studies on the relationships between genera and species were carried out based mainly on morphological characteristics leading to the formulation of numerous classification systems. As there were differences of opinion among the taxonomists, the numbers of species of citrus classified by them were very controversial (Swingle 1943; Swingle and Reece 1967; Tanaka 1976), with Tanaka favoring the naming of many more species than Swingle. Barrett and Rhodes (1976) suggested that there were three true ancestral citrus species, namely, *C. maxima* (pummelos), *C. reticulata* (mandarins), and *C. medica* (citrons); all other species are viewed as introgressions of these ancestral forms. More recently, molecular marker evidence q(Nicolosi et al. 2000) has supported this hypothesis, though there may be other species that might also be considered as ancestral.

Citrus plants are small, spreading, evergreen trees with thorny shoots, growing to about 2–15 m tall. Leaves are unifoliate and alternate with more or less broadly winged petioles. Flowers are fragrant, usually white but sometimes pink or purple pigmented, perfect with 5 petals and 5 sepals, and are borne solitary or in short cymes. Citrus industries in many production areas generate substantial regional revenue. Brazil, the United States, and China (Fig. 1) are the three largest citrus producers in the world (FAO 2003). Citrus is primarily valued for the fruit which can be eaten as a fresh fruit, processed into juice, or added to dishes and beverages. The major types of edible citrus including the ancestral *Citrus* species and their introgressions along with their possible place of origin are listed in Table 1. Citrus is rich in vitamin C, flavonoids, acids, volatile oils, carotenoids, and other microelements.

In a wide genetic perspective, the general term citrus also includes species from two other closely related genera, *Poncirus* (trifoliate orange) and *Fortunella* (kumquat), which are sexually compatible with *Citrus* species. *Poncirus* is the most valuable genetic resource for genetic improvement of *Citrus*. Though its fruits are not edible, *Poncirus* is often used in the production of rootstocks as it possesses many resistance genes that are not found in *Citrus*. Resistance or tolerance to citrus tristeza virus (CTV), *Phytophthora* root rot, citrus nematode, cold accumulation, and other environmental stresses have been explored for use in scion and rootstock genetic improvement via conventional or molecular approaches (Cai et al. 1994; Gmitter et al. 1996; Tozlu et al. 1999a, b; Ling et al. 2000). In addition, the genetically dominant trifoliate leaf of *Poncirus* is a big advantage to develop mapping populations, as it allows the direct identifi-

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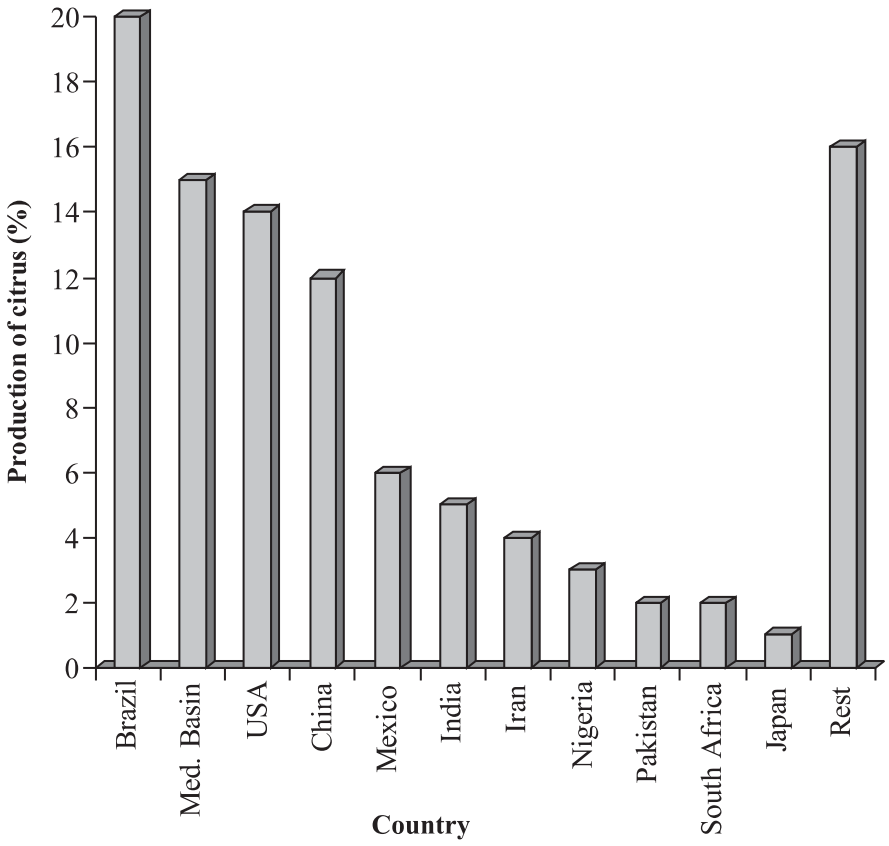

Fig. 1. World production of citrus (FAO, 2003)

cation of zygotic hybrids from true nucellar seedlings. *Fortunella* is an important edible fruit and also a resource for resistance to Asian citrus canker (ACC). In order to have a clear usage of the term "citrus" in this chapter, citrus, in regular font, will be a general term referring to any related species regardless of genus, and *Citrus*, in italic font with initial letter capitalized, will be a genus name representing only the *Citrus* species.

Citrus is vegetatively propagated. Selection of new citrus and related cultivars has been occurring for thousands of years and superior phenotypes have been selected from the wild for cultivation. Citrus has mainly two different breeding targets, viz., scion and rootstock. Yield and fruit quality (both for domestic as well as international market demand) are two of the most important considerations for scion improvement. A superior rootstock is desired to possess broad and durable resistance to pests, diseases, and other environmental stresses (particularly from soil and water). A rootstock should also grow vigorously, be compatible with the scion, and produce maximal numbers of seeds containing true nucellar embryos. Finally, rootstocks substantially influence both yield and fruit quality of the grafted scion cultivars, so these factors also are considered in the evaluation of new candidates. Though the objectives of breeding scion and rootstocks are different, overall breeding objectives may sometime address the aspects of both programs by improving traits such as disease resistance, cold tolerance, etc.

Conventional breeding is a very slow and difficult process due to long juvenility, large tree size, polyembryony, high heterozygosity, and self-incompatibility to some extent. There is a significant lack of knowledge regarding genetic mechanisms controlling the inheritance of agriculturally important traits (most of them may be quantitatively inherited), and only a few of them thus far have demonstrated a single gene inheritance pattern (Davies and Albrigo 1994). Through conventional hybridization, few new *Citrus* cultivars have been produced, although a few rootstocks were developed (Soost and Cameron 1975; Cameron and Soost 1984; Gmitter et al. 1992). Most of the commercially grown cultivars were derived from well-adapted native seedlings/varieties, spontaneous bud mutations, or artificial irradiations. For example, most Satsuma mandarin varieties were from field

Table 1. Four original wild and four hybrid *Citrus* edible species

Name	Scientific name	Possible place of origin
Citron	*C. medica*	India and China
Pummelo	*C. grandis*	Malaysia and India
Mandarin	*C. reticulata*	Southeast Asia
Lime	*C. aurantifolia*	East India
Sour orange	*C. aurantium*	Pummelo × Mandarin. China
Sweet orange	*C. sinensis*	Pummelo × Mandarin. China
Lemon	*C. limon*	Citron × Lime. Unknown, likely in China
Grapefruit	*C. paradisii*	Pummelo × Sweet orange. Barbados island

selection or bud mutation in Japan and China, as were grapefruits and oranges in the US (Hodgson 1967). Chromosomal rearrangements have also been involved in selection for seedlessness and other traits within cultivated citrus (Gmitter et al. 1992). A consequence of the mutational origin and diversification of many of the most important cultivar groups (including oranges, grapefruit, lemons, and certain categories of mandarins such as the Satsuma and Clementine cultivar groups) is that sexual hybridization is excluded as a strategy for genetic improvement. Inbreeding depression and the lack of phenotypic similarity to market expectations and definitions, when hybrids within groups are created, results in plants that are unacceptable to citriculture. A further consequence is that the ability to move useful genes within or among citrus cultivars and germplasm resources, for disease resistance or fruit quality for example, is reliant entirely upon alternatives such as genetic transformation.

Few studies have been conducted to understand the genetics of citrus. Knowledge and understanding of the genetic mechanisms that control important traits such as juvenility/maturity, disease resistance, cold tolerance and aspects of fruit ripening process are clearly lacking (Gmitter et al. 1992). The rapid development of molecular marker technologies has made it possible to investigate gene expression and has helped in construction and integration of genetic and physical maps of the economically important traits. The knowledge and establishment of genomics and bioinformatics have also provided efficient tools for tagging and cloning the genes, and have made the sequencing of the citrus genome plausible. This chapter will summarize the achievements of citrus genome research in the past thirty years, as well as ongoing efforts and planned genomic goals.

14.1.2 Early Knowledge of Citrus Genome and Genetics

Most citrus species, including those from other three distant relative genera, *Microcitrus*, *Eremocitrus* and *Clymenia*, are diploids with nine pairs of chromosomes ($2n = 2x = 18$), although polyploids have been reported. Many spontaneous and induced tetraploids have been used as breeding parents to produce seedless triploid varieties (Gmitter et al. 1992; Gmitter 1994), and numerous tetraploid somatic hybrids have been created as well, by protoplast fusion experiments (Grosser et al. 1996). Many citrus species are outcrossing (Roose et al. 1998). Cytogenetic studies revealed citrus has small but highly variable chromosomes (Naithani and Raghuvanshi 1958; Raghuvanshi 1962; Guerra 1984, 1993). Karyotypes based on Geimsa C-banding (Liang 1988) and staining with the intercalating fluorochromes chromomycin A3 (CMA) and 4′-6-diamidino-2-phenylindole (DAPI, Guerra 1993) show that many chromosome pairs must be heteromorphic. Staining citrus metaphase chromosomes with DAPI and CMA showed that several chromosomes contain large blocks of terminal heterochromatin (Miranda et al. 1997). Factors contributing to chromosomal heterozygosity in citrus include the origin of many accessions by interspecific hybridization and clonal propagation which allow accumulation of karyotypic rearrangements (Roose et al. 1998). The chromosomal identification of different genomes may be an additional and simple method of identifying citrus hybrids and could be of importance for future work on substitution lines. Citrus genome size is also relatively small, and the C value of *C. sinensis* was estimated to be 0.6 picogram per haploid DNA content (Guerra 1984), equivalent to approximately 367 Mb, which is nearly three times the size of *Arabidopsis* genome (125 Mb,

see the International Citrus Genome/Genomics Consortium home page, ICGC).

Most of the knowledge acquired on the inheritance of citrus traits was generally a by-product of the efforts of conventional breeding of rootstocks and scions. The dominance or recessiveness of these morphological traits was speculated according to the segregations of these phenotypes. Some characters such as cold hardiness, fruit acidity, leaf and rind oil, dwarfness, tolerance to chloride stress, resistance to *Phytophthora* and nematodes were roughly described as quantitative trait loci (QTLs). Characters such as polyembryony, trifoliate leaf and polyphenol oxidase-catalyzed browning appeared to be dominant over their allelic phenotypes, monoembryony (single gene), monofoliate leaf (two complimentary genes) and non-browning (single gene) (Soost and Cameron 1975). Rind texture was found to segregate as QTL with a dominant tendency (Yamamoto et al. 1990). According to early inoculation experiments and field survey, resistance to Asian citrus canker (ACC) was also thought to be a dominant trait (Lee 1918) and later, after genetic analysis of dozens of populations, it was assumed to be governed by a single dominant gene (Matsumoto and Okudai 1990). Using ELISA (enzyme-linked immunosorbent assay) on several *Poncirus*-derived populations, resistance to CTV was found to be dominant and was assumed to be controlled by a single gene (Yoshida et al. 1983; Yoshida 1985). By mapping the desired genes, it would be possible to improve the efficiency of conventional plant breeding by carrying out selection not directly on the trait of interest but on molecular markers linked to genes influencing that trait. Efforts to develop localized linkage maps with associated molecular markers will be addressed in the following section.

14.2 Mapping of the Citrus Genome

Plant genome maps may include important linkage relationships among molecular markers and genes that breeders wish to manipulate for cultivar improvement, thereby increasing the efficiency of breeding programs. Long juvenile periods and large plant size combine to hinder conventional breeding of citrus by requiring large investments of time and land for characterization and evaluation of progeny. Mapping and sequencing of a citrus genome would help to elucidate gene function, gene regulation and expression. Genetic maps of citrus may provide the basis for early screening procedures, thus, permitting breeders to make initial selection among very young progeny based on the phenotype predicted by their genotype at molecular loci known to cosegregate with a particular phenotype (Durham et al. 1992).

The first linkage analysis of citrus genome began in the 1980s using isozyme markers. The two linkage groups, one with two markers and another with three markers, were found by a mapping program, Linkage-1 (Suiter et al. 1983) from 37 isozyme genes, using nine families of *C. grandis* Osbeck cv Acidless (pummelo) × *C. jambhiri* Lush. cv Florida and *C. grandis* Osbeck cv Chandler × *P. trifoliata* cv Webber-Fawcett (Torres et al. 1985). Codominant isozyme markers, though very limited, were contin-

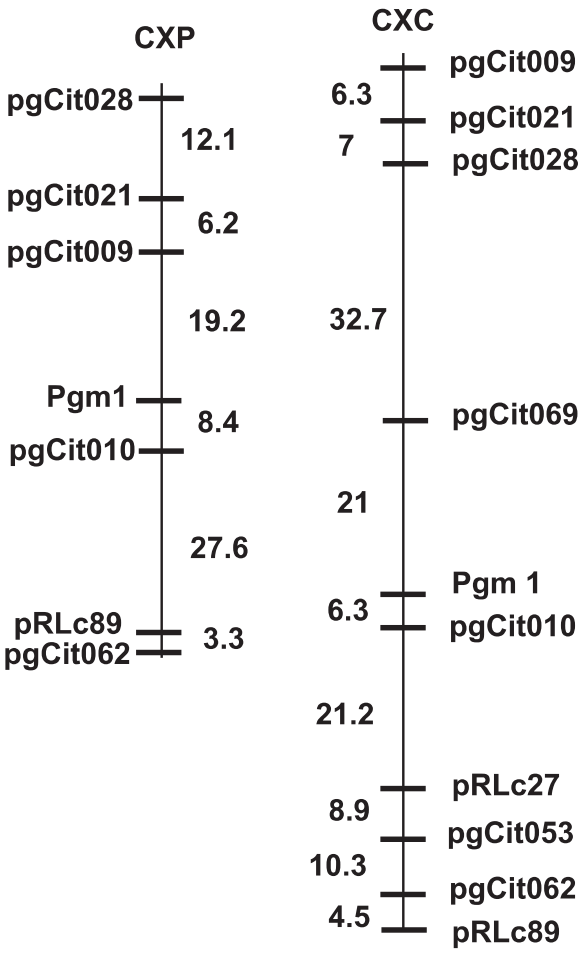

Fig. 2. Citrus linkage map (group 4) deduced from segregation data of two backcross populations of citrus using isozyme and RFLP markers (Durham et al. 1992)

uously used in the later DNA marker-based linkage maps due to their low cost and feasibility (Durham et al. 1992; Garcia et al. 1999). Isozymes may be influenced by the environment as well as by the stage of development of the plant and its organs, thus making the method less reliable; however many citrus isozyme locus linkage studies were conducted using selected systems that were found to be invariant when run on starch gels and using different stages or tissues sources. Throughput of such marker systems was extremely low.

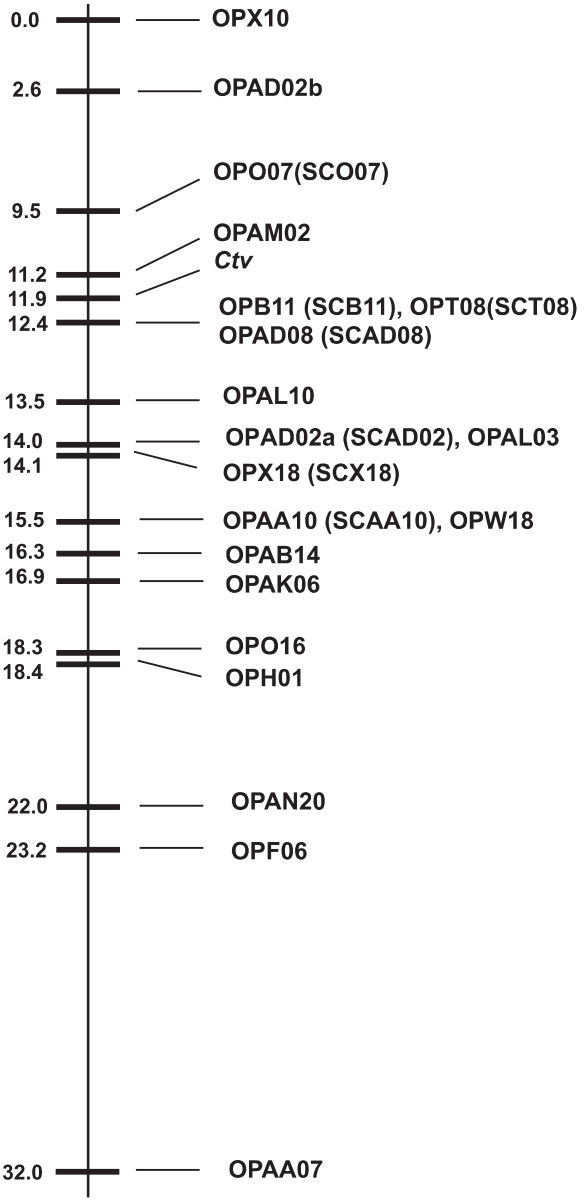

Fig. 3. A localized linkage map of *Ctv* region of *Poncirus trifoliata* constructed using RAPD and SCAR markers (Deng et al. 1997)

Several researchers have used restriction fragment length polymorphism (RFLP), a DNA based marker, for citrus mapping (Liou 1990; Durham et al. 1992; Jarrell et al. 1992; Liou et al. 1996). After cloning and characterizing RFLP markers in *Citrus*, Liou (1990) developed a citrus RFLP-based map, comprising of 29 RFLP and 8 isozyme loci in eight linkage groups. Three other RFLP-based linkage maps have been developed within citrus. Each was constructed from highly heterozygous intergeneric crosses which allowed a range of segregating characteristics to be genetically dissected. One map contained 46 markers (Jarrell et al. 1992) while the other two, constructed within the same cross, had a total of 62 markers (Durham et al. 1992) and each contained 11 linkage groups. Durham et al. (1992) were the first to demonstrate the potential of combining RFLP and isozyme analyses (Fig. 2) for developing a genetic map for citrus and reported that a total of 11 isozymes and 58 RFLPs segregated in a monogenic fashion.

Citrus maps, using other DNA based molecular markers such as randomly amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSRs) and amplified fragment length polymorphism (AFLP) were also constructed for obtaining much higher density of entire genome coverage (Cai et al. 1994; Luro et al. 1994, 1996; Weber and Moore 1996; Simone et al. 1998; Ling et al. 1999; Roose et al. 2000; Sankar and Moore 2001). Several traits of horticultural importance including CTV resistance (Gmitter et al. 1996), nematode resistance (Ling et al. 2000), fruit acidity (Fang et al. 1997) and dwarfing (Cheng and Roose 1995) have been tagged with RAPD markers. The second of the two maps developed by Durham et al. (1992) was extended to include 109 RAPD markers, which condensed to nine linkage groups with a total length of 1,192 cM estimated to cover 70–80% of the *Citrus* genome (Cai et al. 1994).

ISSRs and AFLPs provide a relatively high-throughput polymorphism facilitating the development of dense maps that are effective for identifying markers linked to major genes (Roose 2000). Sanker and Moore (2001) evaluated the usefulness of ISSR analysis in generating markers to extend the genetic linkage map of citrus using a backcross population previously mapped (Durham et al. 1992) with RFLP, RAPD and isozyme markers (Cai et al. 1994). The new map has an improved distribution of markers along the linkage groups with fewer gaps; marker

Table 2. Crosses used for genetic maps of citrus genome

No	Crosses	Cross types	Progeny	Country
1a	*C. grandis* cv. Acidless × *C. jambhiri* cv. Florida	*Citrus* F1	35	USA
1b	*C. grandis* cv. Chandler × *P. trifoliata* cv. Webber-Fawcett	Intergeneric F1	360	USA
2	LB 1-21 (*C. reticulata* cv. Clementine × *C. paradisi* cv. Duncan) × *C. reticulata* cv. Clementine	*Citrus* BC1	65	USA
3	*C. grandis* cv. Thong Dee × USDA 17-40 (*C. grandis* cv. Thong Dee × *P. trifoliata* cv Pomeroy)	Intergeneric BC1	65	USA
4	Sacaton (*C. paradisi* × *P. trifoliata*) × Troyer (*C. sinensis* × *P. trifoliata*)	Intergeneric F1	60	USA
5a	*C. grandis* × *C. grandis*	*Citrus* self F1	52	France
5b	*C. reshni* × *P. trifoliata*	Intergeneric F1	52	France
6	*C. auratium* × *C. latipes*	*Citrus* F1	120	Italy
7a	*C. auratium* × *P. trifoliata* cv Flying Dragon	Intergeneric F1	66	Spain
7b	*C. volkameriana* × *P. trifoliata* cv Rubidoux	Intergeneric F1	80	Spain
7c	Self-pollation of *P. trifoliata* cv Flying Dragon	*Poncirus* self F1	57	Spain
8	*C. sunki* × *P. trifoliata*	Intergeneric F1	80	Brazil

order showed partial or complete conservation in the linkage groups, suggesting that ISSR markers are suitable for genetic mapping in citrus. Fang et al. (1998) identified RAPD and ISSR markers linked to the *Ctv* region in *P. trifoliata*. The genome map of an intergeneric backcross population of citrus, constructed using AFLP, gave 16 linkage groups covering 910.7 cM; when combined with the RAPD-based map, it generated 14 linkage groups covering 1,031.7 cM (Ling et al. 1999). Recupero et al. (2000) reported data on *C. aurantium* and *C. latipes* molecular maps based on a two-way pseudo-testcross mapping strategy using AFLP, RAPD, and RFLP markers.

Dominant markers like RAPDs, ISSRs and AFLPs are useful in the specific population in which they are identified, but are difficult to apply to other populations due to their biallelic nature, which reduces the probability of polymorphism (Roose 2000). To overcome the problems associated with RAPDs, they have also been converted into sequence characterized amplified region (SCAR) markers. This is done by cloning and sequencing RAPD products, designing longer specific primers based on the sequence and amplifying DNA under stringent conditions. SCARs have been developed linked to the *Ctv* resistance gene (Fig. 3) from *P. trifoliata* (Deng et al. 1997). SCAR markers have also been used in the studies on inheritance of citrus nematode resistance (Ling et al. 2000).

Table 2 lists the major crosses that were used in Table 3 for construction of different versions of citrus maps. As these maps (developed in different laboratories) share few common markers, there has generally been very little effort to inter-relate them. Since RAPD or AFLP markers are dominant, they are hardly used as chromosome-anchored markers for general reference and comparative mapping. Hence, recently, locus-specific DNA molecular markers such as simple sequence repeats (SSRs), expressed sequence tags (ESTs), or sequence tagged sites (STS) have also been integrated into these maps (Kijas et al. 1995; Kijas et al. 1997; Ruiz and Asins 2003). Roose et al. (2000) tested nine trinucleotide SSR markers developed by Kijas et al. (1997) in a population derived from *C. taiwanica* × *P. trifoliata* and observed that one of the primer pairs tested had the segregation type desired for combining maps. Cristofani et al. (2000) also used SSRs to construct linkage maps of *P. trifoliata* and *C. sunki*. They further reported a total of 78 RAPD, 3 SSR and 10 AFLP markers to fall into 11 linkage groups of *C. sunki* and 73 RAPD, 4 SSR and 9 AFLP to fall in 11 linkage groups of *P. trifoliata*. The integration of SSR markers into a linkage map of *Citrus* has demonstrated the utility of this marker type for genetic analysis within wide intergeneric crosses and the potential to act as "anchor loci" to align linkage maps from different crosses and laboratories (Kijas et al. 1997). However, their number was still too lim-

Table 3. Genetic maps developed for citrus genomes

References	Marker types	Markers	Linkages	cM	Crosses No[a]
Torres et al. 1985	isozymes	5	2	–	1a, 1b
Liou 1990	RFLP, isozymes	35	8	314	2
Durham et al. 1992	RFLP, isozymes	52	11	533	3
Jarrell et al. 1992	RFLP, isozymes	38	10	351	4
Cai et al. 1994	RAPD, RFLP, isozymes	189	9	1,192	3
Luro et al. 1996	RAPD	34 for *C. grandis*	7	600	5a, 5b
		95 for *Poncirus*	12	1,503	
Kijas et al. 1997	RFLP, ISSR	48	12	410	4
Simone et al. 1998	AFLP, RAPD, RFLP	247 for *C. aurantium*	20	1,000	6
		92 for *C. latipes*	12	600	
Ling et al. 1999	AFLP, RFLP, isozyme	337	11	1,026	3
Garcia et al. 1999	RAPD, RFLP, CAPS, isozyme	69	3	–	7b
Cristofani et al. 1999	RAPD	63 for *C. sunki*	10	732	8
		62 for *P. trifoliata*	8	866	
Roose et al. 2000	RAPD, RFLP, ISSR	156	16	701	4
Sankar and Moore 2001	ISSR, RAPD, RFLP, isozymes	310	9	874	3
Ruiz and Asins 2003	RAPD, SSR, IRAP	48 for *Poncirus*	10	–	7a, 7b, 7c
		120 for *C. auratium*	17		

[a] Cross number is cited from Table 2.

ited at that time to expand their comparative and integrative usage in subsequent citrus genomic exploration.

Recently, ESTs have proven to be powerful tools for gene discovery, gene mapping and for the analysis of quantitative traits. ESTs are generated by large-scale sequencing of randomly picked clones from cDNA libraries constructed from mRNA isolated at a particular development stage and/or tissue; these sequences are available from the public domain such as GenBank (Guo et al. 2004). From the total EST sequence database, a representative set of unigenes are derived and their functions are compared to genes of known function from other organisms. Arrays can be designed using the unigenes to observe the spatial and temporal expression profiles of the available citrus genes. Many ESTs also contain SSR sequences, and through data mining these can be identified and exploited, thus increasing greatly the number of SSRs available as anchoring loci. Development of EST-based genetic maps covering the entire citrus genome is under way and will lay the basis of integration with physical maps for future genome sequencing (Chen et al. 2006).

14.3 Molecular Tagging and Cloning of Specific Genes and QTLs

Molecular marker technologies have provided tools to tag the genes of known phenotypes by developing localized molecular linkage maps, which are essential for a map-based cloning (MBC) approach and marker-assisted selection (MAS) breeding programs. With QTL mapping, the roles of specific resistance loci can be described, race-specificity of partial resistance genes can be assessed, and interactions between resistance genes, plant development and the environment can be analyzed. Resistance to CTV, apparently, was the first phenotype in citrus for which a localized map was developed (Gmitter et al. 1996). The linkage map contained DNA markers associated with its designated conferring gene, *Ctv*. The *Ctv* gene was identified from *Poncirus* and was assumed to be a single dominant gene (Yoshida et al. 1983; Yoshida 1985, 1993). This map was developed using a bulked segregant analysis (BSA) approach (Michelmore et al. 1991) and RAPD markers. Since then, several labs have independently made efforts to

map and clone *Ctv*. Some of these maps shared several common markers (Gmitter et al. 1996; Fang et al. 1998), while others did not (Mestre et al. 1997a; Cristofani et al. 1999). Two BAC contigs (Fig. 4) with integrated fine genetic maps have been constructed (Deng et al. 1997, 2001; Yang et al. 2001), resulting in full length sequencing of the locus spanning several hundreds of kilobases and identification of the candidate genes (Yang et al. 2003; Gmitter et al. unpublished data). These putative *Ctv* gene(s) are now under further confirmation using genetic transformation and complementation tests. However, evidence after prolonged CTV challenge has suggested that there may be more than one CTV resistance genes involved (Mestre et al. 1997b). Under a similar prolonged CTV challenge on a population of citradias (derived from the cross between sour orange and *Poncirus*), one CTV resistance gene was later mapped in a different location within linkage group 4 of *Poncirus*. The change of mapping position was interpreted as a deviation from the single gene hypothesis, which could be QTLs (Bernet and Asins 2003). Further, by QTL analysis of CTV-citradia interactions, considering CTV accumulated titer as a quantitative trait, up to five minor QTLs were detected besides the previously located major *Ctv* gene (Asins et al. 2004). There were other pathogen resistant phenotypes mapped. For example, it was found that a major QTL, designated *Tyr1*, controls resistance to citrus nematode (Ling et al. 2000), and it was adjacent to the *Ctv* region (Ling 1999). Nineteen putative QTLs (8 in *C. volkameriana* and 11 in *P. trifoliata*) controlling the number of fruits per tree were detected in the *C. volkameriana* and *P. trifoliata* progeny (Garcia et al. 2000). Mapping of QTLs associated with freezing tolerance was accomplished using a *C. grandis* × *P. trifoliata* F_1 pseudotestcross population (Weber et al. 2003). Other QTLs controlling some fruit characters and tolerances to salt and cold stress were characterized and DNA linkage maps for these traits have also been constructed (Table 4).

Molecular maps, particularly of those with very closely flanking or co-segregating DNA markers with the gene(s) of interest, may be very useful for fur-

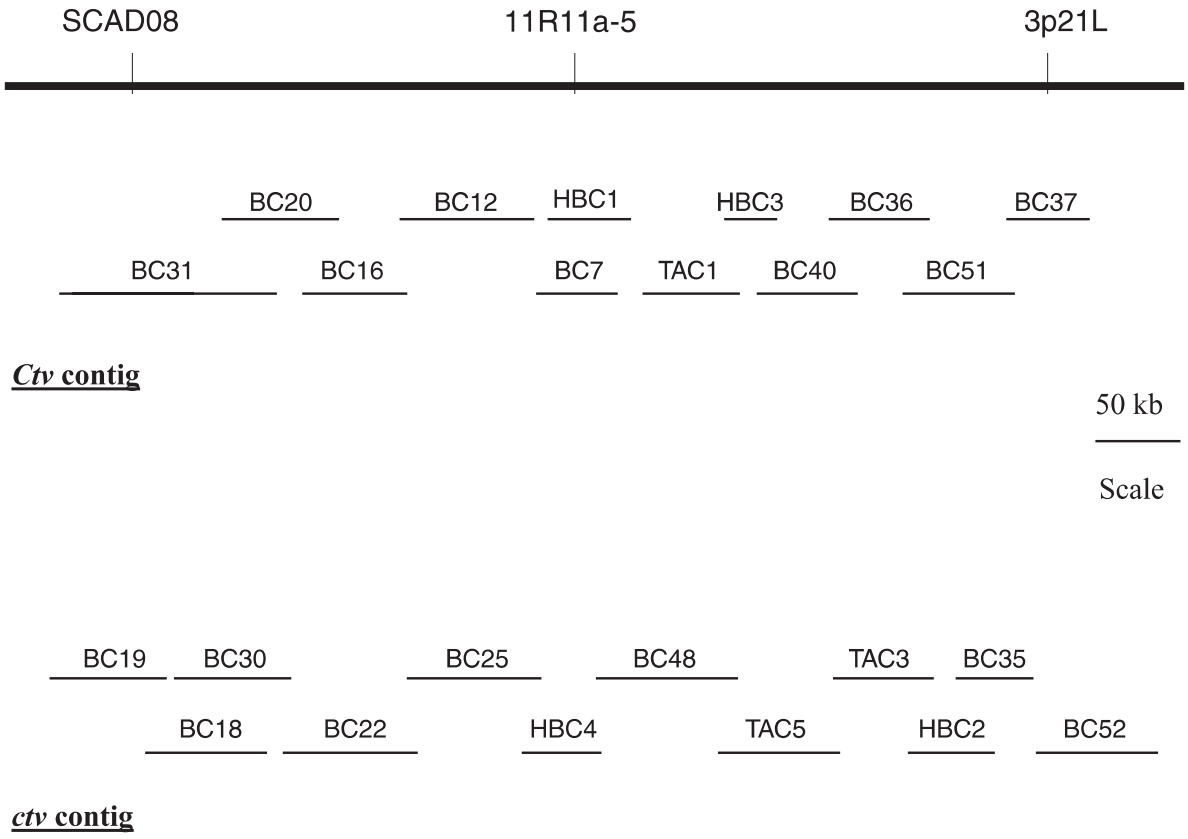

Fig. 4. BAC contigs of CTV resistance gene region (*Ctv* contig) and its allelic susceptibility gene region (*ctv* contig, Deng et al. 2001)

ther genomic manipulation, MBC and MAS breeding programs (Recupero et al. 2000; Asins 2002). MBC, also called positional cloning, is an approach using comprehensive genetics, genomics and bioinformatics tools to isolate gene(s) without prior knowledge of gene product. In theory, flanking or co-segregating DNA markers associated with the gene of interest are identified and a contig covering the target gene region with large insert genomic DNA clones (usually bacterial artificial chromosomes or BACs) are constructed. Sequencing of the spanning physical region and subsequent analysis of the sequence using various gene prediction programs will result in identification of the gene candidate sequences that can be confirmed by complementary test after genetic transformation into the host plant. This strategy has been employed to isolate *Ctv*, a single dominant gene from *P. trifoliata* that confers resistance to CTV (Gmitter et al. 1998; Deng et al. 2001). The resistance gene contig consists of 20 BACs and is approximately 550 kb in length. The susceptibility gene contig, derived from the susceptible citrus chromosome region consists of 16 BACs and extends to about 450 kb (Deng et al. 2001). Although it now takes less than 1 person-year to isolate a gene in *Arabidopsis* (Jander et al. 2002), application of similar tools in cloning citrus gene(s) would take a much longer time to reach the goal. For example *Ctv*, the only citrus gene upon which extensive MBC efforts have been made, has taken about ten years to reach the last step partly due to genetic restraints. However, MBC will make trait-specific improvement of citrus cultivars and rootstocks possible. Isolation and deployment of gene(s) of interest will greatly benefit the citrus industries and would enable a deeper understanding of the citrus genome.

Morphological traits such as dominant trifoliate leaf, considered to be the earliest MAS marker, were easily used to distinguish zygotic hybrids from nucellar seedlings. However, they cannot be used with varieties without such distinct characters. Therefore, common MAS is carried out with the aid of generic biochemical and DNA based markers. Isozymes, RAPDs and EST-SSRs have also been used in the identification of hybrids (Soost and William 1980; Nageswara Rao et al. 2006). The use of DNA based molecular markers to select rootstocks that

Table 4. Mapped citrus phenotypes

Phenotypes	Genotypes	Genetic map
Resistances to		
Citrus tristeza virus (CTV)	Single dominant[a]	Gmitter et al. 1996
Nematode	QTLs	Ling et al. 2000
Citrus variegated chlorosis (CVC)	QTLs	Oliveira et al. 2002
Alternaria	QTLs	Dalkilic et al. 2005
Asian citrus canker (ACC)	QTLs[a]	Choi et al. 2005
Citrus leaf miner (CLM)	QTLs	Bernet et al. 2005
Tolerance to		
Cold accumulation	QTLs	Cai et al. 1994
Na^+ stress	QTLs	Tozlu et al. 1999a
Cl^- stress	QTLs	Tozlu et al. 1999a
Salinity	QTLs	Tozlu et al. 1999b
Freezing	QTLs	Weber et al. 2003
Characters of		
Dwarfing	Single dominant	Cheng and Roose 1995
Acidity	QTLs	Fang et al. 1997
Apomixis	QTLs	Garcia et al. 1999
Nucellar embryony	QTLs	Kepiro and Roose 2000
Yield and seed number	QTLs	Garcia et al. 2000
Rooting	QTLs	Siviero et al. 2003

[a] Different genotypes, which may or may not be from the same locus, were found to have the same phenotype name.

may contain many of the desired resistances to CTV, nematode, *Phytophthora,* etc., will be of very high cost-efficiency as compared to traditional greenhouse or field screening approaches using inoculation. Blind tests of several rootstock selection populations of known phenotypes using DNA markers associated with CTV and nematode resistance genes indicated that MAS is a very promising and highly effective tool for breeding programs (Gmitter et al. unpublished data). In addition, molecular markers have also been widely applied on phylogenetic and taxonomic studies (Herrero et al. 1996; Fang and Roose 1997; Fang et al. 1997; Bret et al. 2001).

14.4 Citrus Genome Plan and Future Trends

The expanding capabilities of genomics and bioinformatics have the potential to revolutionize the entire field of citrus biology and genetics, and they offer promise of greatly improving the cultivars that are grown through precise and targeted manipulations of the genome. The foundations for the future are currently being laid by the international citrus genomics community. Many citrus genetic linkage maps have been developed in different laboratories in the past two decades (Table 3). Due to the use of very few common markers, it is difficult to interrelate the various linkage groups identified on these maps. Hence, it is essential to develop a reference map of the model genotype (Chen et al. 2006). The reference map should include a set of markers that are highly polymorphic and which can be mapped in various populations. This will allow various maps to be compared and combined. Also, more molecular markers are needed to saturate the genome. Long term studies of the nature and mode of inheritance of economically important traits must be pursued to link these difficult-to-evaluate traits more easily to the scored markers (Gmitter et al. 1992). Limited efforts have been made for single-case characterization of a citrus genome in a few areas such as resistance gene candidates (RGCs) (Deng et al. 2000; Deng and Gmitter 2003; Bernet et al. 2004), retrotransposons (Asins et al. 1999; Bernet and Asins 2003), microsatellites (Kijas et al. 1995; Ahmad et al. 2003), satellites (Fann et al. 2001), variations from fragment restriction (Liou et al. 1996), methylation (Cai et al. 1996), and individual gene expressions (Moriguchi et al. 1998; Shimada et al. 2005). Physical maps of citrus, integrated with genetic linkage maps, are also required for efficient localization and isolation of the genes, for studying the organization and evolution of the genome, and as an initial step for efficient whole genome sequencing by serving as the scaffold onto which genomic sequence will be assembled.

Large-scale citrus genome plans have been launched in several countries, and the International Citrus Genome Consortium (ICGC) has been initialized, to provide general guidance and overall goals on a citrus genome sequencing plan. The countries that are leading in planned citrus genome sequencing research are Brazil, US, Spain, Japan, France, Australia, China, Israel and Italy (Machado et al. 2005; Omura et al. 2005; Roose et al. 2005; Talon et al. 2005). Those activities, already done, being done, and to be done, include sequencing ESTs, developing microarray platforms for expression and genotyping studies, constructing genetic maps using ESTs or SNPs, fingerprinting large insert clones to develop physical maps, integrating genetic and physical maps, and eventually the complete sequencing of one or two citrus genome(s). A great number of citrus EST sequences, for example, over 200,000 in Brazil, and over 100,000 in the US, have been acquired (Fig. 5). A citrus GeneChip® from Affymetrix, containing 30,264 citrus unigene probe sets and other important contents, has been recently released (Close et al. 2006). Some are partially or completely released to the public database such as GenBank, and others are still kept for in-house use. Regardless of the availability, these contributors and other citrus genomic research communities have been utilizing comprehensive bioinformatics tools to categorize them, to design gene chips, to explore genetic information such as SSRs and SNPs, for large-scale gene expression and function studies, genetic and physical mapping, and comparison with other advanced genomes (Hisada et al. 1997; Fujii et al. 2003; Boscariol et al. 2005; Terol et al. 2005). Great progress on citrus genome research has been, and is being made, by these efforts eventually leading to the completion of an international citrus genome plan.

Citrus genomic technology is essential for the sustainability and future viability of the world's citrus industries. Most of the critical goals for scion improvement, such as resistance to devastating diseases or quantum changes in fruit quality attributes (color,

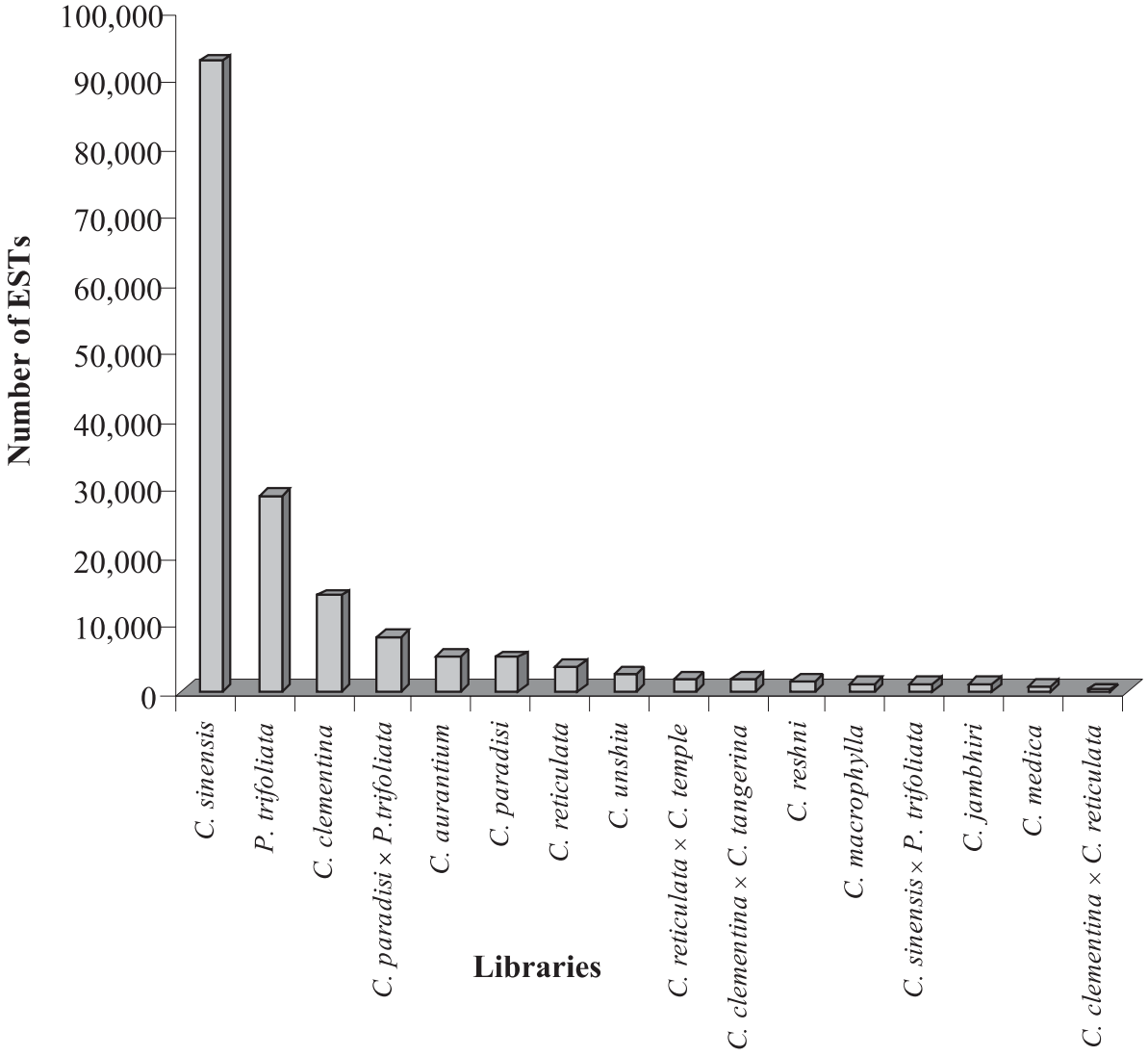

Fig. 5. Current *Citrus* EST entries in GenBank dbEST [Modified from International Citrus Genome Consortium (ICGC)]

flavor, peelability, nutrient content, and phytonutrient value to improve human health), are difficult if not absolutely impossible to approach in any practical sense by conventional breeding strategies. It will be through genomic research that an understanding of fundamental processes can be realized, candidate genes can be identified and cloned, and through some type of genetic transformation these genes and this information will be exploited for the improvement of citrus. Likewise, rootstock improvements will be hastened and maximized through the application of new knowledge and tools developed from it, to make sexual hybridization remarkably more efficient. It is important to keep in mind, however, that the value and utility of new genetic combinations must be demonstrated ultimately by field trials to verify the function and productivity of genetically modified citrus, and the value to consumers of improvements in fruit quality will be proven in the marketplace.

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15 Banana

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15.1 Introduction

Genomics is a rapidly emerging field of research, which came into existence at the end of the last century and promises to become the dominant theme of intellectual activity in the coming decades, revolutionizing our understanding of biology as never before (Maheshwari et al. 2001). It took approximately 100 years from the rediscovery of Mendel's work to the complete sequencing of a higher plant (*Arabidopsis*). The advent of genomics is the direct result of the development DNA sequencing by Sanger et al. (1977) and Maxam and Gilbert (1977). However, genome-wide sequencing could not have proceeded without extensive automation such as high throughput sequencing and the innovation of methods such as fluorescent primers, laser excitation of DNA bands, and the detection of these bands with photomultipliers (Smith et al. 1985, 1986). DNA sequencing makes it possible to decipher the entire blueprint of the development of an organism, its genes and their functions. Genomics can play a vital role in agriculture. The world's population depends to a very large extent on a few crops like rice, wheat, maize, bananas, beans and potato for their food. Understanding the genomics of these crops can lead to enhanced yields and survival under adverse conditions. In the next 20 years the world faces the tremendous challenge of feeding the global population from rapidly diminishing resources. The study of plant genomics helps reveal the alleles and biochemical pathways linked to many processes such as flowering, nutrition, disease and pest resistance, as well as their tolerance to abiotic stresses (Zandstra 2005).

Despite the importance of *Musa* as a food crop for over half a billion people worldwide, genomic research currently undertaken can best be described as a patchwork of initiatives undertaken by a few advanced laboratories. Genetic and physical maps of *Musa* are being developed by CIRAD (Centre de Cooperation Internationale en Recherché Agronomique pour le Developpement), France, and the Institute of Experimental Botany, Czech Republic. The BAC (bacterial artificial chromosomes), BIBAC (binary bacterial artificial chromosomes) libraries and ESTs (expressed sequence tags) have been developed by laboratories in Mexico, France and Europe (Vilarinhos et al. 2003; Ortiz-Vaquez et al. 2005). Segregating populations are being developed by the International Institute of Tropical Agriculture (IITA), Nigeria and Centre Africain de Recherches sur Bananiers et Plantains (CARBAP), Cameroon and CIRAD. The Queensland University of Technology (QUT) in Australia is identifying genes for resistance to *Fusarium* wilt. Genetic transformation of bananas is routinely being done at the Katholieke Universiteit Leuven (KUL), QUT and IITA. Sequencing of BAC clones is being conducted at KUL and The Institute for Genomics Research (TIGR). Activities for discovery of functional genes in *Musa* are being undertaken in Israel (Khayat 2004). This chapter provides an overview of banana breeding and genome analysis of *Musa.*

15.1.1 Botanical Origin and Distribution of Banana

Bananas and plantains (*Musa* spp. L), hereafter referred to as bananas are perennial monocotyledonous herbs that grow well in humid tropical and subtropical regions. There is a wide variety of historic references to bananas and the crop is mentioned in ancient Hindu, Chinese, Greek and Roman texts. The earliest reference to banana dates back to about 500 BC. Some horticulturists suspect that banana was the earth's first fruit. Nevertheless, the origin of bananas is traced back to Southeast Asia in the jungles of Malaysia, Indonesia or Philippines (Simmonds 1966, 1987). Banana originated from two wild diploid ($2n = 22$) species namely, *Musa acuminata* Colla and *M. balbisiana* Colla, with genomic compositions of AA and

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BB, respectively (Cheesman 1948). *Musa accuminata* is a native of the Malay Peninsula and adjacent regions while *M. balbisiana* is found in India eastwards to the tropical Pacific (Simmonds 1966). Many wild varieties still exist in the natural vegetation of Southeast Asia, the center of origin. Although South East Asia and the Western Pacific are believed to be the primary center of origin and domestication of edible bananas (Simmonds 1962; Robinson 1996; Jones 2000), they are also widely distributed in the humid tropical and subtropical world. From Asia, bananas and plantains are believed to have spread throughout the humid tropics (Swennen and Ortiz 1997; Valmayor 2000) solely by humans through suckers (Simmonds 1962). The history of banana cultivation is therefore closely linked to the early movement of human populations. Movement eastwards resulted in the development of a distinct group of AAB bananas, which are cultivated throughout the Pacific Islands. In Africa, banana is thought to have been introduced by Arab traders from India, through Madagascar on the Eastern Coast during the 15th century (Simmonds 1962). The crop was then moved inwards by local migrants and later, from Africa it spread to other parts of the tropical and subtropical world. A great diversity of bananas and plantains now exist in sub-Saharan Africa with various types cultivated in different eco-regions (Swennen and Vuylsteke 1991). The AAB plantains dominate the humid lowlands of West and Central Africa while the AAA cooking and beer bananas prevail in the East African Highlands. The latter two eco-regions harbor the world's greatest diversity of plantains and highland bananas, respectively, and are thus considered the secondary centers of diversification of plantain and bananas (Swennen 1990). The secondary centers of diversification are believed to have enriched the diversity of *Musa* with about 100 clones each (Lescot 2000).

15.1.2 Taxonomy of *Musa*

Banana belongs to the genus *Musa* in the family *Musaceae*, order Zingiberales. It belongs to the subclass *Zingiberidae*, Class *Liliopsida* and Division *Magnoliophyta*. The family *Musaceae* comprises two genera viz., *Musa* and *Ensete* (Fig. 1). The genus *Ensete* consists of monocarpic herbs none of which bears edible fruit. The genus was formally recognized by Cheesman in 1948 comprising 25 species. However, only eight species are now recognized in the genus *Ensete* (Novak 1992). *Ensete* is cultivated in Southern Ethiopia as a major source of food that is obtained from the pseudostem and rhizome (Novak 1992). Only two species, *E. ventricosum* and *E. edule,* are of economic importance as food and fiber crops (Bezuneh and Feleke 1966). Genetic relationships between *Musa* species and *Ensete* clones based on genome size, number of chromosomes and number of 45S rDNA loci showed that *Ensete* is related to *M. beccarii* of section *Callimusa* (Bartos et al. 2005).

The genus *Musa* comprises all the edible bananas and plantains with over 50 species. Five sections namely, *Australimusa, Callimusa, Rhodochlamys, Eumusa* and *Ingentimusa* exist in the genus *Musa* (Stover and Simmonds 1987; Purseglove 1988). These sections vary in the basic chromosomes, i.e. species of *Callimusa* and *Australimusa* have a basic chromosome number of $x = 10$, while species in *Eumusa* and *Rhodochlamys* have a basic chromosome number of $x = 11$. *Ingentimusa* has a single species *M. ingens* with a chromosome number of $2n = 14$. *Musa ingens,* a highland banana in Papua New Guinea, is the largest known herb (Argent 1976). Sections *Callimusa* and *Rhodochlamys* consist of non-parthenocarpic species with no nutritional value and are only important as ornamental crops. *Australimusa* consists of parthenocarpic edible types, collectively known as Fe'i cultivars. The Fe'i bananas have erect fruit bunches and red sap which differentiates this section from other cultivated bananas. Bananas in this section are not only important for food and fiber but their pseudostems also produce a valuable dark red dye which is used in various ways. The origin of Fe'i is controversial among banana authors. Simmonds (1966) suggested that *M. maclayi* is the most likely ancestor, while Cheesman (1950) suggested that Fe'i is closely related to *M. lolodensis*. RFLP analysis by Jarret et al. (1992) revealed that Fe'i was indeed closest to *M. lolodensis* and thus concurred with Cheesman's (1950) findings. *Eumusa* is the largest, most widely distributed, highly diversified and the most important section to which all edible bananas belong. Most cultivars in this section are derived from two species, *Musa acuminata* (A genome) and *M. balbisiana* (B genome). *Musa acuminata* is the most widespread of the *Eumusa* species being found throughout the range of the section with Malaysia (Simmonds 1962) or Indonesia (Nasution 1991; Horry et al. 1997) as the center of diversity.

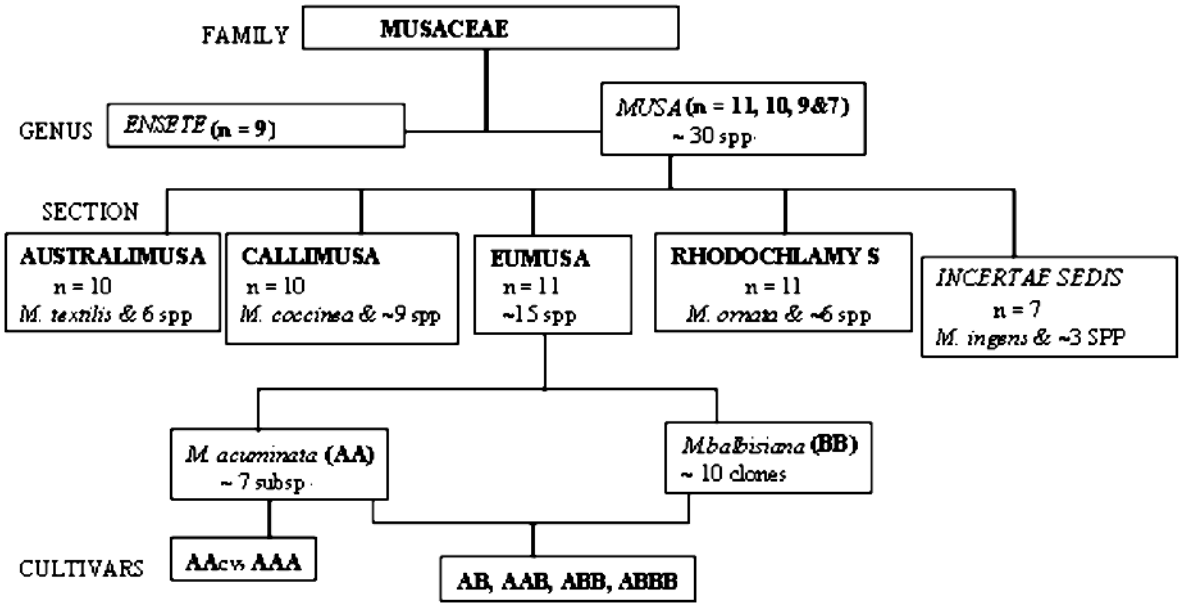

Fig. 1. Classification of Family *Musaceae* showing sectional treatment of the genus *Musa*. Based on Cheesman (1948); Simmonds (1962); Simmonds and Shepherd (1955)

Edibility and subsequent domestication of diploid *M. acuminata* (AA) came about as a result of female sterility and parthenocarpy. Triploid AAA cultivars arose from diploids, perhaps, following crosses between edible diploids and wild *M. acuminata* subspecies, giving rise to a wide range of diverse AAA genotypes such as the AAA dessert bananas and the AAA East African Highland bananas. These two AAA groups of bananas differ markedly in their fruit characteristics with regard to starch content and taste. This suggests that the A genomes of the different subspecies of *Musa* are different from each other. In most parts of South East Asia triploids have replaced the original AA diploids due to their larger fruit and vigorous growth. However in Papua New Guinea, AA diploids remain agriculturally significant and a wide diversity is still found in cultivation.

15.1.3 Botanical Description and Morphology of Banana

East African Highland beer and cooking bananas grow best at altitudes between 1,200 and 1,800 meters above sea level, while dessert bananas and plantains thrive well in the lowlands. Bananas grow well in deep loamy and well-drained soils. The optimum temperature for most cultivated bananas is 26–30 °C (Stover and Simmonds 1987). Temperatures lower than the optimum result in low leaf production, thus limiting the supply of food from the limited photosynthetic leaf area. Banana growth stops at temperatures beyond 38 °C and dies at temperatures below 0 °C. A relative humidity of 60–100% is necessary for proper banana growth, and depending on the evapotranspiration, 25–75 mm of water is required by a banana plant per week which is equivalent to 100 mm rainfall per month. Bananas are prone to wind damage because of weak pseudostems, large leaves that trap wind and shallow root system.

A banana plant is a giant perennial herb with a height of 1.5 to 9 m. It consists of a true stem called corm with roots and a false stem (pseudostem) consisting of leaf sheaths. At maturity, the leaves surround the "heart" that carries the bunch with fruits. The stem (corm) is usually underground and its shape is cultivar dependent. However, in most cultivars the corm is round with the apical meristem at its tip. The meristem remains below the soil level until flowering when it develops into the flower inflorescence axis that bears the bunch. Roots develop from the corm from the region between the inner zone (central cylinder) and the outer zone (cortex) into the soil. Leaves also develop from the meristem of the corm and consist of a sheath, a petiole and lamina or blade. The leaf sheaths' of successive leaves overlap and closely

encircle each other forming the pseudostem or the false stem. The pseudostems of Highland and dessert bananas are green to dark green with many black blotches, while those of plantains are yellowish green with few brown-black blotches. As new leaves develop at the meristem, older leaves are pushed outwards, die and dry out (Simmonds 1962). Most bananas produce approximently 30–40 leaves in its lifetime. After a fixed number of leaves are produced, the meristem gives rise to the flowering stem, which begins to grow upwards through the pseudostem. The flowering stem emerges in the middle of the leaf crown and a complex inflorescence of flower clusters develops. The female flowers appear first and have large ovaries that develop into fruits. As the inflorescence develops, a bulb shaped male bud containing small flowers develops at the end. However, in most cultivated bananas, the fruit develops by parthenocarpy preventing formation of seeds that would otherwise make the fruit unsuitable for human consumption. Three types of flowers are produced on the banana inflorescence. The female (pistillate) flowers develop into the fruit, while the male (staminate) flowers found in the male bud may produce pollen that may or may not be fertile. The third type of flowers called hermaphrodite or neuters are found on the inflorescence axis or rachis between the female flowers and the male bud. They are usually sterile. The female flowers of most cultivated bananas are almost always sterile and the fruits develop by parthenocarpy. In all bananas the growing shoot dies after fruiting once (Simmonds 1962) and its life is perpetuated by means of suckers, which develop from adventitious buds produced on the corm. The suckers are the major form of vegetative planting material and form the subsequent vegetative generation. When the first plant fruits and dies, the maiden sucker (large but non fruiting ratoon with foliage leaves) continues the growth cycle. Bananas are propagated vegetatively through suckers, although wild species can also be propagated by seed (Stover and Simmonds 1987). Sucker development consists of three distinct stages; peeper (young sucker bearing scale leaves only), sword sucker (sucker bearing narrow sword leaves) and maiden sucker (large but non fruiting ratoon with foliage leaves) (Simmonds 1966; Swennen et al. 1984). The cluster formed by the mother plant and the surrounding suckers is referred to as a 'mat'. The number of suckers produced by a plant is very important to farmers in banana production since the crop is vegetatively propagated.

15.1.4 Importance of Bananas and Major Areas of Production

Bananas are the 4th world's most important food crop after rice, wheat and maize, with vast majority of the crop grown and consumed in the tropical and subtropical zones (FAO 2002). The annual world banana production is estimated at 98 million tons of which only 7 million tons enter the world market, suggesting that the crop is more important as food for local consumption than for export. Bananas supply more than 25% of the carbohydrate requirements for over 70 million people in humid forest and mid altitude region of Africa (Robinson 1996), with per capita consumption of approximately 250 kg. Its ability to produce fruits all the year round makes it an important food security crop and cash crop in the tropics (Jones 2000). Bananas are prepared and consumed in a number of ways with each country that produces the crop having its own traditional dishes and methods of processing (Frison and Sharrock 1998). For example, mature unripe bananas are eaten as a starchy food while ripe bananas are consumed raw as a dessert fruit. They can also be consumed boiled, roasted, or fried in ripe or unripe state. Nutritionally, fresh bananas contain 35% carbohydrates, 6–7% fiber, 1–2% protein and fat, besides the major elements such as potassium, magnesium, phosphorus, calcium, iron, and vitamins A, B6 and C (Robinson 1996). Bananas are also used in the manufacture of beer, wine and other products and form an important part of the cultural life of many people (Stover and Simmonds 1987). Other products produced from banana include jam, juice and squashes, banana chips or crisps, sweet banana figs, banana flour, banana powder and starch.

Although, a small proportion of banana production enters the world market, the banana fruit is extremely important as an export commodity especially in Latin America and Caribbean which contribute over 83% of the total banana in the international market (FAO 2002). In Africa, only five countries namely, Ivory Coast, Cameroon, Somalia, Ghana and Cape Verde export approximately 427,000 tons banana and plantain (FAO 2002). The introduction of refrigerated shipment has greatly accelerated the growth of the export trade from Central America and the Caribbean to other parts of the world. Most of the bananas exported are the dessert type from triploid cultivars of *M. acuminata*.

Table 1. Largest producers of banana/plantain in 2004 (FAOSTAT 2004)

Country	Production (metric tons)	Country	Production (metric tons)
India	16,820,000	Nigeria	2,103,000
Uganda	10,515,000	Mexico	2,026,610
Brazil	6,602,750	Thailand	1,900,000
Ecuador	6,552,000	Cameroon	1,830,000
China	6,420,000	Peru	1,660,310
Philippines	5,638,060	Côte d'Ivoire	1,602,423
Colombia	4,400,000	Burundi	1,600,000
Indonesia	4,393,685	Democratic Republic of Congo	1,412,000
Rwanda	2,469,741	Vietnam	1,353,800
Ghana	2,390,858	Guatemala	1,268,000
Costa Rica	2,230,000	Honduras	1,225,066

Because of their high vitamin A and B6 content, bananas are beneficial in the prevention of cancer and heart diseases in humans. Bananas are used to treat diseases such as gastric ulcer and diarrhoea. Vitamin rich nectar sap from banana flower buds is fed to babies and children to strengthen their growth, while potassium helps in boosting brain functioning.

Besides the food and income, banana plays many important roles. For example banana leaves can be used as thatching materials for houses, as plates, tablecloths, umbrellas, sleeping mats, animal feed and in food preparation. Non-fruit parts of the banana plant, including the corm, shoots, pseudostem and male buds are eaten as vegetables in Africa and parts of Asia (Simmonds 1962). The banana pseudostems can also be used as animal feed. Banana leaves and pseudostems contain a high quality fiber which is used for making ropes, handcraft, baskets, carpets and manufacturing of banana paper. In mixed cropping systems, banana plants provide shade for crops that grow better in shade conditions such as cocoa, black pepper, coffee and vanilla. At the system level, bananas maintain the soil structure and cover throughout the year, protecting it from wind and rain erosion. Further more, if the biomass is used as mulch, soil fertility and organic matter remains stable.

Between 1970 and 1997 the annual world banana production increased, from 51 million tons to 88 million tons, an increase of seventy percent (Sharrock and Frisson 1998). At that time banana production was estimated to be growing faster than the production of any other starchy crop in the world. The world's current banana/plantain production is estimated at about 104 million metric tones, grown on about 10 million acres of land in over 100 countries (FAOSTAT 2004). Africa produces 35% of banana and plantains, Asia and Pacific 29%, and Latin America and the Caribbean 35%. India is the world's leading producer of banana and plantain with a production of about 16 million tons followed by Uganda with 10.5 million tons (FAOSTAT 2004). Most bananas produced in Africa are used for local consumption and for local markets than for international trade. The major world banana producing countries are summarized in Table 1.

15.1.5 Genome Groups and Genome Size of *Musa*

Cultivated bananas are grouped on the basis of their genomic origins in relation to *M. acuminata* and *Musa balbisiana* and their ploidy level (Simmonds 1966). Currently, the known cultivars are the diploids (AA), triploids (AAA), and tetraploids (AAAA) forms of *M. acuminata* and diploids (BB), triploids and tetraploids (BBBB) forms of *M. balbisiana* or their hybrids (AB, AAB or ABB) (Simmonds and Shepherd 1955). However, other genomic groups including AAAB, AABB and ABBB from either natural or artificial hybridization are also known to exist (Pillay et al. 2004). The Indian subcontinent is thought to have been the major center of hybridization of 'acuminata' types with the indigenous *M. balbisiana*. The region is

known for its wide variety of AAB, and ABB cultivars (Price 1995). Triploid *M. acuminata* derived (AAA) cultivars are the most common and the most important grown cultivars, and include 'Gros Michel' and 'Cavendish' types (dessert bananas), which constitute most of the world's banana trade. Cooking and beer bananas (AAA) are indigenous to East Africa while plantains (AAB) are very important staple crop in West Africa and some parts of central Africa (Simmonds 1976). The B genome from *M. balbisiana* confers hardiness and resistance to drought observed in the diploid AB and triploid AAB and ABB hybrids (Purseglove 1988). *Musa balbisiana* derivatives show greater variability and produce fruits with more starch and acid, higher dry matter content and more vitamin C. They also give textures and flavors that are not characteristic in *M. acuminata* derived genotypes. *Musa acuminata* has traces of wax on fruits while *M. balbisiana* is often strongly waxy (Stover and Simmonds 1987).

Banana has a small haploid genome of 552–607 Mbp divided among 11 chromosomes. This is only 25% larger than rice, a crop that has been used as a model species in monocotyledon plant genomics studies. Due to its relatively small size, the *Musa* genome is highly amenable to complete functional and sequence analysis and extensive characterization of genes. Banana being one of the few plants with biparental cytoplasmic inheritance namely, mitochondrial paternal inheritance and maternal inheritance of chloroplasts, can act as a good genomic model.

15.1.6 Banana Breeding Objectives

Breeding programs of crops are designed/initiated to address production constraints. Banana production is affected by a wide range of pests and diseases, drought and low yielding cultivars. Therefore, the primary objective of banana breeding programs worldwide is to address these constraints and develop cultivars that are acceptable by farmers. The earliest focus in banana breeding programs was to develop disease-resistant dessert cultivars for export (Rowe and Rosales 1993) following the outbreak of *Fusarium* wilt (Panama disease) in 'Gros Michel'. Shortly thereafter the Cavendish variety that took over as the number one dessert banana replacing 'Gros Michel' was attacked by another fungus, *Mycosphaerella fijiensis*, which caused the Black Leaf Streak (BLS) disease or black Sigatoka. Consequently, breeding efforts for the genetic improvement of Cavendish by developing hybrids resistant to BLS were also initiated. Since then breeders especially in the banana exporting regions have been mainly aiming at developing banana cultivars similar to 'Gros Michel' but with resistance to Panama and leaf diseases (Sathiamoorthy and Balamohan 1993). Besides the diseases, other traits of concern in breeding include high yield, fruit quality (finger length, finger curvature and finger pedicel length), flavor, ripening, plant height (stature) and production efficiency (Stover and Simmonds 1987). Indeed several authors (Simmond 1987; Eckstein et al. 1995; Pillay et al. 2002) described and emphasized the ideotype cultivar as one which is disease and pest resistant, high yielding, photosynthetically efficient, early maturing, display minimum delay between consecutive harvests, short stature, strong roots for optimal nutrient uptake and greater resistance to wind damage.

Breeding for pest resistance especially against the banana weevil has, however, not featured prominently in any breeding program. This is probably because of the absence of good sources of resistance, and lack of a simple screening method for weevil resistance, to enable breeders to rapidly pinpoint resistant line across the available germplasm (Kiggundu et al. 1999). Nevertheless with the advancement of biotechnology, breeding objectives such as resistance to the banana borer, viruses, nematodes, and modification of the fruit ripening patterns are expected to be vigorously pursued.

15.1.7 Limitations of Classical Breeding of Bananas

The overall strategy in banana breeding is to incorporate the desired traits often harbored in wild and cultivated diploids (2×) such as resistance to diseases and pests to the existing cultivars rather than aiming at genetic materials that are completely different from the existing cultivars. However, most of the cultivated clones are triploids (3×) characterized by low male and female fertility (Vuylsteke et al. 1993). The high level of sterility is attributed to uneven chromosome distribution during meiosis that is characteristic of triploids (Adeleke et al. 2004). Other mechanisms of sterility result from morphological errors in post meiotic stages and physiological dysfunction during pollination and fertilization (Simmonds 1962; Pillay et al.

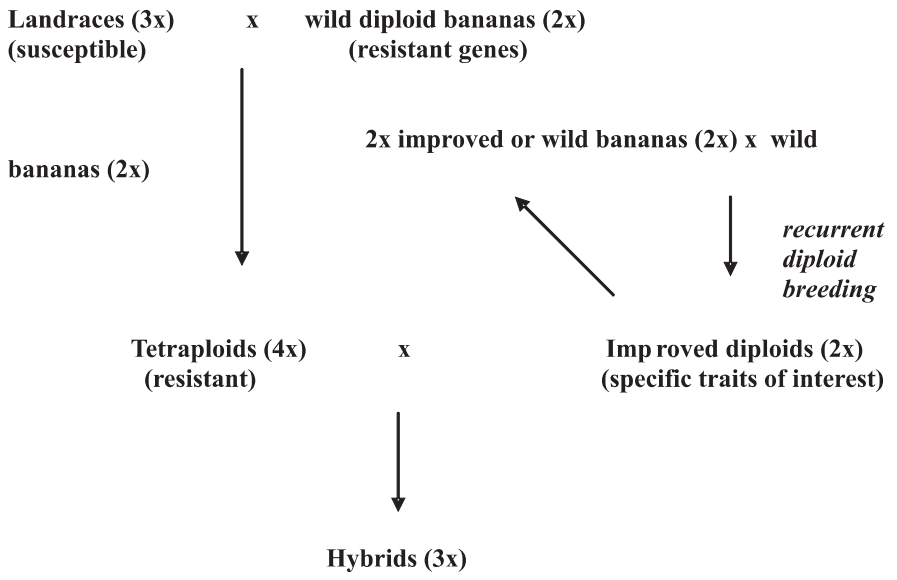

Fig. 2. Simplified *Musa* breeding scheme (adapted from Pillay et al. 2002)

2002). Seed set per bunch in many clones is less than one seed, and germination in soil is less than one percent (Ortiz and Vuylsteke 1996). Bananas also take up to 18 months to mature, which prolongs breeding efforts compared to annual crops. Consequently, breeders currently devolve much of their resources for obtaining, rather than evaluating progeny from crosses. Compounded to this is the fact that the initial steps in genetic improvement of most cultivated bananas involve crossing triploid (3×) accessions to diploid (2×) to produce 4× hybrids as described in Fig. 2 (Pillay et al. 2002). Although the process is conceptually straight forward, complex ploidy and genome arrays which complicate selection can arise. The effect of multiploidy and autopolyploidy chromosome behavior results in unpredictable frequency of aneuploids and undesirable hyperpolyploids (>5×) in addition to 2×, 3×, and 4× euploids (Simmonds 1966). Diploid bananas generally have unacceptably low yield potential, while tetraploid bananas often suffer from premature senescence, fruit drop, short shelf life, weak pseudostem and are prone to undesirable production of seed (Pillay et al. 2002). In addition, banana seeds are large and hard and are not acceptable to most banana consumers (Pillay et al. 2002).

15.1.8 Overcoming *Musa* Breeding Difficulties

Low seed set and germination rates are the major hindrances to hybrid plant production in most triploid *Musa*. This makes it difficult to obtain adequate population sizes to select disease resistant cultivars through crossing. The application of aseptic embryo culture techniques has improved seed germination rates by a factor of 3 to 10 (Vuylsteke et al. 1990). The discovery of seed-fertile landraces capable of producing true seed upon hand pollination (Vuylsteke et al. 1993) and use of advanced breeding populations with improved fertility (Pillay et al. 2000) are sought to further improve the efficiency of banana breeding. Breeders are using in vitro techniques such as shoot-tip culture for multiplication of newly bred genotypes. Micropropagated plants establish faster than conventional suckers and have almost uniform production cycle, which further facilitates establishment and evaluation of hybrids (Robinson 1996). In vitro culture also guarantees safe collection, exchange and conservation of germplasm required for identification of breeding traits and facilitates dissemination and propagation of newly selected cultivars or hybrids. The high in vitro multiplication rates also enable rapid production of clean or disease-free planting material for establishment of large pollination blocks.

In addition, the problems of fertility could be bypassed by using an array of available biotechnological techniques (Pillay et al. 2002). Recombinant DNA technology for instance has been beneficial in the improvement of *Musa* cultivars that are difficult to breed and remains the most promising solution for those varieties that are totally sterile. Biotechnology makes it possible to incorporate genes coding for characters that are not available in the *Musa* gene

pool. Using molecular techniques, novel genes encoding agronomically important traits can be identified, isolated, characterized and introduced into cultivars via a combination of genetic transformation and in vitro regeneration (Sagi 2000; Tripathi 2003). Genetic transformation, i.e. the introduction and stable integration of genes into the nuclear genome and their expression in a transgenic plant, offers a better alternative for the genetic improvement of cultivars not amenable to conventional cross breeding such as Cavendish bananas or Horn plantains (Jones 2000). Some success has been registered in genetic engineering of bananas and plantains, which enabled the transfer of foreign genes into plant cells. Protocols for electroporation of protoplasts derived from embryogenic cell suspensions (Sagi et al. 1994), particle bombardment of embryogenic cells (Sagi et al. 1995; Remy et al. 1998), and co-cultivation of wounded meristems (May et al. 1995; Tripathi et al. 2005) or cell suspension cultures with *Agrobacterium tumefaciens* (Ganapathi et al. 2001; Khanna et al. 2004) are available for bananas and plantains. Particle bombardment (or biolistic transformation) uses accelerated heavy-metal microparticles coated with DNA to penetrate and deliver foreign genes into plant cells, which are then selected and regenerated into plants while *A. tumefaciens*, a soil bacterium, transforms its plant hosts by integrating a segment (T-DNA) of its tumor-inducing plasmid into the plant nuclear genome allowing introduction of virtually any novel gene. Both of these systems have been used successfully on banana. *Agrobacterium*-mediated transformation has been applied to a range of plantain and banana cultivars and synthetic hybrids. The status of research on genetic engineering of banana for disease resistance and future possibilities has been reviewed (Sagi 2000; Tripathi 2003). Since the cultivated banana is largely sterile, its genetically manipulated plants are environmentally safe because the modified genes would not be able to easily escape from the transformed crop.

On the other hand the application of molecular techniques such as RFLP and RAPD in banana breeding can increase the efficiency of identification of promising new genotypes. Early detection of desirable genome combinations significantly improves breeding efficiency and saves field evaluation costs. Faster, precise, none destructive methods for ploidy determination based on the use of flow cytometry (Pillay et al. 2001) have made it easier to detect mixoploidy of especially segregating progeny populations. RAPD and PCR-RFLP markers that are specific for the A and B genomes have been identified (Pillay et al. 2000; Nwakanma et al. 2003a). These molecular methods can be used at any developmental stage of the plant and therefore provide an objective and reliable way for genome classification in bananas and plantains. Indeed the role played by molecular markers in banana and plantain breeding is crucial and inexhaustive.

15.1.9 Banana Breeding Achievements

Though no new banana hybrid has reached the fruit quality of the natural varieties to replace the current Cavendish varieties, plantains, East African Highland and other banana groups in regard to their eating qualities, the recent advances in several breeding programs hold a lot of hope in eventually achieving man-bred acceptable cultivars for commercial production and local consumption through conventional breeding. The initial major challenge that faced banana breeders was developing diploids with combinations of disease resistance and desirable agronomic qualities and identification of seed-fertile triploids for use in breeding desired types of banana. The quest for bred diploids came from the realization that most traits of economic importance are more predictably inherited from the diploid parents than from parents with higher ploidy level (Tenkouano et al. 1999a, b). It is also easier to carry genetic analysis in a diploid background due to disomic inheritance, which facilitates and accelerates breeding. Furthermore, population improvement at the 2× level is effective for eliminating deleterious recessive alleles in selected 2× progenitors for further crosses with cultivated bananas and plantains (Ortiz and Vuylsteke 1996). Hence, the major breeding programs worldwide initially invested in the development of diploid breeding stocks (Ortiz and Vuylsteke 1996) to permit the development of the desired hybrids. Since then several diploid hybrids have been successfully developed by the various breeding programs and registered in the public domain and/or distributed to breeders and geneticists for use in germplasm enhancement and genetic analysis of *Musa* genomes (Rowe 1984; Rowe and Rosales 1993; Vuylsteke 1993; Ortiz et al. 1994; Vuylsteke and Ortiz 1995; Tenkouano et al. 2003). The development of these diploids is undoubtedly a breakthrough which was needed for more rapid progress towards the development of new cultivars. For instance, improved

diploid banana germplasm developed by the Fundacion Hondurenea de Investigacion Agricola (FHIA Honduras) has been successfully utilized as parents of internationally released tetraploid hybrids such as the dessert banana 'FHIA-1' (Goldfinger), 'FHIA-3', 'FHIA 17, 23, 25' (Rowe and Rosales 1993). These improved diploids have also been used to produce East African Highland banana type hybrids (Pillay, unpublished).

Although there have been arguments against tetraploids as potential cultivars because of their weak leaf petioles and theoretical possibility of spontaneous seed setting, several tetraploid hybrids of plantains and other banana types have been developed, some of them having striking resistance to black Sigatoka and good plant and bunch characteristics (Vuylsteke et al. 1993, 1995; Ortiz and Vuylsteke 1998a, b; Jones 2000) and have been globally tested for possible adoption as new cultivars. The yield of most of the tetraploid hybrids is relatively higher than that of their parent landraces. This has been attributed to characteristics such as shorter and robust plant stature, better suckering behavior and at times early maturity, which are all linked with yield. The high yield in plantain tetraploids is particularly attributed to improve ratooning as compared to their parents, which have generally low suckering behavior.

Nevertheless, further ploidy manipulation to reduce the chromosome number to the triploid level to develop male sterile hybrids has been pursued (Ortiz 1997). Major gains in fruit quality have been achieved by restoration of the seedless character in resulting 3× offspring (Tenkouano et al. 1998). For instance, Tropical *Musa* secondary triploid hybrids (here after TM3×) resistant to black Sigatoka have been obtained from tetraploid-diploid crosses and made available to breeders and geneticists interested in germplasm enhancement or for further testing and cultivar release in accordance with the countries specific variety release regulations (Ortiz et al. 1998).

On the other hand, although some important *Musa* subgroups (Cavendish, False Horn plantain) remain recalcitrant to conventional breeding, seed set rates have tremendously improved in many *Musa* hybrids and the germination percentage drastically enhanced using established tissue culture techniques. Consequently, a number of improved genotypes have been widely evaluated and knowledge on genotype-by-environment interaction and stability of the important traits gained. Insight into combining abilities, heterotic groups, and the genetics of qualitative and quantitative traits has been gained and is being applied to make breeding more efficient. A wide array of breeding schemes has been explored, combining conventional and innovative approaches, and producing potential cultivars from primary tetraploids, secondary triploids and other populations (Tenkouano et al. 2003).

15.2 Gene Mapping in *Musa*

Basically, mapping aims at identifying molecular markers genetically (genetic maps) or physically (physical maps) linked to major or minor genes (generally loci) contributing to the expression of a particular trait or continuously varying character (e.g. a QTL). Linked markers can then be exploited to isolate the gene(s) underlying the trait. The isolated genes in turn are used to improve selected genotypes via direct or *Agrobacterium*-mediated gene transfer or, alternatively, the linked markers may serve to select segregants of a cross that carry a desirable trait (marker-assisted selection, MAS). For these reasons genetic and (in a more advanced state) physical maps have now been established for almost all the important crop plants. Most of these maps are integrated maps, i.e. they contain a series of different molecular markers, preferably in a framework of STMS (sequence-tagged microsatellite sites). Genetic mapping in *Musa* is not very far advanced, though a first low-density map of *M. acuminata* was established using isozyme, RFLP, and RAPD markers based on a cross between SF265 (AA) × a *banksii* (AA) segregating for parthenocarpy (Faure et al. 1993). Although a series of crosses segregating for other traits like black Sigatoka resistance, bunch position, chromosome rearrangements have been developed, and mapping projects have been undertaken at CIRAD, no high-density linkage map is yet available. Till now, mapping populations are limited in number, despite the fact that several activities are aimed at developing suitable segregating populations at various Research Institutes. The International Institute of Tropical Agriculture is developing several populations based on the A genome and B genomes. Segregating populations of *M. acuminata* (Calcutta 4) × *M. acuminata* (Calcutta 4), *M. balbisiana* × *M. balbisiana, M. acuminata* × *M. balbisiana* have been developed. Field evaluations of populations

show that the BB segregating populations show very little variation among the progeny while the 'Calcutta 4' × 'Calcutta 4' cross shows a high degree of variation, suggesting that *M. acuminata* (Calcutta 4) may not be homozygous as was previously suggested.

Although genetic mapping of the *Musa* genome obviously lags behind that of other crop plants of comparable market value, several bacterial artificial chromosome (BAC) libraries of the A and B genomes, which will allow the physical mapping of the banana genome, have been established. Genes known only by their phenotypes are best cloned by positional or map-based cloning. This requires the development of large-insert genomic libraries and ordering them into contigs that span the genome region carrying the gene(s) of interest. The BAC system of cloning large DNA fragments is the preferred method for constructing large insert-libraries of genomes (Tomkins et al. 1999). Physical mapping aims at defining the location of a particular gene (or DNA sequence) on a cloned genomic sequence of a size of 100–200 kb. It also allows one to relate genetic distances (cM) between two (or more) markers to physical distances (kb), to align syntenic (and also non-syntenic) regions of two or more genomes from related or non-related organisms to search for homologous, orthologous or paralogous sequences, and to build contigs of specific genomic regions to pinpoint a target gene and to isolate it using map-based cloning approaches (Budiman et al. 2000). The physical mapping methodology involves FISH (fluorescent in situ hybridization) or fiber-FISH to locate sequences on chromosomal preparations (Jackson et al. 1998), or the production of yeast artificial chromosomes, or more efficiently, BAC libraries with large inserts, or transformation-competent artificial chromosomes (TACs). Global physical mapping comprising the entire genome has been achieved for only a few plant species like *Arabidopsis thaliana* and *Oryza sativa* (Kurata et al. 1997; Mozo et al. 1999), but is in progress for many other crop plants.

Although several BAC libraries of different *Musa* species (*M. acuminata*, A library; *M. balbisiana,* B library) have been produced, the clones have not generally been ordered, nor has a tiling path been constructed around interesting regions. However, it is to be expected that the growing awareness of the scientific banana community will catalyse the process of physical mapping, which is the path to the isolation of agronomically interesting genes.

Genomic libraries from *M. acuminata* and *M. balbisiana* accessions have been screened with a variety of repetitive oligonucleotides including $(GA)_{11}$, $(AT)_{11}$, $(CT)_{11}$, $(ATT)_{10}$ and $(CTT)_{10}$ (Jarret et al. 1994). The sequence of selected fragments was then determined and PCR primers designed from sequences flanking the SSR. More than half of the SSR isolated from *M. acuminata* had simple dinucleotide (GA) or (CT) core motifs (Crouch et al. 1997). No simple (AT) repeats were isolated despite their reported abundance in plant species. This is likely to be due to self-annealing of the $(AT)_{11}$ probe. However, several complex SSR which included $(AT)_n$ motifs were isolated by virtue of their association with (GA), (AG) or (CT) motifs. In common with other genera, trinucleotide and tetranucleotide repeats appear to be less abundant in *Musa* than dinucleotide repeats. In this way, approximately 100 useful microsatellite markers have been generated from *M. acuminata* while a similar number is expected to result from parallel work on *M. balbisiana*. Similar microsatellite isolation projects are also ongoing at CIRAD (Lagoda et al. 1995), the University of Frankfurt (Weising et al. 1996) and the University of Saskatchewan while smaller projects have been initiated elsewhere. This is likely to result in the availability of more than 500 microsatellite markers for genetic analysis and molecular breeding in *Musa*.

15.3 Identification of Quantitative Trait Loci (QTL) in *Musa*

Plant characters are often referred to as qualitative or quantitative depending on the number of genes that control them (Fehr 1987). Qualitative characters such as flower color are controlled by one or a few major genes. On the other hand quantitative traits show continuous variation and are controlled by a number of minor genes (polygenes) that are greatly affected by the environment. It is known that genetic and the environmental factors interact to make up the phenotype of a plant. Traditional genetic studies have quantified these factors by using statistical models such as:

$$Y_{ij} = \mu + G_i + E_j + I_{ij} + e$$

Where Y_{ij} is the observed phenotype, μ is the mean phenotype in the population, G_i and E_j are the net effects due to an individual having genotype i and j, I_{ij} is the interaction effect between i and j, and e is the random contribution to the phenotype. These

models can estimate the statistical effects as means variances or covariances of a group of genes, but give very little information about the nature of the polygenes that underlies the trait (Kearsey 2002). In addition, while these models described the effects of genetic and environmental factors, it was not possible to locate the exact genes or chromosomal regions of a plant that contributed to the trait. Locating genes on a chromosome cannot be achieved without gene maps. In the past, classical markers such as pigmentation, morphological traits and isozyme loci were used to generate linkage maps. The general lack of abundance of these types of markers meant that most linkage maps had large intervals between the markers. The first linkage map to be established was that of maize (Sturtevant 1965). Complete genetic maps are essential for studying the genetics underlying quantitative traits. Genetic maps show the ordering of loci along a chromosome and the relative distances between them (Lynch and Walsh 1998). Early 'genetic' linkage maps provided a rough road map to order some genes. Thereafter 'physical' maps provided with landmarks along the whole length of the chromosomes. The physical map was superseded by the concept of molecular maps which rely on molecular markers. Molecular markers such as restriction fragment length polymorphisms (RFLPs), simple sequence repeats (SSRs) and single nucleotide polymorphisms (SNPs) have now made it possible to dissect traits into their genetic components. A chromosomal region that is associated with molecular markers and with a quantitative trait is defined as quantitative trait loci (QTL, Xu 2002). Plant breeders are interested in knowing how many genes are involved in a given trait, the nature of the dominance and epistatic properties of these genes and how they interact with the environment, and whether these genes are structural or regulatory. These questions can only be answered if we have an accurate location of gene(s) on a chromosome. If QTLs could be identified the transfer of traits such as yield, drought tolerance etc. could be accelerated in breeding programs.

Plant populations with different genetic structures have been created for genetic mapping, including F_2/F_3, backcross, doubled haploids, recombinant inbred lines, near isogenic lines, back cross inbred lines and various mutants (Xu 2002). Several advanced statistical methods have been proposed for mapping quantitative trait loci (Lander and Botstein 1989). Much work has also been covered on the theoretical aspects of mapping quantitative trait loci. This is beyond the scope of this chapter.

Several traits in banana such as fruit filling period, bunch weight, and fruit parameters are considered to be quantitative in nature (Ortiz 1995). Studies to identify QTLs in banana are limited. The primary reason for this is the absence of a high density linkage map for *Musa*. A partial linkage map for diploid bananas based on 58 RFLP, four isozyme and 28 RAPD markers was published in 1993 (Faure et al. 1993). A composite linkage map has been constructed by CIRAD from two mapping populations at a LOD score of 4.75. This map covers 1,227 cM and links 373 isozyme, microsatellite, RFLP, RAPD and AFLP markers in 11 linkage groups (unpublished). A QTL for Sigatoka resistance has been placed on the map developed by CIRAD. The identification of QTLs in *Musa* will be of great value for genetic improvement of bananas for polygenic traits such as yield, drought tolerance and others. Published reports and our own unpublished data show that bananas are greatly influenced by the environment. Researchers identifying QTLs in *Musa* must consider environment as a factor. The level of QTLs across environments is trait specific. For example, in soybean out of 11 RFLP markers associated with plant height and eight with lodging, only two markers for plant height and one for lodging were detected in all four locations where the experiments were conducted (Lee et al. 1996).

15.4 Marker-Assisted Breeding in *Musa*

The success of crop improvement programs is highly reliant on the power and efficiency with which the genetic variability can be manipulated. For thousands of years, breeders have been relying on morphological characters to select and cross plants carrying desired traits to finally yield superior cultivars. However, the practice is extremely slow and highly unpredictable often limited by the low number of morphological characters available to them for crop improvement programs. Besides, the expression of morphological characters is affected by environmental conditions and sometimes altered by epistatic and pleiotropic interactions resulting in the difficulty to obtain reliable data. Where breeding goals cannot be achieved using traditional approaches, there is now considerable scope for using molecular or genetic markers to

develop new varieties. Genetic markers offer plant breeders the potential of making genetic progress more precisely and more rapidly than through phenotypic selection. The potential benefits of using markers linked to genes of interest in breeding programs have been obvious for many decades. However, the realization of this potential has been limited by the lack of useful markers. With the advent of DNA-based genetic markers in the late 1970s, the situation changed and researchers could, for the first time, begin to identify large numbers of markers dispersed throughout the genetic material of any species of interest and use the markers to detect associations with traits of interest, thus allowing MAS to finally become a reality. Molecular markers can be conveniently used in plant breeding programs for the characterization of germplasm, assessment of genetic diversity and identification of crop varieties via DNA fingerprinting.

Marker-assisted breeding provides a dramatic improvement in the efficiency with which breeders can select plants with desirable combinations of genes. To date, many markers linked to useful traits have been identified and being utilized in many breeding programs. However, before initiating marker-assisted breeding, it is imperative to define the specific objectives to be achieved through the use of markers, the specific problem and the cost of the technologies to be used, especially in the developmental phase. The success of MAS depends upon several critical factors, including the number of target genes to be transferred, the distance between the flanking markers and the target gene, the number of genotypes selected in each breeding generation, the nature of germplasm and the technical options available at the marker level.

The use of molecular markers in banana breeding is reported for many purposes, such as cultivar identification (Pillay et al. 2001), phylogenetic studies (Kardolus et al. 1998), analysis of recombination between genomes (Osuji et al. 1997a, b), identification of genes controlling traits (Damasco et al. 1996), and assisted selection (Shanmugavelu et al. 1992). Marker-assisted breeding is a powerful tool in many breeding programs and it is being utilized by many breeders to transfer useful genes among species. Thus, MAS offers clear advantages in genetic terms over traditional selection in many circumstances (Crouch et al. 1998). RFLPs of diverse germplasm have been used to study the taxonomy and phylogeny of *Musa* species (Gawel et al. 1992; Jarret et al. 1992; Nwakanma et al. 2003a), and variation in the chloroplast genome within the *Musa* genus (Gawel and Jarret 1991a, b). However, there is only one report of their use to distinguish more closely related material (Bhat et al. 1994). More importantly perhaps, the relatively high cost and technically demanding nature of this technique is not appropriate to routine breeding applications. Thus, researchers have concentrated on applications of the polymerase chain reaction (PCR) for *Musa* genome analysis. All PCR-based molecular markers appear to detect a high level of polymorphism within a range of *Musa* breeding populations. PCR-based assays are amenable to the large-scale throughput demands of screening breeding populations. The RAPD technique has been successfully used to distinguish diverse *Musa* germplasm (Howell et al. 1994; Bhat and Jarret 1995; Pillay et al. 2001). In addition, a molecular linkage map has also been developed using a variety of marker systems including RAPD (Faure et al. 1993). RAPD assays are particularly useful, as they require no prior knowledge of the genome of an organism. RAPD analysis has been used to differentiate *Musa* genome groups (Howell et al. 1994; Pillay et al. 2000), more closely related *Musa* germplasm (Bhat and Jarret 1995) and full-sib hybrids in plantain breeding populations (Crouch et al. 1998, 2000). Teo et al. (2005) used retrotransposon derived markers for identification and characterization of banana cultivars and classification of *Musa* genome constitutions. These reports clearly demonstrate the potential value of this technique for germplasm characterization and cultivar identification but give little insight into the value of the assay for molecular breeding.

Recently the technique of AFLP has been successfully applied in *Musa*. AFLP analysis of *Musa* breeding populations suggests that this technique may be a powerful tool in the molecular breeding of plantain and banana (Ude et al. 2002a, b; Ude et al. 2003). Using AFLP markers, Lheureux et al. (2003) found that 10 markers were co-segregating with the presence and/or absence of BSV infection in *Musa* hybrids. AFLP analysis is clearly a powerful technique in terms of its ability to identify a large number of polymorphic bands without any prior knowledge of the organism. Unfortunately, the information content of these banding patterns is restricted, as they must initially be treated as dominant markers. However, when AFLP analysis is applied to large populations, circumstantial allelic relationships may be sufficient for practical purposes. Software has been developed to distinguish homozygotes and heterozygotes on the basis of band intensity. Yet, such an approach may be frequently confounded by the presence of bands of intermediate intensity.

AFLP assays are also technically demanding and expensive in that they require a number of DNA manipulations and a complex visualization procedure. In addition, they require relatively large amounts of reasonably high quality DNA. The use of poor quality DNA may lead to incomplete digestion which can result in artificial polymorphisms. Nevertheless, AFLP and SSR markers are now being used to identify markers for fruit parthenocarpy, dwarfism and apical dominance in banana and plantain.

Microsatellite markers and AFLP analysis appear to be the most appropriate technologies for marker-assisted breeding in *Musa* (Crouch et al. 1999; Hautea et al. 2005). DNA markers have provided powerful tools for genetic analysis in *Musa* (Crouch et al. 1998, 2000) but may not provide an effective means of predicting progeny performance (Tenkouano et al. 1999b). All marker systems have different advantages and disadvantages in specific applications. Thus, it is important for molecular breeding programs to develop capacity in several assays in order that the most suitable system can be chosen and rapidly applied for any particular application.

15.5 Marker-Assisted Introgression

Marker-assisted selection and aided introgression are being employed by plant breeders mostly to locate and select genes controlling important quality and disease or pest resistant traits (Crouch and Ortiz 2004). The first step however, is the identification of one or more markers linked to the gene(s) to be introgressed and their localization on the molecular map. Though mapping of the *Musa* genome is still in its infancy, the results of functional genomics of model plants will increase the understanding of the basic *Musa* biology as well as the exploitation of genomic information for its improvement. Crouch et al. (1998, 2000) emphasized the fact that introgression of genes for pest and disease resistance from wild germplasm has been and is likely to continue to be a crucial aspect in *Musa* improvement. DNA markers are now being sought for several characters of importance including parthenocarpy, apical dominance, resistance to black Sigatoka, nematodes, and other pests and diseases. Fruit quality parameters (color, texture, ripening) are other candidate traits for DNA markers. RAPD assays have proven to be powerful and efficient means of assisting introgression and backcross breeding. In fact, RAPD markers that are specific for the A and B genomes of *Musa* have been identified and are routinely used. However, RAPD analysis has several disadvantages including the dominant nature of the marker system and reproducibility problems, which may limit their application in marker-assisted selection. Consquently, this led to a focus on the development and utilization of primers for *Musa* microsatellites (Jarret et al. 1994; Kaemmer et al. 1997; Creste et al. 2004), which have been considered optimum markers in other systems due to their abundance, polymorphism and reliability. Simple sequence repeats (SSR) are regions of short tandemly repeated DNA motifs (generally less than or equal to 4 bp) with an overall length in the order of tens of base pairs. SSR have been reported to be highly abundant and randomly dispersed throughout the genomes of many plant species. Variation in the number of times the motif is repeated is thought to arise through slippage errors during DNA replication. Thus, SSRLP may occur even between closely related individuals. Microsatellite markers have been used in plants for fingerprinting, mapping, and genetic analysis. SSRLP analysis has been shown to detect a high level of polymorphism between individuals of *Musa* breeding populations (Crouch et al. 1998, 2000). However, the isolation of microsatellites is time consuming and expensive. Nevertheless, several hundred SSRLP markers have been generated in *Musa* (Jarret et al. 1994; Kaemmer et al. 1997; Crouch et al. 1998). Furthermore, the isolation of SSR is becoming routine with the availability of automated DNA sequencing facilities, improved techniques for the construction of genomic libraries enriched for SSR and improved techniques for the screening of appropriate clones. This has recently allowed the rapid isolation of several hundred microsatellites from the *Musa* B genome (Buhariwalla et al. 2005).

Marker-assisted gene introgression offers an extremely efficient means of precisely identifying rare segregants with the required genome compositions and it is routinely being applied in many breeding programs. While selection theory is the most important tool for the design of breeding programs for improvement of quantitative characters, no general selection theory is available for marker-assisted backcrossing. Its efficiency depends mostly on marker density and position, population size, and selection strategy. Adopting a selection theory approach to predict response to marker-assisted selection for the genetic background of the recurrent parent promises to com-

bine several of the factors determining the efficiency of a gene introgression program into one criterion.

15.6 Map-Based Cloning

The isolation of agriculturally important genes is an important goal in plant molecular biology. Since most agriculturally important genes are known only by phenotype, techniques have been developed to isolate such genes. Currently, map-based cloning (or positional cloning), insertional mutagenesis and subtraction cloning are three of the best-developed strategies. Map-based cloning has been successfully used to isolate plant genes based solely its position on a genetic map. The strategy of map-based cloning is to find molecular markers that are very closely linked to the gene of interest. Those molecular markers can serve as the starting point for chromosome walking or jumping to the gene.

Map-based gene cloning includes four steps:

(a) Target gene mapping: The first step of map-based or positional cloning is to identify a molecular marker that lies close to the gene of interest (5 cM). This procedure typically is done by first finding a marker in the vicinity of the gene. For the initial screening smaller population sizes are used (60–150 individuals).

(b) Physical mapping: The next step is to saturate the region around that original molecular marker with other markers. At this point you are looking for a one that rarely shows recombination with your gene. At this stage, the population size could increase to 300–600 individuals.

The next step is to screen a large insert genomic library (BAC or YAC) with the marker to isolate clones that hybridize to the molecular marker. Once the initial markers that are flanking the target gene have been identified and hybridized to a clone, the position of the gene can be determined. Distance is measured in base pairs other than genetic recombination (in cM). Methods for physical mapping involve FISH, YACs, BACs, STSs (sequence tagged sites).

(c) Chromosome walking or landing:

1. Chromosome walking relies on isolation of a DNA fragment at or near an end of a cloned insert for use as a probe to screen the library and identify more clones. Chromosomal walking was the major map-based cloning method in the past.
2. Chromosomal landing starts with identifying tightly linked molecular markers. The DNA markers are then used to screen a library and isolate (or land on) the clone containing the gene.

Chromosome walking involves creating new markers (usually sequences at the end of the clone) and screening of a segregating population with these new markers. Often this population is large (1,000–3,000 individuals). The goal is to find a set of markers that co-segregate (no recombination) with the gene of interest. Co-segregation means that whenever one allele of the target gene is expressed, the markers associated with that allele are also present. In other words, recombination is not seen between the gene and the markers. If these markers do not co-segregate, new large insert clones should be selected and the process is repeated until the finding of a clone whose markers co-segregate with the gene. To speed the cloning process, it is best to begin with a marker that is tightly linked to the target gene.

(d) Gene identification: Genetic complementation through transformation. DNA fragments between the flanking markers are cloned and introduced into a genotype mutant for your gene by a genetic engineering technique called plant transformation. If the transgenic plant expresses the wild type phenotype, it confirms the presence of the gene of interest on that fragment. At this point the fragment must be sequenced to find a potential open reading frame (ORF), sequences that most likely will encode a gene product. In the best situation, only a single ORF is found, but often this is not the case. Usually several possible ORFs are found and new transgenic plants are created by transforming with a single ORF. Once this ORF is shown to rescue the mutant phenotype, an in-depth molecular and biochemical analysis of the newly cloned gene could then be performed.

RFLP or other molecular genetic markers can be used in chromosome walking procedures. High density genetic maps have been developed or are being prepared in a number of crops. Using RFLPs, the chromosomal location of a particular probe can be determined and a map of various RFLP probe positions

can be constructed. Genes can then be located genetically by their co-segregation with a particular RFLP. The starting gene can be cloned using a closely linked RFLP probe and isolating genomic clones that it corresponds with, then walking from these genomic clones to the gene of interest.

The cloning of genes underlying important agronomic characters offers to revolutionize progress in plant research and breeding, particularly in the area of pest and disease resistance. In addition, map-based cloning of gene(s) responsible for parthenocarpy, dwarfism and albinism in plantains and bananas, would be of great value to both fundamental and applied researchers of many crops.

In fact, only in *Arabidopsis thaliana* have map-based approaches been widely applied (Giraudat et al. 1992; Busch et al. 1996; Lukowitz et al. 1996). In other plants there are only few reports of genes that have been cloned by a map-based approach (Martin et al. 1993; Dixon et al. 1996; BuK Schges et al. 1997).

15.7 Genes and Gene Expression in *Musa*

Although several expressed sequence tag (EST) and cDNA libraries have been established (e.g. for *M. acuminata* ssp. *malaccensis*), no *Musa* EST database can yet be tapped for information about expressed genes (Caetano et al. 2005; Carlos et al. 2005). In addition, the depth of these libraries is not known. Yet full-length cDNA libraries, normalized and representative, would be needed from a whole series of tissues and states (e.g. normal vs. diseased; susceptible vs. resistant; different developmental states). A series of resistance gene analogs have been isolated, using degenerate PCR primers targeting highly conserved regions in proven plant resistance genes (e.g. kinase or transmembrane-encoding domains, or leucine-rich repeat sequences, to name only few). Plant disease resistance genes involved in signal transduction contain domains that are conserved throughout mono- and dicotyledons. Primers have been designed to those domains in the *RPS2* gene of *Arabidopsis thaliana* and the N gene of tobacco. Using these primers for PCR, candidate resistance genes have already been cloned from soybean, potato, rice, barley and *Arabidopsis*. A similar strategy has been applied to clone candidate resistance genes from banana (Wiame et al. 2000). A series of disease resistant genes were isolated from the somaclonal mutant CIEN-BTA-03 (resistant to both *M. fijiensis* and *M. musicola*) and the parent 'Williams' that fall into two classes: nucleotide-binding site-leucine-rich repeat-containing kinases, and serine-threonine protein kinases of the *pto* type (Kahl 2004). All the resistance genes were fully sequenced, and eight of them are also transcribed in the mutant, its parental genotype, 'Pisang Mas' and a tetraploid *M. acuminata*. The researchers at QUT have isolated the complete gene sequence of R gene candidate (RGC-2) from *Musa acuminata* ssp. *malaccensis,* a wild diploid banana segregating for resistance to *Fusarium oxysporum* fsp. *Cubense (*FOC) Race 4. The development of *Fusarium* wilt resistant transgenic banana using this gene is in progress (Dale et al. 2004).

Few genes are targeted, some sequences are known, fewer publications have appeared, but no banana gene has been applied in any way (e.g. for transformation). Also, no attempt has yet been made to design expression chips with families of genes whose sequences are derived from either cDNAs (cDNA microarray), or oligonucleotides, or from clones obtained from related or unrelated plants. Relative success in genetic engineering of bananas and plantains has been achieved recently to enable the transfer of foreign genes into plant cells.

Genetic transformation using microprojectile bombardment of embryogenic cell suspension is now a routine procedure (Sagi et al. 1995; Becker et al. 2000). An efficient method for direct gene transfer via particle bombardment of embryogenic cell suspension has been reported in the cooking banana cultivar 'Bluggoe' and the plantain 'Three Hand Planty' (Sagi et al. 1995). Becker et al. (2000) reported the genetic transformation of the Cavendish banana cv. 'Grand Naine'. *Agrobacterium*-mediated transformation offers several advantages over direct gene transfer methodologies (particle bombardment, electroporation, etc), such as the possibility to transfer only one or few copies of DNA fragments carrying the genes of interest at higher efficiencies with lower cost and the transfer of very large DNA fragments with minimal rearrangement (Gheysen et al. 1998; Hansen and Wright 1999; Shibata and Liu 2000).

Musa was generally regarded as recalcitrant for *Agrobacterium* mediated transformation. Hernandez (1999) has reported that *A. tumefaciens* is compati-

ble with banana indicating the potential for genetic transformation. The recovery of transgenic plants of banana obtained by means of *A. tumefaciens* mediated transformation has been reported. The protocol has been developed for *Agrobacterium* mediated transformation of embryogenic cell suspensions of the banana (Ganapathi et al. 2001; Khanna et al. 2004). At present most of the transformation protocol use cell suspension, however establishing cell suspension is lengthy process and cultivar dependent. The protocol has also been established using shoot tips from various cultivars of *Musa* (May et al. 1995; Tripathi et al. 2002, 2005a). This technique is applicable to a wide range of *Musa* cultivars irrespective of ploidy or genotype (Tripathi et al. 2003, 2005a). This process does not incorporate steps using disorganized cell cultures but uses micropropagation which has the important advantage that it allows regeneration of homogeneous populations of plants in a short period of time. This procedure offers several potential advantages over the use of embryogenic cell suspensions (ECS) as it allows for rapid transformation of *Musa* species.

Currently, the transgenes used for banana improvement have been exclusively isolated from heterologous sources like other plant species, insects, microbes and animals (Tripathi 2003; Tripathi et al. 2004, 2005b). For example the most attractive strategy for serious fungal disease like black Sigatoka control in *Musa* is the production of disease resistant plants through the transgenic approach including the expression of antifungal peptide genes from radish, onion and dahlia. Similarly, research is in progress at IITA for producing bacterial wilt disease (caused by *Xanthomonas campestris* pv. *musacearum*) resistant banana varieties using genes encoding for plant ferredoxin like protein (*pflp)* and hypersensitive response assisting protein *(hrap)* isolated from sweet pepper.

Since most cultivated varieties of banana are sterile and therefore do not set seed, traditional breeding by hybridization is difficult making genetic transformation a viable tool for improving bananas. Although attempts to produce transgenic bananas and plantains are proceeding slowly, public acceptance of these novel plants and their products should be encouraged through sound information and risk assessment studies. The chances of transfer of transgenes from field material to wild species (the major public concern) are expected to be negligible in *Musa* in view of the sterility of many cultivars.

15.8 Future Scope of Works

Bananas are staple food crops for over half a 100 million people in sub-Saharan Africa and over half a billion worldwide. Bananas are the developing world's fourth most important food crop after rice, wheat and corn. Despite these statistics, *Musa* is not included in international genome analysis initiatives. A Global *Musa* Genomics Consortium was established in 2001 with the goal of assuring the sustainability of banana as a staple food crop by developing an integrated genetic and genomic understanding, allowing targeted breeding, transformation and more efficient use of *Musa* biodiversity. Basically, the Consortium aims to apply genomics to the sustainable improvement of bananas. The consortium believes that genomic technologies such as analysis and sequencing of the banana genome, identification of its genes and their expression, recombination and diversity can be applied for the genetic improvement of the crop (Frison et al. 2004). However, large scale funding for this initiative has not been realized as yet.

Banana breeding is a complex procedure that is fraught with constraints such as female and male sterility and long generation times. *Musa* genomics can open up new avenues for more efficient breeding of the crop. It is important to investigate the possibilities via which the primary production and other uses of *Musa* can be promoted for the benefit of the growing world's population.

Strategies for future genomics research in *Musa* include the development molecular markers, construction of genetic and physical maps, identification of genes and gene expression and whole genome sequencing. Sequencing of other plant genomes such as *A. thaliana* and *O. sativa* has provided an enormous amount of data that could reveal unknown features of their genomes. Such data could also be generated for *Musa*. These include sequence composition of various genomic regions, an inventory of the various genic and non-genic sequences (genes and repetitive DNA such as satellites, mini- and macrosatellites, pseudogenes, retropseudogenes, retrotransposons, LINES, SINES, DNA transposons and many others), the distribution of various elements along the chromosomes, potential duplications, translocations, inversions, macro- and microsynteny, structure of centromeres and telomeres, the exact genome size, and number of open reading frames (Kahl 2004). Together with genome

sequencing, the handling of such data must be considered since bioinformatics for banana genomics has not been developed.

Although a number of marker techniques are now available for genomic research, they have not been as widely used in *Musa* as in some of the other crop plants. Markers that co-segregate with a trait can be exploited to accelerate the selection of that trait. This will be especially useful in *Musa* because of its long life cycle. New markers such as SNPs have not yet been applied to banana research and promise to have an impact on protein function. Genetic and physical mapping of the *Musa* genome will make it possible to isolate genes that can be used in genetic transformation. Although a map of a diploid banana is available a much greater effort in developing high density maps to identify QTLs is necessary for *Musa*. The development of ESTs and cDNA libraries are crucial areas of research in *Musa* that needs greater emphasis. In addition, no attempt has been made to design expression chips. Expression chips are being used in many laboratories for other crops. There is enormous potential for genetic manipulation of *Musa* species for disease and pest resistance using the existing transformation systems with the genes isolated from *Musa* genome. The use of appropriate gene constructs may allow the production of nematode, fungus, bacterial and virus-resistant plants in a significantly shorter period of time than using conventional breeding, especially if several traits can be introduced at the same time. It may also be possible to incorporate other characteristics such as drought tolerance, thus extending the geographic spread of banana and plantain production, and thus contributing significantly to food security and poverty alleviation in developing countries. Long-term and multiple disease resistance can be achieved by integrating several genes with different targets or modes of action into the plant genome. Technically, this can be done either in several consecutive steps or simultaneously.

Banana and plantain are regarded as 'orphan crops' or the 'poor man's fruit' with regards to research and the amount of funding devoted to the crop. Yet they are important plants in the subsistence diet of the poor millions. New diseases such as banana bacterial wilt threaten to wipe out the crop in many countries. A whole repertoire of techniques is now available to study the genomes of plants, including *Musa*. Such research will have a tremendous impact in *Musa* breeding and genomics.

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16 Mango

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16.1 Introduction

Mango (*Mangifera indica* L.) is popularly known as the 'king of fruits'. Believed to have originated in Eastern India (Knight 1980), it is an allotetraploid that evolved after interspecific hybridization and subsequent doubling of the chromosome number (Mukherjee 1953). The mango genome is of 4.39×10^8 bp size (Arumuganathan and Earle 1991) and has 20 chromosomes, most of them small. Mango is commercially grown in over 103 countries of the world but nowhere is it so greatly valued as in India. Apart from India the other major mango growing countries in the world are China, Mexico, Pakistan, Indonesia, Thailand, Philippines, Brazil, Australia, Nigeria and Egypt. According to Galan (1993) between 1971 and 1993, the production of mango worldwide, has increased by nearly 50 per cent. Much of this new production has occurred outside traditional centers of mango culture, in South and Central America, Africa and Australia. Mango lends itself to a variety of uses in one form or another, as every part of the tree is valuable and the fruits are rich sources of vitamin A and C. There is a good demand for mango fruits and their processed products in both the national and international market.

There are more than a thousand varieties of mango under cultivation, but only a few of them are grown on a commercial scale. Both polyembryonic and monoembryonic mango cultivars are grown. According to Leroy (1947), adventive embryony is probably due to the effect of one or more recessive genes. Polyembryonic cultivars are grown in Southeast Asia and in tropical Latin America, whereas monoembryonic cultivars are major contributors to commercial production in India and in Florida. Polyembryonic mango cultivars are seed-propagated and exhibit little variability in seedling populations. Monoembryonic mango cultivars are propagated either by inarching or by grafting bud wood onto seedling rootstock.

Not all varieties have been commercially exploited. There are varieties which have some unique characteristics while lacking in others. Therefore, it is necessary to study all the varieties/accessions in the germplasm in order to cope up with the mango crop improvement program. Several crop improvement measures have been taken up in mango with respect to high yield, regular bearing and resistance to certain physiological disorders. Although conventional breeding methods have yielded quite a good number of hybrids and varieties, they are laborious and time consuming. It is necessary that one should know the desirable characteristics of a variety before taking up conventional or molecular breeding. There is very limited work on molecular breeding and mapping of the genotypes in mango as compared to other fruit crops.

16.1.1 Origin and Distribution

The genus *Mangifera*, which belongs to the family 'Anacardiaceae', originated in the Indo-Burma region. According to Mukherjee (1958), the natural spread of the genus is limited to the Indo-Malaysian region, stretching from India to the Philippines and New Guinea in the East. Evidence based on morphological, phytogeographical, cytological, anatomical and pollen studies have indicated that the genus had its origin in the continental region of (i) Burma, Thailand, Indo-China and (ii) the Malayan Peninsula since these happen to be the main centers of species forma-

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tion; the Sunda Islands (Java, Sumatra and Borneo), the Philippines and the Celebes- Timor group forming the secondary centers of development. The highest concentration of *Mangifera* species is reported to be in the Malayan Peninsula, followed by the Sunda Islands and the Eastern Peninsula with many of the species being common between them (Mukherjee 1949a).

The genus which has been sub-divided into two sections, on the basis of the presence or absence of the disc in the flowers (Mukherjee 1949a), is reported to contain 41 species in all but almost all the edible cultivars of mango belong to the single species *Mangifera indica* Linn which originated in the Indian subcontinent. Occurrence of wild forms of *M. indica*, allied species *M. sylvatica* and *M. caloneura*, fossil leaf impressions of *M. pentandra* (a species similar to *indica*) and presence of numerous cultivated and wild varieties in India have been cited as some of the major reasons in favor of *M. indica* having originated in the Indo-Burma region through allopolyploidy, possibly amphidiploidy (Mukherjee 1958). The few other species which contribute edible fruits (though of relatively inferior fruit quality) are *M. caesia, M. foetida* and *M. odorata*, which are confined to the Malaysian region.

The mango, though well-known to the people of the Indian subcontinent for several centuries, was virtually unknown to any botanist until 1605 when Carol Clusius first mentioned about it in his writings (Mukherjee 1949b). Bauhin (1623, 1650), cited in Bose (1985) subsequently, referred to it under the names "Mangas" and "Amba". Bontius (1658) cited in Bose (1985) gave *Mangifera* the name for the first time and he referred to this plant as *arbor Mangifera* (the tree-producing mango). Later, it was mentioned in the literature as *Mangifera indica* Ray, *Mangus dornestica* Hermann or *Mangas sylvatica* Rheede. Linnaeus also referred to it as *Mangifera arbor* in 1747, prior to changing the name to its present form *Mangifera indica* L. in 1753, in his much quoted book 'Species Plantarum' (Mukherjee 1949b).

Besides the Indian subcontinent, the mango is now found in several countries of the tropical and subtropical world where Muslim missionaries, Spanish voyagers and Portuguese explorers introduced it to several places during 15th to 18th century. According to Hayes (1957) mango was being cultivated at the head of the Persian Gulf by the 16th century. It was introduced to Philippines after 1600, to Moluccas in 1665 and to Yemen in the later part of the 18th century (Burns and Prayag 1921). It is also reported that the mango was being grown in England under glass-house conditions as early as 1690 and that the trees at Kew were in fruiting during 1818. In Mexico, it was introduced before 1778 by the Spanish travelers from the Philippines. The Portuguese, who carried mango to South Africa in the 16th century from Goa, were also responsible for introducing it in Brazil by 1700 (Popenoe 1920). It was in cultivation in Barbados in 1742 and in Jamaica in 1782. According to Pope (1929) it was introduced in Florida and the east coast in 1860s and the west in 1870s (Hayes 1957). However, it was only in 1889 that the United State Department of Agriculture (USDA) successfully introduced the grafted plants from India. It reached Azores in 1865 and Queensland in 1870 (Burns and Prayag 1921).

Presently, besides India, it is being cultivated in Pakistan, Bangladesh, Burma, Sri Lanka, Thailand, Vietnam, Malaysia, the Philippines, Indonesia, the Fiji Islands, Tropical Australia, Egypt, Israel, Sudan, Somalia, Kenya, Uganda, Tanzania, South Africa, Niger, Nigeria, Zaire, Madagascar, Mauritius, the USA (Florida, Hawaii, Puerto Rico), Venezuela, Mexico, Brazil and the West Indies Islands.

16.1.2 Taxonomy

Mango *(Mangifera indica* L.) belongs to the dicotyledonous family 'Anacardiaceae'. The nomenclature of *Mangifera* species and mango cultivars has been complicated by the widespread use of synonyms (Lakshminarayana, 1980). This family consists of sixty-four genera, mostly of trees or shrubs, often containing milky or acrid juice, some of which are even poisonous. The leaves are exstipulate, usually alternate and simple. The inflorescence is generally an axillary or terminal panicle or spike bearing small and regular flowers. They may be unisexual (usually male) or bisexual, borne on the same or different trees. Mango possesses a very long taproot. The fruit is usually a drupe and seed is exalbuminous and is located inside the stony endocarp.

The tree is large, spreading, and evergreen, with a dense rounded or globular crown. The trunk is erect, thick, without furrows or buttresses, when old. The bark is thick, sometimes with longitudinal bursts containing a little yellowish transparent gum resin like juice. The wood is reddish grey, often streaked, moderately hard, coarse grained and soft in a young

tree. It is somewhat harder and darker brown on the older trees. Branches are very numerous, the lower ones spreading horizontally to a great extent, the upper ones gradually ascending till they become nearly erect in the center; branchlets are rather thick and robust, often with alternating groups of long and short internodes, glabrous, yellowish green when young, with slightly prominent scars of the fallen leaves.

16.1.3 Crop Improvement

The main objectives of mango breeding are to achieve regular bearing, high yield and resistance to certain physiological disorders like spongy tissue.

16.1.3.1 Mango Breeding

Hybridization, clonal selection, isolation of chance seedlings and mutation breeding are some of the conventional crop improvement methods in mango. After the initiation of hybridization in mango during 1911 at Pune, Maharashtra by Burns and Prayag, till today at least 30 hybrids have been released from different parts of India and abroad.

A clone is one of the basic categories of cultivar (Bricknell et al. 1980) with extremely important applications in horticulture. 'Cloning' may be defined as the vegetative regeneration of a single genotype as represented by a single plant, single growing point, single meristem or single explant. Cloning is a powerful procedure both as a plant selection tool for breeding and as a plant propagation tool for reproduction (Kester 1983). The exploitation of cloning for the selection of superior individuals followed by vegetative propagation has been one of the consistent themes throughout horticultural history. Clones exist in nature and with some species, vegetative multiplication is a major strategy for their adaptation.

16.1.3.2 Crop Improvement in Mango by Hybridization

In India, work on inter-varietal hybridization was initiated in 1911 at Pune, Maharashtra (Burns and Prayag 1921). Subsequently hybridization work was undertaken at many fruit research centers in the country, *viz*., Sabour in Bihar, Anantharajupet and Kodur in Andhra Pradesh, Saharanpur and Central Institute of Subtropical Horticulture, Lucknow in Uttar Pradesh, Krishnanagar in West Bengal, Quaidian in Punjab, IARI (Indian Agriculture Research Institute) in New Delhi, Periyakulam in Tamil Nadu, Paria in Gujarat, Vengurla in Maharashtra and Indian Institute of Horticultural Research, Hessaraghatta, Bangalore in Karnataka. Initial success in hybridization program was very poor because the technique of hybridization was traditional and cumbersome.

Based on a genetic marker, Singh et al. (1980) have shown that the percentage of fruit set from crosses in mango can be doubled simply by doing away with cumbersome process of bagging the panicles again after cross-pollination by hand. This will facilitate the raising of large hybrid populations for selection. As a result of hybridization at least 30 hybrid mango varieties were developed since the initiation of hybridization program in the country. The following are some of the salient research findings with respect to crop improvement in mango by hybridization in different parts of India and Israel.

The hybridization work carried out at the Indian Agricultural Research Institute, New Delhi under the leadership of Dr. R. N. Singh led to the evolution of two improved, regular bearing hybrids namely 'Mallika' (Neelum × Dashehari) and 'Amrapali' (Dashehari × Neelum). The tree of Mallika is semi-vigorous, it is medium to heavy cropper (15 ton ha^{-1}) and has a strong tendency to bear regularly. The fruits have an attractive appearance and the average fruit weight is 307 g. The pulp recovery is high (75%) and it is fiberless and firm and the stone is very thin. Total soluble solids (TSS) is high (25°Brix), and keeping quality of the fruit is better (Singh et al. 1972; Singh 1990).

Amrapali, the other hybrid released from IARI, New Delhi is distinctly dwarf, precocious, highly regular and high yielding (Sharma et al. 1980). It has been utilized for a high-density plantation. An orchard with a density of 1,600 plants ha^{-1} can yield about 11.50 tons in the 4th year and yield reaches about 22.20 tons in the 7th year (Majumder et al. 1982). Besides, Amrapali is very high in vitamin A content (β-carotenoid pigment) and its flesh is deep orange in color. The deep flesh color is important in fruit preservation industry, since addition of artificial color is not required (Singh 1990).

Thirty-nine mango hybrids developed at IARI, New Delhi were studied in order to identify promising plant types on the basis of dwarf growth habit, regularity and prolificacy of bearing. Out of these, two hybrids, *viz*., Hybrid No. 427 and Hybrid No. 411 both of Neelum × Himsagar parentage were found to be dwarf, regular and prolific in bearing. Eight-year-old

hybrid plants of 427 and 411 attained the height of 2.27 and 3.70 m, respectively. The yield per unit volume was 1,433.96 g per cubic meter in Hybrid No. 427 seedling and it was 1,251 g in Hybrid No. 411 (Rangith 1984).

Intensive mango hybridization program was initiated at the Agricultural Experiment Station, Gujarat Agricultural University, Paria, Gujarat in 1968. Thirty hybrids of various parental combinations were assessed for high yielding, regular bearing varieties of mango with improved quality. Of these, three mango hybrids, *viz*., Neelphanso (Neelum × Alphonso), Neeleshan Gujarat (Neelum × Baneshan) and Neeleshwari (Neelum × Dashehari) were found promising and released for commercial cultivation (Sachan et al. 1989). 'Neeleshan' was found to be highly suited for table and juice purposes since fruit quality was excellent (TSS 19.06%; total sugar 13.13%).

'Neeleshan Gujarat' is moderately regular bearer and late maturing type. Fruits are of medium size weighing 318 g with an average yield of 47.75 kg tree^{-1}. Fruit quality is very good (TSS 16.26%; total sugars 11.51%; acidity 0.12%; Vitamin C 16.09 mg 100 g^{-1} pulp). 'Neeleshwari' is dwarf in nature, bearing is moderate and late maturing type. Fruits are medium in size (228 g) and the average yield per tree is 34.95 kg fruits. Fruit quality is very good (TSS 20.53%; total sugars 10.78%; acidity 0.11%; Vitamin C 36.04 mg 100 g^{-1} pulp). Fruit pulp is moderately firm, non-fibrous and suited for table and juice making (Sachan et al. 1989).

Work was initiated at the Regional Fruit Research Station, Vengurla, Maharashtra, in order to evolve a regular bearing variety having Alphonso like qualities. Crosses of Alphonso and Neelum were made since 1970. Out of 40,000 crosses made, nearly 300 hybrid seedlings were obtained and evaluated for desirable characters. Out of the hybrids planted in 1971, No. 13, a cross between Neelum × Alphonso, was found to be the most promising and later this hybrid was released for commercial cultivation under the name 'Ratna' (Limaye et al. 1984). 'Ratna' is a regular bearer with most of the fruit qualities of Alphonso. The fruit is large with good sugar/acid blend, pleasing aroma, long shelf-life, early maturing and free from spongy tissue (Salvi 1983; Salvi and Gunjate 1989). This hybrid is a high yielder and 8, 9, 10, 11 and 12 year old tree has yielded 41.52, 34.66, 30.00, 34.09 and 32.24 kg fruits per tree respectively (Salvi and Gunjate 1989).

Although, 'Ratna' unlike 'Alphonso' is a regular bearer, a good yielder with good fruit qualities and free from spongy tissue, this quality lacks an attractive blush, fruit shape and a typical 'Alphonso' flavor. Hence, intensive work involving back-crossing between 'Ratna' and 'Alphonso' was undertaken with an objective to bring about the desired improvement in 'Ratna'. Parthenocarpic mango Hybrid-117 perhaps the first of its kind was obtained as a result of intensive back-crossing between the hybrid 'Ratna' (Neelum × Alphonso) and Alphonso at RFRS, Vengurla, Maharashtra. This work resulted in the development of Hybrid 117 that was released for cultivation in the Konkan region of Maharashtra with the name 'Sindhu' (Gunjate and Burondkar 1993). 'Sindhu' is comparatively dwarf and a regular bearer having medium sized fruit (215 g), with very high pulp to stone ratio (26:1) and very thin (30 mm) and small stone (6.72 g). Fruits are deep orange in the flesh, fiberless and free from spongy tissue disorder. The fruit pulp is very rich in ascorbic acid (52.22 mg 100 g^{-1} of pulp) and β-carotene (11,850 mg 100 g^{-1}). The non-viable cotyledon free stone makes up only 3.1 per cent of the total fruit weight. The striking character of this hybrid is seedlessness (Gunjate and Burondkar 1993).

Work at Fruit Research Station, Sabour, Bihar on hybridization in mango was initiated as early as 1942 and in the first batch as many as 63 hybrid progenies were obtained. The difficulties faced in hybridization program and probable remedies have also been suggested (Sen et al. 1946). Naresh Kumar (1997) described Sundar Langra (Langra × Sundar Pasand) and Alfazli (Alphonso × Fazli) hybrids developed from Sabour. Sundar Langra hybrid is semi-vigorous, spreading tree, moderate but regular bearer, fruits are medium in size (240 g), fruit pulp was similar to Langra and skin was similar to that of Sundar Pasand.

Work was initiated at the Central Institute for Sub-Tropical Horticulture, Lucknow, UP on mango breeding and two hybrids, *viz*., CISHM-1 and CISHM-2 were released. CISHM-1 is a cross between Amrapali × Janardhan Pasand. Fruits of CISHM-1 are medium in size (225 g), oblong in shape and with slight sinus. Fruit skin is attractive, pulp is firm, high TSS (21%) and suitable for export purpose (Negi et al. 1996). CISHM-2 is a cross between Dashehari × Chausa. Fruits of this hybrid are medium sized, weighing 220 g and oblong in shape. Skin is smooth, tough, yellowish green when ripe. Flesh is firm with scanty fibers and dark yellow in color. TSS of pulp is high (23%). This hybrid has good potential because of its sooty mould free fruit surface even after exposure to heavy rains.

The fruits are similar to Dashehari but mature 15 days later than Dashehari (Negi et al. 2000).

Mango breeding at the Horticultural Research Station, Periyakulam, TNAU resulted in isolation of the Hybrid 2/7 which was later released as PKM-1 in the year 1980. This hybrid is a cross between Chinnasuvernarekha × Neelum. A mean yield of 536 fruits with 102.70 kg was obtained per tree per year with a maximum yield of 1420 fruits weighing 284 kg. The yield has been steady and regular. The hybrid bears fruits on clusters and the fruits are fairly big and very sweet in taste (Shanmugavelu et al. 1987).

An evaluation of four mango hybrids at the Horticultural Research Station, Periyakulam, revealed that a hybrid between Neelum × Mulgoa was high yielder. As many as 48.4 fruits per tree were obtained weighing 25.22 kg. It is a regular bearer with excellent fruit quality. Later this hybrid was released under the name PKM-2 (Shanmugavelu et al. 1987).

Work at the Regional Fruit Research Station, Anantharajupet, Cuddapah district, AP resulted in the release of hybrid, *viz*., Au-Rumani and the Fruit Research Station, Sangareddi released another hybrid, *viz*., Mangera. 'Au-Rumani' is a cross between Rumani × Mulgoa. This hybrid is high yielder and regular bearer. Fruit is apple shaped and resembles 'Rumani' with prominent shoulders. Fruit pulp is firm, sweet and good flavored (Swamy et al. 1970). Mangera is also a cross between Rumani × Neelum varieties. The tree of Mangera is dwarf and hence suitable for high density planting. It is a precocious, regular bearer and high yielder. The fruit is large, roundish oblique in shape. The fruit skin is uniformly yellow with red blush on the shoulders. Quality of fruit is very good (Swamy et al. 1972).

Works on mango breeding at the Regional Fruit Research Station, Kodur, AP resulted in the development of four hybrids, *viz*., Neeluddin, Neelgoa, Neeleshan and Swarnjahangir. 'Neeleshan' is a cross between Neelum × Baneshan. It is a mid-season variety with regular bearing in habit. The fruit size is large and almost similar to Baneshan in shape. It bears in clusters and some trees bear a second crop. Fruits are medium to large in size (300 g). Pulp recovery is high (69%), it is fiberless, fragrant and suitable for canning. Neeluddin (Neelum × Himayuddin) is a regular bearer, but fruits are small in size (220 g). The fruit is pulpy, fiberless, juicy and aromatic.

Work was initiated at the Indian Institute of Horticultural Research, Hessaraghatta, Bangalore in Karnataka during 1970 to develop varieties with regular bearing, high yield, good fruit quality and free from spongy tissue. About 100 F_1 hybrids of different parental combinations were raised and evaluated. Out of which, four hybrids, viz., Arka Aruna (Banganapalli × Alphonso), Arka Puneet (Alphonso × Banganapalli), Arka Anmol (Alphonso × Janardhan Pasand) and Arka Neelkiran (Neelum × Alphonso) have been selected (Iyer and Subramanyam 1993).

Arka Aruna is dwarf in stature, regular, precocious and medium yielder. It bears large fruits (450 g) having attractive peel color with red blush, cream color and fiberless pulp of good flavor, high TSS (22°Brix) and small stone. It has high pulp percentage (78.5) and free from spongy tissue. This is suitable for homestead planting. Arka Puneet is of vigorous plant type with regular and prolific bearing of medium size fruits (225 g) having attractive peel color, good aroma, high TSS (22°Brix), fiberless pulp about 75%, free from spongy tissue and good keeping quality. This hybrid is suitable for table purpose and processing. It is free from fruit fly incidence. The fruits can be stored at room temperature for more than 15 days. Arka Anmol is a semi-vigorous plant type with regular, prolific bearing habit, fruits weighing about 250 g with good table and keeping quality. Attractive golden yellow peel color makes it suitable for export. Arka Neelkiran is a regular bearer, fruits are medium in size, attractive and quality of fruit is better than Neelum.

In Israel mango breeding is in progress at the Agricultural Research Organisation (ARO), Bet Degan from where two hybrids, *viz*., 'Tango' and 'Shelly' have been released. 'Tango' is a cross between Naomi × Keitt. Tango tree is medium in size, the inflorescence is pyramidal, 35 cm long and is densely flowered. The flowering season begins in late February to early March. Fruits are oblong with pointed beak and shallow sinus. The dorsal shoulder is higher than the ventral one and the apex is tombed. The fruit size is uniform with an average weight of 380 g. The skin of the mature fruit is thin and smooth and its color is orange, blushed with a brilliant red. The orange colored pulp is juicy and tender with scarce fibers. It has a mild, sweet and sour flavor with a mild, pleasant aroma. The fruit can be stored for 13 days at 20 °C and 21 days at 14 °C. Preliminary data suggest that the yield of 'Tango' is slightly above average of parents (Lavi et al. 1997b).

'Shelly' was selected from a cross between 'Tomy Atkins' and 'Keitt' and is a late ripening hybrid. Fruits are round with no beak or sinus, apex is rounded. Average fruit weight is 500 g (ranging from 350 to 700 g).

The peel of the mature fruit is of medium thickness and smooth and its color is orange with a red blush. The pulp is orange with a juicy, firm texture and scarce fibers. The flavor is mild and slightly sweet. Mature fruit have remained firm and attractive up to 30 days after picking at room temperature. Preliminary data reveals that the yield of Shelly hybrid is well above average of parents (Lavi et al. 1997a).

16.1.3.3 Crop Improvement in Mango by Clonal Selection

Clonal selection within cultivars has yielded valuable results and hence appears to be worth pursuing particularly in countries where certain cultivars are in cultivation for a long time. When a cultivar is grown for a long period, though originated through vegetative propagation, variation may occur due to mutation at micro or macro level. This mutation is a possibility in a variety when cultivated for a prolonged period.

Alphonso mango is an export quality cultivar of India. Pandey (1998) studied different clones of this cultivar, *viz*., 'Alphonso of Behat' in Saharanpur (UP), 'Alphonso Batli' of Kirkee, Pune (Maharashtra), 'Alphonso Punjab', 'Alphonso White' of North Kanara district of Karnataka, and found that they differ from one another in more than one character. Pandey (1984, 1998) described seven different clones in Alphonso mango, *viz*., Alphonso Behat, Alphonso Batli, Alphonso Bihar, Alphonso Black, Alphonso Bombay, Alphonso Punjab and Alphonso White. The origins of all these clones are not known but these clones are indigenous to different parts of India.

In perennial trees like mango, asexual propagation renders preservation of accumulated mutations (both macro and micro) which normally would be sieved out by sexual propagation. This offers the scope for the selection of good clones within a cultivar (Iyer and Mukunda 1998). Exploitation of natural variability through selection of superior clones of commercial mango cultivar has been undertaken by several workers. Naik (1948) observed significant variation among the trees of the same variety in an orchard with regard to fruit shape, size, color and quality of the fruits which was ascribed to bud mutations. Oppenheinmer (1956) based on a survey in many mango orchards in India reported wide variability in the performance of the trees of the same variety in the same orchard. Singh (1971) and Naik (1971) have emphasized on the role of clonal selection in mango improvement based on their extensive observations. Iyer and Dinesh (1997) and Iyer and Degani (1997) have emphasized on the need for great caution while identifying new clones. It is necessary to test the new clones under replicated trials to compare them against standard commercial varieties to confirm the distinctiveness and superiority.

Work on clonal selection at the University of Agricultural Sciences, Bangalore has led to the identification of two superior clones in the cultivars Alphonso and Raspuri (Mukunda 1996; Anon 1998). HSA-4 (Alphonso clone) has fruits of larger size (410 g) with attractive fruit color. NPP-5 (Raspuri clone) is a high yielder, regular bearer, has better fruit shape and TSS of 21% (Mukunda 1996; Iyer and Mukunda 1998).

Studies carried out on the evaluation of certain clones of mango cv. Alphonso at the University of Agricultural sciences, Bangalore have revealed that 'MA-1' clone was an outstanding progeny, as the plants were vigorous in growth habit; panicle density was highest and produced largest sized panicles (Mukunda 2004). Sex ratio in this clone was highest and as such fruit set was also highest. Fruits of 'MA-1' clone were medium to large in size, firm pulp, highest pulp recovery, thin stone and medium peel. Highest TSS, moderate acidity and excellent TSS/acid blend was noticed in the fruit pulp of 'MA-1' clone. Fruits of 'MA-1' clone had attractive skin color with red blush on the shoulders; flavor was delightful and superb in taste. Fruits of 'MA-1' were tolerant to spongy tissue.

A regular bearing and high yielding mango clone, "Dashehari-51" has been released by the Central Institute for Sub-tropical Horticulture, Lucknow, UP. This clone produces good crop every year without off-bearing rhythm, the per year productivity being 38.8% more than "Dashehari". Even in an 'off' year, 'Dashehari-51' clone produced an average yield of 43.4 kg fruits per plant per year while Dashehari tree produced very poor fruits (Chadha et al. 1993; Rajput et al. 1996; Ghosh 1997; Anon 1999; Negi 2000).

Desai and Dhandar (2000) studied a large number of mango varieties of Goa state which are important either from commercial or from breeding point of view, for their physico-chemical and morphogenetic variations. These varieties differ in respect of bearing habit, fruit size, fruit color, flesh contents, pulp color and quality. Pulp contents depending on fruit size varied from 67.56% in Bemcorado to 83.21% in RC-MS-1 a clonal selection of Bemcorado. In general, the varieties namely Mankurad selection and Bomcarado selection were observed to be promising as

these clones possessed most of the desired commercial traits meeting international standard.

Chaikiattiyos et al. (2000) conducted the progeny test of '320' Kaew clones collected from the North, North-east and Central Thailand, and selected a number of clones with superior horticultural traits for further evaluation. Of these, the 'SK007' clone produced the highest fruit yield of 65.4 kg per tree at the age of 7–8 years. A subsequent comparison yield trial of the previously selected clones indicated that the 'SK007', officially recommended by the Department of Agriculture, Bankok, Thailand and currently known as 'Kaew Sisaket', has an average yield of 25.5 kg per tree at year 5–6 with the average fruit weight of 252 g, 81% flesh and 0.049% citric acid. The fine texture, low fiber content and firm flesh offer good quality for pickling.

Jintanawongse et al. (1999) made an attempt to improve the existing commercial mango cultivars in Thailand by the clonal selection in Nam Dok Mai, Khiew Sawoey, Rad and Nang Klang Wan cultivars. Since 1990, the growth behavior, yield and quality of fruit characteristics such as fruit size, flesh color, sweetness, aroma and texture were evaluated. The data showed 'Clone No. 01' of Nam Dok Mai, 'Clone No. 04' and '05' of Khiew Sawoey, Clone No. 03 of Rad and 'Clone No. 03' of Nang Klang Wan clones were superior over the other clones. In addition, DNA fingerprinting was also made for all these five clones. These clones are to be released as recommended and certified cultivars by the Department of Agriculture in the near future.

Singh (1999) reported a new variety 'MDCH-1' (Madhulika) out of 41 species found in the world. Studies made from 1990 onwards at ICAR, Manipur Centre, Imphal showed that this new species was the most promising one of the region besides its resistance to stone-weevil which is the common pest for the region. Among the 17 genotypes studied in foothill conditions of Manipur, the genotypes MDCH-1 and STH-1 have been found to be very promising with most desirable characteristics such as regular bearer, dwarf, better fruit quality and resistance to stone-weevil and diseases. Thus Singh (1999) was of the opinion that the new genotypes, MDCH-1 and STH-1, would be 'wonder mangoes' of the 21st century, and may be able to revolutionize the mango production in India and abroad.

Survey of local mango genotypes at Goa state has enabled collection of 68 different varieties. Big-fruited clones of 'Hilario' and 'Udgo' with outstanding fruit quality were screened (Anon 1994). During 1985–1995, mango clone Cardozo Mankurad was selected from the Mankurad variety of Goa. This new clone was described in detail and compared with the parent variety Mankurad. Cardozo Mankurad is regular bearer and produces large fruits of attractive color, high quality and yield (Mathew and Dhandar 1997).

Intensive survey made by Ramaswamy (1989) in the North-Western region of Tamil Nadu comprising Salem and Dharmapuri districts led to the isolation of three selections of 'plus trees'. Parameters like regular bearing, dwarfness, high yield, good fruit quality and field tolerance to pests and diseases formed the basis of selection. Dwarf clonal selections, one each in Rumani and Bangalora, and a high yielding elite Neelum clone were isolated. The estimated bark phenol contents were from 4,300 to 4,800 μg per gram of fresh weight. This physiological parameter appears to be linked with dwarfness. The selected clones lend scope for high density planting at a density of 500 trees per ha. The average weight of fruit in these elite clones of Rumani (362 g), Neelum (404.20 g) and Bangalora (639.40 g) clones was high and the pulp recovery was the highest (89.26, 87.74 and 93.93%, respectively) and TSS of the fruit pulp was medium to good (16.85, 22.34 and 16.96%, respectively).

Vijayakumar et al. (1991) made an intensive survey in Dharmapuri district of Tamil Nadu and exploited the natural variability present in Rumani and Neelum varieties of mango. 'DPI 55' a clone of Rumani and 'DPI 45' a clone of Neelum were dwarf in nature and suitable for high density planting. These clones possess precocity, regular bearing, attractive good quality fruit with high productivity. Vijayakumar et al. (1992) developled "Paiyur 1" mango, a clonal selection from Neelum variety of Tamil Nadu. This new clone is a dwarf plant, low spreading in nature and thus suitable for high density planting at a close spacing of 5 m × 5 m accommodating 400 trees per hectare. During the ninth year of planting the fruit yield of 'Paiyur 1' clone was 22.30 kg per tree accounting for 8,920 kg of fruits per hectare. The mean fruit weight was 121 g with 68 per cent pulp recovery and good taste.

An assessment of various vegetative and fruit characters was made by Subramanyam and Iyer (1989) in mango germplasm collected from different regions of India, to select suitable parents for hybridization. Accordingly they identified two dwarf plants from collections made in Kerala state, which they named as 'Kerala Local' and 'Local Kalapady'. These selec-

tions have potential for incorporating dwarfness in vigorous growing commercial varieties as well as using as new clones to establish high density orchards.

Mukherjee et al. (1983) based on detailed surveys of mango orchards in eastern India have identified many superior clones. Based on their survey 23 elite clones were identified. Some of the clones which ranked excellent grade by them are: 'Dawadi', 'Emrat Bhog', 'Shah Pasand', 'Sadik Pasand', 'Kali Bhog' (all from Murshidabad) and 'Misrikanta' (from Maldah). They also suggested that it would be worth putting these clonal selections in a varietal trial for comparison with the standard commercial variety for releasing as new superior selections for commercial exploitation and for utilization in the hybridization program.

In Punjab, several selections of sucking mango with good sucking qualities, abundant juice, less fiber, small stone and red blush on cheeks have been made at the Regional Fruit Station, Gangian. These are numbered as GN 1, GN 2, GN 3, GN 4, GN 5, GN 6 and GN 7 (Sandhu et al. 1990).

The 'Davis-Haden' is an example of a bud sport originated in Florida (USA) from 'Haden' variety. The fruit of 'Davis-Haden' is somewhat larger than 'Haden' and its season of maturity is about a month later. Such mutations occur frequently in some plants though in mango nothing is known of the frequency with which spontaneous mutations occur (Young and Ledin 1954). One of the mutant varieties reported in mango from Peru is 'Rosica'. It is a bud mutant of the local Peruvian cultivar "Rosado de Ica". In trials it was found to be precocious and regular bearing, giving good yield of high quality fruit. Unlike other local cultivars, it did not produce small seedless fruit, and it was monoembryonic (Medina 1977). Li Gueiheng et al. (1996) reported that 'Hongmang 6' is a mutant derived from Zill variety of mango from China. Production of Hongmang 6 is very high, 7-year-old trees producing yields of 43.50 kg fruits per tree. Fruits are of good eating quality and red to purplish-red in color while flesh is dark yellow and juice with TSS of 15.8%. Roy and Sharma (1960) observed a bud-sport in the local variety Hirasonia at the Bihar Agricultural College, Sabour, which bore virtually fiberless fruits, larger than those of parent variety and differing in other respects. The mutant was considered to be of potential economic value. Sharma et al. (1981) obtained some very interesting plant types in the VM_1 generation by stabilizing the changes induced through the heading back technique. Amongst these are plants with very long and very short extension growths, very thick and very thin shoots; multiple branching shoots; very large and very small lanceolate leaves and small leaves with highly wavy margins and plants which appear to be compact in growth habit. A few of these induced plant types, both from the varieties Dashehari and Mallika, appear to be promising from the point of view of dwarfness and hence, may prove their immense value. Higher TSS and better sugar/acid blend, than the standard Dashehari (as a control), were also observed in few plants.

A clone of mango 'Totapuri' has been selected at the Gujart Agricultural University, Paria, Gujrat (Anon 1997). This clone was identified from a private farmer's mango orchard of Babubhoi Bhagvanbhai Patel of Goima village, Pardi taluk, Valsad district. The age of the mother plant is 35 years and vigorous in nature with irregularly spreading primary and secondary branches. This clone flowers and fruits twice in a year, first week of October and second week of March. It yields 24,000 fruits per tree per year weighing 700 kg per tree (Naik 2000).

In North-Eastern region, selections Manipur-I and Manipur-II have been identified. These clones are dwarf, precocious, polyembryonic and regular bearer (Chadha and Yadav 1996). Chadha (1998) also reported about clonal selection in mango ('Sunderraja') made at Rewa, Madhya Pradesh. Two clones one each of Banganapalli, 'Rati Banganapalli' and clone ('Nuzvid') selected earlier, performed well (Anon 1999). Further, one clone each of Alphonso, Totapuri and Banganapalli has been selected at the Regional Fruit Research Station, Vengurla, Maharashtra; the Fruit Research Station, Anantharajupet, Andhra Pradesh, and the Agricultural Experimental Station, Paria, Gujrat, respectively (Anon 1999).

Studies on evolving improved plant types through physical and chemical mutagens are, in general, lacking in fruit trees, more particularly in mango. The study conducted by Sharma et al. (1983) showed that the LD_{50} values (S-irradiation) for the mango cultivars Neelum, Dashehari, and Mallika were 3.9, 2.9 and 2.4 Krad respectively. The effective dosages of EMS and NM for cvs. Dashehari and Neelum were 1.5% and 0.05%, respectively. Primary effects of both physical and chemical mutagens were found to be more or less the same. Some interesting changes in vegetative characters have also been established. Out of these a few plants appeared to be promising for

dwarfness whereas in some others, fruit quality improved.

A survey was initiated by Singh et al. (1985) between 1982 and 1985, to find out relative superiority of selected clones from different orchards, *viz*., Pilkhini, Motijhil and Nawalpur around Varanasi. One of the selected mango clones of 'Banarasi Langra' mango on the basis of overall performance, the 'clone No. 2' from Pilkhini orchard and 'clone No. 1' from Motijhil orchard were found to be most promising clones because of less fruit drop, better yield, light bottle green color of the skin with fairly high fruit quality and negligible incidence of malformation.

Kher (1961) gave a detailed account of the morphology and anatomy of the leaf, stem and fruit of a variegated mango plant from Malihabad region. The variation appears to be genetically controlled and results from a mutation in the middle histogenic layer. Singh and Ranganath (1997) surveyed 85 locally available clones in South and North Andaman, and six clones having regular and early flowering habit and bearing good yield of quality fruits were selected for further study. Two years after planting of grafts in the field, it was noticed that clone No. 6-1-3 showed early flowering (early October), and in its first year of fruiting about 100 fruits were harvested in March. The fruits were of good quality with minimum thickness of peel and had minimum stone weight.

Rukayah Aman (1989) reported that mango clone MA-125 which is known to be difficult to flower can be induced chemically using flower inductants. Both paclobutrazol and uniconazole between 5 and 10 g a.i. per tree and 0.50 to 1.50 g a.i. per tree, respectively increased flowering and fruiting in this clone. However potassium nitrate was not effective for this clone.

Siller Cepeda et al. (1994) studied the fruit quality and post-harvest behavior of four mango clones, *viz*., 'Osteen', 'Palmer', 'Fabian' and 'S1-25' in comparison with 'Kent' the commercial variety from Mexico. 'Osteen' and 'Kent' had similar fruit characteristics. 'Osteen' had the largest fruits and 'Fabian' the smallest (516 and 387.5 g per fruit, respectively). After storage 'Osteen' had the highest pulp percentage (83.2) and the lowest percentage of seeds (5.8) and peel (10.9). 'Osteen' and 'Fabian' had a yellowish pulp color while the other clones had a more orange colored pulp. It was concluded that 'Osteen' has potential as a commercial cultivar because when compared with 'Kent', it showed a comparable or higher values of fruit weight, pulp weight, sweetness and sugar/acid ratio. The post-harvest behavior of Palmer indicated that it could be used for longer storage periods, because it maintained high firmness and a low sugar/acid ratio after the storage. 'Fabian' had a high percentage of latent anthracnose which was expressed during storage.

Strains within 'Kensington Pride' have been identified in Australia and one of them, Grosszmann even having improved resistance to bacterial black spot (Mayers et al. 1988; Whiley et al. 1993).

16.1.3.4 Limitation of Traditional Breeding Methods

The main disadvantage of traditional breeding is that it is laborious and consumes lot of time. A superior mango type can be isolated through selection, clonal selection and by hybridization. It involves extensive field and paper work; and collection of at least ten years' data to confirm its quality. One can release a variety through these methods in his lifetime. The juvenile period present in most of the tropical fruits also affects quick assessment of a variety. Therefore, it is necessary to look for the important objectives in mango improvement program and use the available molecular approaches to address these problems. Selection of unique plants/genotypes/clones can be easily achievable using molecular approach. Markers can be used as a tag to isolate unique types using marker-assisted selection. Even linkage distance studies among genotypes can be taken up using these markers. Using molecular approach one can isolate desirable genes from one plant and incorporate into another. With this one can genetically manipulate a plant to have desirable characters. It can be easily achievable in less time. However, molecular studies in mango are limited when compared to other fruit crops.

16.2 Application of Molecular Markers for Genetic Analysis in Mangoes

There are hundreds of mango cultivars, of which only some 25 to 40 are of commercial importance (Chadha and Pal 1986). Commercially grown cultivars have been identified on the basis of leaf, panicle, fruit, and stone characteristics; however, these characters may change with environmental conditions (Laksh-

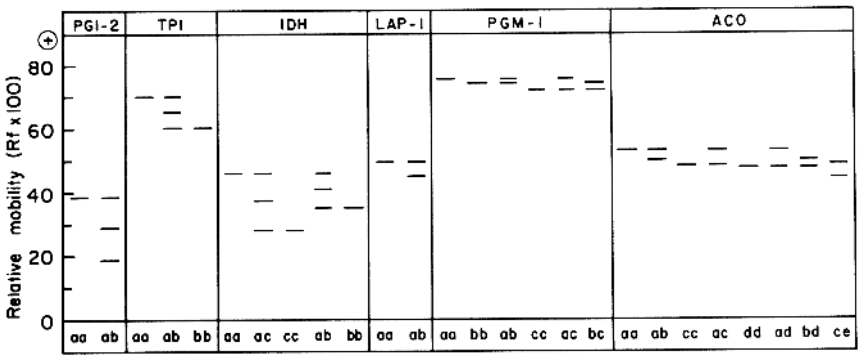

Fig. 1. Schematic zymograms of representative phenotypes for PGI, TPI, IDH, LAP, PGM And ACO isozymes in mango. Relative mobility ($R_f \times 100$) on the *left*; *O* = Origin. All enzymes migrated anodally (toward the top of the figure) (With permission from J Am Soc Hort Sci)

minarayana 1980). Furthermore, the actual identity of some cultivars is still in question, because similar cultivars grown in different areas often have different names (Lakshminarayana 1980). Having reliable means of cultivars identification and verification is important. Therefore, identification of genetic markers is of great value in this regard.

16.2.1 Isozymes

In the recent years, enzyme polymorphism has been used successfully to identify cultivars in various fruit species. However, isozymes can be affected by stage of development and tissue used for extraction (Feret and Bergmann 1976). Leaf isozymes of esterases, aspartate amino transferase, acid phosphatases, and alkaline phosphatases were used to detect possible genetic variation among individual mango clones (Gan et al. 1981). Degani et al. (1990) have used isozyme systems aconitase, isocitrate dehydrogenase, leucine aminopeptidase, phosphoglucose isomerase, phosphoglucomutase, and triosephosphate isomerase to characterize 41 mango (*Mangifera indica* L.) cultivars. The outcross origin of some of the mango cultivars was supported by the isozymic banding patterns (Fig. 1). Reported parentage of some other cultivars was not consistent with their isozymic banding patterns.

Isozyme systems were also used to detect zygotic seedlings from five polyembryonic cultivars of mango. Significant differences were found between cultivars for the percentage of zygotic and nucellar seedlings detected (Schnell and Knight 1992). They were able to determine the off-types using isozymes and concluded that this procedure can be used to help certify rootstock mother trees (Fig. 2).

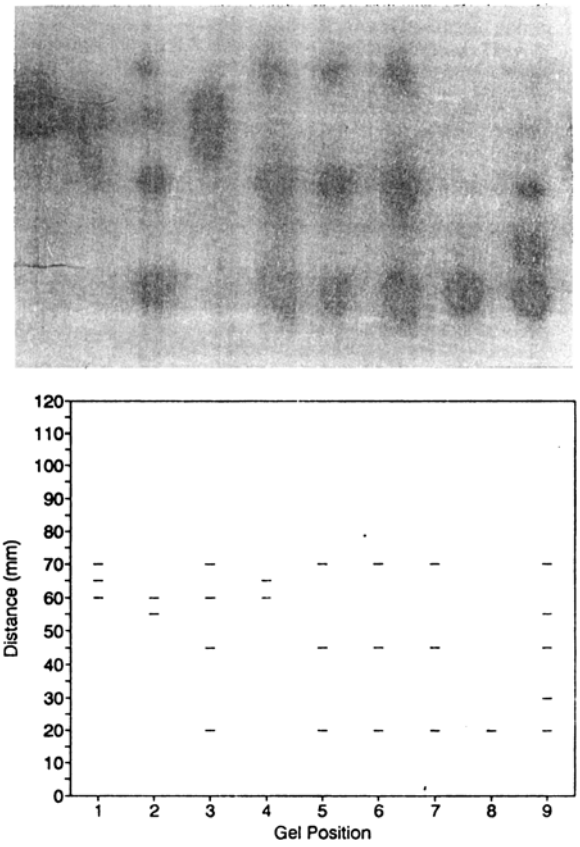

Fig. 2. Isozyme banding patterns (photograph and corresponding diagram) of glucose-6-phosphate iosmerase among rootstock cultivars and seedlings. Gel position: 1) Madoe, 2) Madoe RSP 1, 3) Madoe RSP 11, 4) Madoe RSP 18, 5) 13-1, 6) Turpentine, 7) Turpentine RSP 2, 8) Turpentine RSP 9 and 9) Golek RSP 13 (With permission from HortScience)

On the contrary, Gazit and Knight (1989) used one enzyme system, glucose-6-phosphate isomerase (GPI), and gas chromatography to detect zygotic plants among open-pollinated seedling populations from polyembryonic mango cultivars. Gas chro-

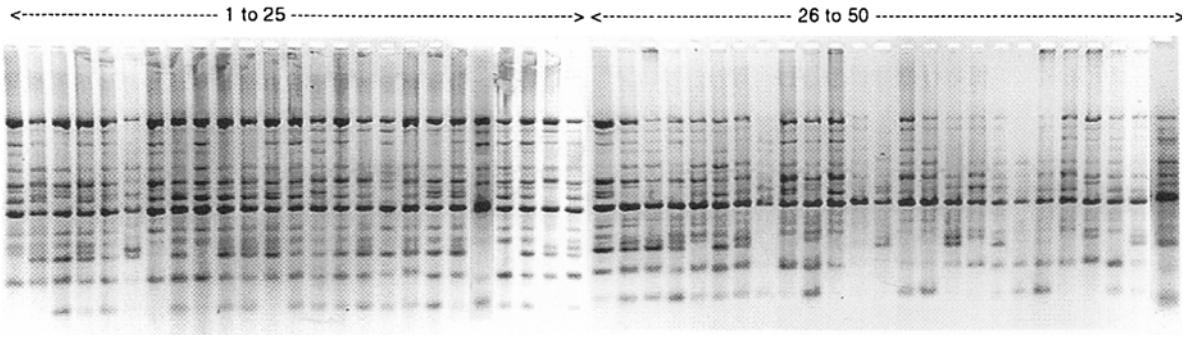

Fig. 3. RAPD gel profile of 50 mango cultivars using Operon primer D1. *Lanes* 1–10: Raspuri, Neelum, Baneshan, Ratna, Mulgoa, Dashehari, Hamlet, Alphonso and Totapuri, *Lanes* 11–20: PKM-1, Rumani, Sindhu, Mallika, Amrapali, Neeleshan, Neelgoa, Neeluddin, PKM-2 and Himayuddin, *Lanes* 21–30: Kesar, Goamunkur, Suvarnarekha, Vanraj, Cherukurasam, Arka Aruna, Jehangir, Svarna Jehangir, Kuddus and Kalapadu, *Lanes* 31–40: Arka Puneet, Vikrabad, Arka Anmol, Pulihora, Rajgira, Achar Pasand, Fazli, Khas-Ul-Khas, Nekkere and Bombay Green, *Lanes* 41–50: Langra, Janardhan Pasand, Willard, Allumpur Baneshan, Rajapuri, Zardalu, Kishen Bhog, Tenneru, Ratnagiri Alphonso and Dilpasand

matograms were too cumbersome for analysis of large populations; the isozymes system proved to be simple, repeatable, and cost effective. However, enzyme polymorphism in mango has not been examined systematically.

16.2.2 DNA Markers

As the efficiency of a selection scheme or genetic analysis on phenotype is a function of heritability of the trait, factors like environment, traits of multigenic and quantitative inheritance, or partial and complete dominance often confound the expression of genetic traits. Many of these complications of a phenotype-based assay can be overcome through direct identification of genotypes with DNA (deoxyribose nucleic acid) based diagnostic assay. Genomic fingerprinting has been accomplished traditionally through the use of isozymes, and more recently through restriction fragment length polymorphism (RFLP), variable number tandem repeats (VNTRs), or a combination of both. While these methods have been very useful in cultivars identification, they have a number of disadvantages, including a limited number of isozyme loci and the time, expense, and use of [^{32}P] for labeling with RFLPs and VNTRs. Polymerase Chain Reaction (PCR) based method overcomes these disadvantages and is used in several crops. For this reason, DNA-based genetic markers are being integrated into several plant systems and are expected to play an important role in future plant improvement programs in mango.

16.2.2.1 Random Amplified Polymorphic DNA (RAPD)

PCR technology has led to the development of several novel genetic assays based on selective DNA amplification (Krawtez 1989; Innis et al. 1990). A genetic assay was developed independently by two laboratories (Welsh and McClelland 1990; Williams et al. 1990). RAPD assay detects nucleotide sequence of polymorphisms in DNA using only a single primer of arbitrary nucleotide sequence. The protocol is also relatively quick and easy to perform and uses fluorescence instead of radioactivity. Because the RAPD technique is an amplified-based assay, only nano-gram quantities of DNA are required. One of the strengths of this new assay is that they are more amenable to automation than conventional techniques. It is simple to perform and is preferable to experiments where the genotypes of large number of individuals are to be determined at a few genetic loci.

The use of RAPDs to determine genetic relationships has been demonstrated in several crops. Within *Mangifera* (mango) species, RAPDs have been used to determine phylogenetic relationships (Schnell and Knight 1993). RAPD generated clusters did not agree with the taxonomic classification in mango based on phenotypic traits (Kostermans and Bompard 1993) into *Mangifera* and *Limus*. When the two subsections of the genus were analyzed separately, the classification agreed more closely with the traditional taxonomic analysis. This technique has been successfully used to identify 25 accessions of mango and to validate their genetic relationships (Schnell et al. 1995). Genetic relatedness of traditional Indian mango cul-

tivars grown in commercial scale was studied using RAPD makers (Ravishankar et al. 2000). Results of the study indicated that cultivars from a particular geographical region were closely related. In India, it is very difficult to distinguish wild trees from cultivated ones as they spread all over Indian peninsula, the result clearly indicate that majority of the commercial cultivars evolved from local germplasm and later they were selected and propagated vegetatively. Fifty commercial mango cultivars were screened using RAPD markers to estimate the genetic diversity (Kumar et al. 2001). A high degree of genetic variation was observed among the cultivars and the variety 'Mulgoa' was found to be very distinct (Figs. 3 and 4).

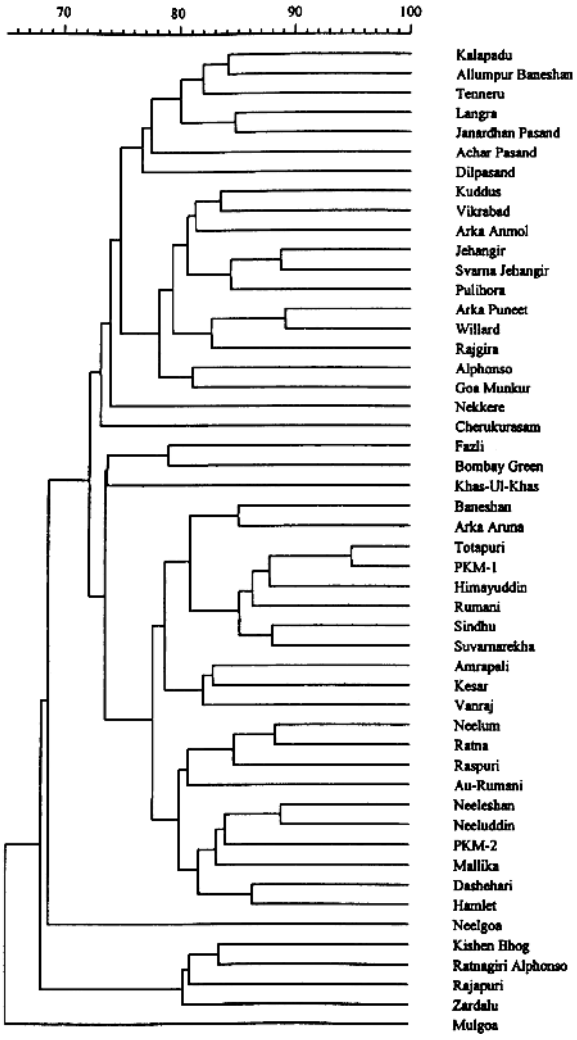

Fig. 4. Association among mango genotypes revealed by UPGMA cluster analysis from RAPD data of 139 amplification products generated by 10 primers

Along with this paternity analysis of 14 mango hybrids was also carried out. The cluster analysis revealed the relationship of hybrids with their parents. The progenies were placed close to one of their parents whose characters resemble with it.

Lopez-Valenzuela et al. (1997) reported that RAPD marker can distinguish mangoes based on embryonic types and their geographical origin. The genetics of polyembryony in mango (*Mangifera indica* L.) was studied by Arnon et al. (1998) and they suggested that it is determined single dominant gene. The segregation pattern obtained by RAPD of individuals originating from selfing of several monoembryonic cultivars and one polyembryonic line indicated that the polyembryony in mango was of genetic nature. All the plants originating from monoembryonic bear monoembryonic fruit. An one-monoembryonic to three-polyembryonic segregation pattern was observed among individuals originating from the polyembryonic line, indicating that polyembryony in mango is under the control of a single dominant gene. This was also proved by Ravishankar et al. (2004) where they used both RAPD and chloroplast DNA for PCR-RFLP analysis to detect the genetic bases of Indian polyembryonic and monoembryonic mango cultivars. The cluster analysis of both markers revealed that eventhough the embryonic types are intercrossable, the polyembryonic types group separately indicating diverse genetic base. This suggested that polyembryonic types might have been introduced from other parts of Southeast Asia and is unlikely to have originated from India.

The long juvenility period of mangoes (up to 5 years) would make RAPD markers an extremely useful tool for the identification of cultivars during propagation and growth. The ability to identify mango cultivars using RAPD markers would also aid in the management of germplasm collections as identical cultivars often have different names. However, RAPD markers suffer from low reproducibility between laboratories.

16.2.2.2 Simple Sequence Repeats (SSRs)

This is widely used as a versatile tool in plant breeding programs as well as in evolutionary studies because of their ability for showing diversity among cultivars (Adato et al. 1995; Mhameed et al. 1996; Levi and Rowland 1997). SSRs, also known as microsatellites, are an efficient type of molecular marker based on tan-

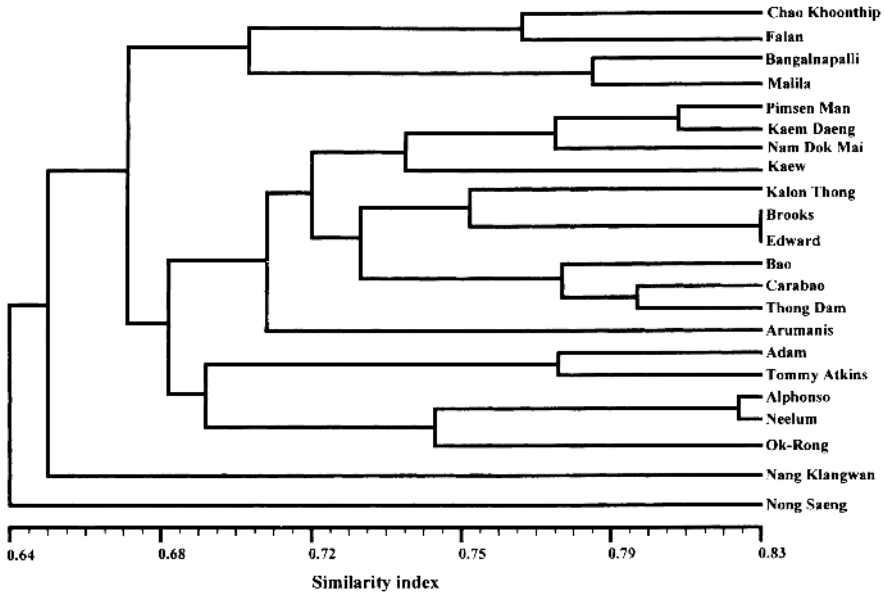

Fig. 5. Dendrogram illustrating the phylogenetic relationship among 22 mango cultivars based on UPGMA cluster analysis (With permission from Elsevier)

dem repeats of short (2–6 nucleotides) DNA sequence (Charters et al. 1996). These repeats are highly polymorphic, even among closely related cultivars, due to mutations causing variation in the number of repeating unit (Brown et al. 1996). SSRs, therefore, target highly variable and numerous loci.

Using Jeffrey's minisatellite probe Adato et al. (1995) identified and analyzed genetic variations among mango genotypes. They were able to trace back the offspring to one of the parents using banding pattern, demonstrating the reliable application of the system for paternity dilemmas. On the contrary SSR anchored primers were used to identify and to validate genetic variation among Thai cultivars (Eiadthong et al. 1999), but could not separate the cultivars according to their embryonic types, nor the types eaten as ripened fruit or unripe fruits (Fig. 5).

Recently, Sequence Tagged Microsattelite Sites (STMS) markers are being developed by many laboratories for mango. The main advantage of these markers are precise quantification of allele length and they are amenable for automation using automated DNA sequencer. Viruel et al. (2005) reported the sequence and variability parameters of 16 microsatellite primer pairs obtained from two mango genomic libraries after digestion of DNA of the cultivar Tommy Atkins with *Hae*III and *Rsa*I and enrichment in CT repeats. The polymorphism revealed by those microsatellites was evaluated in a collection of 28 mango cultivars of different origins. The SSRs studied allowed unambiguous identification of all the mango genotypes studied. They suggested that this discrimination can be carried out with just three selected microsatellites. UPGMA cluster analysis and principal coordinate analysis grouped the genotypes according to their origin and their classification as monoembryonic or polyembryonic types reflecting the pedigree of the cultivars and the movement of mango germplasm. A similar attempts is being made to develop SSR markers for mango in France (Duval et al 2005), Japan (Honso et al. 2005) and in India (Ravishankar 2006. Personal communication). These SSR markers are going to help extensively in mango genome mapping.

16.3 Linkage Mapping

Genetic linkage and QTL (Quantitative Trait Loci) mapping experiments involve large volumes of data. These include pedigree details, genotypes and trait data all of which must be combined in different forms to suit the nuances of each analysis program. Such experiments frequently also consist of collaborations between several groups making data sharing and concurrency a key concern. Many good software modules for statistical analysis of genomic data are offered in the public domain like MapMaker for linkage map construction, MapMaker/QTL for interval mapping for experimental crosses and others.

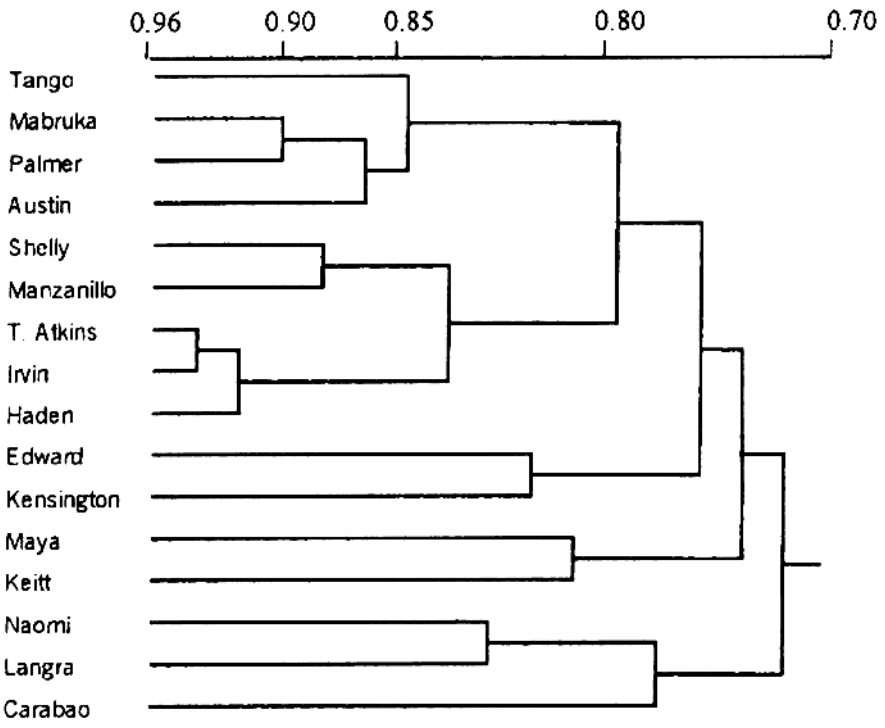

Fig. 6. Analysis of 16 mango cultivars using 42 various AFLP bands (With kind permission from Springer Science and Business Media)

An important development during the last decade in quantitative genetics was the ability to identify genome regions responsible for variation of a trait due to the advent of molecular markers (Paterson et al. 1988). The term QTL has come to refer to polygenes underlying a quantitative trait. In genetics, the distance between genes on the genome is assessed on the basis of the frequency of recombination of the genes, estimated from scoring genotypes of progeny of a cross (Kearsey and Pooni 1996). Mapping quantitative traits is difficult because the genotype is never unambiguously inferred from the phenotype. Classical quantitative genetics pursues a different approach, using statistical concepts such as means, variances, correlations, heritabilities (h^2), built on assumptions, e.g., that effects of individual genes on a trait are small and additive. This assumption sheds little light, if any, on the individual genes themselves (Prioul et al. 1997).

Major gene mutants, however, are scarce and may not exist in a population under study. Because QTL may occur throughout the genome, a large number of gene markers are required to locate them. Early studies of quantitative traits suffered from the lack of major-gene markers that could make a complete map. This problem was overcome with the realization that maps could be constructed using pieces of DNA as markers.

The advent of complete genetic linkage maps consisting of codominant DNA markers like RFLP, AFLP and SSR, that has stimulated interest in the systematic genetic dissection of discrete Mendelian factors underlying quantitative traits in plants. A marker linkage map can be used to localize QTL for a quantitative trait, as first demonstrated by Paterson et al. (1988). The basis of all QTL detection is the statistical analysis of associations between markers and trait values. Statistical techniques for using a marker map to detect QTL have reached a fairly high level of sophistication, but improvements are still being made(Kearsey and Farquhar 1998). A widely used method was intervalmapping (Lander and Botstein 1989). Other approaches, e.g., the multiple QTL method (Jansen 1995), were developed to detect multiple linked QTL. However, a QTL detected by any technique is not a true gene, only the indicated genome region that most likely contains gene(s) for the trait under study.

Two complementary uses of the QTL approach have emerged: the fundamental and the applied (Prioul et al. 1997). The first use, which is of interest to physiologists, targets QTL by determining their contribution to physiological components of macroscopic traits. Not only does the QTL approach provide unequivocal answers to a range of physiological questions, it also generates new insight into the causality

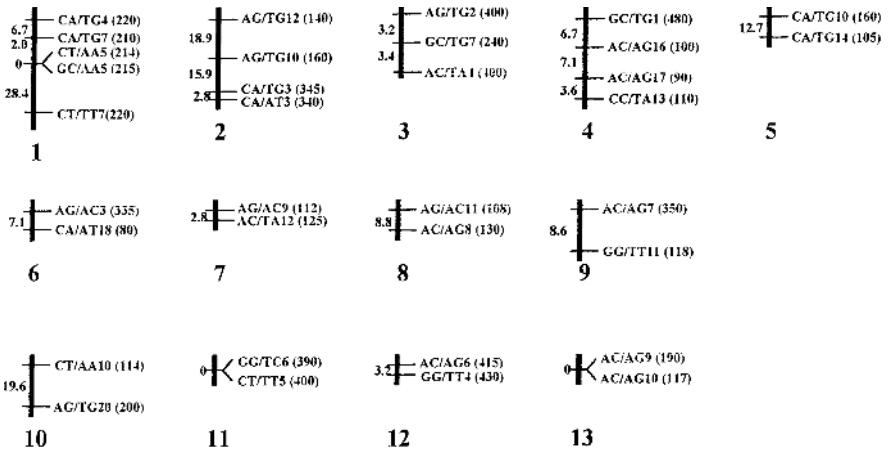

Fig. 7. Mango genetic linkage map. The various marker names refer to the primer combinations used for AFLP analysis. The numbers in parentheses indicate the size of the markers in base pairs (bp). Distances between markers are in centiMorgans. (With kind permission from Springer Science and Business Media)

between components that would have been difficult to obtain by conventional physiological approaches (Simko et al. 1997). The second use of the QTL studies, which is of interest to breeders, is marker-assisted breeding (MAB). This approach uses markers for tagging QTL of interest so as to pyramid favorable QTL alleles and break their linkage with undesirable alleles (Lee 1995; Ordon et al. 1998; Ribaut and Hoisington 1998).

The AFLP technique is based on the detection of genomic restriction fragments by PCR amplification, and has been applied to various plant species. This PCR-based technique permits inspection of polymorphism at a large number of loci within a very short period of time and requires very small amount of DNA. This method is robust for efficient DNA fingerprinting of the mango genome. A great majority of the AFLP markers (85%) are transmitted in a Mendelian fashion. Thus these markers could be used for genetic analysis.

Kashkush et al. (2001) used AFLP technique to identify mango cultivars, for studying the genetic relationship among 16 mango cultivars (Fig. 6) and seven mango rootstocks, and for the construction of a genetic linkage map. A preliminary genetic linkage map of the mango genome was constructed, based on the progeny of a cross between 'Keitt' and 'Tommy-Atkins'. Each of the two parents and 29 progeny were genotyped using 105 AFLP bands. The segregation of the alleles from each marker was examined for deviation from Mendelian expectation. The combined map consists of 13 linkage groups and 34 markers (Fig. 7). They reported that the genetic map consists only of markers that behave in a Mendelian fashion. Linkage analysis was carried out using both the MAPMAKER and LINKEM software. Both the programs provided identical results. They finally conclude that AFLP markers are suitable for cultivar identification, estimating genetic relationships and mapping QTLs in mango.

This is the only reported work on genetic linkage mapping in mango. Plant Genetics group at United States Department of Agriculture, Agricultural Research Service, Miami, Florida are working to solve the problems associated with the evaluation, enhancement, and preservation of subtropical/tropical fruit, using linkage maps. They have maintained germplasm repository. Their main objective is to develop and apply new or improved methods for elucidating the genetic bases for important horticultural traits, genetic marker-based approaches for genetic diversity assessment, and selection of improved germplasm and also identifying genes involved with horticulturally and agronomically important traits using Candidate Gene Approach (CGA). Mapping QTL for fruit characteristics, yield and disease in an F_2 population in mango in under progress. More focus has to be given towards this area of research that helps in tagging the genes of desirable traits.

16.4 Gene Isolation and Analysis

Gene expression studies in mangoes are limited. It is desirable to identify the genes uniquely expressed for particular traits. This would help in genetic manipulation of the plant to derive superior types having desirable characters. In mango, genes related to ripening have been characterized biochemically, but at molecular level only very few genes have been studied like

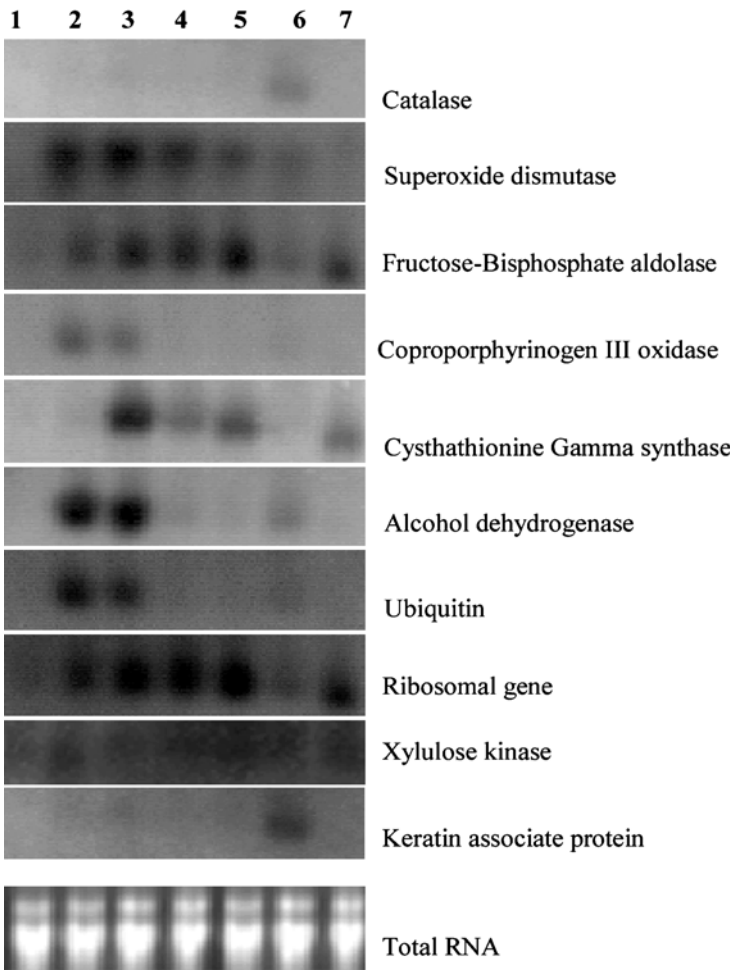

Fig. 8. Autoradiogram of northern analysis of the ten selected genes. 20 μg of total RNA from tissues of matured unripe fruites, RNA from 1, 2, 3 and 4 d fruits after ethylene treatment and from both spongy and healthy tissues. *Lane* 1–5 represent different stages of fruit ripening starting from matured fruits and *lane* 6–7 represent RNA from both spongy and healthy tissues

peroxisomal thiolase mRNA (Bojorquez and Gomez-Lim 1995), alternative oxidase (AOX) and uncoupling proteins (UCP) (Considine et al. 2001). AOX and UCP play an important role in post-climacteric senescent processes.

The next step in the molecular analysis of fruit is the construction of a gene library. This approach has already yielded valuable information in other fruit crops, where the genes coding for several ripening-related enzymes have been isolated. In mango, a cDNA library has been recently constructed and several ripening-related genes have been isolated (Gomez-Lim et al. unpublished data). Among the genes identified are those coding for cellulase, ACC synthase and the alternative oxidase, an enzyme involved in the cyanide-resistant respiration in fruits (Day et al. 1980). Preliminary expression studies of these genes show that they are predominantly expressed during fruit ripening, a fact consistent with their function and with studies in other fruits. In addition, other ripening related genes from mango have been cloned (Gomez-Lim et al. unpublished data) but their identity is still unknown. They also show predominant expression during ripening. Sequence analysis of these genes is in progress together with further studies to try and elucidate their function or identity.

At molecular level, no studies have been carried out to isolate genes involved in pest and disease resistance, and physiological disorders. This has been successfully isolated in several other crops. Very recently RAPD analysis was used to determine the genetic diversity on mango malformation pathogens (Zheng and Ploetz 2002). Vasanthaiah (2006) successfully isolated the differentially expressed genes specific to spongy tissue in Alphonso mango cultivar using subtractive hybridization. Thirty-seven genes from both spongy and healthy tissues were cloned and characterized. Higher expression of catalase, ubiquitin, coproporhyrinogen III oxidase and keratin genes were noticed in the tissue indicating

the existence of oxidative stress in spongy tissue (Fig. 8). It is also evident from the earlier studies that high temperature, high humidity, high respiration and low transpiration (Shivashankara and Mathai 1999) result in spongy tissue. These conditions elevate temperature and free radical levels in the fruit, which are toxic to the cell and resulting in cell membrane damage. Because of this the activity of enzymes like amylase, glutamate dehydrogenase, glutamate oxaloacetate transaminase, peroxidase were reduced. This influences sugar metabolism making the tissue hard and affecting the normal ripening. This study (Vasanthaiah 2006) indicated that oxidative stress is the probable cause for the spongy tissue formation.

Recently, in mango fruit cv Alphonso, 26 differentially regulated cDNAs from ripening tissue were isolated using PCR based subtraction at Indian Institute of Horticultural Research, Bangalore. These cDNA sequences were analysedwith NCBI database to assign putative identification. Expression patterns of major latex protein,cytokinin oxidase, omega-3-fatty acid desaturase, chitinase, putative flavanone 3-beta hydroxylase, putative auxin regulated protein, lipoxygenase, succinate dehydrogenase, protein phosphatase-2C and Acetyl-CoA acyltransferase were studied using RNA blot analysis at different stages of ripening. Majority of the identified genes were up-regulated during ripening process. A few of the identified genes have not been characterized in mango. The genes identified by differential expression are involved in changes associated with fruit ripening process like surge in respiration, ethylene biosynthesis, softening of mesocarp tissue, accumulation of pigments, development of characteristic aroma, change in color of the fruit and defense response (Ravishankar 2006 personal communication).

16.5 Gene Manipulation by Genetic Engineering

Limited reports exist on genetic manipulation in fruit crops. Recent experiments have shown that it is possible to turn-off the expression of certain genes in transgenic plants by introducing a gene constructed to generate antisense RNA (Eguchi et al. 1991). This allows expression of specific genes to be diminished, permitting their identification and assessment of their function during ripening. Genetic transformation has also been employed to achieve this goal. In mango a number of enzyme activities have been detected during mango fruit ripening (Selvaraj and Kumar 1995). Some of these may or may not be directly involved in the softening process. The results of the genetic transformation studies clearly indicated that correlative data linking enzyme activity and fruit softening might not accurately predict enzyme function.

The genetic transformation studies have also shown their potential for prolonging fruit shelf life. These procedures are universal and can be applied to many crops. Recent achievements in the transformation techniques will permit testing the function of specific hydrolytic enzymes for extending mango fruit shelf life. This development is particularly relevant because it will probably be the first tropical fruit whose ripening pattern may be genetically manipulated.

16.6 Future Scope

Mango has comparatively small haploid genome size and is about three times as large as *Arabidopsis* (Armuganathan and Earle 1991). This fact should be helpful for the application of other molecular techniques like Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), SSR, Differential Display RT-PCR (Reverse Transcriptase-PCR) and Subtractive Hybridization technique to assess genetic diversity, to construct linkage maps and to identify genetic markers linked to a trait of interest among different mango cultivars. This will help in isolating distinct types that are regular and precocious bearer. However, these techniques can also be used in isolating desirable genes responding to pest and disease incidence, and physiological disorders, which can be utilized to genetically manipulate mango plant to make it disease resistant. Very recently, Amplified Fragment Length Polymorphism (AFLP) information was used for identification of mango (*Mangifera indica* L.) cultivars to study genetic relationship among mango cultivars and rootstocks for the construction of a genetic linkage map (Kashkush et al. 2001). The development PCR based SSR markers from various laboratories will further strengthen molecular mapping activities and help in fine mapping

of mango genome. The molecular techniques also provide information on the genetic variability of mango species to build database on mango genetic diversity. Finally, all these molecular techniques have a potential for developing a superior genotypes with desirable characteristics in a shorter time when compared to conventional breeding methods.

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17 Avocado

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17.1 Introduction

17.1.1 Crop Production

Avocado (*Persea americana* Mill.) is a high-value specialty crop enjoying widespread cultivation in the far corners of the world. Mexico (Michoacán State) is the primary producer, with over 200,000 acres yielding almost 2.1 billion lbs of avocados, followed by the United States (68,000 acres, 531 million lbs; 90% from California) and Chile (65,000 acres, 388 million lbs), and significant contributions (cited in order of decreasing fruit production) also by Peru, South Africa, Israel, Spain, Australia, and New Zealand (figures for 2004/2005 season; *http://www.avocadosource.com*). By far the most widely grown cultivar is 'Hass', a black-skinned form adapted to a Mediterranean climate and combining high yields with excellent flavor. The exemplary nutritive composition of avocado – including lutein, vitamin E, monounsaturated ("good") fat and folate – has also contributed to its growing popularity.

17.1.2 Botanical Races

Three botanical races are often distinguished to circumscribe subgroups combining particular fruit characteristics and ecological adaptations: the Mexican, Guatemalan and West Indian races. The Mexican botanical race includes cultivars with thin, black skin and anise-scented leaves. Cultivars with thick, rough, green skin belong to the Guatemalan botanical race and have a level of cold tolerance somewhat inferior to the Mexican race. Cultivars of the West Indian race – a misnomer as these genotypes appear to have arisen in coastal Guatemala – are cold-sensitive, often relatively salt-tolerant, and have fruit with low oil content. Wild *Persea* relatives bear little resemblance to their cultivated counterparts, but cultigens have been selected by local people throughout Central America, Mexico, and farther afield, which has led to a plethora of forms that are assigned to one of the three botanical races based on the best morphological and ecological "fit". A discontinuity between wild and selected avocado forms suggests that any intermediate genotypes have long disappeared, and that human selection has been in progress for some considerable time. Indeed, the first archaeological evidence from the Mexican state of Puebla dates back to ca. 8000 BC (Smith 1966, 1969; Whiley et al. 2002).

17.1.3 Classical Breeding

The process of avocado breeding today continues to rely on open-pollination of promising varieties and – until very recently – without knowledge of the pollen source and unassisted by molecular techniques. This selection approach is the consequence of several obstacles to traditional breeding that are related to the biology of the avocado tree itself. The first and foremost among these is the inability to perform controlled pollinations (avocado trees produce well over a million tiny flowers that are abundantly shed; reviewed in Davenport 1986). The bulky size of the avocado tree makes large-scale experimental trials land- and labor-intensive. The widespread use of rootstocks introduces additional cost, conflicting breeding strategies, and can even confound data collection when the rootstock outgrows its scion. Finally, trees are slow to mature, and five or more years elapse before reliable estimates of fruit yield and quality are obtained.

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17.2 Molecular Beginnings

Faced with these substantial challenges, attempts have been made over the past 25 years to adopt molecular approaches. Various molecular studies have focused on the development of markers, including isozymes (Torres and Bergh 1980), RFLPs (Furnier et al. 1990; Davis et al. 1998), microsatellites (SSRs) (Lavi et al. 1994; Ashworth et al. 2004), VNTRs (Mhameed et al. 1996), and RAPDs (Kobayashi et al. 2000). Nonetheless, molecular technologies have been slow to take hold.

Several factors have so far conspired to delay the application of molecular tools to avocado breeding, in addition to those pertaining to the biology of the plant. Most importantly, the public sequence databases have been largely devoid of any suitable gene regions or ESTs. Moreover, avocado is a member of family Lauraceae that lacks major crop species and represents an ancestral lineage predating the origin of the Eudicots (Stevens 2001 onwards). The phylogenetically distant position of avocado decreases the utility of sequences from the major model organisms, e.g., *Arabidopsis,* which in turn has discouraged the pursuit of alternative molecular approaches, such as candidate genes and comparative gene expression. Similarly, linkage and genetic mapping studies (Lavi et al. 1991, 1992, 1993; Mhameed et al. 1996; Sharon et al. 1997, 1998), though providing preliminary insight into associations between such traits as skin color and fiber presence in the fruit flesh, are limited in scope due to a relative shortage of markers in relation to the number of chromosomes ($x = 12$), a lack of experimental replication, and limited baseline cytogenetic work. It therefore seems unlikely that genes will be localized to particular chromosomes in the near future.

Our laboratory is currently involved in a QTL study that, unlike previous research, attempts to control for genetic variation across sites and environments. Our aim is to work toward marker-assisted selection that will eliminate the delay between genotype selection and evaluation of maturity-dependent productivity traits. In parallel, we have been pursuing a comparative sequencing project to study haplotype diversity for several genes in various avocado cultivars and wild relatives/cultigens.

17.2.1 SSR Markers

The development of molecular marker technology (primarily SSR markers) has benefited the breeding process in the short-term by enabling the study of genealogical relationships among varieties and cultigens (e.g., Furnier et al. 1990; Davis et al. 1998; Ashworth and Clegg 2003; Schnell et al. 2003) and of parentage/pollen movement in avocado orchards (Goldring et al. 1987; Degani et al. 1989; Davenport et al. 1994; Kobayashi et al. 2000; Garner et al. 2006). SSRs are now the markers of choice for avocado in studies devoted to pollen flow and varietal differentiation, having replaced some of the more costly or less informative markers. These and other marker applications detailed below have fuelled the need for a greater number of markers. One of our research priorities has been to address this need by screening two genomic DNA libraries of 'Hass' for dinucleotide and trinucleotide markers, yielding some 150 useful marker loci. Of these, we routinely use some 30 loci (see Ashworth et al. 2004 for primer sequences), and the remainder has been screened for ease of interpretation and utility in distinguishing a panel of ca. 5–10 cultivars. In relation to the overall number of library clones sequenced (ca. 1,500), the yield of SSR markers has been exceedingly low, due primarily to clone redundancy, an apparent scarcity of loci, and poor interpretability of the banding profiles.

SSR Applications: Genealogical Studies These impediments notwithstanding, considerable information is beginning to accumulate on cultivar relationships, the delimitation of the three botanical races, and pollen movement within avocado groves.

Building on previous studies that used RFLP markers (Furnier et al. 1990; Davis et al. 1998), we analyzed genealogical relationships between 35 cultivars using 25 SSR markers (Ashworth and Clegg 2003). Consistent with prior research, heterozygosity was high, particularly for the SSR markers (73.4 and 52.6% for dinucleotide and trinucleotide markers, respectively). Low statistical support (bootstrap) for the assemblages denoting the three botanical races, even after (known) hybrid genotypes had been excluded from the analysis, suggested ancient hybridization or a more recent origin of the botanical races than previously thought. Indeed, the hybrid nature of most extant cultivars is probably responsible for homogenizing some of the racial differences.

One practical conclusion from our genealogical study is that botanical race assignments and geographic origin of a cultivar are often confused or applied inconsistently. For example, a "Guatemalan" cultivar may be so called because it has a rough thick green skin (characteristic of the Guatemalan botanical race) or because it was collected in Guatemala. Even when the geographic origin is disregarded, race assignment is based on a somewhat vague formula combining fruit skin thickness and color, presence or absence of anise scent, and origin (if known) of the maternal parent, with several other criteria added based on breeder experience. Clearly, this convention does not readily accommodate genotypes that have lost one or more of the key parental (maternal) traits (e.g., skin color, thickness; anise scent) through genetic segregation, for which ample evidence exists (e.g., Storey et al. 1984; Bergh 1966, 1967; Bergh and Whitsell 1974, 1975), or having a complex hybrid status (multiple backcrossing). Consequently, SSR markers are now also being used to eliminate or change the assignment of mislabeled material (Schnell et al. 2003) or to unravel parentage (Ashworth and Clegg 2003). These SSR studies show that phenotype tracks genotype in an unpredictable manner, and that greater rigor is needed in the delimitation of botanical races and in the description of avocado cultivars.

Another conclusion emerging from our work with SSR markers (Ashworth et al. 2004) is that not all markers developed using the 'Hass' genomic DNA library are useful for typing more distant genotypes, especially the more divergent rootstocks or the related species *P. schiedeana* and *P. steyermarkii*. Conversely, loci that show insufficient variation (one or two alleles) within a core group of cultivars sometimes harbor additional alleles when applied to the more distant genotypes. These observations have important implications for marker development strategies, particularly with respect to the cut-off applied to the number of repeat units that qualifies a sequenced clone for inclusion in the subsequent round of marker development.

SSR Applications: Pollination The co-dominance of SSRs means that allelic segregation can reveal paternity of a given progeny genotype, a major advance in a crop that defies controlled pollination and where paternal origin has hence always been subject to conjecture. The application of SSRs to pollination studies has therefore received considerable attention, not only from breeders, but particularly from avocado growers who are interested in the relationship between pollination and yields: 'Hass' fruit commands a far higher market price than alternative cultivars that are conventionally integrated into an orchard for the sole purpose of enabling cross-pollination. This planting practice stems from the observation that 'Hass' trees adjacent to certain "pollinizer" cultivars produce a profusion of fruit that often far exceeds that of 'Hass' trees located only one row farther away. However, this yield boost is not always observed, and growers are eager to embrace marker technology to better understand the relationship of selfing and cross-pollination on yield in order to calculate the trade-off between planting a 'Hass' monoculture versus interplanting 'Hass' with less profitable "pollinizer" cultivars. In a multi-location study, RAPD markers (Kobayashi et al. 2000) suggested that while progressive distance from a "pollinizer" cultivar strongly reduced the percentage of outcrossed fruit, the positive correlation between outcrossing and yield is only weak, with other factors accounting for most of the yield differential.

There continues to be considerable interest in deploying SSR markers to tease apart the roles of pollen source and competing environmental factors. However, as important insights into pollen flow within commercial avocado orchards are starting to accumulate, new conundrums are also revealed. Thus, recent SSR studies (Davenport et al. 2005; Garner et al. 2006) are corroborating earlier work based on RAPD markers and isozymes, showing that pollen (mediated by bees) does not move far from its source tree, yet consensus continues to be elusive with regard to yield differences between selfed and outcrossed fruit.

Sometimes, contradictory findings may have humble origins, and it is possible that differential sample collection methodologies are at least partially responsible. The prevailing wisdom stipulates that selfing reduces yields and that prematurely shed fruit is likely to be selfed whereas outcrossed fruit is more likely to be retained on the tree to maturity (Degani et al. 1997). Consequently, the sampling of marble-sized fruit – a common practice in experiments involving large-scale DNA extraction – may be skewing yield data on the assumption that it is likely to over-represent the fraction of selfed fruit relative to the more mature fruit harvested during commercial operations. Molecular studies would thus often be tracking a different (more highly selfed) pool of fruit than that collected during a typical harvest. For example, two studies in commercial

orchards (Kobayashi et al. 2000; Garner et al. 2006) report a very low outcrossing percentage (based on sampling marble-sized fruit), yet our own work (with trees raised from fruit that had been picked at maturity) bespeaks much higher rates of outcrossing (see below). Further studies will be needed to resolve the relationship between sampling stage and the impact of selfing and outcrossing on fruit yields.

17.3 QTL Analysis

The traditional method of breeding avocado still relies on the selection of progeny from a maternal source interplanted with pollen donors having desirable properties, followed by prolonged field trials to ascertain agronomic value. With a view to placing the process of avocado improvement onto a molecular footing, we have begun a QTL study that will feed into a marker-assisted method of selection. SSR markers associated with desirable (quantitative) traits will be used to preselect among seedlings: those that carry the markers will be retained, while genotypes lacking the marker will be eliminated from the outset.

Our QTL study is centered on 200 'Gwen' progeny genotypes that are clonally replicated twice at each of two environments (coastal and inland Southern California; 800 trees overall) and grafted onto a uniform 'Duke 7' rootstock. Growth and yield-related traits (tree height, stem girth, canopy diameter and several fruit yield and quality traits) have been measured once or twice a year since 2001, and the collection of SSR marker data from over 100 loci is in progress. Preliminary marker data indicate that virtually all our 'Gwen' progeny genotypes are outcrossed. Paternity of about one quarter is unclear, but the remaining three quarters are sired by 'Fuerte', 'Bacon', and 'Zutano' in approximately equal proportions. This genetic composition will furnish valuable information on the influence of the pollen source against a uniform maternal genetic background under replicated experimental conditions.

17.4 Comparative Sequencing Study

In order to explore the avocado genome at multiple levels, our lab has sequenced four genes (cellulase, chalcone synthase, flavonol-3-hydroxylase, and serine-threonine kinase) in a panel of 54 *Persea* genotypes (33 cultivars and 21 wild relatives/cultigens from Mexico, Ecuador, Costa Rica, and the West Indies). The nucleotide diversity (θ_W) for the four genes ranges from 0.003 to 0.016. Nucleotide diversity is moderate compared with other plants such as maize and barley, averaging 0.0157 and 0.0134 in the cultivars and germplasm, respectively, for flavonol-3-hydroxylase. These diversity values suggest that, subject to a more prolific representation of avocado DNA sequences on public databases, SNP development will be feasible, resulting in a much enlarged pool of genetic markers and paving the way for a more diversified approach to molecular studies in avocado.

17.5 Prospects

Happily, ESTs and other DNA sequences are now starting to accumulate, as avocado has been selected as an exemplar representing the basal angiosperms in the Floral Genome Project (*http://fgp.bio.psu.edu/fgp/*). Already, some 10,000 ESTs have been generated. More elaborate studies are proposed that will focus additionally on comparative gene expression in fruit development and on genetic mapping (Douglas Soltis, personal communication). The synergy between a fortified sequencing effort, gene expression studies, and existing molecular tools will open up new opportunities and prospects for avocado breeding.

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18 Pineapple

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18.1 Introduction

18.1.1 Economic Importance

Pineapple (*Ananas comosus* (L.) Merrill) is the third most important tropical fruit crop, after bananas and mangoes (citrus being considered mainly subtropical). Although cultivated in all tropical and subtropical countries, mostly between 30°N and 30°S, minor plantations can be found beyond these latitudes in areas with mild climates, often under protective shelter (Nakasone and Paull 1998). According to the Food and Agriculture Organisation (FAO) statistics (http://apps.fao.org), world pineapple production increased from 3,833,137 tons in 1961 to 15,287,413 tons in 2004. Five countries, namely Thailand (17,000,000 t), the Philippines (1,650,000 t), Brazil (1,435,600 t), China (1,475,000), and India (1,300,000) contributed with about half of the world production in 2004. A second group of significant producers that includes countries as disparate as Nigeria, Mexico, Costa Rica, Indonesia, Kenya, Colombia, Ivory Coast, Venezuela, Vietnam, Malaysia, United States, and South Africa supply about one third of the total world production.

Approximately 70% of the produced pineapple is consumed as fresh fruit in the country of origin (Loeillet 1997). The world pineapple trade consists mainly of processed products as canned slices, chilled fresh-cut chunks and spears, juice and juice concentrates. For example, worldwide exports of concentrated juice represent more than US$ 250 million and the value of exported canned pineapple more than US$ 600 million. Even so, the value of the fresh fruit market is rapidly increasing, particularly the chilled, fresh-cut fruit market (Rohrbach et al. 2003). Pineapple is also a source of bromelain, used as a meat-tenderising enzyme, and high quality fiber. The waste resulting from industrial processing is used for animal feed.

18.1.2 Taxonomy

Pineapple is a perennial monocot belonging to the order Bromeliales, family Bromeliaceae, subfamily Bromelioideae. The Bromeliaceae comprise 56 genera with 2,921 species (Luther 2002), classified into three subfamilies: Pitcarnioideae, Tillandsioideae and Bromelioideae. This last subfamily shows a tendency towards the fusion of floral parts, a trait most developed in *Ananas*, the only genus whose flowers and bracts are completely merged into a single sorose-type parthenocarpic fruit formed by 50 to 200 coalescent berries (Coppens d'Eeckenbrugge et al. 1997).

Pineapple taxonomy was recently revised by Coppens d'Eeckenbrugge and Leal (2003). Until then, the accepted taxonomy and nomenclature of pineapple was that of Smith and Downs (1979), which described nine species distributed in two genera, *Ananas* and *Pseudananas*, the latter being monotypic. It was first amended by Leal (1990) who invalidated *A. monstrosus*.

Most quantitative traits used in the Smith and Down's classification are not clearly discriminative and are excessively dependent on environmental conditions. The qualitative traits used for classification, such as leaf spininess, are determined by one or few loci (Cabral et al. 1997), which mutate and segregate within species as well as between species.

Many distinctions, particularly those related to spininess, fruit size and fertility, appeared to be the direct result of human selection in the course of domestication. In addition, crosses among the

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species of Smith and Down are successful and the resulting hybrids are fully fertile. Even the diploid *Ananas* and the tetraploid *P. sagenarius* (Arruda da Câmara) Camargo can be hybridized experimentally, mainly producing self-fertile tetraploid hybrids and a few triploids. Available data on biochemical and molecular diversity also indicated an incipient speciation process within *Ananas* (Leal and Coppens d'Eeckenbrugge 1996; Coppens d'Eeckenbrugge et al. 1997).

Coppens d'Eeckenbrugge and Leal (2003) proposed a simplification of the classification, downgrading the two genera and eight species into two species (with the restoration of *A. macrodontes* Morren instead of *P. sagenarius*) and five botanical varieties of *A. comosus*. Since then, more recent molecular marker analyses have refined our understanding of pineapple diversity and speciation, and have provided new data on the relationship between the two pineapple species, without affecting the new classification.

A. macrodontes is a vigorous self-fertile tetraploid ($2n = 4x = 100$), with spiny leaves, 2–3 m long and 7 cm wide, propagating by elongate basal stolons. The syncarp lacks the leafy crown typical of *A. comosus*. The latter species is generally diploid ($2n = 50$), self-incompatible, and propagates vegetatively by suckers (borne on the stem), slips (borne on the peduncle), and the fruit crown(s). The pineapple cultivated for the fruit corresponds to the botanical variety *A. comosus* var. *comosus*. Its leaves are relatively wide (more than 5 cm), spiny, partially spiny or smooth, and its strong peduncle bears a fruit whose size may reach several kilograms. *A. comosus* var. *ananassoides* (Baker) Coppens & Leal (formerly *A. ananassoides* (Baker) L.B. Smith and *A. nanus* (L.B. Smith) L.B. Smith) corresponds to the most common wild form, with thinner spiny leaves and a much smaller fruit on a long, slender scape. Another wild form is *A. comosus* var. *parguazensis* (Camargo & L.B. Smith) Coppens & Leal (formerly *A. parguazensis* Camargo & L.B. Smith), with wider leaves, constricted at their base, antrorse and retrorse spines, and a globose fruit. The two remaining botanical varieties are cultivated. *A. comosus* var. *erectifolius* (L.B. Smith) Coppens & Leal (formerly *A. lucidus* Miller *sensu* Smith & Downs) is very similar to *A. comosus* var. *ananassoides*, except for its smooth fibrous leaves, which are used by Amerindians to make hammocks, fishing lines and nets. *A. comosus* var. *bracteatus* (Lindl.) Coppens & Leal (grouping the former *A. bracteatus* (Lindley) Schultes f. and *A. fritzmuelleri* Camargo) is a very vigorous and spiny plant, producing a medium-sized fruit with long bracts. It is cultivated as a living fence. Its fruit was also collected for juice and it is still found as a sub-spontaneous plant in ancient settlements of southern South America. A variegated variant has become a common ornamental of tropical gardens.

18.1.3 Natural Habitat and Origin

Both *Ananas* species (*A. comosus* and *A. macrodontes*) have a natural distribution confined to the South American sub-continent. Nevertheless, long before the arrival of Christopher Columbus to Guadeloupe in 1493 and this first European contact with this crop, the Native Americans had already domesticated and dispersed the pineapple throughout South and Central America, the Antilles and the Caribbean region (Leal and Coppens d'Eeckenbrugge 1996; Coppens d'Eeckenbrugge et al. 1997). The pineapple cultivation very probably initiated with var. *comosus* and var. *erectifolius* evolving from var. *ananassoides* and/or var. *parguazensis* in the region north of the Amazon river (Orinoco and Rio Negro basins, and Guiana shield), where a wider morphological and molecular variability is found in wild and cultivated types (Duval et al. 2003). *A. comosus* var. *bracteatus*, and *A. macrodontes* originated in the South of the continent (Paraguay and southern Brazil) (Leal and Coppens d'Eeckenbrugge 1996).

18.1.4 Chromosome Number and Genome Size

The most common chromosome number for the subfamily Bromelioideae is $2n = 50$, (Cotias-de-Oliveira et al. 2000). It is also the most common among the *A. comosus* varieties and cultivars. Nevertheless, triploid and tetraploid clones have also been identified in var. *comosus* and tetraploid clones in var. *ananassoides* (Sharma and Ghosh 1971; Lin et al. 1987; Dujardin 1991; Cotias-de-Oliveira et al. 2000). *A. macrodontes* is tetraploid ($2n = 100$) (Lin et al. 1987). Arumuganathan and Earle (1991) estimated the haploid genomes size at 444 Mbp for *A. comosus* var. *bracteatus* and 526 Mbp for var. *comosus*.

18.1.5 Propagation and Floral Biology

The propagation of pineapple is mainly vegetative, by stem suckers, peduncle slips, and fruit crown. *A. macrodontes* produce underground stolons. Natural genetic diversity studies indicate that clonal propagation is also the most common mode of multiplication in the wild, as seeds germinate slowly and/or young seedlings rarely survive under natural conditions (Coppens d'Eekenbrugge et al. 1997).

All botanical varieties of *A. comosus* possess a gametophytic self-incompatibility system. The self-rejection reaction is variable in intensity and generally stronger in the cultivated var. *comosus*, which is probably a result of the domestication process and selection for seedless fruits (Coppens d'Eeckenbrugge et al. 1993). In contrast, *A. macrodontes* is self-fertile and the self-progenies are very homogeneous, indicating that this species is highly homozygous and autogamous (Collins 1960). The main vectors for natural cross-pollination are humming birds, while bees and ants may play a secondary role. Wind pollination has never been reported. Seeds lack dormancy and can retain germination capacity for at least six months.

While no apparent depression was detected in formation or germination of self-seeds, severe inbreeding depression has been observed among self-progenies of several *Ananas* cultivars in later stages of development, rendering almost impossible the continuation of the inbreeding process beyond the first or second generation of selfing (Collins 1960; Coppens d'Eeckenbrugge et al. 1993; Cabral et al. 2000).

The success of the intraspecific and interspecific crosses in the genus *Ananas* was referred to above. References to intergeneric hybrids involving *Ananas* and other genera as *Aechmea*, *Cryptanthus* and *Neoreglia*, exclusively for ornamental purposes, can be found in Grant (1998) and, for example, on the web page of the Florida Council of Bromeliad Societies (http://fcbs.org/articles/Bigenerics.htm).

18.1.6 Pineapple Breeding

Hybridization and clonal selection, mainly involving elite cultivars, have been used in modern pineapple breeding for over a century. Nevertheless, the heterozygous nature of pineapple cultivars, and the consequent strong segregation and recombination, have strongly limited the success of hybrid breeding. Until recently, the severe selection among millions of seedlings resulted in cultivars of only average quality or of local importance (Coppens d'Eeckenbrugge et al. 1997). This quite desperate situation ended in the late 1990s with the commercial success of 'Golden Ripe', a new cultivar that stirred the world market of fresh pineapple and awakened the interest in cultivar diversification.

Included among the goals of pineapple breeding programs are the introgression of resistances to diseases such as *Phytophthora* and *Fusarium,* the prevention of disorders such as internal browning (blackheart) and the control of specific traits such as early natural flowering, in elite cultivars. To avoid the lottery of segregation and recombination, genetic engineering appears to be a promising breeding strategy since it allows transferring a single gene, or a few genes, without substantially altering the initial genome. Efficient procedures for genetic transformation (Sripaoraya et al. 2001; Espinosa et al. 2002) and in vitro regeneration and propagation (Escalona et al. 1999; Firoozabady and Gutterson 2003; Sripaoraya et al. 2003) have already been established. The first field and greenhouse trials of genetically transformed pineapple clones exhibiting reduced expression of polyphenol oxidase (PPO) and of 1-aminocyclopropane-1-carboxilate (ACC) synthase or expressing the bialaphos resistance (*bar*) gene have already been carried out (Rohrbach et al. 2000; Sripaoraya et al. 2001; Sripaoraya et al. 2006; Botella and Fairbairn 2005; Trusov and Botella 2006).

In this respect, the construction of dense genome maps of molecular markers is of paramount importance for the further isolation, via positional cloning, of genes of interest for pineapple improvement. This is of particular significance regarding those genes that are uniquely known and uniquely detected by their phenotypic expression in plants (e.g. resistance genes).

18.2 Molecular Systematics

Pineapple molecular studies have been carried out with a variety of techniques, including isozymes (García 1988; Aradhya et al. 1994), RAPD (Ruas et al. 2001), AFLP (Kato et al. 2004; Paz et al. 2005), RFLP (Duval

et al. 2001) and cpDNA PCR-RFLP (Duval et al. 2003). All of them clearly support the separation between *A. comosus* and *A. macrodontes*, as well as the low level of genetic differentiation among the former *Ananas* species. Thus, Aradhya et al. (1994) observed that the variation among the five botanical varieties accounted merely for 14% of the total isozymic variation and Duval et al. (2001) reported a continuous RFLP variation within *A. comosus*. Another consistent observation (Duval et al. 2001) was a wide variation within *A. comosus* var. *ananassoides* and a close genetic relationship between this and other varieties, particularly the wild *parguazensis* and the cultivated *comosus* and *erectifolius*, confirming that the cultivated types were directly derived from their wild relatives.

The existence of clones in the Guianas, morphologically intermediate between *comosus* and *ananassoides*, with haplotypes also found in both these varieties, suggests a recent domestication and a continuing introgression process. Concerning the *erectifolius* variety, the RFLP and PCR-RFLP data indicate multiple domestication events, involving convergent selections from different *ananassoides* genotypes for a few morphological traits (smooth and fibrous leaves).

According to isozyme, RFLP and AFLP data, *A. comosus* var. *bracteatus* appears relatively uniform and better differentiated from the other varieties. Isozyme and RFLP markers indicate a particular affinity with *A. macrodontes*. The study of cpDNA by PCR-RFLP identified a unique haplotype for the common representatives of var. *bracteatus* shared with all the other varieties. On the other hand, a unique accession, formerly classified as *A. fritzmuelleri* Camargo, presents a haplotype that is almost identical to the one typical of *A. macrodontes*. Thus molecular data suggest a special position of *A. comosus* var. *bracteatus* in relation to *A. macrodontes*, as it appears to be constituted by two combinations of nuclear genes from both species with chlorotypes from one or the other species. Whether these combinations are the product of a rare introgression event during the evolution of *Ananas*, an "accidental genotype" maintained by vegetative propagation, or the testimony of an intermediate stage in its evolution, is difficult to ascertain.

18.3 Construction of Genetic Maps

Available information on ongoing genome mapping programs in pineapple is very scarce. The unique pineapple genome maps published so far are the genetic maps of molecular markers including the morphological trait 'piping', constructed by Carlier et al. (2004, 2006). To our knowledge no other qualitative trait or QTLs have been mapped, and no results of physical mapping have been reported.

18.3.1 F_1-Based Genetic Maps

As mentioned above, except for breeding purposes, *Ananas* is usually propagated vegetatively either for cultivation or under natural conditions. Genotypes well adapted to a particular natural environment, as well as those selected for human needs, tend to be fixed in a heterozygous state. In addition, heterozygosity is reinforced by different degrees of self-incompatibility and lack of sexual barriers, which promote crosspollination, and by a strong inbreeding depression.

The high level of heterozygosity allows the use of F_1 progenies as segregant populations for genetic mapping employing the "two-way pseudo-testcross" or "double pseudo-testcross" strategy (Grattapaglia and Sederoff 1994; Hemmat et al. 1994), since the alleles of a heterozygous polymorphic locus present in one parent and absent in the other are expected to segregate in a 1:1 Mendelian ratio among the F_1 population.

The first genetic maps of pineapple, one for *Ananas comosus* var. *bracteatus* and a second one for *Ananas comosus* var. *comosus* were recently published (Carlier et al. 2004). The mapping population used for the construction of both maps consisted of 46 F_1 plants derived from a cross between var. *comosus* (cv. Rondon – clone BR 50) and var. *bracteatus* ("Branco do mato" – clone BR 20), carried out in Martinique. Map construction was twice more efficient for var. *bracteatus* than for var. *comosus*, a consequence of the higher average heterozygosity of the former and, also, of the fact that it possesses a large number of specific DNA-markers not present in var. *comosus*.

The F_1-based map of *A. comosus* var. *bracteatus* is constituted by 335 DNA markers (60 RAPD, 264 AFLP and 11 ISSR), assembled in 50 linkage groups: 26 groups gathering at least four markers each, six groups of three markers each and 18 pairs of markers. This map spans over 2,111 cM, which corresponds to 57.2% of the 3,693 cM genome length estimated according to Chakravarti et al. (1991).

The A. *comosus* var. *comosus* map gathers 156 molecular markers (33 RAPD, 115 AFLP and 8 ISSR) in 30 linkage groups spanning over 1,311 cM, corresponding to 31.6% of the 4,146 cM long genome. The locus *P*, whose dominant allele determines the 'piping' morphological trait, was also included in this map. The ratio between physical and genetic distances is approximately 120 kbp/cM for var. *bracteatus* and 127 kbp/cM for var. *comosus* (Carlier et al. 2004).

18.3.2 F_2-Based Genetic Map

One of the F_1 plants used for the construction of the first genetic maps was selfed in Martinique and leaves of 142 F_2 plants were sent to Portugal for the construction of a new, F_2-based, genetic map. Among the 451 molecular markers analysed, 52 molecular markers (16 from var. *comosus* and 36 from var. *bracteatus*) showed a less pronounced distortion $\left(\chi^2_{0.01} > \chi^2 \geq \chi^2_{0.05}\right)$ while 43 other markers showed a more skewed segregation $\left(\chi^2 > \chi^2_{0.01}\right)$.

The first genetic map constructed on the basis of an F_2 segregating population assembles 412 genetic markers (311 AFLP, 66 RAPDs, 34 ISSRs and one morphological trait, piping) in 50 linkage groups. Thirty-nine markers remained unlinked (Carlier et al. unpublished).

In order to estimate the total span of the map, each linkage group was enlarged at each extremity (Marques et al. 1998) with the average distance between adjacent markers 5.32 cM. The total span of the F_2-based map is 2,458 cM, which corresponds to 62.7% of the average pineapple map length calculated on the basis of the above-mentioned F_1-based maps.

Unfortunately, the F_2 population used to construct this map showed a very strong inbreeding depression. Most of the plants exhibited a very weak and unhealthy phenotype and died before fruit setting. Therefore, the segregation analysis of morphological traits, with the exception of the piping phenotype, could not be performed.

18.3.3 Integrated Genetic Maps of Pineapple

One of the main goals of the construction of the F_2-based genetic map was to use it for the construction of an integrated genetic map. Such an integrated map would join the F_2-based and the F_1-based maps and markers that had previously remained unlinked.

The first integrated genetic map of molecular markers in pineapple was published recently by Carlier et al. (2006). This map gathered 574 markers (454 AFLP, 79 RAPD and 41 ISSR) in 46 linkage groups, spanning more than 2,421 cM, and covering 62% of the genome, the genetic size of which, 3,919 cM, was calculated as the arithmetic average of the previous estimations (Carlier et al. 2004) for the genomes of var *bracteatus* and var. *comosus*.

Presently, the integrated map (Fig. 1) assembles 659 DNA markers (506 AFLP, 113 RAPD and 40 ISSR), one isozyme locus (PGM) and one morphological trait locus (piping). Thus far, this map is constituted by: (a) 17 linkage groups integrating molecular markers of var. *bracteatus* and var. *comosus* (15 of these integrate markers from the F_1-based and F_2-based genetic maps, while two of them gather only markers analysed in the F_2 population); (b) 11 linkage groups integrating markers of F_1- and F_2-based maps, but only of var. *bracteatus*; and (c) eight linkage groups that, in the same way, integrate only markers from var. *comosus*.

18.4 Germplasm Resources and GeneBank Data

Pineapple genetic resources (e.g., for plant genetic improvement, production of segregating populations or construction of cDNA or BAC genome libraries) are maintained by CIRAD in Martinique (French West Indies); by the Brazilian National Genetic Resources and Biotechnology Research Centre (EMBRAPA/CENARGEN), in Brasilia; by the National Cassava and Tropical Fruit Research Centre (EMBRAPA/CNPMF), Cruz das Almas, Brazil; and by the US Pacific Basin Agricultural Research Center, USDA, Hawaii. These represent the most diverse germplasm collections. Other important collections are maintained for breeding purposes by national institutions in Malaysia, Australia, Cuba, Japan, and other producing countries, as well as by private companies (Coppens d'Eeckenbrugge and Duval 1999).

Functional genomic studies in pineapple are very scarce but their number is increasing rapidly. Thus far, have been cloned and functionally characterized genes that encode: an ACC synthase and an ACC oxydase (Cazzonelli et al. 1998); a NAD^+-dependent

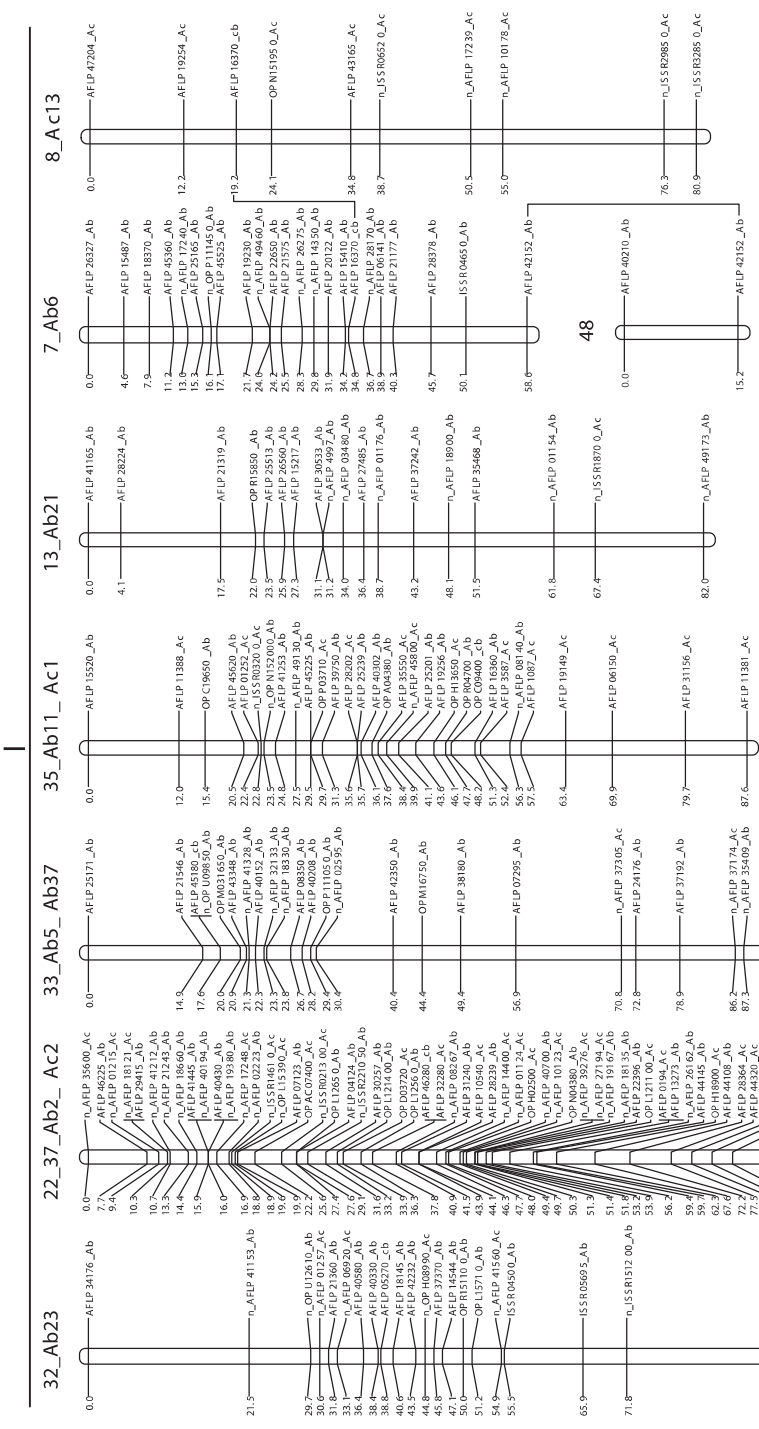

Fig. 1. Integrated genetic map (F_1-based and F_2-based genetic maps) of *Ananas comosus* (pineapple). *I* – Linkage groups that integrate molecular markers of var. *bracteatus* and var. *comosus*; *II* – Linkage groups integrating markers only of var. *bracteatus*; and *III* – Linkage groups that integrate markers only of var. *comosus*. Identification of the integrated linkage groups: e.g. 22_37_Ab2_Ac2 – numerals 22 and 37 refer to the linkage groups 22 and 37 of the F_2-based genetic map; Ab 2 – refers to the linkage group 2 of the F_1-based genetic map of var. *bracteatus*; Ac2 – refers to the linkage group 2 of the F_1-based genetic map of var. *comosus*. Molecular markers are identified as previously described (Carlier et al. 2004)

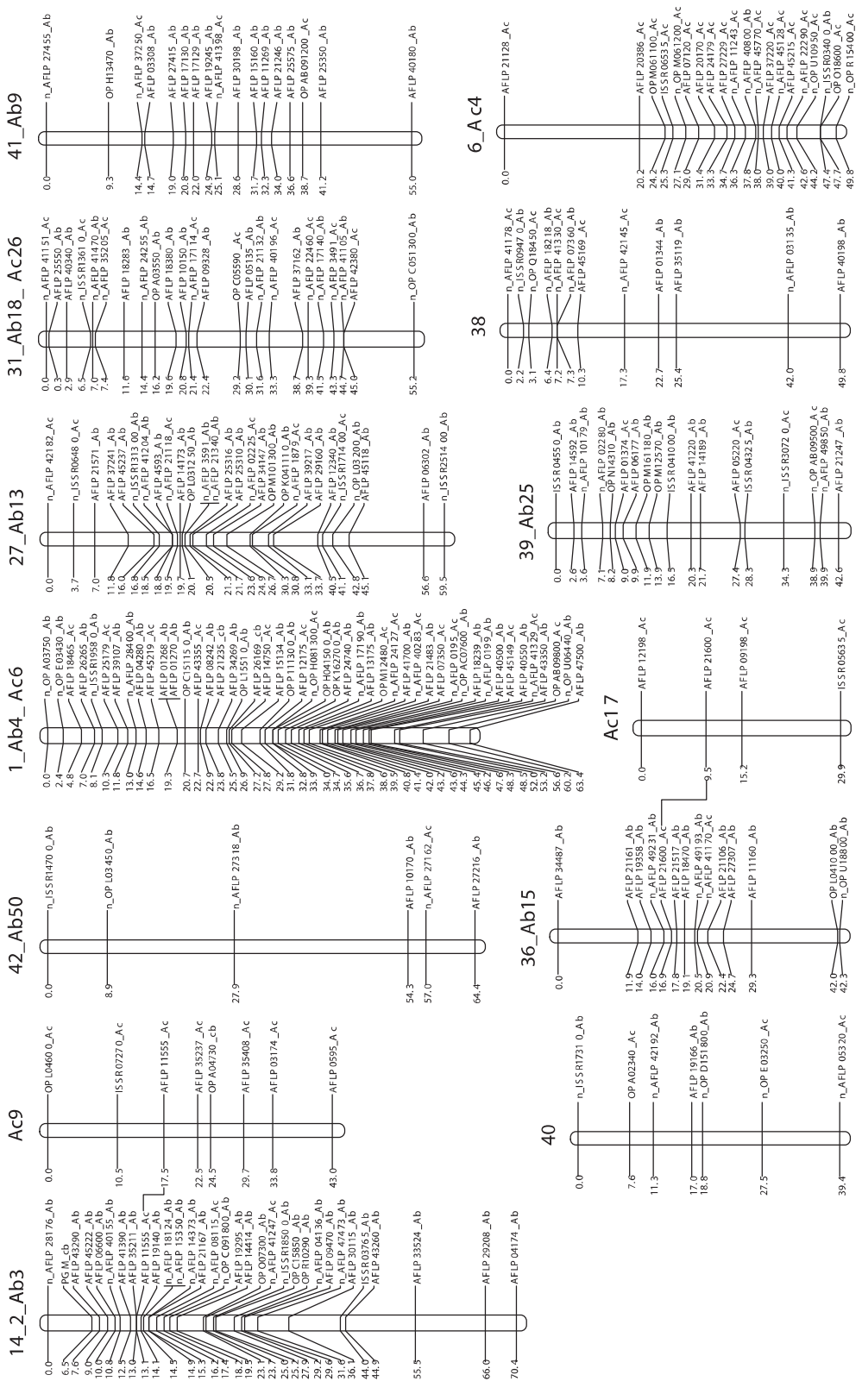

Fig. 1. (continued)

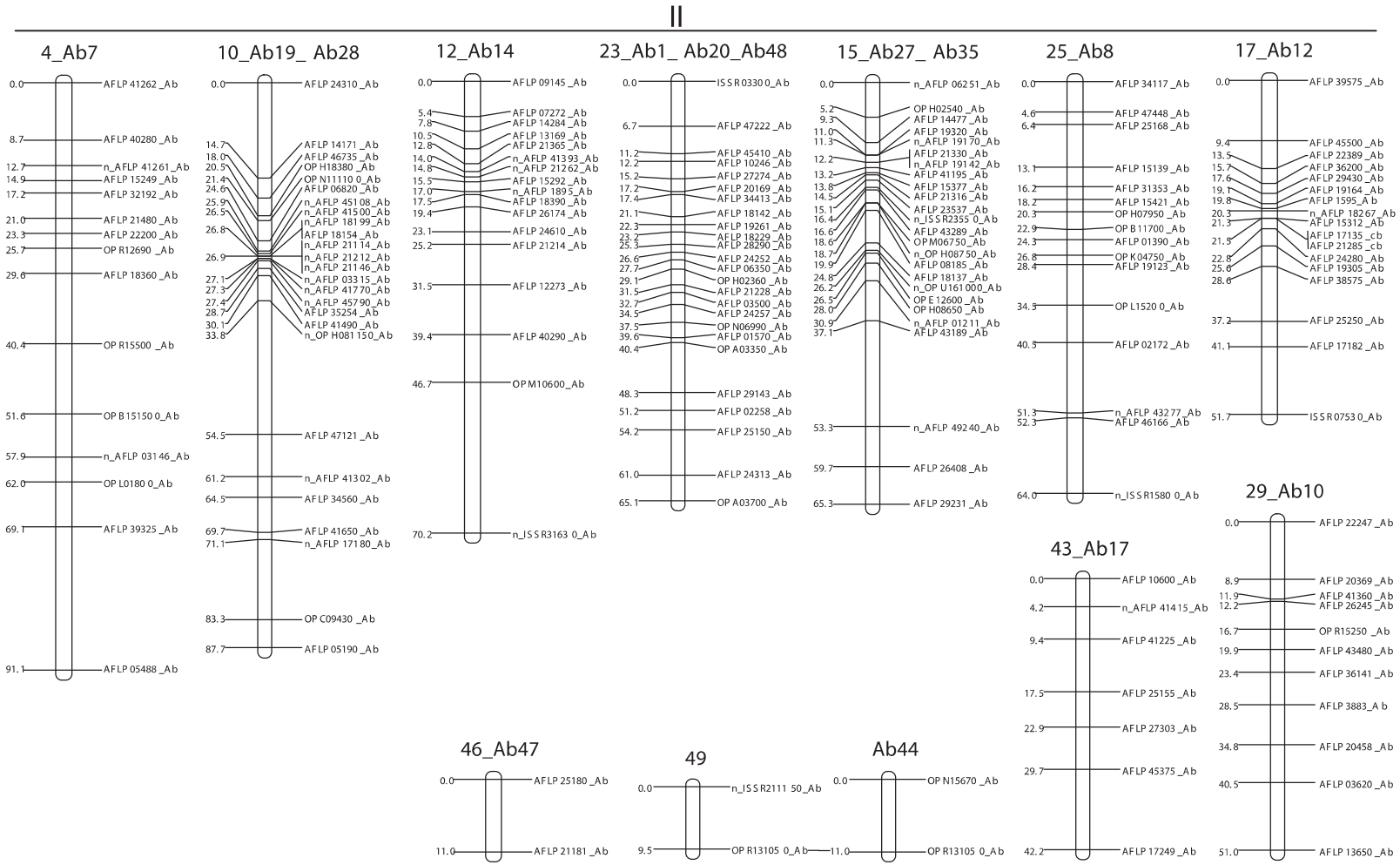

Fig. 1. (continued)

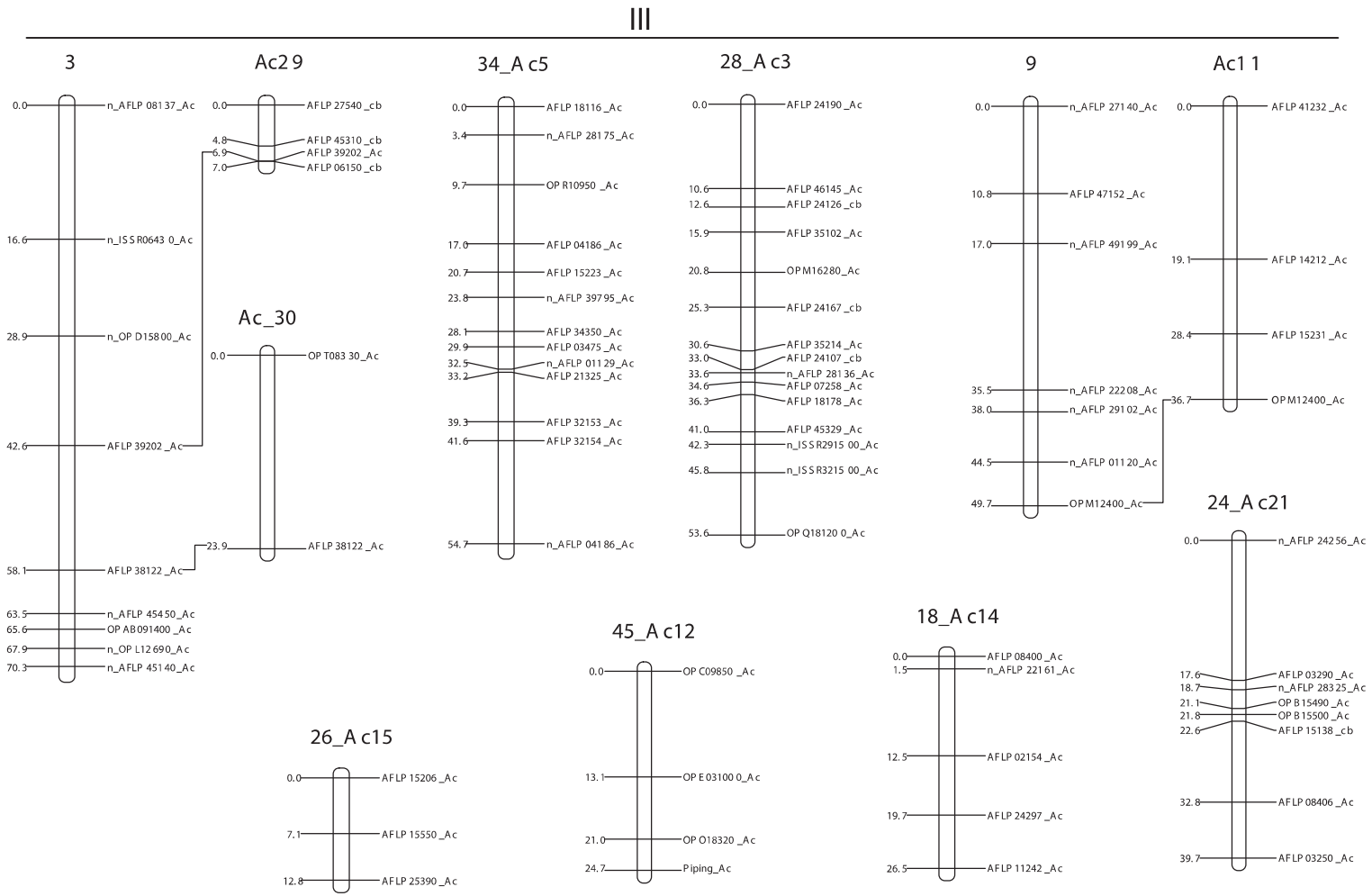

Fig. 1. (continued)

malate dehydrogenase (Cuevas and Podestá 2000); ananain (Carter et al. 2000); a Cu/Zn-superoxide dismutase (Lin et al. 2000); two distinct polyphenol oxidases (Stewart et al. 2001); and the cysteine protease inhibitor cistatin (Shyu et al. 2004). A retroposon-like sequence, repeatedly integrated in the genome in multiple variable sequences and putatively capable of transposing (Thomson et al. 1998), and the genomic sequence coding for bromelain inhibitors (Sawano et al. 2002), have also been isolated and characterized. Moreover, recent studies on genes involved in root development (Neuteboom et al. 2002) and in fruit ripening and nematode-root interaction (Moyle et al. 2005a, b, 2006) have resulted in a very large number of sequenced ESTs.

The amount of genomic data in databases is still scarce but has been rapidly increasing, particularly during the last two years. Presently, a search for pineapple genomic data through the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) results in about 60 microsatellite and other DNA marker loci from var. *bracteatus* and over 5,700 ESTs from var. *comosus*. About 5,650 of these EST sequences have been contributed by Moyle et al. (2005a, b, 2006) who have clustered 408 green fruit, 1,140 yellow fruit, 343 root tip, 1,298 early nematode infection and 246 late nematode infection related ESTs into 3,383 contigs. This research group has created an online pineapple bioinformatics resource: PineappleDB (www.pgel.com.au), periodically updated with gene expression data arising from the pineapple microarray project they are implementing.

The TropGENE-DB (http://tropgenedb.cirad.fr) is an information system created by CIRAD, France, to store genetic, molecular and phenotypic data, particularly data on genetic resources, molecular markers, genetic and physical maps, sequences, genes, etc. Presently, information is available only on banana, cocoa, coconut, coffee, cotton, oil palm, rice, rubber tree and sugarcane, but the extension of the database and the inclusion of pineapple and other tropical crop species is expected soon.

18.5 Future Prospects

The pineapple integrated genetic map (Fig. 1) is incomplete and requires further study in order to assemble nearly all analysed markers into 25 linkage groups corresponding to the $n = 25$ chromosomes of the species. Two tasks appear more urgent to accomplish this goal as described below.

The first task is to include in the map some of the already published microsatellite markers, contributing to its further improvement by the integration of new markers and groups of markers from the F_1-based and F_2-based maps in linkage groups established and ordered with higher statistical significance. Additionally, due to the genetic similarity between both *Ananas* species and among the botanical varieties, the microsatellite markers and other polymorphic sequence tagged site (STS) markers are expected to be useful for the integration of genetic markers from other *Ananas* genotypes. Eventually, genetic markers could also be integrated from other *Bromeliaceae* species.

The second task is to clone, sequence and transform into sequence characterized amplified region (SCAR) markers an array of mapped RAPD, AFLP and ISSR markers strategically distributed along the genome.

A very dense and integrated genetic map of molecular markers, complemented by microsatellite and SCAR markers covering almost all the pineapple genome, will constitute a major scientific tool. It will allow the rapid location of any genetic locus and, consequently, the rapid identification of molecular markers linked to any gene of interest. Such markers can be very useful in marker-assisted selection (MAS) and in gene isolation via map-based cloning programs. In addition, such a map would act as a basic framework of the genome in future physical mapping programs or pineapple genome sequencing projects.

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19 Papaya

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19.1 Introduction

Papaya belongs to family Caricaceae. This family consists of six genera including *Carica* a monotypic genera, *Jacaratia* with seven species, *Jarilla* with three species, *Cylicomorpha* with two species, *Horovitzia* with one species and *Vasconcellea* with 21 species (Badillo 2000). All members of the Caricaceae examined cytologically are diploid with $2n = 2x = 18$ (Darlington and Ammal 1945). The genus *Carica* is characterized by a unilocular ovary and is represented by a single species *C. papaya,* while the genus *Vasconcellea* comprises the remaining *Carica* species possessing pentalocular origin. *Carica* is the only genus of Caricaceae containing domesticated species *papaya*, which is by far the most economically important having a distribution throughout the tropics and subtropics of the world. Papaya probably originated in the lowland of Central America, between southern Mexico and Nicaragua. However, it is now cultivated in many tropical and subtropical part of world (Storey 1969). Papaya is a major tropical fruit grown commercially in India, Brazil, Mexico, Australia, Hawaii, Thailand, South Africa, Philippines, Indonesia and Taiwan. India is the largest producer of papaya contributing 25% of the total world production. It thrives well under tropical climate. However, papaya can be grown in frost-free subtropical climate as well.

Intensive papaya improvement program in India, Hawaii, Mexico, Brazil and Philippines gave rise to a large number of improved hybrids and selections such as Kapoho Solo, Sun Rise, Sun Set, Waimanalo, Laie-Gold, Kamiya (USA), Pusa Delicious, Pusa Nanha, Pusa Dwarf, Pusa Majesty, Surya, Coorg Honey Dew, CO 1, 2, 3, 4, 5, 6 and 7, Pant Papaya-1 (India), Cavite Special (Philippines), Sainampueng, Kak Dum (Thailand), and Improved Peterson, Guinea Gold, Sunnybank and Arline-57 (Australia). However, none of these varieties are close to an ideal variety. Moreover, papaya cultivation is hampered severely due to problems like prevalence of papaya ring spot virus (PRSV), papaya leaf curl virus (PaLCuV), fungal diseases such as foot rot and fruit anthracnose, stamen carpelloidy and summer sterility. Papaya is a polygamous plant, and sex forms constitute the basis for papaya breeding program. Some of the prevalent genetic problems are related to sex forms such as summer sterility and stamen carpelloidy (Giacometti 1987). Storey (1984) considered elimination of ambisexual andromonoceous forms that tend to become sterile at certain climatic conditions or show a tendency towards stamen carpelloidy by developing a heterozygous andromonoceous form, M_2M_2 by possible elimination of the zygotic lethal factor. Due to fairly large size of papaya petiole (75–100 cm), high density planting of papaya is not feasible. Papaya improvement program has led to development of the mutant 'Solo' line with short petiole (45–60 cm) that are positioned obliquely upright and trees can be planted at 0.9–1.2 m apart in the row. Breeding program for developing ring spot resistant variety in papaya is going on for long time.

A great deal of work on intergeneric hybridization has been reported from India, Venezuela, Hawaii, Brazil, Taiwan and Australia (Horovitz et al. 1958; Padnis et al. 1970; Gama et al. 1985; Manshardt and Wenslaff 1989a, b; Chen et al. 1991; Drew et al. 1998). In order to transfer the gene conferring resistance to PRSV, *C. papaya* was crossed with *V. cauliflora*, *V. cundinamarcensis, V. quercifoila, V. stipulata, V. goudatiana* and *V. parviflora*. Few hybrids were developed through the embryo rescue technique. However, most of these crosses turned out to be sterile. Incompatibility between C. *papaya* and *Vasconcellea* species has been a major bottleneck in production of useful intergeneric hybrids. Now it is understood that postzygotic barriers, i.e., embryo abortion and lack of endosperm development (Manshardt and Wenslaff

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1989a, b), were mainly responsible for incompatibility between papaya and its wild species. Recent biotechnological investigations revealed that *V. cauliflora* and *C. papaya* are genetically very distant and unfortunately a lot of efforts have gone into hybridization work involving these species (Jobin-Décor et al. 1996). Hybrids between these two species lack vigor, rarely survive till flowering, and if they do, are infertile (Manshardt and Wenslaff 1989a, b). Similarly, hybrids between *papaya* and *stipulata* were reported to lack vigor and viability (Horovitz and Jimenez 1967). Interestingly, intergeneric hybridization program in Australia with *C. papaya* crossed with *Vasconcellea* species *pubescens, quercifolia, parviflora* and *goudotiana* led to the development of vigorous hybrids (Drew et al. 1998). PRSV resistance has been reported often in crosses between *C. papaya* and *V. cauliflora* (Moore and Litz 1984; Vegas et al. 2003). However, the postzygotic barrier in these hybrids has prevented further backcrossing. Interestingly, Khuspe (1980) reported production of viable F_1 and F_2 populations that were resistant to PRSV in the F_1 population and segregated for PRSV resistance in the F_2 population with a 3:1 ratio. However, his work did not confirm development of papaya hybrid resistant to PRSV. Interspecific hybrids between *C. papaya* and other *Vasconcella* species have also demonstrated resistance to PRSV. All *V. pubescens* hybrids were resistant to PRSV when manually inoculated three times at two-weekly intervals in a glasshouse (Drew et al. 1998). A large population of *C. papaya* × *V. quercifolia* hybrids were manually inoculated using the same procedure. Of those tested, two thirds were resistant and the remaining produced symptoms. However, progressing past intergeneric F_1 hybrids has been very difficult, and the only successes have resulted from backcrosses from *C. papaya* × *V. quercifolia* to *C. papaya*. Embryo culture has produced no F_2 progeny to date and only limited progenies in backcrosses with papaya. In Hawaii, F_1 hybrids contributed only unreduced gametes in backcrosses, yielding sequidiploid plants that were very sterile indicating that meiosis did not function normally in those hybrids (Manshardt and Drew 1988). The first fertile backcross plants have been reported from Australia (Drew and O'Brien 2001).

The tolerance-breeding program has enabled farmers to grow papaya with reasonable fruit production despite plants becoming infected with PRSV, and it has also been helpful to farmers for obtaining good quality, reasonably priced papaya seeds. In the same way, other tolerant lines have been developed. In Taiwan, Lin et al. (1989) reported the development of the hybrid Tainung No. 5, from the cross of FL 77-5 (from Florida) and Costa Rica Red, with good level of tolerance and horticultural characteristics. It has a strong trunk and shows early fruit bearing and ripening. The height of the first fruit from the base of trunk is about 56–60 cm. The use of tolerant papayas has not resolved the virus problem in the long term and development of genetically resistant cultivars is considered the only reliable solution to PRSV control. In Thailand, a series of papaya lines developed by crossing the Florida tolerant and local variety 'Khakdum' followed by recurrent selections is the result of an on-going breeding program since 1987 (Prasartsee et al. 1998). 'Khakdum' is a popular Thai cultivar with desirable fruit characteristics, but it is very susceptible to PRSV (Nopakunwong et al. 1993). Previous trials at the Khonkaen Horticultural Experiment Station (Prasartsee et al. 1998) showed that Florida tolerant papaya produced acceptable amounts of fruit despite being infected with PRSV. Thus, reciprocal crosses were made between the Florida tolerant variety and 'Khakdum' in an effort to produce hybrid lines that are PRSV-tolerant and have acceptable horticultural characteristics. Only one cross was made initially between 'Florida Tolerant' and 'Khakdum'. Subsequent crosses were made from within the progeny population. The first priority was to maintain a high level of PRSV tolerance followed by selection of desirable horticultural characteristics. These progenies were named Thapra 1, Thapra 2 and Thapra 3. In 1997, 'Thapra.2' was released as 'Khakdum Thapra' tolerant to PRSV in Thailand.

19.2 Molecular Characterization

Molecular markers are being used in phylogenetic studies of various taxa adding new dimension to evolutionary pathways. Different molecular markers (RAPD, RFLP, AFLP, IISR-PCR) have been employed to measure genetic variation within and between species, varieties and related genera of *Carica*. Badillo (2000) has rehabilitated *Carica* as a monotypic genera consisting one species and elevated *Vasconcellea* as a separate genus with 21 species. Many studies pertaining to molecular characterization are clearly indicating the wide genetic distance between *Carica* and *Vasconcellea*. Sharon et al. (1992) used mi-

crosatellite and minisatellite probes to evaluate genetic relationship among *Carica* species. Genetic analysis of DNA finger-print bands revealed no linkage or allelic relationship among the bands analyzed indicating that these loci are not clustered in *Carica* genome. The phylogenetic relationship of 12 wild and cultivated species of *Carica* was analyzed by Aradhya et al. (1999) using restriction fragment length variation in a 3.2 kb-PCR amplified intergeneric spacer region of cpDNA. The evolutionary split in *Carica* strongly suggests that *C. papaya* diverged from the rest of the species in the early period of evolution of the genus and evolved in isolation probably in Central America. The chloroplast and mitochondrial DNA diversity of 61 genotypes belonging to 18 *Vasconcellea* species using PCR-RFLP revealed higher level of interspecific variation in two cpDNA regions than analysis with mtDNA which supported the monophyly of *Carica*. Further, cpDNA analyses showed two basic evolutionary lineages within the genus *Carica*, one defined by cultivated *C. papaya* and another consisting of the remaining wild species from South America in a well resolved but poorly supported monophyletic assemblage. This may indicate a higher level of inter-fertility for *Vasconcellea* species from the latter clade in interspecific crossing with papaya. A reticulate evolution for *Vasconcellea* has therefore been suggested. Finally, intraspecific cpDNA variation was detected in *V. microcarpa* thus providing molecular evidence for the high diversity previously indicated by morphological observation (Droogenbroeck et al. 2004). AFLP markers have been used to study the genetic relationship among 71 papaya accessions and related species with nine *Eco*RI/*Mse*I primer combinations. Genetic diversity among *papaya* cultivars derived from the same or similar gene pool was smaller such as Hawaiian solo hermaphrodite cultivars and Australian dioecious cultivars with genetic similarity at 0.921 and 0.912, respectively. Self-pollinated hermaphrodite cultivars were as variable as open-pollinated dioecious cultivars. *C. papaya* showed the least genetic similarity with these species. AFLP markers supported the notion that *C. papaya* diverged from the rest of *Carica* species early in the evolution of this genus (Kin et al. 2002). This theory was further strengthened by the study on phylogeny of *Vasconcellea* and *Carica* species native to Ecuador using AFLP markers (Droogenbroeck et al. 2002). A total of 95 accessions belonging to three genera were evaluated. Both cluster and PCO analysis clearly separated the species of three genera and illustrated the large genetic distance between *C. papaya* accessions and *Vasconcellea* group. The specific clustering of highly diverse group of *Vasconcellea* × *heilbornii* accessions also suggests that these genotypes may be the results of bi-directorial introgression events between *Vasconcellea stipulata* and *V. cundinamarcensis.* Papaya is also grown widely in India. Many improved papaya varieties from various parts of India have been developed. Recently, Saxena et al. (2005) have measured genetic diversity among 10 commercially important papaya cultivars using three SPAR techniques namely RAPD, IISR and DAMD and concluded that IISR-PCR is probably the best technique for assessing papaya germplasm. Papaya germplasm was found to be quite narrow. However, least genetic variation was observed between CO 2 and CO 3, whereas CO 4 and CO 7 and Coorg Honey Dew and Red Fleshed were found to be genetically distant.

19.3 Marker-Assisted Selection

Papaya is conventionally propagated by seeds and dioecious papaya varieties do not ensure the right sex type. As a result, 50–60% of seeds produce male plants which need to be uprooted after six to seven months of planting. The resources (fertilizer, water, weeding, land, labor and time) used in development and weeding out male plants makes papaya cultivation cumbersome and uneconomical. Efforts to distinguish sex of papaya at juvenile stage through morphological and biochemical markers have not met with success. A loose linkage between flower morphology and sex type has been identified, but sex determination based on flower morphology is not possible until four months. In an open-pollinated species such as papaya, the selection of the appropriate sex type of the progeny for commercial planting would be beneficial, since only the female and hermaphrodite plants are grown for fruit. Knowledge of the sex type of papaya is important in selecting parents for use in hybridization work. Crosses between females and hermaphrodites will give all fruit-bearing progenies. Sex expression in papaya is controlled by a single gene with three alleles which have a pleiotropic effect (Hofmeyer 1941; Storey 1953). The sex homologues were designated as M for male, MH for hermaphrodite and m for female. All combinations of dominant alleles, such as

MM, MHMH and MMH, are lethal to the zygote. This makes all males and hermaphrodites into enforced sex heterozygotes. One-fourth of the seeds in their fruits are nonviable. The genotypes for sex are MM for male, MHm for hermaphrodite and mm for female. Using these sex genotypes, there are eight possible cross combinations that could be made with various segregation ratios. Self-pollination in males, cross-pollination between males and females and cross-pollination between male and hermaphrodites can all be done using the sexually ambivalent males (SAMs) that produce perfect flowers during certain periods of the year. Male and hermaphrodite trees undergo various degrees of sex reversal, depending on seasonal changes and climate (Awada 1958). To make the cultivation profitable it is necessary to grow more female:male plants. To discriminate between male and female plants, sex specific molecular markers have been identified in a few dioecious species such as *Silene* and *Pistachio*. In papaya, RAPD and microsatellite markers linked to sex have been reported (Soundur et al. 1996; Parasnis et al. 1999). Two RAPD markers, T12 and T1C each mapped 7 cM apart from the SEX 1 locus (Soundur et al. 1996). Parasnis et al. (2000) have also reported sex diagnostic. They have developed a male specific SCAR marker in papaya by cloning a male-specific RAPD (831 bp) fragment and designing longer primers. The potential of this SCAR marker is further exploited to develop a simplified and highly accurate sex diagnostic assay by including an internal PCR control following a single step DNA extraction procedure, optimizing the PCR condition to simultaneously amplify male-specific and control bands from the crude leaf extract. This diagnostic approach is of great commercial significance of papaya growers as well as to seed companies and plant nurseries for early identification of female seedlings of dioecious species. In principle, this experimental design could easily be applied to molecular analysis of any agriculturally important trait for which specific DNA probes could be identified and hence opens new avenues of research in the field of genetic diagnostics of plants. In 2002, a group in Japan (Urashaki et al. 2002 a, b) has reported that the random amplified polymorphic DNA (RAPD) technique was used to determine the sex of a dioecious species, *Carica papaya* L. with three sex types, male, female and hermaphrodite. A 450 bp marker fragmented PSDM (Papaya Sex Determination Marker) was used for all male and hermaphrodite plants but not in the female plants so far analyzed. Recently embryo induction of papaya by anther culture has been reported and identification of the sex of plantlets derived from embryos using a sex diagnostic PCR was done. Anthers, containing approximately 80% pollen, were collected from 10 to 14 mm long male flower buds. They were pre-treated with agar (0.8%) or in liquid medium for 1 – 5 days at 25-35^{0}C, then transferred to agar medium with 0.1 mg l^{-1} BA and 0.1 mg l^{-1} NAA. Agar and liquid media used for the pre-treatment contained only water or MS nutrients with or without Sucrose (2.0%). On the agar medium, no embryos were induced. At 35 °C embryo induction rate tended to increase up to about 4% when anthers were treated in water for 1 day or MS medium with Sucrose for 3 or 5 days. The sex of plantlets established through anther culture was analyzed using a sex-diagnostic PCR. All plantlets were determined as female. From these results it was suggested that all plantlets established through anther culture were of microspore origin and then the anther culture technique is useful for breeding of female papaya.

19.4 Construction of Genetic Maps

Papaya is an ideal fruit crop for genomic research because of its relatively small genome size (372 Mbp) (Arumugunathan and Earle 1991). However, not much information has been generated so far. High-density genetic maps are prerequisite for isolation and cloning of genes of interest, genomic dissection, marker-assisted selection etc. Genetic mapping of many crops has been accomplished. However, genetic map of papaya has only recently been developed. Ma et al. (2004) constructed a high-density genetic map of papaya using 54 F_2 plants derived from Kapoho and Sun Up cultivars with 1,501 markers including 1,498 AFLP markers, PRSV cp markers, morphological sex types and fresh fruit color. These markers map to 12 linkage groups at a LOD score of 5.0 and recombination fraction of 0.25. The 12 major linkages groups covered a total length of 3,294.2 cM, with an average distance of 2.2 cM between adjacent markers. This map revealed severe suppression of recombination around the sex determination locus with a total of 22.5 markers co-segregating with sex type. The cytosine bases were found to be highly methylated in this region on the basis of distribution of methylation-sensitive and methylation-insensitive markers (Fig. 1).

BACs are the most commonly employed vectors for carrying large DNA fragments. Ming et al. (2001) reported construction of bacterial artificial chromosome (BAC) library from papaya. The BAC library consists of 39,168 clones from two separate ligation reactions. The average insert size of library is 132 kb. The entire BAC library was estimated to provide 13.7× papaya genome equivalents, excluding the false positive and chloroplast clones (Table 1). High-density filters were made containing 94% or 36,864 clones of the library with 12.7× papaya-genome equivalents. Eleven papaya cDNA and 10 *Arabidopsis* cDNA probes detected an average of 22.8 BACs per probe in the library.

Liu et al. (2004) fine-mapped the sex determination gene (Table 2) with the help of 4,380 informative chromosomes, two SCAR markers (W11 and T12), three cloned sex linked AFLP markers (cpsm10, cpsm31 and cpsm54) and one BAC end (cpbe 55). No recombinants were detected. They reported the discovery of an incipient Y chromosome in papaya of which 10% is a non-recombining, rapidly evolving, sex-determining region flanked by normal autosome-like regions that comprise the remaining 90% of chromosomes. This proves that sex chromosome evolve from autosome. The severe suppression of recombination and excessive divergence between homologues in the region containing the papaya sex-determining genes indicate that this is an incipient sex chromosome. On the basis of size of present contig map (2.5 Mb) and 57% of cpsm markers that have been accounted for, the physical size of MSY is estimated at 4–5 Mb or 10% of papaya's primitive chromosome. The incipient sex chromosomes of papaya may yield insights about earlier stages of sex chromosome evolution. The small physical size of MSY region and the mosaic arrangements of sequence degradation indicate a recent origin of the papaya sex chromosomes.

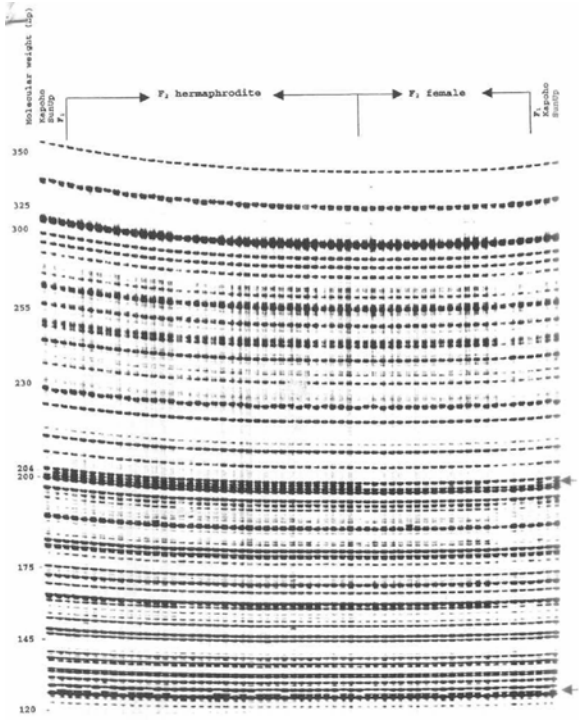

Fig. 1. AFLP products amplified by the primer pair E-GCT/M-AG. The Sun Up dominant marker between 200 and 204 bp is cosegregating with sex (Source: Genetics 166:419–436)

19.5 Recombinant DNA Technology

Recently, DNA recombinant technology has opened up new vistas for development of virus resistant papaya. Pathogen derived resistance (PDR) has been proved to be an effective tool in combating plant viruses. Genetic engineering for virus resistance has been found effective whereby transgenic plants expressing virus genome sequence resist attack by corresponding viruses. Coat protein mediated resistance (CPMR) was first reported in 1986 in tomato. Subsequently, a large number of transgenic lines (citrus, papaya, potato, peanut, squash, sugar beet) containing CP transgene have been produced. There are several mechanisms involved in CPMR. However, it is largely believed that resistance is RNA mediated *via* post-transcriptional gene silencing.

PRSV resistant transgenic papaya has been developed and commercialized in 1998 in Hawaii, USA by Dr. Dennis Gonsalves and his team (Gonsalves 1998). SunUp and Rainbow cultivars of transgenic papaya have been developed by cloning CP gene of mild strain of PRSV from Hawaii. Dennis Gonsalves and his group used PDR concept in 1986 by cloning the CP gene of PRSV HA 5-1 (a mild strain) from Hawaii. Because of various technical difficulties and the requirement that the gene be expressed as a protein, the gene was engineered as a chimeric protein containing 17 amino acids of cucumber mosaic virus at the N terminus of the full-length CP gene of PRSV HA 5-1 (Ling et al. 1991). Dr. Maureen Fitch, Scientist

Table 1. Results of screening of papaya BAC library with homologous and hetrologous cDNA, rDNA and cpDNA. Source: Ming et al. (2001) Theor Appl Genet 102:892–899

Probes	No. of bands	Ligation 1	Ligation 2	Total
AEST9	2	5	8	13
AEST18	2	5	7	12
AEST36	2	6	14	20
AEST37	6	0	0	0
AEST47	2	10	17	27
AEST48	3	6	17	23
AEST63	4	8	17	25
AEST64	2	9	9	18
AEST69	4	16	47	63
AEST127	2	19	29	48
CPF9A1	5	8	7	15
CPF9A2	3	0	0	0
CPF9A3		6	5	11
CPF9A4		9	11	20
CPF9A5		4	6	10
CPF9A6		3	25	28
CPF9A7		10	11	21
CPF26A3		3	3	6
CPF26A7		18	19	37
CPF26A4&5		19	27	46
Total	44	154	279	433
18sPXp108		18	43	61
rop B and *teunk*		211	293	504

Table 2. Fine mapping with SCAR markers in MSY region of papaya. Source: Liu et al. (2004) Nature 427:348–352

Population	Progeny	Hema-phrodite	Female	SCAR markers	Recom-binant
Kapoho × SunUp	F_2	335	150	W11	0
Kapoho × SunUp	F_2	335	156	W11, T12, cpbe55	0
Kapoho × SunUp	F_2	481	274	W11, T12, cpbe55	0
Kapoho × Saipan Red	F_2	175	49	Cpsm31, cpsm 10	0
AU0 × SunUp	F_2	170	65	W11, T12, cpsm54	0
Total	2,190	1,496	694		0

at USDA, Hawaii took the challenge of transforming papaya in 1987. The red-fleshed Sunrise, Sunset (a sib selection of Sunrise), and the yellow-fleshed Kapoho, were chosen as target cultivars. The embryogenic tissue was bombarded with tungsten particles coated with engineered DNA construct of the PRSV HA 5-1 CP gene using the gene gun. Fifteen months later, transgenic plants were obtained and grown in the greenhouse (Fitch et al. 1990, 1992). R_0 transgenic lines were screened for virus resistance against severe strain of PRSV HA from Hawaii at greenhouse at Cornell University. R_0 micropropagated plants of the first line were further characterized as it showed excellent resistance to PRSV HA (Fitch et al. 1992) among all R_0 lines. Line 55-1 was female and thus progenies could not be obtained directly from the R_0 plants, unlike a hermaphrodite. In order to know whether 55-1 is resistant to PRSV, a trial was laid down using R_0 plants.

R_1 plants were obtained by crossing line 55-1 with nontransgenic Sunset under greenhouse conditions. These plants were screened in the greenhouse for resistance to PRSV isolates from around world. Analysis clearly showed that 50% of the progenies were transgenic. This confirmed that transgenic plants had one insert of the *npt*II gene and, the CP gene. Field experiment was conducted to evaluate transgenic plants under natural field condition. The transgenic papaya showed excellent resistance throughout the two-year trial (Lius et al. 1997). Nearly all (95%) of the nontransgenic plants and those of a transgenic line that lacked the CP gene showed PRSV symptoms. The homozygous line 55-1 was later named SunUp. The hybrid made from the cross of the transgenic SunUp and the non-transgenic Kapoho was named Rainbow.

Efforts are being made to generate PaLCuV resistant transgenic papaya using both sense and antisense *Rep* gene as well as CP gene of PaLCuV in India. The first report of papaya leaf curl disease in India to be caused by a geminivirus was published by Saxena et al. (1998a, b). Recently the first report of papaya leaf curl virus (PaLCuV) infecting papaya plants in Taiwan is also published (Chang et al. 2003). Papaya cultivation is severely threatened by PaLCuV. The disease is transmitted by the vector whitefly (*Bemisia tabaci*), and characterized by severe curling, downward cupping and crinkling of leaves. Early infection leads to severe reduction in yield and management of disease is urgently required. Developing transgenic papaya plants resistant to PaLCuV seems to be most promising considering several points as discussed above regarding genetics and breeding program of papaya. Nucleotide sequence and intergeminiviral homologies of the DNA-A of PaLCuV from India have already been reported by Saxena et al. (1998c) and further molecular characterization of papaya leaf curl geminivirus (PaLCuV) and its isolates is currently under studies.

Transformation of plants with viral genes has been proven in many cases to produce resistance to the virus from which the genes were derived. The technology has been successfully used to produce resistance in papaya with respect to PRSV and trials with PaLCuV are undergoing. The benefit of transgenic virus resistance includes increased yield, reduced pesticide use to control the vectors of viruses, i.e. whitefly in case of PaLCuV and improved crop as well as food quality. The coat protein (CP) gene is most often used to confer resistance. In some cases, the expression of CP correlated with resistance, and strong evidence for prevention of uncoating was shown. However, reports indicate that coat protein mediated resistance is not successful in case of geminiviruses, and most of the strategies for genetically engineered resistance to geminivirus involve the replication-associated protein (*Rep*) sequences (Sinistera et al. 1999; Yang et al. 2004). For some viruses there can be both CP and RNA mechanism that can confer resistance in transgenic plants. In case of PaLCuV, it is being speculated that high levels of resistance can be produced in papaya plants transformed with a viral replicase gene, which includes the full length gene as well as various deletions or sequence modifications. The mechanism of resistance in replicase-expressing plants is complex and may involve expression of a protein that blocks virus replication and/or movement, as well as post-transcriptional gene silencing. Further, development of transformation technique in different commercially significant papaya cultivars broadens the possibility of use of engineered virus resistance in papaya breeding.

It has been observed in case of several viruses including geminivruses that resistant plants did not confer resistance to other isolates (if present) of the same virus. Resistance to virus on transgenic plants expressing CP or *Rep* gene was shown to be dependent on the sequence homology between the CP or *Rep* transgene expressed in the plant genome and the CP or *Rep* gene from the incoming virus. Therefore, knowledge of the degree of homology among the CP and *Rep* gene from the distinct PaLCuV isolates which are present in a given area is important to guide the development of transgenic papaya for the control of PaLCuV. To address this problem a comprehensive plan is to be developed in case of PaLCuV and already work on genetic variability of PaLCuV isolates is currently going on in India (S Saxena, personal communication). The CP and *Rep* genes from different isolates of PaLCuV (collected from different geographic locations from North India) were cloned and sequenced. The sequences revealed a substantial amount of variability in different PaLCuV isolates implicating the need to look for most homologous region in CP or *Rep* gene to be used as putative transgene and also the need for using genes from local isolates in generation of PaLCuV resistant transgenic papaya. If successful the technology would improve the yield and quality of papaya fruit.

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20 Persimmon

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20.1 Introduction

Persimmon, *Diospyros kaki* Thunb., is a deciduous fruit tree, native to the East Asia. It is believed to have originated in the mountain area of southern China and has been cultivated as an important fruit crop in China, Korea, and Japan from prehistoric times. Persimmon fruit containing high amount of vitamin C, dietary fiber, carotenoids and polyphenols (tannins) is usually consumed as a fresh or dried fruit that was one of the important nutrition sources in old times. Young fruit has been used for obtaining tannins (persimmon oil), which is of great value for industrial uses.

Persimmon is a typical oriental fruit and less known in non-Asian countries. Most part of persimmon production is from East Asia. In 2004, the global production of persimmon totaled 2,518,123 metric tones, 72.3% from China, 11.9% from Korea and 9.2% from Japan (FAO 2004). Following these main producing countries, Brazil, Italy, and Israel are producing substantial amounts, and Australia and New Zealand are producing persimmon mainly for export. Recently, remarkable expansion in persimmon production has occurred in Spain, though persimmon statistics of FAO does not include Spanish production (Llácer and Badenes 2004). Thus, persimmon is gaining popularity as a new fruit crop in the non-Asian countries in recent years.

The genus *Diospyros* L. consists of approximately 400 species, found mostly in the tropics of Asia, Africa and Central-South America (Yonemori et al. 2000). Only few species, including *D. kaki*, are native to the temperate zone. Most wild species of the genus *Diospyros* are diploid ($2n = 2x = 30$) or tetraploid ($2n = 4x = 60$), while *D. kaki* is basically a hexaploid ($2n = 6x = 90$) (Ng 1978; Tamura et al. 1998; Choi et al. 2003a, b). Some of the seedless cultivars of *D. kaki* have been reported as nonaploid ($2n = 9x = 135$) (Zhuang et al. 1990; Tamura et al. 1998). Therefore, single or several diploid and/or tetraploid species must be involved in the polyploidization of persimmon, but so far, there is no consensus as to how persimmon acquired a high chromosome number and whether it is an auto- or allo-polyploid. In an earlier study based on morphological, geographical and cytological analysis, Ng (1978) suggested a hypothesis that *D. kaki* had originated directly from *D. roxburghii* (syn. *D. glandulosa*) through polyploidy, cultivation and selection. However, phylogenetic study based on DNA variation in the special region of cpDNA (rbcL-ORF106 and trnT-trnF) indicated that *D. glandulosa* is closely related to *D. oleifera*, which is native of the temperate region of China, and may not be the direct progenitor of *D. kaki* (Yonemori et al. 1998). Recently, phylogenetic analysis using DNA sequences of ITS and *mat*K region of some *Diospyros* species revealed that *D. galandulosa* and *D. oleifera* were relatively close to *D. kaki*, but direct relationship between *D. galandulosa* and *D. kaki* has not been proved (Yonemori et al. submitted). On the other hand, close relationship between *D. kaki* and *D. lotus* was shown in both studies (Yonemori et al. 1998; Yonemori et al. in preparation). *D. lotus* is a diploid species, widely distributed in temperate Asia and consumed as fresh or dried fruits. The molecular data indicates that *D. lotus* or its ancestral species would be associated with the speciation of *D. kaki*.

It is difficult to define and characterize the sets of homologous chromosomes based on chromosome observation under a light microscope because somatic chromosomes of *Diospyros* species are too small (Tamura et al. 1998). Recently, however, fluorescent in situ hybridization (FISH) has been applied as a new useful tool for analyzing karyotypes and phylogenetic relationships of some *Diospyros* species (Choi et al. 2003a, b). When FISH using an rDNA probe was performed, four homologous chromosomes and non-

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homologous two pairs of chromosomes carrying 45S rDNA were observed (Choi et al. 2003a). The presence of non-homologous two pairs of chromosomes bearing 45S rDNA indicates that *D. kaki* might be an allohexaploid. However, four homologous chromosomes with 45S rDNA might mean that *D. kaki* may be an autoallohexaploid or at least some chromosomes are homoeologous among the different structural genomes of *D. kaki*. Thus, the genomic composition of *D. kaki* might be an allo- or autoallo-hexaploid rather than autohexaploid, although further analysis will be required to clarify the polyploid nature of persimmon. Previously, Zhuang (1990) hypothesized that *D. kaki* might be an allohexaploid, since bivalent formation occurred regularly and few multivalents were observed in the meiosis of pollen mother cell. However, the possibility of polysomic polyploidy in persimmon genome cannot be ruled out because lack of multivalent formation does not necessarily indicate a disomic polyploid in case of species with short chromosomes (Krebs and Hancock 1989; Wolf et al. 1989; Qu et al. 1998). As discussed bellow, segregation analysis of molecular markers indicated the existence of polysomic inheritance in *D. kaki* (Kanzaki et al. 2001).

The polyploidy nature in persimmon makes genetic linkage analysis difficult and, so far, no effort has been made to develop a genetic map for persimmon. Recently, we have developed molecular markers associated with the trait of natural astringency-loss in persimmon fruit and the markers are practically useful in persimmon breeding programs (Kanzaki et al. 2001). Through the analysis of the markers, a possible explanation has given about genetic nature of the trait of natural astringency-loss. In this chapter, we focus on the trait of astringency-loss and describe the possibility of polysomic inheritance in persimmon.

20.2 Nature of Natural Astringency-Loss in Persimmon Fruit and Its Inheritance

Generally, a persimmon fruit accumulates high amount of soluble tannins and tastes extremely astringent. However, some cultivars are genetically defined to lose astringency naturally on the tree as fruit development and are called as 'non-astringent persimmon' or 'sweet persimmon'. Strictly speaking, persimmon cultivars are classified into four types based on the relationship between astringency in the fruit at harvest, presence of seed, and flesh color (Hume 1914; Kajiura 1946; Yonemori et al. 2000). These four types are: 1) pollination-constant non-astringent (PCNA), 2) pollination-variant non-astringent (PVNA), 3) pollination-variant astringent (PVA), and 4) pollination-constant astringent (PCA). Among these four types, the only inborn non-astringent type is PCNA-type because PVNA-type fruits lose astringency only when they have a sufficient number of seeds. The decisive difference between PCNA and the other three types (non-PCNA-type) is the pattern of tannin accumulation in fruits (Yonemori and Matsushima 1985). PCNA-type fruits stop to accumulate tannins at the early stage of fruit growth, while non-PCNA-type accumulates tannins until the middle stage of fruit development. Therefore, PCNA-type fruits contain much less tannins than non-PCNA-type at maturity and low amount of tannins results in easy deastringency in PCNA-type fruit. In other words, PCNA-type lacks the ability to accumulate high amount of tannins in the fruits. The PCNA/non-PCNA trait is qualitatively inherited to the progenies and PCNA-type is recessive to non-PCNA-type (Ikeda et al. 1985; Yamada and Sato 2002). According to their reports, crosses among PCNA-type plants yielded only PCNA-type offspring and all F_1 hybrids between PCNA and non-PCNA-type cultivars become non-PCNA-type offspring. When these F_1 hybrids were backcrossed to PCNA-type cultivars/selections, only around 15% of PCNA-type offspring were segregated in the backcross population. Thus, it can be said that PCNA-type is a recessive mutant in which the mutation has occurred on the gene(s) controlling tannin accumulation (called *Ast*, for astringency). The low ratio of PCNA-type offspring in the backcrossed population might be caused by polyploid nature of persimmon. Assuming that persimmon is an allohexaploid (or disomic hexaploid), Ikeda et al. (1985) suggested a hypothesis that each of three structural genomes would have single *Ast* locus (*Ast1*, *Ast2*, and *Ast3*) and PCNA phenotype can be expressed only in recessive genotype with triplicate genes (*ast1 ast2 ast3*). In such a case, the expected ratio of PCNA-type offspring in the backcross population would be 12.5% if the donor parent (non-PCNA-type) was homozygous dominant genotype with the three genes. Recently, however, based on the segregation pattern of molecular markers

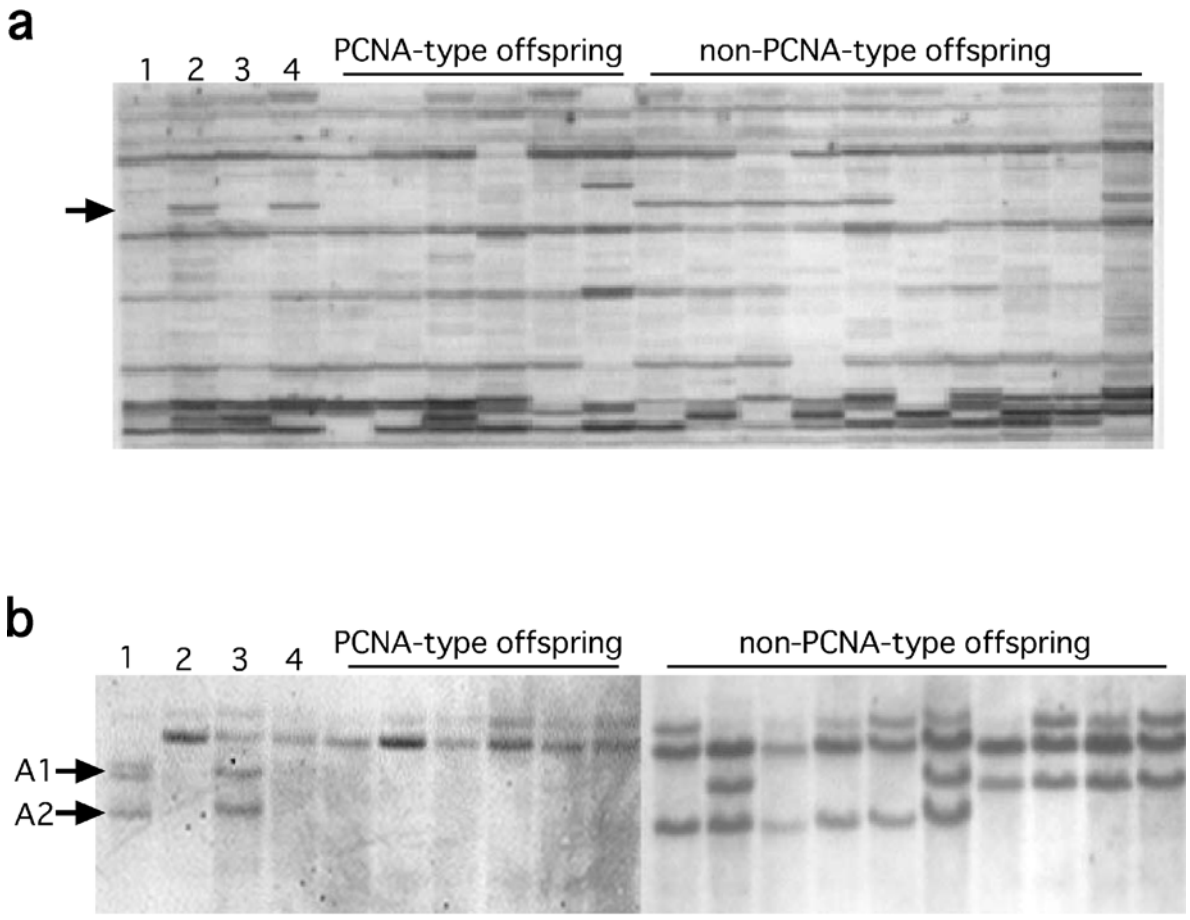

Fig. 1. (**a**) A part of AFLP fingerprints using primer combination EACC/MCTA. *Lanes*: 1, PCNA bulk; 2, non-PCNA bulk; 3, PCNA parent; 4, non-PCNA parent; following PCNA and non-PCNA offspring used for bulked segregant analysis. *Arrow* indicates the AFLP marker, EACC/MCTA-400. (**b**) RFLP analysis of genomic DNAs digested with *Hin*dIII, using EACC/MCTA-400 as a probe. *Lanes*: 1, non-PCNA donor parent; 2, PCNA parent for F_1 hybrid; 3, F_1 hybrid; 4, PCNA parent for backcross; following PCNA and non-PCNA offspring used for bulked segregant analysis. *Arrow* indicates the two RFLP markers (A1 and A2) linked to *Ast* locus

linked to the PCNA/non-PCNA trait, Kanzaki et al. (2001) suggested a new hypothesis that the mode of inheritance of *Ast* gene appeared to be polysomic rather than disomic.

20.3 Identification of Molecular Markers Liked to the PCNA/non-PCNA Trait and Polysomic Segregation of the Markers

AFLP analysis was conducted to identify molecular markers linked to the trait of natural astringency-loss in PCNA type (Kanzaki et al. 2001). A total of 128 primer combinations were used in a bulked segregant analysis and a candidate marker linked to one of the dominant alleles conferring non-PCNA trait was identified (Fig. 1a). This marker (EACC/MCTA-400) was absent in all PCNA-type offspring tested and was present in half of the non-PCNA-type offspring. When the EACC/MCTA-400 fragment was isolated and used as a probe for RFLP analysis, two polymorphic markers (A1 and A2) were detected (Fig. 1b). The segregation pattern of A2 marker in RFLP analysis was the same as that of EACC/MCTA-400 obtained by AFLP analysis. A1 marker could be detected in all A2-negative non-PCNA-type offspring and some A2-positive non-PCNA-type offspring. These results indicate that EACC/MCTA-400 and A2 markers are linked to one dominant allele and A1 marker is linked to another dominant allele. In our breeding population, all non-PCNA-type offspring could be distin-

Table 1. Segregation analysis of the RFLP markers associated with PCNA/non-PCNA trait. F_1 progeny is derived from the cross PCNA[a] × non-PCNA and backcross progeny is derived from PCNA × non-PCNA-type F_1

	Observed segregation of RFLP markers[b]	Genotype of non-PCNA parent	Expected segregation of RFLP markers	χ^2	P
F_1 progeny	8 A1 : 22 A1A2 : 10 A2				
	Disomic model	*Ast1/Ast1 Ast2/Ast2*	All A1A2	**	**
		Ast1/ast1 Ast2/ast2	1 A1: 1 A1A2: 1 A2 : 1 a	24.8	< 0.01
		Ast1/Ast2 Ast1/Ast2	1 A1: 2 A1A2 : 1 A2	0.6	0.74
	Tetrasomic model	*Ast-1/Ast-1/Ast-2/Ast-2*	1 A1 : 4 A1A2 : 1 A2	2.75	0.25
Backcross progeny	14 A1A2 : 37 A1 : 27 A2 : 23 a				
	Disomic model	*Ast1/ast1 Ast2/ast2*	1 A1A2 : 1 A1: 1 A2 : 1 a	10.8	0.013
	Tetrasomic model	*Ast-1/Ast-2/ast/ast*	1 A1A2 : 2 A1: 2 A2 : 1 a	4.39	0.22

[a] Assume that genotype of PCNA parent is *ast1/ast1 ast2/ast2* (disomic model) or *ast/ast/ast/ast* (tetrasomic model).
[b] Four RFLP patterns are represented as A1(showing only A1 marker), A2 (showing only A2 marker), A1A2 (showing both A1 and A2 markers), and a (showing neither markers).

guished from PCNA-type offspring by the presence of either RFLP marker or both. This suggests that there are two DNA fragments which are associated separately with gene(s) conferring the non-PCNA trait, and that the gene linked with each fragment is able to express the same non-PCNA trait. Contrary to the Ikeda's hypothesis (1985) that triplicate genes could be associated with the trait, our results indicated that PCNA/non-PCNA trait would be controlled by duplicate genes. Here, we suggest a possible hypothesis about the inheritance of the trait based on the segregation analysis of these RFLP markers in F_1 and backcross progenies.

Assuming that persimmon is a disomic poplyploid (allohexaploid) and homeoalleic *Ast* genes are separately associated with the trait, two RFLP markers (A1 and A2) linked to each *Ast* gene (*Ast1* and *Ast2*) should be segregated independently in a progeny. For example, if the genotype of non-PCNA-parent is homozygous for two loci (*Ast1/Ast1 Ast2/Ast2*), all F_1 hybrids between PCNA-parent (genotype: *ast1/ast1 ast2/ast2*) must show both A1 and A2 markers (Table 1). If the non-PCNA-parent has heterozygous state for both loci (*Ast1/ast1 Ast2/ast2)*, A1 and A2 bands will segregate independently and 25% of F_1 progeny should present neither A1 nor A2 markers. These assumptions, however, did not fit to the observed segregation of these markers in F_1 progeny (Table 1). It might be possible that both A1 and A2 markers are linked to homozygous dominant alleles at each locus. Assuming that *Ast1* and *Ast2* form two allele pairs at different loci (*Ast1*:*Ast2 Ast1*:*Ast2*), the expected segregation of A1 and A2 markers in F_1 progeny should be A1:A1A2:A1 = 1:2:1, and this ratio seems to fit to the observed segregation (Table 1). However, when the segregation in backcross progenies was considered, disomic model did not fit to the observed ratio (Table 1). Thus, it would not be most likely that duplicate *Ast* loci with a disomic nature control the PCNA/non-PCNA trait.

On the other hand, assuming tetrasomic inheritance of the *Ast* gene seemed to be more likely (Table 1). If persimmon is an autoallohexaploid and the PCNA/non-PCNA trait is controlled by tetra-alleic *Ast* locus in the polysomic genome, segregation of the RFLP markers linked to the locus would follow tetrasomic segregation. The observed segregation of the RFLP markers in both F_1 and backcross progenies did not deviated from the expected tetrasomic segregation (pure chromosome segregation) (Table 1) and this result suggests the polysomic nature of the locus. In addition, the expected ratio of PCNA plants in backcross progeny would be 16.7% (tetrasomic pure chromosome segregation) and this seemed to be consistent with the ratio of PCNA-type plants in the breeding population previously reported (Ikeda et al. 1985; Yamada and Sato 2002).

There exists no conclusive evidence that persimmon is an autoallohexaploid. Both of cytogeneic analysis (Choi et al. 2003a) and segregation analysis of RFLP markers (Kanzaki et al. 2001) indicates that persimmon may have four chromosomes in a homologous group. To elucidate the genomic composition, further cytogenetic studies and segregation analysis using codominant molecular markers will be required. Genetic studies on persimmon would not progress without solving this issue.

20.4 Future Scope for Persimmon Breeding

PCNA type is the most desirable for fresh consumption. Thus, to develop and release commercially attractive PCNA-type cultivars have been the main breeding objective in Japan. So far, several PCNA type cultivars have been released from the breeding programs in National Institute of Fruit Tree Science (Yonemori et al. 2000). In the Japanese breeding programs, PCNA-type cultivars/selections have been used as both parents to obtain PCNA-type offspring exclusively in the progenies. However, as crossings among PCNA-type cultivars/selections were repeated, inbreeding depression becomes a serious problem (Yamada 1993). Using non-PCNA-type cultivars/selections as a source of breeding is the better way for extending the genetic pool of breeding population, but it had been an impractical and inefficient strategy to develop PCNA-type cultivars. All F_1 hybrids between PCNA and non-PCNA type cultivars become non-PCNA type and only around 15% of PCNA-type offspring is obtainable even in backcross population. For making such a strategy more practical, marker-assisted selection using the RFLP markers linked to *Ast* locus is a useful system. We are developing an easy PCR-based selection system based on the DNA sequences of adjacent region of the RFLP markers because RFLP analysis is a relatively laborious and inconvenient in a practical work.

It had been believed that PCNA type was uniquely developed only in Japan. Recently, however, a PCNA-type cultivar, 'Luo Tian Tian Shi', was found growing in Luo Tian prefecture of China (Wang 1982; Wang et al. 1997). Phylogenetic tree based on AFLP analysis showed distant relationship between 'Luo Tian Tian Shi' and Japanese PCNA-type cultivar (Kanzaki et al. 2000a) and it would indicate independent occurrence of each Chinese- and Japanese- PCNA type. Also, the genetic nature of Chinese PCNA trait seemed to be different from that of Japanese PCNA trait. The RFLP marker linked to *Ast* locus could be detected in 'Luo Tian Tian Shi' (Kanzaki et al. 2000b) and Ikegami et al. (2004) reported that hybrids between 'Luo Tian Tian Shi' and Japanese PCNA-type cultivar segregated into PCNA and non-PCNA plants. These results suggest that the gene controlling Chinese PCNA trait should be different from *Ast* gene of Japanese cultivar, although genetic nature of Chinese-PCNA trait has not been understood well. However, as the cross between 'Luo Tian Tian Shi' and Japanese non-PCNA cultivar yield PCNA-type offspring in F_1 generation (Ikegami et al. 2006), Chinese PCNA cultivar will be an important breeding source for persimmon breeding project in the future.

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