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Bo Liu *Editor*

The Plant Cytoskeleton

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The Plant Cytoskeleton

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Preface

This book is blessed with 14 wonderful chapters aimed at summarizing in part the current knowledge on the structure and function of plant microtubules and actin filaments. Since the initial discovery of microtubules in plant cells in 1963 and the visualization of green algal actin filaments in 1974, their dynamic behaviors and their roles in specific cellular functions have been embraced one after another. While plant cell biologists continue to be excited by the beautiful cytoskeletal network in plant cells, their understanding of the function behind the network has been greatly advanced by discoveries of novel proteins that interact with the network. Recent progress has benefited from technological advances in areas like live cell imaging, genetic screening, and the tools of genomics and proteomics.

In this book, the first six chapters visit the molecular basis of the plant cytoskeleton. Since the first plant genome was sequenced, the number of genes encoding cytoskeletal proteins has stunned us. From isolating actin and tubulin genes to uncovering those encoding myosins and kinesins, plant biologists have made tremendous progress in the past decade or so. Nineteen years ago when microtubule-translocating activities were first demonstrated in isolated phragmoplasts, no one could have predicted that the little Arabidopsis plant would have more microtubule-based motor kinesins than are encoded in the human genome. In the post-genomic era, new avenues have opened and are leading to explosive discoveries made by mining sequenced genomes and characterizing the functions of proteins encoded by novel cytoskeletal genes. Undoubtedly, characterizing the proteins that interact with the plant cytoskeletal network becomes a task that is integral for our understanding of plant evolution.

The second part of the book includes three chapters that cover how microtubules are arrayed during plant cell division. It is both puzzling and fascinating that plant cells are able to organize microtubules into arrays like the preprophase band, the bipolar spindle, and the phragmoplast in the absence of a structurally defined organizing center. Fortunately, the molecular mechanisms underlying the organization of these arrays are emerging. Again, advances made in this area will provide many clues regarding how land plants have evolved.

The book ends with a section devoted to connecting the cytoskeleton with plant growth and development. These five chapters summarize current knowledge on the mechanisms that regulate different patterns of cell growth as well as on how the

growth of whole organs is regulated by the cytoskeleton. Plants demonstrate remarkable patterns of growth, and their tissues and organs are built on cells of splendid shapes. How plant cells acquire their distinct shapes is an intriguing question. After their birth in the meristem, plant cells respond to internal and external cues that direct the acquisition of specific roles after differentiation. Once these cues are read, the cytoskeletal network is remodeled and it guides changes to the cell wall that influence cell shape. These changes, ultimately, determine the shape of the plant and they reflect adaptations to environmental conditions that plants have made over millions of years.

This book is not intended to cover every aspect of the plant cytoskeleton. Besides serving as a convenient reference, it is intended to generate enthusiasm amongst young scientists to join the efforts to dig out the root of the plant cytoskeleton. The plant cytoskeleton is no longer a subject reserved for cell biologists. Its impacts on plant growth and development can no longer be overstated. I hope that the chapters included in this book inspire additional in-depth studies focused not only on the cytoskeleton, but also on its links with developmental phenomena and plant responses to the environment.

Much of what we have learned about the plant cytoskeleton has been inspired by the careful observations of many pioneers in the field of plant cell biology. I am particularly indebted to my mentor Prof. Barry A. Palevitz who has been an inspirational source for me and others through his discoveries and visions.

Davis, CA Bo Liu

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Part I
Molecular Basis of the Plant Cytoskeleton

Chapter 1
Actin Functions in the Cytoplasmic and Nuclear Compartments

Richard B. Meagher, Muthugapatti K. Kandasamy, and Lori King

1.1 Introduction

Eukaryotes share conserved roles for the actin cytoskeleton in cell polarity determination, cell division, vesicle transport, organelle movement, and newly described nuclear activities. In plants and animals, the number of genes encoding components of the actin system is expanded and individual genes are specialized to participate in directing multicellular development. With more than a dozen gene families encoding multiple actin and actin binding protein (ABP) variants, actin cytoskeletal proteins have a particularly high degree of combinatorial complexity in angiosperms, rivaling or exceeding that in mammals. A genome wide duplication event several hundred million years ago in a lycopod-like ancestor likely generated the two most divergent classes found within most families: the vegetative or constitutive protein variants and the reproductive protein variants. Subsequent gene duplication events allowed further subfunctionalization of the plant cytoskeletal gene and protein variant families. A major scientific focus of our research program and a theme throughout this review is the importance of dynamic interactions among ancient actin and ABP gene and protein family members resulting both from their differential regulation and from the differential protein-protein interactions of multiple protein variants.

This review will focus on recent genetic and molecular cell biological studies that have advanced our understanding of plant actin cytoskeletal dynamics and its role in various cellular functions including establishment of cell polarity, elongation of cells, signaling within cells, movement of cargo, and control of both genetic and epigenetic regulation. Divergent actin, actin depolymerizing factor (ADF/cofilins), profilin, and myosin variants have independent and often non-overlapping protein activities supporting different aspects of cell and organ development.

Recent studies have better resolved cell biological functions for the actin cytoskeleton. For instance, plant organelles tethered to actin filaments move independently

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around plant cells on myosin motors in a relatively stationary cytoplasm. In some elongating plant cells, growth from the barbed end of actin filaments, filament severing, and changes in filament convolution are the dominant activities that reshape the cytoskeleton, undoubtedly affecting cargo movement. Actin and ADF variants have direct roles in glucose signaling, pathogen response, day-length control of flowering, cold response, gravity detection and response, drought stress signaling of guard cell closure, and control of the cell cycle.

This article will also address the nuclear functions of actin cytoskeletal proteins, proteins once thought to be purely cytoplasmic (e.g. actin, ADF, profilin, myosin). Conventional cytoplasmic actins and several ABPs are found at relatively high concentrations in plant nuclei with possible roles in nuclear cargo movement, transcription, regulation of chromatin structure, and epigenetic control of gene expression. Strictly nuclear components like the nuclear plant actin-related proteins (ARPs) and numerous actin-interacting proteins that function only in the nucleus such as Swi2/Snf2-related DNA dependent ATPases will be considered only tangentially and have been dealt to a significant extent in previous reviews [22, 96, 101, 103, 145]. To maintain focus on the roles of cytoplasmic and nuclear actins and their dynamics in the control of plant cell and organismal development, the functions of the plant ARP2/3 complex will not be addressed here, in spite of a new rich literature on this complex's role in plant growth and morphogenesis [11, 37, 77, 84, 133].

1.2 Evolutionary Origin and Phylogeny of Plant Actins and Actin Binding Proteins

Even in the compact Arabidopsis genome, the actin cytoskeletal genome is complex; it comprises more than 130 known genes divided into nearly a dozen gene families, each encoding between 3 and 21 proteins variants [95]. The estimated sizes of the actin and most ABP families in Arabidopsis are listed in Table 1.1. Since their common ancestry with the earliest land plants like moss ~600 MYA (million years ago), ancient genome-wide duplication events played a significant role in the evolution of this complex system of cytoskeletal genes in higher plants. In contrast to the higher plants, a brief survey of the literature and gene sequence databases suggests that the actins and many ABPs in the moss *Physcomitrella patens* are encoded by smaller or less divergent gene families or by gene singlets [5, 152, 154] (unpublished observations) as compared to Arabidopsis (Table 1.1).

In the history of Arabidopsis, at least three genome-wide duplication events occurred at an estimated 400, 150, and 40 MYA as summarized in Fig. 1.1a [14, 28, 51, 164]. Estimating the dates of these events and correlating them with plant morphological and gene family evolution is useful to understanding gene function. With the essentially complete genome level sequence data now available from Arabidopsis, Populus, Rice, Sorghum, Selaginella, and Physcomitrella and some green alga, we can expect even more precise dating of genome-wide and cytoskeletal gene duplication events in the near future.

Table 1.1 The plant actin cytoskeletal components are encoded primarily by gene families: Estimated family sizes in *Arabidopsis*[a]

Gene	# Members
Actin	8[b]
ADF/cofilin	11
Profilin	5
Formin	21
Annexin	9
ARPs (actin-related proteins)	8
Capping protein α and β subunits	2
CAP1	1
Myosins VIII	4
Myosins XI	13
Filamins	1
Fimbrins	5
Villins/gelsolins	5
Rho/Rop	11
Total	134

[a]Estimates of family size from TAIR and the literature [15, 29, 95, 114]
[b]Likely pseudogenes *ACT5* and *ACT9* are not included [93]

For more than 25 years, our laboratory has addressed the role of gene duplication in the evolution of actin cytoskeletal gene families [54, 95, 97, 102, 138]. Several of our independent estimates for the time of actin gene duplications reasonably agree with newer estimates of genome wide duplications (Fig. 1.1a), although we have not yet confirmed that the cytoskeletal gene duplication events and the genome duplication events were concurrent. One of the earliest gene duplication events (#1 in Fig. 1.1a) generated vegetative and/or nearly constitutive (V) and reproductive (R) classes of genes. We've argued that the fixation of these cytoskeletal paralogs from a genome-wide duplication was contingent upon the co-evolution of the first leaves as new structures derived from sterilized reproductive structures such as sporangia [107]. Novel or more restricted cytoskeletal functions in leaves may have provided the selective pressure for the subfunctionalization of these new classes of actins and ABPs. From this early event, approximately 350–500 MYA, we have proposed that all higher plants have separate vegetative and reproductive classes of actins and actin binding proteins such as Actin Depolymerizing Factors (ADF/cofilin) and profilins (PRF) (Fig. 1.1b–d). Genes in these separate classes are differentially regulated and their encoded protein variants are so divergent that they often do not fully complement mutants in the same complementary class of proteins [18, 64, 67, 104].

The actin, ADF/cofilin and profilin families and many of the ABP gene families listed in Table 1.1 encode ancient reproductive members that are most highly expressed in pollen and/or in pollen and ovules. In the Arabidopsis lineage, the third and most recent genome-wide duplication (24–65 MYA) is thought to have duplicated co-expressed, closely related pairs of actin and ABP genes in each family (Fig. 1.1a, #3) [58, 93, 98, 106, 107, 132]. The impact of this more recent event on

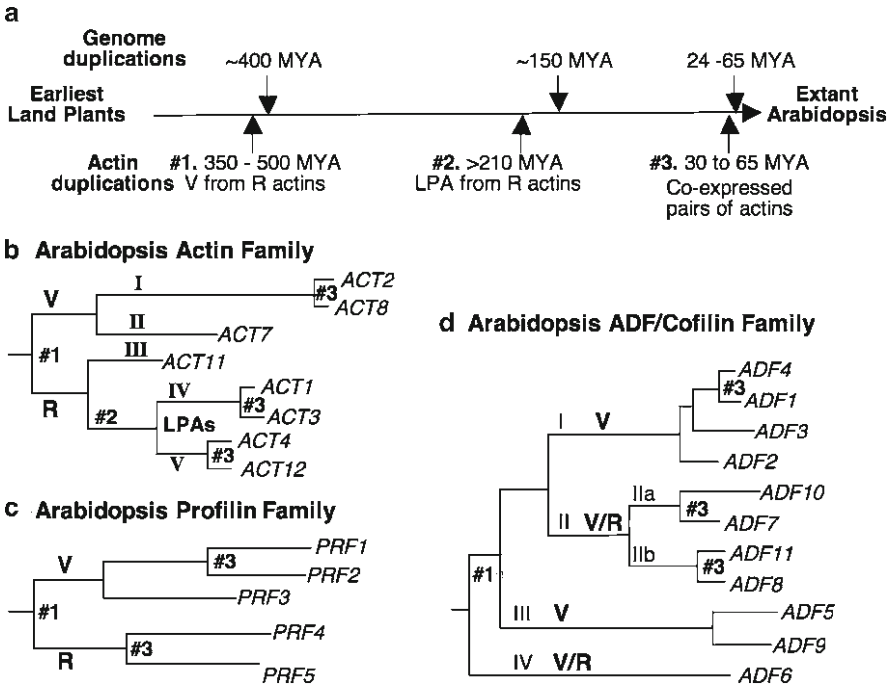


Fig. 1.1 Origin of cytoskeletal gene families by gene duplication. (**a**) Genome duplications vs. *ACTIN* gene duplications predicted in the history of Arabidopsis. Events #1 and #3 appear in the history of nearly all cytoskeletal gene families. Event #2, the origin of the late pollen actins (LPA), occurred in a shared ancestor separating dicots (e.g., Arabidopsis), monocots, and gnetales from gymnosperms. Dates of common ancestry in (**a**) were interpolated from published values [51, 100, 110, 161, 164]. (**b–d**) Three *Arabidopsis* cytoskeletal gene families, actin (**b**), profilin (**c**), and ADF/cofilin (**d**), which encode 8, 5, and 11 protein isovariants, respectively, are each shown in a neighbor-joining tree. Each family contains ancient subclasses that are differentially expressed in patterns defined as vegetative (all but pollen or ovules) and/or constitutive (all but pollen) (V) and reproductive (R). The duplication events numbered in (**a**) (#1, 2, 3) are used to label likely corresponding events in (**c**) and (**d**)

expression patterns and protein variant differences is less obvious and undoubtedly less significant.

Evidence for the second duplication comes from our analysis of the reproductive actin proteins; using an epitope-specific antibody that identifies the late pollen actins (LPAs), we demonstrated that an additional duplication event separated the LPAs from what is likely the basal reproductive actin more than 210 MYA (Fig. 1.1a) [66]. This event may also have separated the constitutively expressed vegetative actins (ACT2 and ACT8, Fig. 1.1b) from a more basal and highly regulated vegetative actin (ACT7, Fig. 1.1b). We are uncertain if the intermediate event(s) duplicating these actins was concurrent with the intermediate genome wide duplication described in Fig. 1.1a (*top*) or an endoduplication of an actin gene. Many of the other families of ABPs (Table 1.1) have interesting phylogenetic structures that need further quantification and dissection.

1.3 The Actin Cytoskeleton in Plant Cell Polarity and Elongation

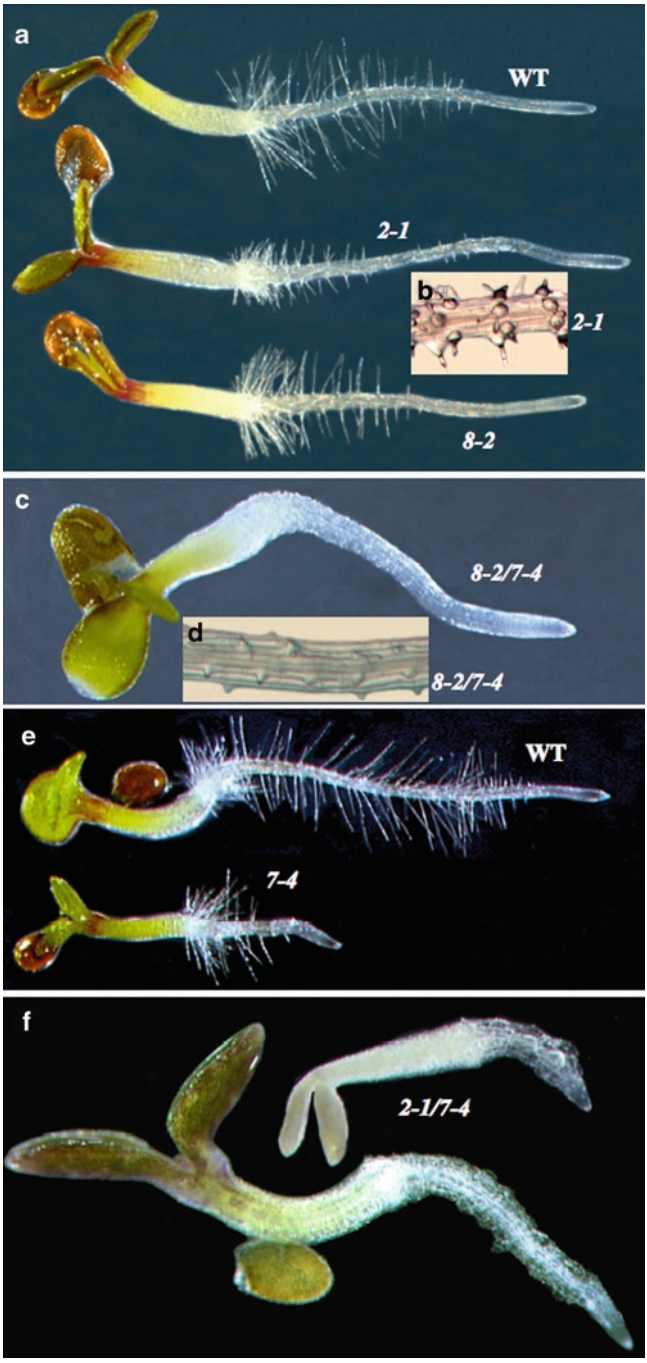
A combination of studies using fluorescent microscopy, histochemistry, and genetics demonstrate that actin and numerous ABPs are necessary for normal plant cell polarity and elongation [13, 19, 39, 52, 122, 154]. Actin filament nucleation, elongation, severing, turnover, and the dynamics among diverse cytoskeletal protein variants are thought to be at the heart of the process in plants, but only recently have scientists in the field begun to determine the importance of these components.

1.3.1 Genetic Studies Demonstrate the Role of Actin and ABP Variants in Cell Polarity and Elongation

Recent studies in Arabidopsis have been essential in determining the role of particular actin and ABP variants in plant cell polarity and elongation. Considering that the small Arabidopsis genome encodes eight actins and more than 120 ABPs in several families (Table 1.1), the protein variant dynamics of different protein-protein interactions is likely to be quite complex [95, 99, 104, 106]. Genetic dissection has been an essential and fundamental tool in separating the different functions of gene family members, especially when combined with biochemical, immunochemical, and cell biological approaches.

Subclass I vegetative actins (ACT2 and ACT8) constitute about 60 and 40% of the total actin expressed in wild type shoot and root tissues, respectively. Yet single mutants in ACT2 or ACT8 (Fig. 1.1b) have wild type levels of total actin in shoots and roots because of the up-regulation of ACT7 [69]. However, these single mutants are still defective in root hair cell elongation and tip growth with the two mutants producing root hairs about ½ to ¾ the length of wild type, respectively, as shown in Fig. 1.2a, b. The *act2-1/act8-2* double mutant has approximately 90 and 80% of total actin levels in shoots and roots, respectively, again because of increased levels of ACT7. Trichoblast cells in the double mutant roots do not develop past the trichoblast initiation and bulge formation stage (Fig. 1.2c, d). Although somewhat dwarfed at early seedling stages, the aboveground organ structures in the adult plants are normal [69]. Expression of ACT2 or ACT8 variants from the ACT2 promoter can fully complement the dwarfing, root hair growth, and cell polarity phenotypes in the double mutant. Thus, although subclass I actins ACT2 and ACT8 are the most highly expressed actins and essential for root hair elongation, they may not be indispensable to most of plant development due to the up-regulation of ACT7.

Arabidopsis subclass II actin ACT7 makes up about 40 and 60% of the total actin in shoot and root tissues, respectively. ACT7 mutant roots have about 40% of the level of total actin found in wild type [69], because neither ACT2 or ACT8 is sufficiently upregulated to compensate for the loss of ACT7. Thus, ACT7 mutants have highly dwarfed roots (Fig. 1.2e), but also produce relatively normal, albeit slightly dwarfed, aboveground adult organs. The polarity of ACT7-deficient root cells is highly skewed resulting in severely disorganized root cell files, abnormal alignment of nuclei, and poor cell elongation [45, 69].


a
WT
2-1
b
2-1
8-2
c
8-2/7-4
d
8-2/7-4
e
WT
7-4
f
2-1/7-4

Double vegetative actin mutant plants lacking both subclass I ACT2 and subclass II ACT7 express only 15–25% of total normal actin protein levels. These double mutant plants are extremely small and have severely dwarfed organs and pleiotropic phenotypes, making apportionment of particular functions difficult [44, 69]. For example, root epidermal cells are bulbous and no longer recognizable as trichoblasts or atrichoblasts in these double mutants (Fig. 1.2f).

Single knockout mutants in 11 of the 17 Arabidopsis myosins had no discernable aboveground plant morphological phenotype under laboratory growth conditions, suggesting there is some functional redundancy among these genes for their aboveground activities. Knockout mutants in myosin *XI-K* or *XI-2* have defective root hair elongation similar to *ACT2* and *ACT8* mutants [121]. Comparisons of single and double myosin mutants suggest that myosin *XI-K*, *XI1*, and *XI-B* have partially redundant and additive functions essential to normal root hair elongation.

Microscopic and genetic studies suggest that other Arabidopsis ABP gene families like those encoding the profilins, ADF/cofilins, and formins also participate in cell elongation and root hair development [10]. However, with a few exceptions, genetic analysis of genes from these three families is too preliminary to draw any definite conclusions about their roles in cell elongation and/or polarity. An Arabidopsis regulatory mutant *prf1-1* expressing about ½ the normal levels of PROFILIN1 (PRF1, PFN1) produces long hypocotyls and hypocotyl cells approximately 1.5 times the length of wild type and extra root hairs [94]. The *prf1-1* mutant phenotypes may speak more to the regulation of profilin than what occurs in a profilin defective plant. Moreover, the two most closely related constitutive profilins, PRF1 and PRF2, have different poly(L) proline and actin binding properties and are differentially localized in plant cells [156]. We need a more extensive genetic analysis of profilin variants to further characterize their molecular and developmental activities. The moss *Physcomitrella patens* has three profilin genes. The simultaneous silencing of all three greatly reduced cell tip growth and resulted in dwarf plants. The expression of any moss or lilly profilin complements the phenotype [152]. Hence, in moss, profilin is essential for tip growth, but particular pofilin variants may not have distinct activities in tip growth.

Subclass II ADFs (Fig. 1.1d) are diverged from the other ADFs and thought to be involved in rapid cell elongation. In monocots like rice, subclass II variants are expressed in both pollen and roots, but in dicots gene duplication and subfunctionalization has further subdivided the subclass II variants into root hair-specific and pollen specific genes [88, 132]. Arabidopsis subclass IIb ADFs ADF8 and ADF11 are expressed exclusively in early trichoblast stage root cells and root hairs and are likely to play a role

Fig. 1.2 Protein variant-specific function of vegetative class actins in the control of root hair and root growth in *Arabidopsis*. (**a**–**d**) ACT2 and ACT8 control root hair growth. (**a**) 84 h-old wild-type (WT) and mutant (*act2-1*, *act8-2*) seedlings. (**b**) A Portion of *act2-1* mutant primary root depicting strong defects in root hair development. (**c**) 72 h-old *act2-1/act8-2* double mutant seeding showing a complete lack of root hair development. (**d**) An enlarged portion of the bald root of *act2-1/act8-2* double mutant. Note the bulges but no elongation of trichoblasts into root hairs. (**e**, **f**) ACT7 is involved in control of root growth. (**e**) 96 h-old wild-type and *act7-4* mutant seedlings. (**f**) 5-day-old *act2-1/act7-4* double mutant seedlings. Note the stunted roots with swollen epidermal cells and no root hairs

in root hair development [132] (Ruzicka and Meagher, unpublished data). By contrast, subclass IIb ADFs ADF7 and ADF10 are expressed exclusively in mature pollen and extending pollen tubes and are likely to be necessary for pollen tube growth [132].

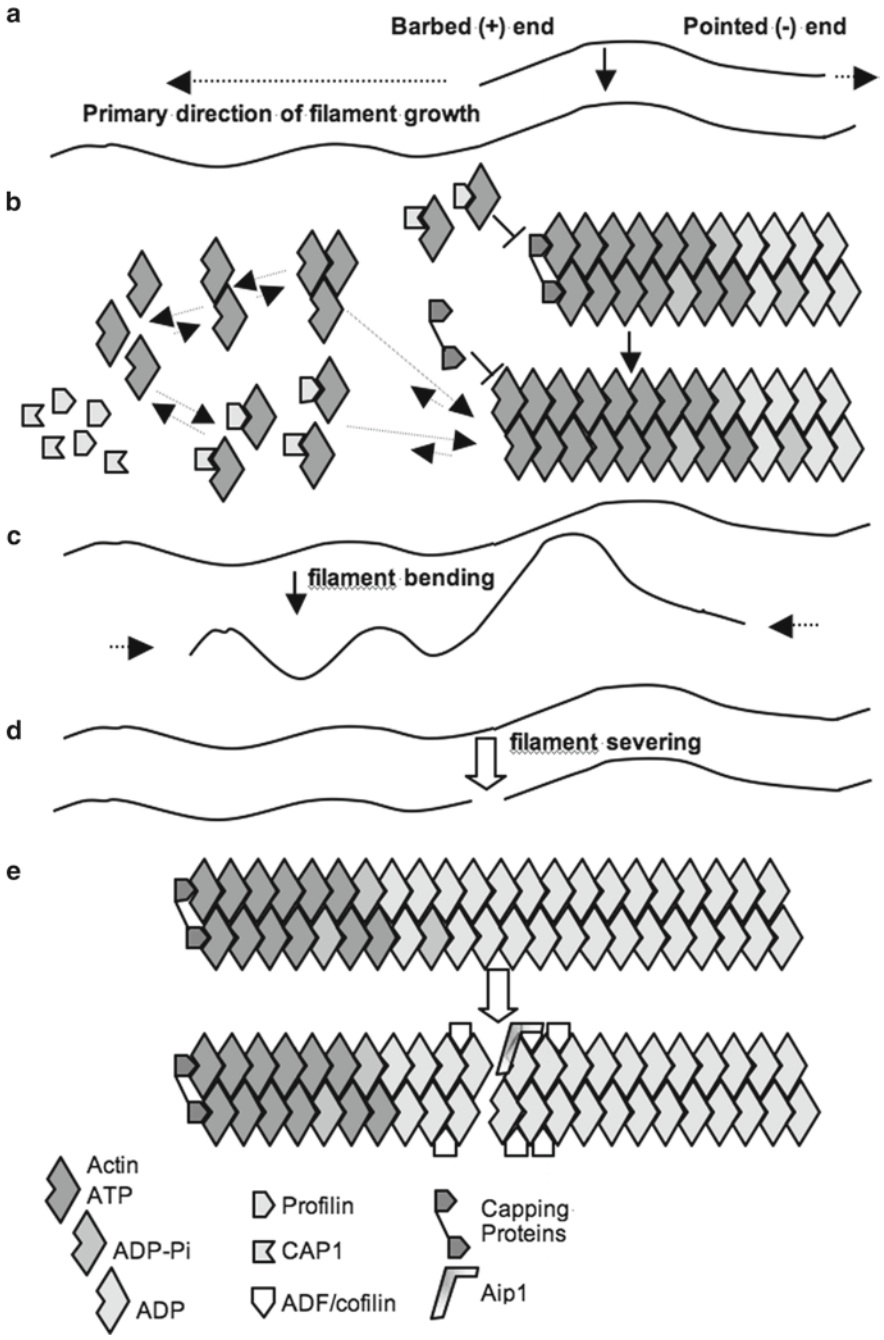
Formin, the membrane bound actin and profilin binding protein, is encoded by a family of 21 genes in Arabidopsis (Table 1.1). Expression of the N-terminal half of Arabidopsis formin FH4 (i.e., the two membrane anchor domains, the poly-L-proline profilin binding domain, and the first of two Formin Homology motifs FH1) fused to GFP produces a dominant negative loss of root hair phenotype [32]. These data strongly suggest that FH4 is a normal part of root hair cytoskeleton, and that the C-terminal domain is necessary for normal FH4 activity.

1.3.2 F-Actin Filament Dynamics and Cell Elongation

Recently, observations of single filament dynamics in living plant cells became possible with the development of novel fluorescent reagents and microscopic tools. Fluorescent live cell imaging shows that rapid movement of actin filaments is an important part of plant cell division, elongation, and differentiation [53]. Earlier studies used GFP-tagged talin to observe the organization of F-actin filaments in elongating live cells or to show the association of F-actin filaments with plastid stromules [75, 76, 82]. In addition, powerful reporters for imaging actin filaments in live plant cells were made from green fluorescent protein (GFP) fusions tagging either the N- or C-terminus or both of the second actin-binding domain from *Arabidopsis* FIMBRIN1 (fABD2) [139, 158]. As compared to the earlier TALIN GFP-mTn reporters, fABD2 reporter plants have fewer detrimental phenotypes [55, 74]. Living cells from transgenic plants expressing the doubly tagged *35S*:*GFP-ABD2-GFP* construct reveal brightly labeled single actin filaments and actin bundles [159]. Chris Staiger's laboratory (Purdue University) recently demonstrated they could follow the dynamics of single actin filaments in live plant cells by using both plants expressing *35S*:*GFP-ABD2-GFP* and time-lapse variable-angle epifluorescence microscopy (VAEM) for rapid sensitive imaging [143]. They focused their studies on the epidermal cells from the hook region of the hypocotyl, which are some of the most rapidly elongating cells in Arabidopsis.

As expected from in vitro and in vivo studies in other organisms, actin filaments in these epidermal cells elongate primarily from one end (Fig. 1.3) [143]. Growing

Fig. 1.3 Actin filament dynamics in elongating plant cells. (**a**) In rapidly elongating hypocotyl cells, actin filaments grow rapidly from the one end [143]. (**b**) Filament growth from the barbed end is modeled using a modification of the illustrations in Staiger and Blanchoin [141]. In Arabidopsis, ADENYLYL CYCLASE-ASSOCIATED PROTEIN1 (CAP1) may provide the primary filament capping activity to prevent filament elongation [21]. (**c**) F-actin filaments rapidly change their effective length by bending or unfolding, a property quantified as convolutedness, and undoubtedly involved in accurately repositioning bound cargo [143]. (**d**) Plant F-actin filaments are shortened primarily by severing. (**e**) The severing activity of actin filaments in (**d**) is modeled at the molecular level, with existing data suggesting that ADF/cofilin variants are the major effectors of severing in plants


a
Barbed (+) end
Pointed (-) end
Primary direction of filament growth
b
c
filament bending
d
filament severing
e
Actin
ATP
ADP-Pi
ADP
Profilin
CAP1
ADF/cofilin
Capping
Proteins
Aip1

filaments generally elongate at rates greater than 1 μm/s. The rate is consistent with levels of polymerizable actin monomer in the range of 50 μM and dose-dependent inhibition by the G-actin binding compound, Latrunculin B. As modeled in Fig. 1.3b, growth of the filaments probably occurs by the addition of ATP bound G-actin monomers to the barbed filament end [141].

We once thought that plants sequestered ATP-actin monomers as profilin-actin complexes that delivered actin subunits to the filament barbed ends. Plant profilin concentrations are relatively high, perhaps high enough to sequester most of the actin monomer pool. However, plant profilins lack the necessary activity to regenerate ATP-actin from ADP-actin, unlike animal and fungal profilins. Arabidopsis ADENYLYL CYCLASE-ASSOCIATED PROTEIN1 (CAP1) accelerates the rate of nucleotide exchange on actin monomers [21] and can therefore enhance F-actin filament growth from monomers in vitro. With a CAP1 to actin ratio of 1–3, CAP1 is an abundant protein in plants, and may play a significant role both complementing and augmenting the role of profilin as an actin monomer sequestering protein. Chaudhry et al. [21] propose a new model for actin filament turnover in plants in which CAP1, "serves as an intermediate between the severing/depolymerizing activity of ADF/cofilin and the assembly promoting function of profilin at filament barbed ends" (Fig. 1.3b).

We might have expected from work in non-plant systems that actin filament turnover in plants would proceed by rapid removal of monomers or small numbers of subunits from the ends of filaments [143]. Instead, Staiger et al. [143] observed in live elongating hypocotyl cells a remarkably high rate of filament severing, as summarized in Fig. 1.3d, e. In 5–10 s, they observed many single filaments cleaved at multiple locations. The lifetime for a typical filament was less than 30 s. Because the fragments can't be tracked unambiguously for more than several successive frames, the authors could not determine how the numerous small fragments generated by severing activity are further depolymerized to replenish the monomeric actin pool for subsequent re-polymerization. Staiger et al. predict a major role for ADF/cofilin in this F-actin severing and turnover from biochemical activities and its abundance in plants [142].

Another activity observed for actin filaments that contributes to their dynamic behavior is a rapid and significant filament bending and unbending as modeled in Fig. 1.3c [143]. Some filaments have a traced contour length twice their end-to-end length and some filaments changed their shape multiple times in a 30 s interval. The parameter of "*convolutedness*" was defined to describe the ratio of a filament's traced contour length to its end-to-end length as measured by the long side of an enclosing rectangle. In rapidly elongating hypocotyl cells, the average F-actin filament changes its *convolutedness* at a rate of about 8% per second. Thicker filaments, presumably actin bundles, did not significantly change their *convolutedness* or changed only slowly compared with single filaments. Imagining how the *convolutedness* of F-actin filaments might accommodate the rapid and accurate positioning and repositioning of organelles and other bound cargo within a growing cell is easy. Using the myosin inhibitor 3-Butanedione Monoxime (BDM), the authors provide preliminary evidence for myosin-dependent filament buckling and straightening.

The genetic tools to test this hypothesis directly are now in place. The Dolja laboratory has characterized a series of double and triple myosin XI mutants in Arabidopsis [121, 125] that may be useful in parallel microscopic studies to dissect the role of myosins in actin filament behavior.

1.3.3 Protein Variant Differences vs. Gene Regulation

Actin is a multifunctional protein encoded by moderately large and divergent gene families in vertebrates and angiosperms, with the best-studied actin families in flies, mice, humans, and Arabidopsis. The various actin protein variants produced in these organisms are most often expressed in a tissue-specific fashion that is conserved across distantly related species, suggesting that the presence of a particular variant in those cell types may be functionally relevant. However, only a handful of studies have attempted so far to explore in detail the relative importance of specific actin variants for various functions in any organism.

Genetic experiments by Fyrberg et al. [40] and Roper et al. [129] have demonstrated functional specialization among the classes of *Drosophila* actin variants. 88F is a gene encoding an adult muscle actin. Flies with a null 88F allele are flightless, a phenotype which can be rescued by a wild type copy of the 88F gene or suppressed by another flight muscle paralog, 79B. However, the flightless phenotype is not suppressed by larval muscle actin paralogs or any of the cytoplasmic actin paralogs, suggesting they have functional differences. Furthermore, the two highly conserved cytoplasmic actins (ACT5C and ACT42A) have only two amino acid differences, but Wagner et al. [155] have shown that only the regulated expression of ACT5C is important for fly development.

The mammalian genome encodes at least six distinct actin variants [50, 123] and the expression of these variants is spatially and temporally regulated throughout development and in the adult organism [149]. The variants are also often differentially distributed within a cell and some are specifically associated with certain subcellular structures such as mitochondria, costameres, neuromuscular junctions, and microvascular pericytes, implying that the cell recognizes different actin variants as functionally distinct entities [130]. As reviewed in Bulinski [17, 73], Karakozova et al. [73] have recently shown that the differential chemical modification (arginylation) of co-expressed, but differentially distributed, beta and gamma actin variants facilitate movement in non-muscle cells. Actin variant specific functions have been proposed for muscle contraction, cell migration, endo- and exocytosis and the maintenance of cell shape, but the specific functions for each of the actin variants during mammalian development likely remain unknown [149].

Arabidopsis researchers are also striving to establish whether the different actin genes and their encoded protein variants are specialized to perform a subset of the many essential actin functions in different organs and cell types. As detailed above, Arabidopsis actin is encoded by eight functional genes, grouped into ancient vegetative and reproductive classes with five subclasses based on phylogeny and expression

patterns (Fig. 1.1b). Several lines of evidence suggest that actin protein variants encoded by the two major classes (V & R, Fig. 1.1b) or the different subclasses (I, II, III, IV, V) of actin genes are functionally different.

First, promoter-*GUS* reporter gene expression studies [2, 3, 56, 57, 92, 107] and protein blots using actin class- or subclass-specific monoclonal antibodies [65, 66, 106] revealed the differential expression of actin variants during cell morphogenesis and organismal development. The vegetative actin genes are so named because they are specifically active in vegetative organs such as leaves, stems, roots and floral organs like sepals, petals, stigma, style and ovary wall, whereas the reproductive actins are predominantly active only in mature pollen and/or ovules, especially the embryo sac [106, 107].

During microsporogenesis, expression shifts from the vegetative actins to the reproductive actins. In microspore mother cells and microspores, actin expression is primarily vegetative, but once the microspore differentiates into mature pollen, actin expression changes so that all five reproductive actins are abundantly expressed [66]. Thus, upon germination the fast tip-growing pollen tubes are equipped with specific expression of reproductive-class actins.

Regulation of ACT7, one of the vegetative actins, is also specifically influenced by environmental and physiological cues, and this distinguishes ACT7 from the other vegetative actins ACT2 and ACT8. For instance, *ACT7* gene expression and ACT7 protein levels are strongly up-regulated in response to various plant hormones and pathogen attack, and its expression is essential for regeneration of callus on hormone-containing medium [65, 92]. Further, as mentioned earlier, ACT7 protein levels are up-regulated in *ACT2*-, *ACT8*, and *ACT2/ACT8*-defective plants.

Second, ectopic expression studies support the functional difference between the two classes of plant actin variants. To illustrate, when a reproductive actin, ACT1, is ectopically overexpressed in vegetative tissue, it severely disrupts the organization of the actin cytoskeleton which affects the development of the plant as a whole, resulting in abnormally dwarf plants with aberrant organs and cell types. However, when a vegetative actin, ACT2, is overexpressed to similarly high levels in vegetative tissue, it has only a mild effect on both actin organization and plant morphology [67]. Clearly, the vegetative and reproductive actin variants are functionally distinct. Given the functional distinction of the actin variants, we hypothesized that an associated paucity of the right class of interacting actin binding proteins (ABPs) for the reproductive class actin in the vegetative tissue might have affected actin dynamics and thereby the development of tissues and organs in the ACT1 misexpression plants.

Third, genetic characterization, complementation, and suppression studies of actin mutants further support the functional difference between different subclasses of actin variants. For instance, and as described above, single actin subclass I knockouts are affected mainly in root hair growth (Fig. 1.2a, b) [44, 69, 128]. Double mutant plants are completely devoid of any root hairs (Fig. 1.2c, d). However, ACT2 and ACT8 variants, but not ACT7, fully rescued the root hair growth defects of the double mutants [69]. The complete rescue of root hair growth by high levels of any of the two subclass 1 actins suggest that they are redundant, but again, the partial

rescue of root hair growth by the subclass 2 ACT7 shows that the ACT7 variant is functionally different from the other two vegetative actins.

The mutant phenotype, complementation, and suppression data for subclass 2 actin ACT7 are distinct from subclass I. ACT7 knockout mutants are drastically affected in root growth, epidermal cell specification, cell division, and root architecture (Fig. 1.2e) [45, 69], yet overexpression of almost any actin class or subclass variant (e.g., ACT1, ACT2, ACT8, or ACT7) from the ACT7 promoter can fully complement the root elongation and cell polarity phenotype of ACT7 knockout. These data suggest that the ACT7 variant has lost functions needed to fully complement ACT2 or ACT8, but other plant actin variants have all the functions contained in the ACT7 variant. To test this hypothesis [69], overexpressed the ACT8 variant from multiple actin regulatory sequences in an *act2-1 act7-4* double mutant background. The resulting plants had normal morphology. We conclude from these studies that differences in both regulation and sequence of actin paralogs are essential for normal plant development.

As is the case for actin (Fig. 1.1, Table 1.1), most of the ABPs in plants are also encoded by gene families and at least two of them, profilins and ADF/cofilins, exhibit phylogenetic grouping into vegetative (or nearly constitutive) and pollen-specific classes [68, 90, 132]. Like the actins, the protein variants reveal both class-specific functional and biochemical differences. For instance, the constitutively expressed profilins of maize have higher affinity for poly-L-proline, sequester more monomeric actin, and disrupt the actin cytoplasmic architecture in live cells more rapidly than pollen-specific profilin [78]. Like the two ADF/cofilin variants, UNC-60A and UNC-60B, encoded by *Caenorhabditis elegans unc-60*, the Arabidopsis reproductive (e.g. PRF4) and vegetative (e.g. PRF2) profilins bind differently to plant and vertebrate actins (C.J. Staiger, personal communication). In *C. elegans*, the most dramatic difference between UNC-60A and UNC-60B is that, at pH 7.0, UNC-60A binds more weakly to filamentous actin than UNC-60B and therefore has different depolymerizing activity [117, 118].

The class-specific activity of ADF/cofilins not only applies to their customary function in depolymerizing actin filaments, but also other novel functions in gene regulation and signaling. For example, in Arabidopsis, which encodes 11 ADFs, ADF4 is a component of the plant-signaling pathway that provides resistance against *Pseudomonas syringae* [148]. ADF9 regulates the expression of essential regulators of flowering time [18].

In addition to different protein variant functions, some of the *ADF* genes also exhibit distinct patterns of gene expression, being only active in specific cell types such as trichoblast and root hair cells (ADF8 and ADF11) and mature pollen and pollen tubes (ADF7 and ADF10) [132]. Thus, families of actins and ABPs in multicellular eukaryotes have functional diversity manifested through differences in gene regulation, amino acid sequence, and protein-protein interaction, all of which control normal multicellular development. Our understanding of the development of different tissues and organs and possibly the human diseases caused by mutations in different actin and ABP genes would be enhanced by further genetic proof of the functional necessity for the various actin and ABP variants (Table 1.1).

1.3.4 Protein–Protein Interactions Among Actin and ABP Families

The major classes of profilins and ADF/cofilins, in particular, show the tissue-specific expression and corresponding phylogenetic grouping into vegetative or constitutive and reproductive classes (Fig. 1.1). In recent ectopic coexpression studies, we have examined if there is a class-specific, preferential interaction between actin and ABP variants expressed in the two major plant tissues. For instance, in Arabidopsis, ectopic overexpression of a reproductive ACT1, but not overexpression of a vegetative ACT2, in vegetative tissues causes severe dwarfing of plants and abnormal actin cytoskeletal structures. We hypothesized that the misexpression of a pollen-specific ACT1 in vegetative cells adversely alters plant development by changing actin dynamics because of inappropriate or poor interaction with endogenous vegetative ABPs [67]. We tested this hypothesis by ectopically coexpressing reproductive profilin (PRF4) or ADF variants (e.g., ADF7) with ACT1 [64].

We found that coexpression of reproductive, but not vegetative, ABP variants considerably suppressed the ectopic ACT1 expression phenotypes, thus restoring wild-type stature and organ structure and normal actin cytoskeletal architecture in the double transgenic plants. Cells from vegetative tissue in these rescued plants contained high levels of both reproductive actin and reproductive ABPs. We conclude that in these cells the reproductive profilin or ADF properly interacts with ACT1, which compensates for the excess of reproductive ACT1 monomers and prevents formation of aberrant actin structures. These co-expression plants contain excessive amounts of actin, but it is organized into normal arrangements of filaments.

In plants misexpressing ACT1 alone or coexpressing ACT1 and a vegetative profilin or ADF variant, both actin filament organization and plant development were extremely abnormal. We hypothesize that aberrant actin filament structures and plant morphogenesis occurred because of the poor or inappropriate interaction of the endogenous or overexpressed vegetative ABPs with the misexpressed, excessive reproductive actin monomers. We conclude that actins and ABPs have evolved class-specific, protein–protein interactions that are essential to the normal actin cytoskeletal dynamics and plant development. Biochemical evidence for the differential binding of different classes of ABPs with the two major classes of plant actins would support the above model.

The macroevolution of organs and tissues in higher plants and animals may have been contingent upon the expansion of numerous cytoskeletal gene families encoding interacting proteins [98, 104, 107]. Once gene family members evolve compartmentalized expression, protein variants are free to evolve new interactions with partners that may be incompatible with protein networks in other compartments. Ancient classes of actin and actin-binding proteins, which elaborate intercellular structures influencing organismal development, are clear examples of such coevolving networks. The above described ectopic expression and suppression data provide evidence for the co-evolution of organ-specific protein-protein interactions. Understanding the contingent relationships between the evolution of organ-specific protein variant networks and organ origination may prove key to explaining multicellular development.

1.3.5 *Organelle Streaming and Vesicle Movement Within a Relatively Stationary Cytoplasm*

The beauty of chloroplasts spinning around in plant and algal cells has lured many plant cell biologists into the field. Although debated for more than 50 years, the most widely accepted model for this activity says that organelles, including chloroplasts, mitochondria, peroxisomes, and some golgi stacks, are carried around the cell in a streaming cytoplasm [62, 63, 140]. An alternative model states that forces within the cytoplasm act directly on organelles and power their movement. This flow of organelles itself generates the force necessary to carry along some adjacent cytoplasm, giving the illusion of cytoplasmic streaming.

Supporting the latter model is the observation that actin filaments and bundles are tightly bound to many organelles, suggesting organelles are individually tethered to and moved along the cellular actin/myosin system. For example, a basket of F-actin surrounds chloroplasts, with single actin filaments and bundles often extending from these baskets into the surrounding cytoplasm [71, 72]. Chloroplasts are attached to the cytoskeleton by a nuclear-encoded chloroplast outer membrane protein CHUP1 that interacts with actin and profilin [136]. Until recently, however, tests for the above described models for organelle movement around plant cells were too complex to interpret, and a deep understanding of cytoskeletal-organelle dynamics remained elusive. Using digital microscopy to analyze the independent movement of large numbers of fluorescently tagged organelles in single cells is beginning to help to clarify these processes [113].

Valerian Dolja and his colleagues at Oregon State recently demonstrated that many individual organelles move independently, different organelles move at different rates, and occasionally some organelles move in opposite directions from the bulk of organelles [6, 7, 121, 125, 135]. They concluded that organelles move independently in a relatively stationary cytoplasm. The illusion of a fully streaming cytoplasm is created by the predominant movement of organelles in one direction around the cell periphery. Therefore, neither of the older models appears to be strictly correct.

Because organelles and their surrounding cytoplasm are rich in actin filaments and myosin and because many studies support the inhibition or enhancement of organelle movement by actin inhibitors like cytochalasin, phalloidin, and latrunculin, the theory that actin and myosin motors power organelle movement has seldom been in doubt [20, 38, 52, 153, 160]. The most definitive data demonstrating the role of actin/myosin motors in organelle movement come from molecular genetic analysis in the Arabidopsis system in Dolja's laboratory and in Andreas Nebenfuhr's laboratory at the University of Tennesee.

Arabidopsis encodes 17 myosins, falling into two ancient classes, named VIII and XI to distinguish them from myosins in other kingdoms [127]. Knockout mutants in myosin *XI-K* or *XI-2*, but not other myosins, showed reduced transport of Golgi stacks, peroxisomes, and mitochondria [121]. Figure 1.4a illustrates the movement of a Golgi stack in mutant cytoplasm compared to a Golgi stack in wild type cytoplasm. Conclusions drawn from these studies were made by summing

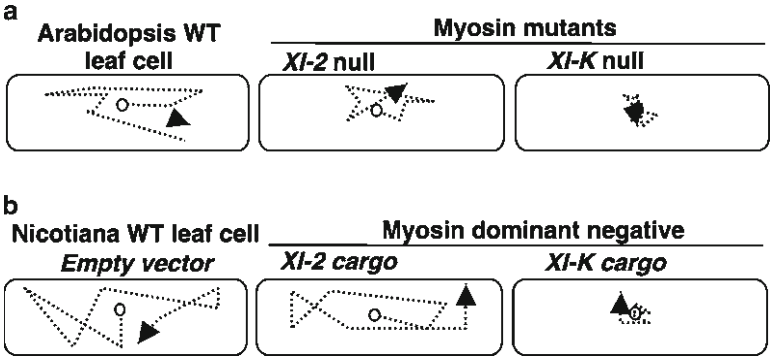


Fig. 1.4 Myosin defects disrupt organelle trafficking. (**a**) This illustration shows the restricted path of Golgi vesicle transport observed in two myosin null mutants deficient in XI-2 and XI-K as compared to wild-type *Arabidopsis* Columbia leaves. Golgi location in a leaf vein cell was monitored at 2 s intervals and plotted relative to a common origin [121]. The origin is shown with a *circle* and the end point with an *arrowhead*. Thousands of such individual golgi, mitochondria, and peroxisomes were monitored in different studies to demonstrate the role of different myosins. (**b**) Restricted path of Golgi vesicle transport in *Nicotiana benthamiana* leaf cells in the presence of transgene expressing the *N. benthamiana* myosin *XI-K-cargo* domain as compared to plants expressing the *XI-2-cargo* domain or and empty vector [7]. *N. benthamiana* is a close relative of tobacco

similar quantitative observations on tens of thousands of individual Golgi. Comparisons of single and double mutants suggest that myosin *XI-K*, *XI-1*, *XI-2*, and *XI-B* have partially redundant and additive functions essential to organelle movement and normal root hair elongation. Double knockout mutants showed less than 10% of the mean organelle velocity of wild type [7, 125]. These data suggest that individual myosin variants are necessary for organelle transport. Molecular genetic studies are beginning to define the specificity of individual myosin globular tail domains in transport [86, 87].

To test if these data are specific to the Arabidopsis system, further experiments were done in a distant angiosperm, *Nicotiana benthamiana.* As illustrated for one Golgi stack in Fig. 1.4b, the authors show that, indeed, over expression of the cargo-carrying tail domain of XI-K myosin in *N. benthamiana* produces a dominant-negative phenotype by reducing the transport of populations of Golgi, peroxisomes, and mitochondria [7].

In a process requiring class VIII myosins, plant virally encoded Heat shock protein 70 (Hsp70) proteins are localized to plasmodesmatal complexes through the ER and endocytic vesicles. Using overexpression of the tail (cargo) domain of class VIII myosins, but not the motor domain, to generate dominant negative phenotypes [6], show that a few Arabidopsis VIII myosins (e.g., VIII-1, VIII-2, VIII-B) participate in this process, but based on mutant analysis, XI-class myosins are not involved.

Specialized actin filaments are attached to and bundled around chloroplasts [61, 71] and probably determine the chloroplast's movement in the cytoplasm and orientation to the light. Drug treatments confirm a role for actin/myosin motors, but not tubulin, in chloroplast movement with response to light intensity [163]. Blue light plays the most significant role in chloroplast relocation through the photoreceptors Phototropin1 and 2 [80, 81]. However, none of the above mentioned

VIII or XI myosins were shown essential for chloroplast movement [7, 121]. Myosin gene and protein function may be extremely redundant, which would require higher-order mutant plants to obtain a chloroplast movement-defective phenotype. Or a novel motor may have made the identification of chloroplast-specific motors elusive. Recently, researchers discovered that silencing *myosin XI* in *N. benthamiana* caused abnormal chloroplast positioning, implicating this class of myosin motors in plastid movement [134].

1.4 Nuclear Actin

1.4.1 History of Nuclear Actin Research

More than 30 years ago, actin was reported in the nuclei of frog (*Xenopus leavis*) and slime mould (*Physarum polycephalum*) [24–26, 60]. Giant Xenopus oocyte nuclei have a volume 100,000 times that of somatic cell nuclei, and actin appears essential to their structural integrity [16, 41]. Actin filaments are observed in Xenopus nuclei and these filaments contact nucleoli, spherical bodies and the nuclear pore complexes [112]. In normal sized plant and animal cell nuclei, nuclear actin is distributed in a diffuse manner throughout the nucleus and may form short filaments, but is also concentrated in nuclear speckles [27, 43, 137]. In these smaller plant and animal nuclei, neither have long F-actin filaments been seen, nor has a role for actin in nuclear structural integrity been confirmed.

1.4.2 Plant Actin and ABPs in the Nucleus

A focus of our research program is in expanding our general knowledge about the genetic and epigenetic activities of nuclear actin in plants. We hypothesize that ancient and divergent actin variants have different nuclear functions. In sharp contrast to the actin filaments and bundles seen in the cytoplasm (Fig. 1.5a), we have recently shown that Subclass I ACT2 and ACT8 (Fig. 1.5b, c) and Subclass II ACT7 are distributed relatively evenly throughout the nucleoplasm; however, the ACT7 variant is more highly concentrated in nuclear speckles and is also detected in the nucleolus (Fig. 1.5d, e) [70]. Furthermore, we demonstrated that a number of novel monoclonal antibodies, not just the well-characterized 2G2 monoclonal, were effective at detecting all nuclear actins. In addition, we used subclass specific monoclonal antibodies to show that Subclass I or II actin is absent from the nuclei of mutants specifically deficient for each subclass of actin. We are interested in using actin-subclass specific mutants to explore the role of different actin variants in the genetic and epigenetic control of gene expression and perhaps in maintaining the structural integrity of plant nuclei.

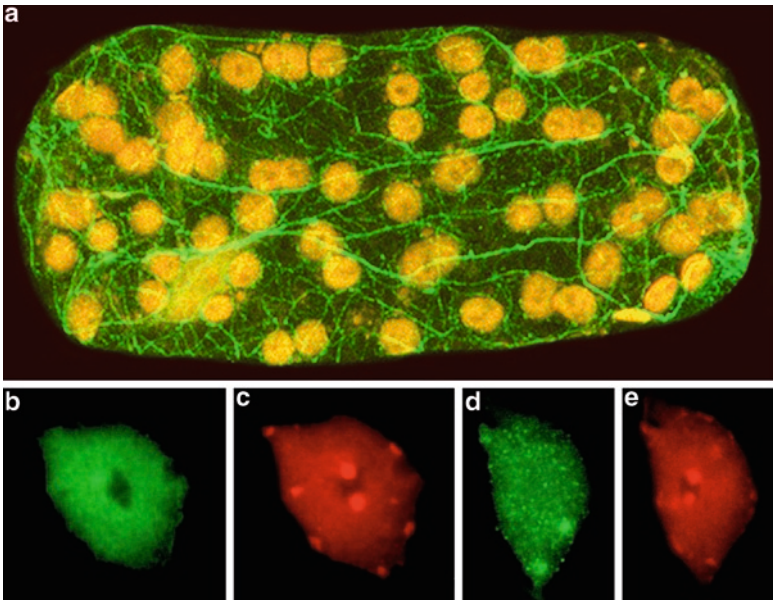


Fig. 1.5 Nuclear and cytoplasmic localization of actin. (**a**) Immunofluorescence staining of actin with a general actin monoclonal antibody MAbGPa in a cryofixed and freeze substituted leaf cell. Actin filaments in the cytoplasm were visualized with FITC-conjugated secondary antibody and the chloroplasts were imaged using autofluorescence, and a merged confocal image is shown in (**a**). (**b–e**) Immunofluorescence staining of actin in paraformaldehyde-fixed and isolated leaf nuclei. Imaged with a conventional fluorescence microscope. DAPI images of DNA in the nuclei (**c**, **e**). (**b**) Nuclus labeled with MAb13a, which recognizes ACT2 and ACT8 in vegetative cells. (**d**) Labeling of ACT7 with MAb2345a. Note the ACT7 staining is diffuse as well as concentrated in speckles and is localized to the nucleolus

Myosin, ADF/cofilin, profilin, RhoGap, spectrin, filamin, tropomyosin, actinin, and paxillin are well-characterized monomeric and polymeric actin binding proteins once thought to be purely cytoplasmic but which have now been found in the nucleus, as well [1, 35, 43, 115, 150]. We have shown that in some Arabidopsis cells, ADF and profilin variants are found in higher concentration per unit area in the nucleus than in the cytoplasm [68, 132]. This is not generally the case in animal cells, where, in unstressed cells, lower nuclear to cytoplasmic ratios of these proteins are found. Our data may reflect the greater number of gene family members and protein variants in plants, which allows some encoded protein variants to evolve more specialized nuclear functions. As with subclass I and II actin variants, plant profilin variants are found throughout the nucleoplasm and in the nucleoli from paraformaldehyde fixed tissues. In contrast, ADF proteins were more concentrated in the nucleoplasm [70]. Yet, neither the ADF/cofilin variants, nor profilin variants assayed appear concentrated in nuclear speckles, as is the case with ACT7. Perhaps other variants not yet assayed will have the speckled phenotype.

1.4.3 Molecular Genetic Functions of Nuclear Actin

Based on actin's association with several protein complexes, a number of functions for nuclear actin, other than structural maintenance, have been identified or can be implied. Actin participates in transcriptional initiation through binding with RNA polymerase (Pol) I, II, and III and interactions with pre-messenger RNA complexes that prepare chromatin for transcription [43, 47, 89, 108, 120, 162]. Actin is found in the nucleoli and, along with myosin, translocates ribosomal subunits from their site of assembly in the nucleolus through nuclear pores to the cytoplasm [23]. Actin is also localized to plant and animal Cajal bodies, where it may position target genes transcribing small nuclear RNAs and export ribonuclear particles (RNPs) and *small RNA*s [27, 36, 42].

Nuclear actin may be functionally and structurally distinct from cytoplasmic actin because a nuclear actin conformation-specific monoclonal antibody 2G2 reacts with paraformaldehyde fixed nuclear, but not fixed cytoplasmic actin [137]. Nuclear actin appears to be dynamic in its nuclear concentrations and activity(s). A few studies have reported that a member of the importin beta-superfamily, EXPORTIN6, is essential to animal actin nuclear export and to the maintenance of necessary levels of nuclear actin [16, 144]. EXPORTIN6 most likely acts in actin export via the two well-conserved nuclear export signals (NESs) located in the junction between structural domains IIa and IIb of actin in all kingdoms.

1.4.4 Epigenetic Functions for Actin in the Assembly of Chromatin Remodeling and Modifying Complexes

Common activities for nuclear actin have become evident in assembling chromatin remodeling machines, chromatin modifying machines, and perhaps other nuclear complexes. With the exception of a few highly specialized protists that possess little epigenetic machinery, these activities are likely to be conserved among all eukaryotes [46, 105]. Biochemical experiments have shown actin to be a binding partner in most of the best-characterized chromatin remodeling and modifying complexes in mammals and yeast.

Actin is a constituent of ATP-dependent chromatin remodeling complexes, such as SWI/SNF, SWR1, RSC, INO80, and p400 and the modifying histone acetylation complexes like NuA4 HAT [22, 79, 109, 116]. These machines are composed of between 10 and 13 subunits and so are quite complex. The genomes of higher plants and animals encode multiple paralogs of the subunit variants in these complexes. Plants, like mammals and unlike yeast, encode tens to hundreds of isoforms of these complexes, possibly allowing for more combinations of isoforms to accommodate more refined epigenetic control over development [101].

Most chromatin remodeling complexes that contain a very large Swi2-related DNA dependent ATPase subunit also include an actin subunit and one or more

ARPs, such as ARP4, ARP5, ARP6 or ARP8 [145]. A few exceptions exist, like the yeast SWI/SNF and RSC complexes that contain ARP7 and ARP9 yet do not appear to contain any conventional actin subunits [146].

Various molecular genetic studies have demonstrated that actin, together with an ARP, bind an N-terminal domain of the large Swi2-related subunit, forming, at minimum, a trimer that initiates assembly of the much larger active remodeling complex [96, 105]. However, the Swi2-subunit binding site for actin and ARP is not well conserved and was not identified until recently. All Swi2-related proteins share a rapidly evolving HSA (helicase SANT-associated) domain and an adjacent post-HSA domain. The HAS and post-HAS domains together are approximately 100 amino acids in length and are generally within a few hundred amino acids of the N-terminus of these several hundred to two thousand amino acid long proteins [105, 145]. Szerlong et al. [145] demonstrated that actin and ARP bind together to this HSA domain. Figure 1.6 models the assembly of one isoform of the mammalian Swi/Snf BRG chromatin remodeling complex that contains approximately 13 subunits. An actin and ARP subunit join together with the Swi2-related subunit

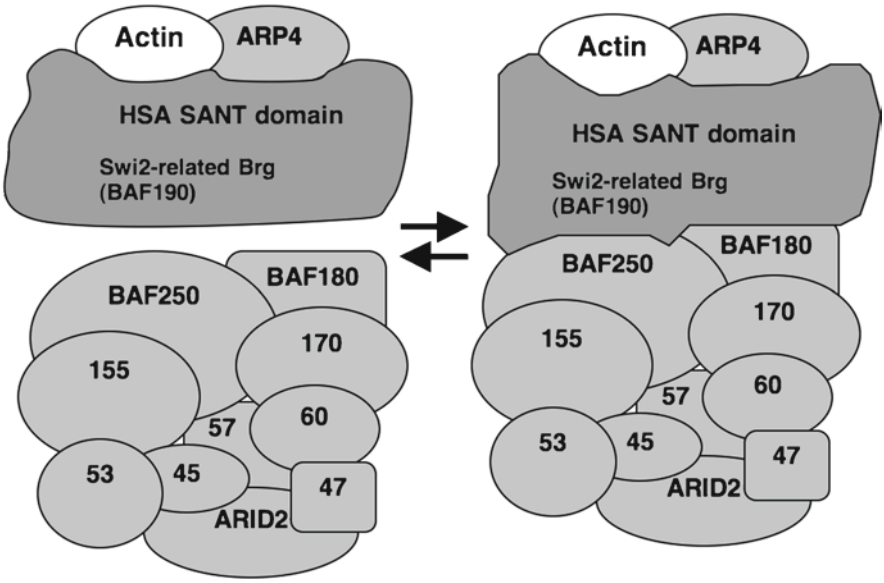


Fig. 1.6 Nuclear ARP4 (Baf53) and actin bind the HSA domain of the Swi2-related Brg DNA dependent ATPase in the mammalian Swi/Snf BRG chromatin remodeling complex. This model illustrates that β-actin and ARP4 bind Brg, and then the β-actin/ARP4/Brg sub-complex binds to a second sub-complex containing several other Brg proteins to form an active chromatin-remodeling machine [83, 145]. A related model may be proposed for a large number of chromatin-active complexes. Two nuclear ARPs or a nuclear ARP and actin bind as dimmers or heterodimers to the helicase-SANT (HSA) domain of the large Swi2-related DNA dependent ATPase subunit in chromatin remodeling complexes or the Vid21-related helicase subunit in chromatin modifying complexes. Again, the ARP-containing sub-complex then binds a second sub-complex with a larger number of subunits

and the actin/ARP/Swi2 trimeric complex thus formed is essential to the initiation of fully functional BRG class complexes. Nucleosome movement is powered by ATP hydrolysis mediated by the BRG Swi2-related ATPase [30]. Arabidopsis and human genomes encode approximately 30–40 Swi2-related proteins that are presumably assembled into multiple isoforms of chromatin-active complexes.

Actin is also a subunit of histone acetylation complexes (HAT), like yeast NuA4 and mammalian TIP60 complexes that lack a Swi2-homolog. Szerlong et al. [145] show that the Eaf1 subunit of NuA4 and other Vid21-related subunits of fungal HAT complexes also contain an HSA domain. Moreover, they show that the HSA domain of Eaf1 binds actin and ARP4. Hence, an alternate HSA domain protein interacts with two actin-related sequences in HAT complexes. Sequence conservation among HSA domains is weak even among fungal proteins, so structural homologs in other kingdoms may be difficult to identify based on sequence alone. For example, Eaf1 has some weak and previously undetected homology in its HSA and SANT domains to a few Swi2-related proteins, such as human p400/Domino [4]. Actin and an ARP may interact in many other HAT complexes via poorly conserved HSA domain proteins that are as yet unidentified.

Tang et al. [147] identified RNA helicase A (RHA) as an actin-interacting protein, while trying to determine the role of actin in the transcription pre-initiation complex (PIC). β-actin, usually thought to be strictly cytoplasmic, is pulled down in experiments precipitating RHA or DNA dependent RNA polymerase II (PolII) proteins. Furthermore, using immuno-histochemical methods, the authors showed β-actin and RHA colocalized to the same domains within the nucleus. RNA silencing of RHA demonstrated that actin's interaction with PolII required RHA. Just as the helicase domains of Swi2 and Vid21 proteins are poorly conserved among family members, no obvious homology exists in RHA among the HSA or actin-binding domains. Possibly, actin recognizes a structural similarity shared among many DNA and RNA helicase domains. Perhaps actin assists in the movement of helicases along the DNA duplex by ATP driven breakage of hydrogen bonds between paired bases.

1.5 Actins and ADFs in Signal Transduction

Given that actin is such a multifunctional protein, it is un-surprising that the actin system participates in signal transduction cascades controlling gene expression in both plants and animals. Recent publications using the Arabidopsis model indicate that actin and possibly some ABPs play direct roles in glucose signaling, pathogen response, day-length control of flowering, cold response, gravity detection and response, drought stress signaling of guard cell closure, and control of the cell cycle as summarized in Table 1.2 [48, 49, 85, 91, 111]. Through vesicle transport and the positioning of receptors and signaling proteins, the plant actin cytoskeletal system participates indirectly in information cascades, including roles in pathogen response, cell polarity determination, exocytosis, and endocytosis (Table 1.2) [12, 33, 34, 59, 157]. A few examples of actin's direct role in signaling are discussed below.

Table 1.2 Recent examinations of the possible role of the actin cytoskeleton in signal transduction

Signaling pathway	Implicated target gene or protein	References
Likely direct role		
Glucose	HXK1 and actin	[8, 9]
Pathogen response	ADF4, RP28.2 PRF1, PRF1	[148, 156, 157]
Day length and flowering	ADF9	[18]
Cell proliferation	BBM, ADF9	[119]
Gravity	ARG1, ARL2	[48, 49, 111]
ABA and guard cell response	MAP kinase likely	[85, 91]
Likely indirect role		
Vesicular transport and cell polarity	PINs	[33]
Inhibition of endocytosis	EHD1, EDH2	[12, 34]
Intracellular vesicular transport	ROP1, REN1	[59]

One general signaling model emerging in plants and animals suggests that activities stimulating F-actin polymerization or depolymerization may release cytoplasmic actin-bound nuclear cofactors. For example, human Cofilin 1 functions as a repressive cofactor of glucocorticoid receptor (GR). Through cofilin's ability to promote F-actin cleavage and turnover, cofilin releases cytoplasmic GR bound to an actin-hsp90 complex, promotes the redistribution of GR to the nucleus, and causes the activation of GR target gene expression [131].

Conversely, activities that stimulate F-actin polymerization release cytoplasmic G-actin-bound MAL, a family of transcriptional cofactors that function as co-activators of serum response factor SRF [151]. MAL proteins interact with monomeric or G-actin via RPEL (RPXXXEL) motifs. When actin is stimulated to polymerize, G-actin releases MAL, which then localizes to the nucleus and binds to SRF [124].

The plant glucose signaling pathway is consistant with a model in which actin modulates signal transduction cascades. Arabidopsis HEXOKINASE1 (HXK1) is a structural homolog of actin that controls glucose signaling and phosphorylation. In collaboration with our laboratory, Brandon Moore's research group (Clemson, SC) has shown that HXK1 binds subclass I actins ACT2 and ACT8 (Fig. 1.1b). Nuclear signaling by HXK1 requires an intact F-actin cytoskeleton because signaling is compromised in actin mutants and by chemicals that cause depolymerization [8, 9].

Our lab has published studies implicating the actin cytoskeleton in signaling that changes gene expression. First, we show that mutants in Arabidopsis *ADF9* (Fig. 1.1d) flower early [18]. Early flowering is rescued by the expression of ADF9 transgenes, but is not suppressed by transgenes expressing other ADF protein variants. These data suggest a unique function for ADF9 in flowering time control (Meagher laboratory, unpublished data). As might be expected by some models for ADF function, the high level repressor of flowering *FLC* and its closest relatives *MAF4*, and *MAF5* are all expressed at two to tenfold lower levels in ADF9-deficient

mutants than in wild type, and loss of their activity is sufficient to account for the flowering time phenotype [126]. We presented data suggesting that loss of ADF9 caused a defect in the epigenetic control of these three MADS box factors. For example, the spacing and density of nucleosomes at the 5′ end of *FLC* is altered in ADF9-defective plants [18].

Second, Brad Day (Michigan State) has shown ADF4 (Fig. 1.1d) to be a component of the plant defense response pathway that provides resistance to the pathogenic bacterium *Pseudomonas syringae*. ADF4 responds to an effector protein, AvrPphB [31, 148], and ADF4 mutant plants support more pathogenic bacterial growth, showing almost no hypersensitive response to AvrPphB relative to wild type plants. The ADF4 variant in particular is required for resistance triggered by the effector AvrPphB because even the very closely related ADF3 variant fails to respond to AvrPphB. These data suggest that actin dynamics is essential to plant defense signaling pathways and significant protein variant specificity is necessary in the process.

Whether ADF's participation in signaling fits the general model of cytoskeletal involvement in signaling or if signaling proceeds by some other mechanism(s) remains unclear. Considering the high nuclear concentrations observed for some plant ADF variants [132], they may function directly within the nucleus and not through cytoskeletal activities, but such nuclear activities and mechanisms remain to be identified and elucidated.

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References

1. Abe H, Nagaoka R, Obinata T (1993) Cytoplasmic localization and nuclear transport of cofilin in cultured myotubes. Exp Cell Res 206:1–10
2. An YQ, Huang S, McDowell JM, McKinney EC, Meagher RB (1996) Conserved expression of the *Arabidopsis ACT1* and *ACT3* actin subclass in organ primordia and mature pollen. Plant Cell 8:15–30
3. An YQ, McDowell JM, Huang S, McKinney EC, Chambliss S, Meagher RB (1996) Strong, constitutive expression of the *Arabidopsis ACT2/ACT8* actin subclass in vegetative tissues. Plant J 10:107–121
4. Auger A, Galarneau L, Altaf M, Nourani A, Doyon Y, Utley RT, Cronier D, Allard S, Cote J (2008) Eaf1 is the platform for NuA4 molecular assembly that evolutionarily links chromatin acetylation to ATP-dependent exchange of histone H2A variants. Mol Cell Biol 28:2257–2270
5. Augustine RC, Vidali L, Kleinman KP, Bezanilla M (2008) Actin depolymerizing factor is essential for viability in plants, and its phosphoregulation is important for tip growth. Plant J 54:863–875
6. Avisar D, Prokhnevsky AI, Dolja VV (2008) Class VIII myosins are required for plasmodesmatal localization of a closterovirus Hsp70 homolog. J Virol 82:2836–2843
7. Avisar D, Prokhnevsky AI, Makarova KS, Koonin EV, Dolja VV (2008) Myosin XI-K Is required for rapid trafficking of Golgi stacks, peroxisomes, and mitochondria in leaf cells of Nicotiana benthamiana. Plant Physiol 146:1098–1108

8. Balasubramanian R, Karve A, Kandasamy M, Meagher RB, Moore BD (2007) A role for F-actin in hexokinase-mediated glucose signaling. Plant Physiol 145:1423–1434
9. Balasubramanian R, Karve A, Moore BD (2008) Actin-based cellular framework for glucose signaling by Arabidopsis hexokinase1. Plant Signal Behav 3:322–324
10. Baluska F, Salaj J, Mathur J, Braun M, Jasper F, Samaj J, Chua NH, Barlow PW, Volkmann D (2000) Root hair formation: F-actin-dependent tip growth is initiated by local assembly of profilin-supported F-actin meshworks accumulated within expansin-enriched bulges. Dev Biol 227:618–632
11. Bannigan A, Baskin TI (2005) Directional cell expansion – turning toward actin. Curr Opin Plant Biol 8:619–624
12. Bar M, Sharfman M, Schuster S, Avni A (2009) The coiled-coil domain of EHD2 mediates inhibition of LeEix2 endocytosis and signaling. PLoS One 4:e7973
13. Belanger KD, Quatrano RS (2000) Polarity: the role of localized secretion. Curr Opin Plant Biol 3:67–72
14. Blanc G, Hokamp K, Wolfe KH (2003) A recent polyploidy superimposed on older large-scale duplications in the Arabidopsis genome. Genome Res 13:137–144
15. Blanchoin L, Staiger CJ (2010) Plant formins: diverse isoforms and unique molecular mechanism. Biochim Biophys Acta 1803:201–206
16. Bohnsack MT, Stuven T, Kuhn C, Cordes VC, Gorlich D (2006) A selective block of nuclear actin export stabilizes the giant nuclei of *Xenopus oocytes*. Nat Cell Biol 8:257–263
17. Bulinski JC (2006) Cell biology. Actin discrimination. Science 313:180–181
18. Burgos-Rivera B, Ruzicka DR, Deal RB, McKinney EC, King-Reid L, Meagher RB (2008) *ACTIN DEPOLYMERIZING FACTOR9* controls development and gene expression in *Arabidopsis*. Plant Mol Biol 68:619–632
19. Cardenas L, Lovy-Wheeler A, Kunkel JG, Hepler PK (2008) Pollen tube growth oscillations and intracellular calcium levels are reversibly modulated by actin polymerization. Plant Physiol 146:1611–1621
20. Cardenas L, Lovy-Wheeler A, Wilsen KL, Hepler PK (2005) Actin polymerization promotes the reversal of streaming in the apex of pollen tubes. Cell Motil Cytoskeleton 61:112–127
21. Chaudhry F, Guerin C, von Witsch M, Blanchoin L, Staiger CJ (2007) Identification of *Arabidopsis* cyclase-associated protein 1 as the first nucleotide exchange factor for plant actin. Mol Biol Cell 18:3002–3014
22. Chen M, Shen X (2007) Nuclear actin and actin-related proteins in chromatin dynamics. Curr Opin Cell Biol 19:326–330
23. Cisterna B, Necchi D, Prosperi E, Biggiogera M (2006) Small ribosomal subunits associate with nuclear myosin and actin in transit to the nuclear pores. Faseb J 20:1901–1903
24. Clark TG, Merriam RW (1977) Diffusible and bound actin nuclei of *Xenopus laevis* oocytes. Cell 12:883–891
25. Clark TG, Merriam RW (1978) Actin in *Xenopus oocytes*. J Cell Biol 77:427–438
26. Clark TG, Rosenbaum JL (1979) An actin filament matrix in hand-isolated nuclei of *X. laevis* oocytes. Cell 18:1101–1108
27. Cruz JR, Diaz M, de la Espina S (2009) Subnuclear compartmentalization and function of actin and nuclear Myosin I in plants. Chromosoma 118:193–207
28. Cui L, Wall PK, Leebens-Mack JH, Lindsay BG, Soltis DE, Doyle JJ, Soltis PS, Carlson JE, Arumuganathan K, Barakat A, Albert VA, Ma H, dePamphilis CW (2006) Widespread genome duplications throughout the history of flowering plants. Genome Res 16:738–749
29. Cvrckova F, Novotny M, Pickova D, Zarsky V (2004) Formin homology 2 domains occur in multiple contexts in angiosperms. BMC Genomics 5:44
30. Dang W, Bartholomew B (2007) Domain architecture of the catalytic subunit in the ISW2-nucleosome complex. Mol Cell Biol 27:8306–8317
31. Day B, Graham T (2007) The plant host pathogen interface: cell wall and membrane dynamics of pathogen-induced responses. Ann N Y Acad Sci 1113:123–134
32. Deeks MJ, Cvrckova F, Machesky LM, Mikitova V, Ketelaar T, Zarsky V, Davies B, Hussey PJ (2005) *Arabidopsis* group Ie formins localize to specific cell membrane domains, interact

with actin-binding proteins and cause defects in cell expansion upon aberrant expression. New Phytol 168:529–540
33. Dhonukshe P, Grigoriev I, Fischer R, Tominaga M, Robinson DG, Hasek J, Paciorek T, Petrasek J, Seifertova D, Tejos R, Meisel LA, Zazimalova E, Gadella TW Jr, Stierhof YD, Ueda T, Oiwa K, Akhmanova A, Brock R, Spang A, Friml J (2008) Auxin transport inhibitors impair vesicle motility and actin cytoskeleton dynamics in diverse eukaryotes. Proc Natl Acad Sci U S A 105:4489–4494
34. Dhonukshe P, Tanaka H, Goh T, Ebine K, Mahonen AP, Prasad K, Blilou I, Geldner N, Xu J, Uemura T, Chory J, Ueda T, Nakano A, Scheres B, Friml J (2008) Generation of cell polarity in plants links endocytosis, auxin distribution and cell fate decisions. Nature 456:962–966
35. Dingova H, Fukalova J, Maninova M, Philimonenko VV, Hozak P (2009) Ultrastructural localization of actin and actin-binding proteins in the nucleus. Histochem Cell Biol 131:425–434
36. Dundr M, Ospina JK, Sung MH, John S, Upender M, Ried T, Hager GL, Matera AG (2007) Actin-dependent intranuclear repositioning of an active gene locus in vivo. J Cell Biol 179:1095–1103
37. Dyachok J, Shao MR, Vaughn K, Bowling A, Facette M, Djakovic S, Clark L, Smith L (2008) Plasma membrane-associated SCAR complex subunits promote cortical F-actin accumulation and normal growth characteristics in *Arabidopsis* roots. Mol Plant 1:990–1006
38. Foissner I, Wasteneys GO (2007) Wide-ranging effects of eight cytochalasins and latrunculin A and B on intracellular motility and actin filament reorganization in characean internodal cells. Plant Cell Physiol 48:585–597
39. Frank MJ, Cartwright HN, Smith LG (2003) Three *Brick* genes have distinct functions in a common pathway promoting polarized cell division and cell morphogenesis in the maize leaf epidermis. Development 130:753–762
40. Fyrberg EA, Fyrberg CC, Biggs JR, Saville D, Beall CJ, Ketchum A (1998) Functional non-equivalence of *Drosophila* actin isoforms. Biochem Genet 36:271–287
41. Gall JG (2006) Exporting actin. Nat Cell Biol 8:205–207
42. Gedge LJ, Morrison EE, Blair GE, Walker JH (2005) Nuclear actin is partially associated with Cajal bodies in human cells in culture and relocates to the nuclear periphery after infection of cells by adenovirus 5. Exp Cell Res 303:229–239
43. Gieni RS, Hendzel MJ (2009) Actin dynamics and functions in the interphase nucleus: moving toward an understanding of nuclear polymeric actin. Biochem Cell Biol 87:283–306
44. Gilliland LU, Kandasamy MK, Pawloski LC, Meagher RB (2002) Both vegetative and reproductive actin isovariants complement the stunted root hair phenotype of the *Arabidopsis act2-1* mutation. Plant Physiol 130:2199–2209
45. Gilliland LU, Pawloski LC, Kandasamy MK, Meagher RB (2003) *Arabidopsis* actin gene *ACT7* plays an essential role in germination and root growth. Plant J 33:319–328
46. Gordon JL, Sibley LD (2005) Comparative genome analysis reveals a conserved family of actin-like proteins in apicomplexan parasites. BMC Genomics 6:179
47. Grummt I (2006) Actin and myosin as transcription factors. Curr Opin Genet Dev 16:191–196
48. Harrison B, Masson PH (2008) ARG1 and ARL2 form an actin-based gravity-signaling chaperone complex in root statocytes? Plant Signal Behav 3:650–653
49. Harrison BR, Masson PH (2008) ARL2, ARG1 and PIN3 define a gravity signal transduction pathway in root statocytes. Plant J 53:380–392
50. Hartman AL, Sawtell NM, Lessard JL (1989) Expression of actin isoforms in developing rat intestinal epithelium. J Histochem Cytochem 37:1225–1233
51. Heckman DS, Geiser DM, Eidell BR, Stauffer RL, Kardos NL, Hedges SB (2001) Molecular evidence for the early colonization of land by fungi and plants. Science 293:1129–1133
52. Hepler PK, Vidali L, Cheung AY (2001) Polarized cell growth in higher plants. Annu Rev Cell Dev Biol 17:159–187
53. Higaki T, Sano T, Hasezawa S (2007) Actin microfilament dynamics and actin side-binding proteins in plants. Curr Opin Plant Biol 10:549–556
54. Hightower RC, Meagher RB (1986) The molecular evolution of actin. Genetics 114:315–332
55. Holweg CL (2007) Living markers for actin block myosin-dependent motility of plant organelles and auxin. Cell Motil Cytoskeleton 64:69–81

56. Huang S, An YQ, McDowell JM, McKinney EC, Meagher RB (1996) The *Arabidopsis thaliana ACT4/ACT12* actin gene subclass is strongly expressed throughout pollen development. Plant J 10:189–202
57. Huang S, An YQ, McDowell JM, McKinney EC, Meagher RB (1997) The *Arabidopsis ACT11* actin gene is strongly expressed in tissues of the emerging inflorescence, pollen, and developing ovules. Plant Mol Biol 33:125–139
58. Huang S, McDowell JM, Weise MJ, Meagher RB (1996) The *Arabidopsis* profilin gene family. Evidence for an ancient split between constitutive and pollen-specific profilin genes. Plant Physiol 111:115–126
59. Hwang JU, Vernoud V, Szumlanski A, Nielsen E, Yang Z (2008) A tip-localized RhoGAP controls cell polarity by globally inhibiting Rho GTPase at the cell apex. Curr Biol 18:1907–1916
60. Jockusch BM, Becker M, Hindennach I, Jockusch H (1974) Slime mould actin: homology to vertebrate actin and presence in the nucleus. Exp Cell Res 89:241–246
61. Kadota A, Yamada N, Suetsugu N, Hirose M, Saito C, Shoda K, Ichikawa S, Kagawa T, Nakano A, Wada M (2009) Short actin-based mechanism for light-directed chloroplast movement in Arabidopsis. Proc Natl Acad Sci U S A 106:13106–13111
62. Kamiya N (1960) Physics and chemistry of protoplasmic streaming. Annu Rev Plant Physiol 11:323–340
63. Kamiya N, Kuroda K (1956) Velocity distribution of the protoplasmic streaming in *Nitella* cells. Bot Mag Tokyo 69:544–554
64. Kandasamy MK, Burgos-Rivera B, McKinney EC, Ruzicka DR, Meagher RB (2007) Class-specific interaction of profilin and ADF isovariants with actin in the regulation of plant development. Plant Cell 19:3111–3126
65. Kandasamy MK, Gilliland LU, McKinney EC, Meagher RB (2001) One plant actin isovariant, ACT7, is induced by auxin and required for normal callus formation. Plant Cell 13:1541–1554
66. Kandasamy MK, McKinney EC, Meagher RB (1999) The late pollen-specific actins in angiosperms. Plant J 18:681–691
67. Kandasamy MK, McKinney EC, Meagher RB (2002) Functional nonequivalency of actin isovariants in *Arabidopsis*. Mol Biol Cell 13:251–261
68. Kandasamy MK, McKinney EC, Meagher RB (2002) Plant profilin isovariants are distinctly regulated in vegetative and reproductive tissues. Cell Motil Cytoskeleton 52:22–32
69. Kandasamy MK, McKinney EC, Meagher RB (2009) A Single Vegetative Actin Isovariant Overexpressed Under The Control Of Multiple regulatory sequences is sufficient for normal *Arabidopsis* development. Plant Cell 21:701–718
70. Kandasamy MK, McKinney EC, Meagher RB (2010) Differential sub-localization of actin variants within the nucleus. Cytoskeleton, in press
71. Kandasamy MK, Meagher RB (1999) Actin-organelle interaction: association with chloroplast in arabidopsis leaf mesophyll cells. Cell Motil Cytoskeleton 44:110–118
72. Kandasamy MK, Meagher RB (2000) Cytoskeleton-organelle interaction: Higher plant chloroplasts are contained in actin baskets and attached to actin filaments and bundles. In: Microscopy and microanalysis, 6, G. Bailey, S. McKernan, R. Price, S. Walck, P.-M. Charest, and R. Gauvin, eds (New York: Springer-Verlag), pp. 296–297
73. Karakozova M, Kozak M, Wong CC, Bailey AO, Yates JR III, Mogilner A, Zebroski H, Kashina A (2006) Arginylation of beta-actin regulates actin cytoskeleton and cell motility. Science 313:192–196
74. Ketelaar T, Anthony RG, Hussey PJ (2004) Green fluorescent protein-mTalin causes defects in actin organization and cell expansion in Arabidopsis and inhibits actin depolymerizing factor's actin depolymerizing activity in vitro. Plant Physiol 136:3990–3998
75. Ketelaar T, de Ruijter NC, Emons AM (2003) Unstable F-actin specifies the area and microtubule direction of cell expansion in Arabidopsis root hairs. Plant Cell 15:285–292
76. Kost B, Spielhofer P, Chua NH (1998) A GFP-mouse talin fusion protein labels plant actin filaments in vivo and visualizes the actin cytoskeleton in growing pollen tubes. Plant J 16:393–401

77. Kotchoni SO, Zakharova T, Mallery EL, Le J, El-Assal Sel D, Szymanski DB (2009) The association of the Arabidopsis actin-related protein2/3 complex with cell membranes is linked to its assembly status but not its activation. Plant Physiol 151:2095–2109
78. Kovar DR, Drobak BK, Staiger CJ (2000) Maize profilin isoforms are functionally distinct. Plant Cell 12:583–598
79. Krogan NJ, Keogh MC, Datta N, Sawa C, Ryan OW, Ding H, Haw RA, Pootoolal J, Tong A, Canadien V, Richards DP, Wu X, Emili A, Hughes TR, Buratowski S, Greenblatt JF (2003) A Snf2 family ATPase complex required for recruitment of the histone H2A variant Htz1. Mol Cell 12:1565–1576
80. Krzeszowiec W, Gabrys H (2007) Phototropin mediated relocation of myosins in *Arabidopsis thaliana*. Plant Signal Behav 2:333–336
81. Krzeszowiec W, Gabrys H (2009) The role of actomyosin in blue light-induced chloroplast movements. Postepy Biochem 55:187–195
82. Kwok EY, Hanson MR (2004) In vivo analysis of interactions between GFP-labeled microfilaments and plastid stromules. BMC Plant Biol 4:2
83. Lessard J, Wu JI, Ranish JA, Wan M, Winslow MM, Staahl BT, Wu H, Aebersold R, Graef IA, Crabtree GR (2007) An essential switch in subunit composition of a chromatin remodeling complex during neural development. Neuron 55:201–215
84. Leung DW, Otomo C, Chory J, Rosen MK (2008) Genetically encoded photoswitching of actin assembly through the Cdc42-WASP-Arp2/3 complex pathway. Proc Natl Acad Sci U S A 105:12797–12802
85. Li S, Assmann SM, Albert R (2006) Predicting essential components of signal transduction networks: a dynamic model of guard cell abscisic acid signaling. PLoS Biol 4:e312
86. Li JF, Nebenfuhr A (2008a) Inter-dependence of dimerization and organelle binding in myosin XI. Plant J 55:478–490
87. Li JF, Nebenfuhr A (2008b) The tail that wags the dog: the globular tail domain defines the function of myosin V/XI. Traffic 9:290–298
88. Li XB, Xu D, Wang XL, Huang GQ, Luo J, Li DD, Zhang ZT, Xu WL (2010) Three cotton genes preferentially expressed in flower tissues encode actin-depolymerizing factors which are involved in F-actin dynamics in cells. J Exp Bot 61:41–53
89. Louvet E, Percipalle P (2009) Transcriptional control of gene expression by actin and myosin. Int Rev Cell Mol Biol 272:107–147
90. Maciver SK, Hussey PJ (2002) The ADF/cofilin family: actin-remodeling proteins. Genome Biol 3:reviews 3007
91. MacRobbie EA, Kurup S (2007) Signalling mechanisms in the regulation of vacuolar ion release in guard cells. New Phytol 175:630–640
92. McDowell JM, An YQ, Huang S, McKinney EC, Meagher RB (1996) The *Arabidopsis ACT7* actin gene is expressed in rapidly developing tissues and responds to several external stimuli. Plant Physiol 111:699–711
93. McDowell JM, Huang S, McKinney EC, An YQ, Meagher RB (1996) Structure and evolution of the actin gene family in *Arabidopsis thaliana*. Genetics 142:587–602
94. McKinney EC, Kandasamy MK, Meagher RB (2001) Small changes in the regulation of one *Arabidopsis* profilin isovariant, PRF1, alter seedling development. Plant Cell 13:1179–1191
95. Meagher R, Fechheimer M (2002) The arabidopsis cytoskeletal genome. In: Meyerowitz E, Somerville C (eds) Arabidopsis, www.aspb.org/publications/arabidopsis/toc.cfm. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp 1–26
96. Meagher R, Kandasamy M, Smith A, McKinney E (2010) Nuclear actin-related proteins at the core of epigenetic control. Plant signal Behav 5:1–5
97. Meagher RB (1991) Divergence and differential expression of actin gene families in higher plants. Int Rev Cytol 125:139–163
98. Meagher RB (1995) The impact of historical contingency on gene phylogeny: Plant actin diversity. In: Hecht M, MacIntyre R, Clegg M (eds) Evolutionary biology, vol 28. Plenum Press, New York, pp 195–215
99. Meagher RB (2002) Post-genomics networking of biotechnology for interpreting gene function – editorial overview. Curr Opin Plant Biol 5:135–140

100. Meagher RB, Berry-Lowe S, Rice K (1989) Molecular evolution of the small subunit of ribulose bisphosphate carboxylase: nucleotide substitution and gene conversion. Genetics 123:845–863
101. Meagher RB, Deal RB, Kandasamy MK, McKinney EC (2005) Nuclear actin-related proteins as epigenetic regulators of development. Plant Physiol 139:1576–1585
102. Meagher RB, Hightower RC (1983) Plant actin is encoded by diverse multigene families. In: Downey K, Voellmy RW, Ahmad F, Schultz J (eds) Advances in gene technology: molecular genetics of plant and animals. Miami Winter Symposium, Academic Press, pp 171–189
103. Meagher RB, Kandasamy MK, Deal RB, McKinney EC (2007) Actin-related proteins in chromatin-level control of the cell cycle and developmental transitions. Trends Cell Biol 17:325–332
104. Meagher RB, Kandasamy MK, McKinney EC (2008) Multicellular development and protein-protein interactions. Plant Signal Behav 3:333–336
105. Meagher RB, Kandasamy MK, McKinney EC, Roy E (2009) Chapter 5. Nuclear actin-related proteins in epigenetic control. Int Rev Cell Mol Biol 277:157–215
106. Meagher RB, McKinney EC, Kandasamy MK (1999a) Isovariant dynamics expand and buffer the responses of complex systems: the diverse plant actin gene family. Plant Cell 11:995–1006
107. Meagher RB, McKinney EC, Vitale AV (1999b) The evolution of new structures: clues from plant cytoskeletal genes. Trends Genet 15:278–284
108. Miralles F, Visa N (2006) Actin in transcription and transcription regulation. Curr Opin Cell Biol 18:261–266
109. Mizuguchi G, Shen X, Landry J, Wu WH, Sen S, Wu C (2004) ATP-driven exchange of histone H2AZ variant catalyzed by SWR1 chromatin remodeling complex. Science 303:343–348
110. Moore MJ, Bell CD, Soltis PS, Soltis DE (2007) Using plastid genome-scale data to resolve enigmatic relationships among basal angiosperms. Proc Natl Acad Sci U S A 104:19363–19368
111. Morita MT (2008) Sensing of the direction of gravity in gravitropism. Annu Rev Plant Biol 61:705–720
112. Morozova KN, Kiseleva EV (2008) Changes in *Xenopus* oocyte nucleus and cytoplasm organization after actin filaments depolymerization by latrunculin. Tsitologiia 50:394–405
113. Nelson BK, Cai X, Nebenfuhr A (2007) A multicolored set of in vivo organelle markers for co-localization studies in Arabidopsis and other plants. Plant J 51:1126–1136
114. Nibau C, Wu HM, Cheung AY (2006) RAC/ROP GTPases: "hubs" for signal integration and diversification in plants. Trends Plant Sci 11:309–315
115. Ohta Y, Nishida E, Sakai H, Miyamoto E (1989) Dephosphorylation of cofilin accompanies heat shock-induced nuclear accumulation of cofilin. J Biol Chem 264:16143–16148
116. Olave IA, Reck-Peterson SL, Crabtree GR (2002) Nuclear actin and actin-related proteins in chromatin remodeling. Annu Rev Biochem 71:755–781
117. Ono S, Baillie DL, Benian GM (1999) UNC-60B, an ADF/cofilin family protein, is required for proper assembly of actin into myofibrils in *Caenorhabditis elegans* body wall muscle. J Cell Biol 145:491–502
118. Ono S, Benian GM (1998) Two Caenorhabditis elegans actin depolymerizing factor/cofilin proteins, encoded by the unc-60 gene, differentially regulate actin filament dynamics. J Biol Chem 273:3778–3783
119. Passarinho P, Ketelaar T, Xing M, van Arkel J, Maliepaard C, Hendriks MW, Joosen R, Lammers M, Herdies L, den Boer B, van der Geest L, Boutilier K (2008) BABY BOOM target genes provide diverse entry points into cell proliferation and cell growth pathways. Plant Mol Biol 68:225–237
120. Percipalle P, Visa N (2006) Molecular functions of nuclear actin in transcription. J Cell Biol 172:967–971
121. Peremyslov VV, Prokhnevsky AI, Avisar D, Dolja VV (2008) Two class XI myosins function in organelle trafficking and root hair development in *Arabidopsis*. Plant Physiol 146:1109–1116
122. Perroud PF, Quatrano RS (2006) The role of ARPC4 in tip growth and alignment of the polar axis in filaments of *Physcomitrella patens*. Cell Motil Cytoskeleton 63:162–171

123. Pollard TD (2001) Genomics, the cytoskeleton and motility. Nature 409:842–843
124. Posern G, Treisman R (2006) Actin' together: serum response factor, its cofactors and the link to signal transduction. Trends Cell Biol 16:588–596
125. Prokhnevsky AI, Peremyslov VV, Dolja VV (2008) Overlapping functions of the four class XI myosins in *Arabidopsis* growth, root hair elongation, and organelle motility. Proc Natl Acad Sci U S A 105:19744–19749
126. Ratcliffe OJ, Kumimoto RW, Wong BJ, Riechmann JL (2003) Analysis of the Arabidopsis *MADS AFFECTING FLOWERING* gene family: MAF2 prevents vernalization by short periods of cold. Plant Cell 15:1159–1169
127. Reddy AS (2001) Molecular motors and their functions in plants. Int Rev Cytol 204:97–178
128. Ringli C, Baumberger N, Diet A, Frey B, Keller B (2002) *ACTIN2* is essential for bulge site selection and tip growth during root hair development of *Arabidopsis*. Plant Physiol 129:1464–1472
129. Roper K, Mao Y, Brown NH (2005) Contribution of sequence variation in *Drosophila* actins to their incorporation into actin-based structures in vivo. J Cell Sci 118:3937–3948
130. Rubenstein PA (1990) The functional importance of multiple actin isoforms. BioEssays 12:309–315
131. Ruegg J, Holsboer F, Turck C, Rein T (2004) Cofilin 1 is revealed as an inhibitor of glucocorticoid receptor by analysis of hormone-resistant cells. Mol Cell Biol 24:9371–9382
132. Ruzicka DR, Kandasamy MK, McKinney EC, Burgos-Rivera B, Meagher RB (2007) The ancient subclasses of *Arabidopsis ACTIN DEPOLYMERIZING FACTOR* genes exhibit novel and differential expression. Plant J 52:460–472
133. Saedler R, Jakoby M, Marin B, Galiana-Jaime E, Hulskamp M (2009) The cell morphogenesis gene *SPIRRIG* in *Arabidopsis* encodes a WD/BEACH domain protein. Plant J 59:612–621
134. Sattarzadeh A, Krahmer J, Germain AD, Hanson MR (2009) A myosin XI tail domain homologous to the yeast myosin vacuole-binding domain interacts with plastids and stromules in Nicotiana benthamiana. Mol Plant 2:1351–1358
135. Schepetilnikov MV, Solovyev AG, Gorshkova EN, Schiemann J, Prokhnevsky AI, Dolja VV, Morozov SY (2008) Intracellular targeting of a hordeiviral membrane-spanning movement protein: sequence requirements and involvement of an unconventional mechanism. J Virol 82:1284–1293
136. Schmidt von Braun S, Schleiff E (2008) The chloroplast outer membrane protein CHUP1 interacts with actin and profilin. Planta 227:1151–1159
137. Schoenenberger CA, Buchmeier S, Boerries M, Sutterlin R, Aebi U, Jockusch BM (2005) Conformation-specific antibodies reveal distinct actin structures in the nucleus and the cytoplasm. J Struct Biol 152:157–168
138. Shah DM, Hightower RC, Meagher RB (1983) Genes encoding actin in higher plants: intron positions are highly conserved but the coding sequences are not. J Mol Appl Genet 2:111–126
139. Sheahan MB, Staiger CJ, Rose RJ, McCurdy DW (2004) A green fluorescent protein fusion to actin-binding domain 2 of *Arabidopsis* fimbrin highlights new features of a dynamic actin cytoskeleton in live plant cells. Plant Physiol 136:3968–3978
140. Shimmen T (2007) The sliding theory of cytoplasmic streaming: fifty years of progress. J Plant Res 120:31–43
141. Staiger CJ, Blanchoin L (2006) Actin dynamics: old friends with new stories. Curr Opin Plant Biol 9:554–562
142. Staiger CJ, Poulter NS, Henty JL, Franklin-Tong VE, Blanchoin L (2010) Regulation of actin dynamics by actin-binding proteins in pollen. J Exp Bot 61:1969–1986
143. Staiger CJ, Sheahan MB, Khurana P, Wang X, McCurdy DW, Blanchoin L (2009) Actin filament dynamics are dominated by rapid growth and severing activity in the *Arabidopsis* cortical array. J Cell Biol 184:269–280
144. Stuven T, Hartmann E, Gorlich D (2003) Exportin 6: a novel nuclear export receptor that is specific for profilin–actin complexes. Embo J 22:5928–5940

145. Szerlong H, Hinata K, Viswanathan R, Erdjument-Bromage H, Tempst P, Cairns BR (2008) The HSA domain binds nuclear actin-related proteins to regulate chromatin-remodeling ATPases. Nat Struct Mol Biol 15:469–476
146. Szerlong H, Saha A, Cairns BR (2003) The nuclear actin-related proteins Arp7 and Arp9: a dimeric module that cooperates with architectural proteins for chromatin remodeling. Embo J 22:3175–3187
147. Tang W, You W, Shi F, Qi T, Wang L, Djouder Z, Liu W, Zeng X (2009) RNA helicase A acts as a bridging factor linking nuclear beta-actin with RNA polymerase II. Biochem J 420:421–428
148. Tian M, Chaudhry F, Ruzicka DR, Meagher RB, Staiger CJ, Day B (2009) *Arabidopsis* actin-depolymerizing factor AtADF4 mediates defense signal transduction triggered by the *Pseudomonas syringae* effector AvrPphB. Plant Physiol 150:815–824
149. Tondeleir D, Vandamme D, Vandekerckhove J, Ampe C, Lambrechts A (2009) Actin isoform expression patterns during mammalian development and in pathology: insights from mouse models. Cell Motil Cytoskeleton 66:798–815
150. Uribe R, Jay D (2009) A review of actin binding proteins: new perspectives. Mol Biol Rep 36:121–125
151. Vartiainen MK, Guettler S, Larijani B, Treisman R (2007) Nuclear actin regulates dynamic subcellular localization and activity of the SRF cofactor MAL. Science 316:1749–1752
152. Vidali L, Augustine RC, Kleinman KP, Bezanilla M (2007) Profilin is essential for tip growth in the moss Physcomitrella patens. Plant Cell 19:3705–3722
153. Vidali L, McKenna ST, Hepler PK (2001) Actin polymerization is essential for pollen tube growth. Mol Biol Cell 12:2534–2545
154. Vidali L, van Gisbergen PA, Guerin C, Franco P, Li M, Burkart GM, Augustine RC, Blanchoin L, Bezanilla M (2009) Rapid formin-mediated actin-filament elongation is essential for polarized plant cell growth. Proc Natl Acad Sci U S A 106:13341–13346
155. Wagner CR, Mahowald AP, Miller KG (2002) One of the two cytoplasmic actin isoforms in *Drosophila* is essential. Proc Natl Acad Sci U S A 99:8037–8042
156. Wang F, Jing Y, Wang Z, Mao T, Samaj J, Yuan M, Ren H (2009a) *Arabidopsis* profilin isoforms, PRF1 and PRF2 show distinctive binding activities and subcellular distributions. J Integr Plant Biol 51:113–121
157. Wang W, Wen Y, Berkey R, Xiao S (2009b) Specific targeting of the *Arabidopsis* resistance protein RPW8.2 to the interfacial membrane encasing the fungal Haustorium renders broad-spectrum resistance to powdery mildew. Plant Cell 21:2898–2913
158. Wang YS, Motes CM, Mohamalawari DR, Blancaflor EB (2004) Green fluorescent protein fusions to *Arabidopsis* fimbrin 1 for spatio-temporal imaging of F-actin dynamics in roots. Cell Motil Cytoskeleton 59:79–93
159. Wang YS, Yoo CM, Blancaflor EB (2008) Improved imaging of actin filaments in transgenic *Arabidopsis* plants expressing a green fluorescent protein fusion to the C- and N-termini of the fimbrin actin-binding domain 2. New Phytol 177:525–536
160. Williamson RE (1975) Cytoplasmic streaming in Chara: a cell model activated by ATP and inhibited by cytochalasin B. J Cell Sci 17:655–668
161. Yoon HS, Hackett JD, Ciniglia C, Pinto G, Bhattacharya D (2004) A molecular timeline for the origin of photosynthetic eukaryotes. Mol Biol Evol 21:809–818
162. Zheng B, Han M, Bernier M, Wen JK (2009) Nuclear actin and actin-binding proteins in the regulation of transcription and gene expression. FEBS J 276:2669–2685
163. Zheng M, Beck M, Muller J, Chen T, Wang X, Wang F, Wang Q, Wang Y, Baluska F, Logan DC, Samaj J, Lin J (2009) Actin turnover is required for myosin-dependent mitochondrial movements in Arabidopsis root hairs. PLoS One 4:e5961
164. Zimmer A, Lang D, Richardt S, Frank W, Reski R, Rensing SA (2007) Dating the early evolution of plants: detection and molecular clock analyses of orthologs. Mol Genet Genomics 278:393–402

Chapter 2
Plant Myosins

Etsuo Yokota and Teruo Shimmen

2.1 Introduction

Myosin is a molecular motor capable of producing motive force along actin filaments using the energy from ATP hydrolysis. A myosin molecule is generally composed of heavy and light chains. Most of the myosin heavy chains identified thus far have basically the N-terminal motor domain with ATP-hydrolysis and actin-binding sites, a neck domain with light chain binding sites, and a C-terminal tail region in which primary structures and sizes are diverse between myosin classes. The motor domain together with neck domain is often referred to as myosin head. The neck domain occupied by light chains works as a lever arm in the motor function. On the basis of sequence similarity of motor domain, myosins are divided into at least 24 classes [22]. Among them, three classes of myosins, VIII, XI and XIII, are plant specific [73, 113]. The *Arabidopsis thaliana* genome encodes seventeen myosins, four myosin VIII and thirteen myosin XI classes, while twelve myosin XI and two myosin VIII classes in *Oryza sativa* [37, 74].Two genes encoding Myosin XIII, Aclmyo1 and Aclmyo2, is found only in green alga *Acetabularia* [107]. Isoforms of myosin XI and myosin VIII of land plants are divided into 5 and 2 groups, respectively, by the phylogenic analysis [3]. In *Arabidopsis*, some isoforms of myosin X (XI-A to XI-E and XI-J) and myosin VIII (ATM2 and myosin VIIIB) are exclusively expressed in pollen, while other isoforms, MYA1 (XI-1), MYA2 (XI-2), XI-I, XI-K (myosin XI) and ATM1 (myosin VIII) in vegetative organs at relatively high levels [4]. The plant myosins are believed to be involved in various cellular functions in such as cytoplasmic streaming or nuclear, organelle and vesicle transport, cytokinesis, membrane trafficking, signal transduction and intercellular communication through the plasmodesmata. Recently, plant myosins are also shown to be utilized for the intra and intercellular movement of some kinds of plant viruses in the host plant cells [2, 28].

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The investigation of plant myosin had begun from attempting to elucidate the molecular mechanism of characean cytoplasmic streaming [82]. In 1970–1990, several proteins with ATPase activities had been isolated biochemically and reported as plant myosins (cited in [84]). However, none of these results have been reproduced. Myosin VIII designated as ATM1 [45] and myosin XI as MYA1 [44] were first identified and cloned from *Arabidopsis thaliana*. Because of similarity in overall domain structures, at the first discovery MYA1 was grouped with myosin V, phylogenically most closed to plant myosins [22, 62]. At the same time, native myosins, the 170-kDa myosin with 170-kDa heavy chain from lily pollen [117], the *Chara* myosin with 225-kDa heavy chain from *Chara* internodal cell [33, 114] and the 175-kDa myosin with 175-kDa heavy chain from tobacco cultured cell BY-2 [122], were isolated, and then identified as myosin XI later [41, 63, 85, 99]. These native myosins showed the motile activity in vitro and an actin-activated ATPase activity. In addition, the recombinant head of *Chara* myosin XI [35, 36] and MYA1 [27] possessing the motile activities, were prepared. The biochemical and biophysical properties of native myosin XI and its recombinant forms were analyzed. On the other hand, the motile and the ATPase activity of myosin VIII and myosin XIII have not yet been reported, because neither the native molecules from plant materials nor functional recombinant forms were prepared. However, ATP- and actin-binding sites in the motor domain and light chain binding sites in the neck domain are also conserved in these myosins.

In the past two decades, antibodies against animal myosin, frequently myosin II, were used for identifying the myosin from plant materials [73]. However, neither the antibody against the lily pollen 170-kDa myosin XI nor *Chara* myosin XI cross-reacted with the myosin II, visa verse [114, 117]. The 175-kDa polypeptide recognized by an anti-myosin II antibody in germinating lily pollen was found to be clathrin [93], indicating necessity of reevaluation of plant myosins identified by the antibody against animal myosin.

The molecular biological approach with the mutant and expression analyses and the immunocytochemical studies with specific antibodies against individual myosin class revealed the intracellular function of plant myosins. Myosin XI is generally associated with organelles through its tail region and is involved in their transport and the cytoplasmic streaming in plant cells. The lily pollen 170-kDa myosin XI is co-localized with various organelles and vesicles [119] including mitochondria and vesicles [77] in pollen tubes. This myosin is also found in subapical regions of tubes [60]. One of the cargos transported by tobacco 175-kDa myosin XI [124] is endoplasmic reticulum (ER). Myosin VIII is localized in cell plate and plasmodesmata [7,75], and its tail region is also targeted to the endosomes [25, 80]. Myosin XIII is found on organelles and vesicles, preferentially on chloroplasts at cell apex of growing conical tip in *Acetabularia* [107]. The organelles and vesicles transport by plant myosins is documented in detail in the Chapter.

In this Chapter, we focus mainly on the biochemical and biophysical properties and the role of heavy chain domains of myosin XI. Furthermore, we describe their relevance to physiological and cellular function, such as the cytoplasmic streaming or oragelle and vesicle transport. We also discuss the mechanism of cytoplasmic

streaming in plant cells in order to further insight into its molecular basis and the role, based on a recently proposed model of cytoplasmic streaming in *Arabidopsis* [104]. The cytoplasmic streaming in *Arabidopsis* is indicated to participate in the organization of actin bundles and in the integration of cytoplasmic strands.

2.2 Domain Structures of Plant Myosin Heavy Chains

The molecular weight of plant myosin heavy chains deduced from their sequences ranges from 120 to 140-kDa for myosin VIII and from 150 to 270-kDa for myosin XI [113]. The heavy chains of plant myosins are composed of motor and neck domains and tail region. Figure 2.1 shows the schematic domain structures of heavy chains of plant myosins. In the N-terminus before the motor domain in myosin VIII, an extra peptide consisted of around 90 amino acids is present, although its function is unclear thus far [45, 73]. A PEST motif is included in this peptide [7, 106]. This motif is cleaved in vitro by a protease, calpain [65] and is thought to be responsible for degradation of the proteins including itself. This motif may be involved in the turnover of myosin VIII. In myosin XI heavy chain, the existence of putative Src homology 3 (SH3)-like subdomain is revealed in its N-terminus before the motor domain [37, 62, 109]. The function of this subdomain is also unclear at present.

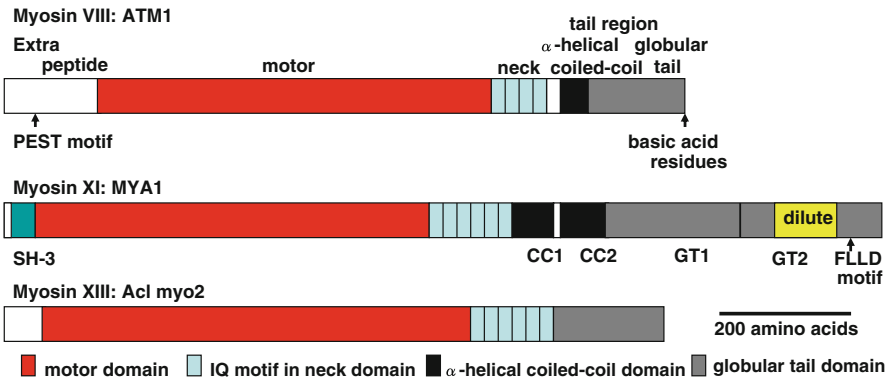

Fig. 2.1 Schematic domain structures of plant myosins, myosin VIII (ATM1), myosin XI (MYA1) and myosin XIII (Aclmyo2) (adopted from [62]). Before the motor domain, N-terminus extra peptides exist in plant myosins. In this peptide region of ATM1 or MYA1, PEST motif [7] or SH-3 like subdomain [37, 109] is present, respectively. In extreme C-terminus of ATM1, a group of basic amino acid residues are present [45]. The α-helical coiled-coil domain in MYA1 is divided into two segments, CC1 and CC2 [56]. Between them, a non-helical loop consisting of 10–15 amino acids, probably braking the α-helical coiled-coil structure, is inserted [56, 99]. The globular tail domain in MYA1 is composed of two subdomains, GT1 and GT2 [55]. In GT2 subdomain, a dilute domain, signature element of this myosin and myosin V [57], is present. FLLD motif is putative AtRabC2a binding site [30]. In myosin XIII, α-helical coiled-coil domain lacks

This domain identified in animal myosin classes is considered to interact with other proteins possessing the SH3 binding domain or with motor domain itself [62]. In *Dictyostelium* myosin II, this subdomain is indicated to communicate with the functional regions in the motor domain [23]. In neck domain of myosin VIII and myosin XI heavy chains, 3–4 and 4–6 tandem repeats of IQ motifs, respectively, responsible for binding of calmodulin (CaM) or its related proteins as light chains, are present. The tail region of myosin VIII and myosin XI is further divided into two domains, α-helical coiled-coil domain in its N-terminus and globular tail domain in the C-terminus. Within the C-terminus in the globular tail domain of myosin XI, a dilute domain designated from a mutant "dilute" mouse that encodes the heavy chain of myosin Va, is contained and is signature element of this myosin and myosin V [57]. At the very C-terminus in myosin VIII, a putative phosphorylation site by protein kinase A and C [7] and a group of basic residues [45] are present. Although plant myosins were classified based on sequence comparison of motor domains, the tail regions between myosin isoforms belonging in each class are also conserved to some extents, like non-plant myosins [49]. Two myosin XIII heavy chains, Aclmyo1 and Aclmyo2, are also consisted of motor and neck domain with 4 or 7 IQ motifs and tail region [107]. But, two unique features are found in the tail region. First the α-helical coiled-coil domain is lacking in both myosin XIII, and second the length of Aclmyo1 tail region is so short, only 18 amino acids are contained, whereas Aclmyo2 has long tail composed of 178 amino acids.

2.3 The Motor Domain

The motor domain is implicated in hydrolyzing ATP, interacting with actin filaments, and then generating the motive force along acin filaments cooperatively with the neck domain. The ATP binding pocket containing phosphate binding loop (P-loop) and surface loops for interacting with the actin are conserved in plant myosins, despite of several divergences in each class plant myosin, also in isoforms of each myosin class [41, 63, 74, 109]. Recombinant motor domain of *Chara* myosin XI [35, 36] and MYA1 [27] fused to artificial lever arm, instead of their intrinsic neck domain, can translocate F-actins in vitro, revealing that the motor domain of myosin XI actually possesses and exerts the motile activity. The motile activity of myosin VIII and XIII has not yet been reported. Similarly to the head containing motor and neck domains of MYA2 [109], a head of myosin VIII isoform, ATM2, expressed in tobacco leaf cells decorates the actin filaments [80]. The expression of tail region of tobacco myosin VIII or ATM1 inhibits the targeting of virus protein to the plasmodesmata in tobacco cells [2] or the movement of certain endosomes [25], respectively, in a dominantly negative manner. These results likely indicate the motor activity in myosin VIII.

The motile activity of myosin is usually measured and evaluated by using the motility assay in vitro. In a simple assay, the fluorescently labeled F-actin with ATP is introduced onto the myosin-coated glass surface in a flow cell and is monitored

by high sensitive camera [84]. By this assay, native lily pollen 170-kDa myosin XI [117], tobacco 175-kDa myosin XI [122] and *Chara* myosin XI [33, 114] showed the translocation of the F-actin with a velocity comparable to that of cytoplasmic streaming or organelle transport observed in living pollen tubes, BY-2 cells and characean internodal cells, respectively. The *Chara* myosin XI can slide the F-actin at the velocity of 50–60 μm/s, the fastest molecular motor in the world. The sliding velocity of F-actin generated by the recombinant MYA1 head in vitro is estimated to be 3.2 μm/s, corresponding to the velocity of the organelle movements in *Arabidopsis* [27]. The F-actin prepared from skeletal muscle is usually utilized for monitoring and analyzing the motile activity in vitro owing to its easy large scale preparation. No significant difference was shown in the sliding velocity induced by the lily pollen 170-kDa myosin XI between plant F-actin prepared from BY-2 cells and animal one [34]. Furthermore, a subfragment-1 (S1) of skeletal muscle myosin II, the single myosin head fragment, translocated both plant and animal F-actin with similar velocity. These evidences demonstrate the involvement of myosin XI in the cytoplasmic streaming or organelle transport, and that the myosin XI in different plant spices has an intrinsic motile activity to slide actin filaments with different velocity. In tobacco BY-2 cells, other myosin XI isoform, which is composed of 170-kDa heavy chain cross-reacted with an antibody against the lily pollen 170-kDa myosin XI heavy chains, translocates the F-actin with different velocity, 3–4 μm/s, from that of tobacco 175-kDa myosin XI, an average velocity of 9 μm/sec [122, 124]. Incidentally, the lily pollen 170-kDa myosin XI translocates F-actin in vitro at an average velocity of 7 μm/s. These observations suggest that the myosin XI isoform in a given plant spice also possesses the different motile activity. Which differences in myosin head or other domains contribute to the distinct sliding velocity between myosins XI, and in particular how *Chara* myosin XI is able to have such fast sliding velocity remain to be elucidate, and are now in progress [32, 113].

2.4 The Directional Determinant of Organelle Transport by the Polarity of Acitn Filaments

The actin filament and its bundle work as a track for myosin motors. The filament is a polar polymer; one end is usually referred to as barbed end (+ end) and the other as pointed end (– end), because the myosin II head, S1 or HMM (heavy meromyosin, two headed myosin fragment of skeletal muscle myosin II), decorates it as arrowhead fashion. The polymerization rate of barbed end is faster than that of pointed end, when the monomer G-actin is added to a pre-exist actin filament. On the other hand, the depolymerization rate on the pointed end is higher than that on barbed end (see Chapter). The individual class of myosin moves unidirectionally along an actin filament, and the most of myosin classes thus far move toward the barbed end, with an exception of myosin VI moving toward the pointed end [110]. Similarly to myosin II and myosin V, the tobacco 175-kDa myosin XI is shown to move along an F-actin toward the barbed end, but not opposite direction [99].

The cytoplasm in the characean cell streams toward barbed ends of actin filament cables visualized by their decoration with HMM [42]. When organelles isolated from lily pollen are introduced on actin cables of *Nitella* internodal cell, they can move along actin cables in the same direction as that of the myosin II-coated beads or as that of endogenous cytoplasmic streaming in *Nitella* [46, 48]). These evidences support that the *Chara* myosin XI and the lily pollen 170-kDa myosin XI, maybe even other myosins XI, are also barbed end-directed motors, and imply that the direction and polarity of the cytoplasmic streaming or organelle and vesicle movement generated by myosin XI are determined by the polarity of actin filaments or bundles, but not myosin XI. Like in characean cell, the cytoplasmic streaming occurs toward barbed ends of actin bundles in a root hair cell of *Hydrocharis* [98] and in a pollen tube of *Haemanthus* [54]. In some tip growing cells, the cytoplasmic streaming shows a reverse fountain pattern. The streaming is directed to the tip in the periphery of tubes, while to the base in the transvacuolar strand penetrating vacuole in the center of tubes. In the periphery and the transvacuolar strand, arrowheads of HMM on actin filaments in the bundles point to base and tip, respectively. These evidences strongly support the above implication. In an individual actin bundle in pollen tube [54] and root hair cell [98] and in an actin cable in characean cells [68, 111], the polarity of actin filaments shows uniform polarity. An actin binding protein, villin, which is able to arrange the actin filaments into a bundle with uniform polarity [118, 123], is colocalized with the actin bundles or cables in such plant cells [98, 105, 120, 123]. Hence, villin is one of the actin-filament bundling factors determining the polarity of actin filaments in their bundles. The electron microscopic images showing that the actin bundles are frequently linked to cortical microtubules in the periphery of pollen tube [52] and root hair cell [98], in which the organization of actin bundles is disturbed by the depolymerization of microtubules [96]. It is speculated that the cortical microtubules play a role in the polar arrangement of actin bundles in the periphery of cells. On the other hand, the other mechanism as described below is proposed for those polar arrangements in the transvacuolar strand, in which the microtubules are absent.

2.5 The Neck Domain

The neck domain in plant myosin heavy chains has several IQ motifs, interacting sites with CaM or its related proteins even in the absence of Ca^{2+}. Therefore, CaM is expected to be associated with the heavy chains as the light chain in plant myosins. CaM was identified to be light chain in lily pollen 170-kDa myosin XI [121] and tobacco 175-kDa myosin XI [122]. However, it is not certain whether all IQ motifs are occupied with CaM. The detail sequence analysis of IQ motif in *Chara* myosin XI with Pfam protein families database [36] or CaM target database [32] provided a possibility that other factors, instead of CaM are targeted to IQ motifs in this myosin. In chicken brain Va [14, 19] or yeast myosin V, Myo2p [91], essential light chains of myosin II, LC17 and LC23 or Mlc1p respectively, bind to the

neck domain, in addition of CaM. It is possible that other protein(s), besides CaM, is also associated with IQ motifs as light chains in other myosin XI.

It is generally accepted that the neck domain occupied by light chains acts as a lever arm for power stroke and force transduction (lever arm model) [101]. The step size, the displacement generated by myosin per one ATP hydrolysis cycle, is determined in part by the length of lever arm and is expected to become larger when the length of lever arm is longer. Myosin V, in which the neck domain is composed of 6 IQ motifs, can move along an F-actin with 36 nm step size. This size was shown to decrease with reducing the lever arm length, the number of IQ motifs [79]. The sliding velocity is also expected to be proportional to the lever arm length [101]. *Chara* myosin XI motor domain fused to the artificial lever arms with various lengths, such as no neck domain with 3 nm length of lever arm, two α-actinin repeats with 14 nm length that also acts as lever arm in myosin motor, and the neck domain of mouse myosin V containing 6 IQ motifs with 24 nm length, can translocate F-actins in vitro, and their sliding velocity is also proportional to the length of lever arm in some extents [35, 36]; at velocity of around 24 μm/s by the motor domain with longest lever arm, while 8 μm/s with shortest one. The lever arm model also appears to be applied to the myosin head of myosin XI. Similarly to myosin V, the tobacco 175-kDa myosin XI having 6 IQ motifs in the neck domain can move along F-actin with 35 nm step size, which was revealed by an optical trap analysis [99]. The length of 35 nm matches the half-pitch of F-actin helix, about 36 nm, indicating that tobacco 175-kDa myosin XI moves more or less straight, neither spirally nor rotationally, along the longitudinal axes of the actin filament. On the other hand, the step size of *Chara* myosin XI is estimated to be 19 nm [43], shorter than expected value from 6 IQ motifs. This discrepancy may be due the dissociation of light chains from neck domain during isolation steps, or the step size may not simply and solely be determined by the length of neck domain in native *Chara* myosin XI.

2.6 α-Helical Coiled-Coil Domain in the Tail Region

From the presence of α-helical coiled-coil domain, myosin VIII and myosin XI heavy chains have been predicted to form a dimer [44, 45]. The electron microscopic studies support this prediction. The *Chara* myosin XI [115] and tobacco 175-kDa myosin XI molecule [99] have two heads connected by stalk or rod structure, and a tail with two small globular structures (Fig. 2.2a). There are two remarkably morphological differences among these myosins XI. In the head, the shape and size of *Chara* myosin XI is similar to that of myosin II, while the tobacco 175-kDa myosin XI to myosin V. Second difference is the length of stalk. The tobacco 175-kDa myosin has 25 nm stalk, consistent with whole length of α-helical coiled-coil domain, about 24 nm, deduced from its primary sequence (about 170 amino acids) of MAY1 heavy chain [56]. This domain in other higher plant myosins XI is also composed of similar number of amino acid residues. On the other hand, *Chara* myosin XI has a very long rod structure, about 50 nm [115], reflecting the long

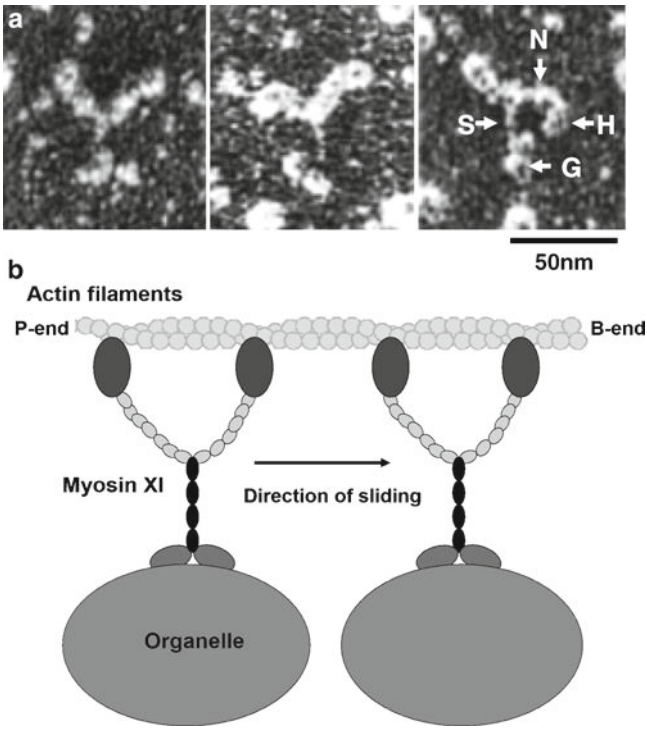

Fig. 2.2 The electron microscopic images of tobacco 175-kDa myosin XI (**a**) (adapted from [99]) and a schematic model showing the transport of organelles by myosin XI (**b**) (adapted from [85]). (**a**), The motor domain (*arrow H*), neck domain containing 6 IQ motifs (*arrow N*), a stalk (*arrow S*) or rod constructed from dimerized α-helical coiled-coil domains, and two small globular tail subdomains (*arrow G*), are visualized. (**b**) Myosin XI is associated with organelle through its globular tail domain. The organelles are trasnlocated by the sliding of myosin head along actin filaments from their pointed end (P-end) toward the barbed end (B-end)

α-helical coiled-coil domain, in which around 750 amino acids are included [32, 67]. The first 191 residues exhibit relatively higher similarity with the sequence in the α-helical coiled-coil domain of MYA1 [32].

The α-helical coiled-coil domain in higher plant myosin XI is further divided into two segments, CC1 and CC2 [56]. Between these segments corresponding to the center of α-helical coiled-coil domain, there is a non-helical loop consisting of 10–15 amino acids, probably braking the α-helical coiled-coil structure [56, 99]. Expressed α-helical coiled-coil domains of MAY1 in *Arabidopsis* leaf epidermis show the weak interaction between them, conceivably the dimerization [56]. The weak interaction between α-helical coiled-coil domains is dependent predominantly on the CC1 segment. This segment appears to be highly conserved in other higher plant myosins XI, predicting their similar properties in the dimerization. Interestingly, the interaction between α-helical coiled-coil domains is stabilized by the organelle targeting though the globular tail domain as described below.

Despite the absence of α-helical coile-coil domain in myosin XIII heavy chain [107], the situation in situ, monomeric or dimeric, is not known. While native myosin VI is monomeric, its dimerization via the tail domains is promoted by associating with adaptor/receptor proteins [71, 126] or phosphatidyl inositol 4,5 bisphosphate (PIP_2) [89] on the cargo. This may be true for myosin XIII.

2.7 Processive Movement of Higher Pant Myosin XI

The most important feature of motor proteins responsible for the transport of cargo is processivity; a single myosin motor moves along an actin filament for a long distance without detaching during the multiple mechanochemical cycles [11, 26, 100]. Hence, even small numbers of processive myosin is able to transport the cargo effectively along the actin filament for long distance. In animal cells, myosin V, but not all, and myosin VI have been known as the processive motors, which shows properties in their mechanochemical cycles different from those of non-processive myosins, for instance, the rate-limiting release of ADP and a high duty ratio, meaning that myosin heads spend the majority of their overall ATPase cycle strongly bound to actin filament. The linkage of two heads with the stalk is absolutely important for prossecive movement, in which at least one of two heads remains bound to the actin filament at all times during the movement. Yeast myosin V, Myo4p, kinetically possesses pocessive properties, the high duty ratio, however this myosin moves non-processively because it is monomeric, single headed myosin (cited in [10]). The non-processive myosins, for example myosin II, are dissociated from actin filament after a single mechanochemical cycle. To exert the function of non-processive myosins, in some cases, multiple motor complex or cluster is formed and assembled, for a famous example, the thick filament of myosin II in the muscle. The kinetical analyses in ATPase cycle and a single myosin-coated bead assay using the optical trap reveal the tobacco 175-kDa myosin XI is a fastest processive motor able to slide along an F-actin at the velocity of 7 μm/s with 35 nm step in the known motor proteins [99]. To explain how myosin V is able to move processivity, a hand-over-hand model was proposed [81, 100]. The one head called as trailing head and the other as the leading head exchange coordinately and alternatively the position along an actin filament like as “walking” on the filament, and one head remains bound to the actin filament when the other head detaches from it. The similar mechanism is reliably applicable for the motion of tobacco 175-kDa myosin XI [99]. The recombinant head of MYA1 shows the kinetic processivity [27], ADP release as rate-limiting step and the high duty ratio. The dimerization of MYA1 via α-helical coiled-coil domain as described above, likely confers the processivity to this myosin.

In the bead assay using the optical trap, a force generated by single tobacco 175-kDa myosin XI is estimated to be 0.5 pN [99]. Hydrodynamic analysis has proposed that the motive force of cytoplasmic streaming per unit interface was 0.3–0.4 pN/μm^2 in characean cells [125]. If the similar situations, such as the viscosity of cytoplasm

described below, are also applied to tobacco BY-2 cells, a few molecules of this myosin is assumed to be sufficient for transport of a spherical organelle with 1 μm diameter [99]. ER is indicated to be one of cargos translocated by this myosin in BY-2 cells [124]. This organelles forms strands and reticular structures and streams dynamically and continuously in the cytoplasm. The processivity of tobacco 175-kDa myosin XI, also of other higher plant myosins XI, may make a possible to effective transport of ER and other organelles.

On the other hand, whether the *Chara* myosin XI is the processive motor is controversial and remains to be elucidated (supporting non-processive in [6], processive in [32, 92]). The bead assay coated with a single myosin molecule with the optical trap shows the non-processivity in *Chara* myosin XI [43]. Even if this myosin is a non-processive motor, a multiple and high density of myosin molecule on the cargos along an actin filament, comparable with thick filament of myosin II, may enable *Chara* myosin XI effectively to transport cargos [113]. This situation is hypothesized to be one mechanism for fast sliding velocity in this myosin [43].

2.8 Regulation of Higher Plant Myosin XI Through CaM Light Chain

CaM is well known to regulate the activity of its target proteins in a Ca^{2+}-dependent manner. Hence, the CaM light chains are expected to confer the Ca-sensitivity to the myosin XI and to regulate its motile activity. In the case of lily pollen 170-kDa myosin XI [121] and tobacco 175-kDa myosin XI [122], their motile activities in vitro are suppressed by Ca^{2+} at concentrations higher than 10^{-6} M. In lily pollen 170-kDa myosin XI, the relation of inhibitory effect of Ca^{2+}on its motile activity with the behavior of CaM light chain was analyzed in some details. Between 10^{-6} and 10^{-5} M Ca^{2+}, the suppression of motile activity of lily pollen 170-kDa myosin XI is reversible, when the Ca^{2+} concentrations is reduced. The CaM remains associated with the heavy chain in this range of Ca^{2+} concentrations, seeming that Ca^{2+} in this concentration range causes reversible inhibition by an allosteric interaction of the heavy chain and the CaM light chain. In contrast, at higher than 10^{-5} M, Ca^{2+} induces an irreversible inhibition of the motile activity, and the dissociation of the CaM from the lily pollen 170-kDa myosin XI heavy chain. The impaired activity is restored by lowering Ca^{2+} concentrations to some extents in the presence of exogenous CaM prepared from the lily pollen. According to the lever arm model, the mechanism of CaM-dissociation-induced inhibition is suggested to be caused by reduction of the structural integrity in the lever arm. The dissociation of CaM light chains from the heavy chain by the high concentrations of Ca^{2+} had been indicated in myosin V and also caused the inactivation of motile activity in this myosin [50]. In living plant cells, however, it is improbable that the myosin encounters Ca^{2+} concentrations higher than 10^{-5} M.

2.9 Regulation of Cytoplasmic Streaming or Organelle Transports in Pollen Tubes

The individual organelle and vesicle show the characteristic moving patterns in the reverse fountain streaming, and positioning in the growing pollen tubes (see the Chapter). A steep tip-focused Ca^{2+} gradient is generally formed in a growing pollen tube, and the concentrations of Ca^{2+} are shown to be higher than 10^{-6} M in the extremely apical region of the tube [31]. The relatively large organelles, amyloplast and vacuole, turn back to the grain near the basis of the tip. Although the mitochondria and ER move to and locate in a subapical domain of the tip, they do not invade into the apical region ([60, 69]; and see Chapter). When the Ca^{2+}-gradient is diminished, these organelles stream into the apex, indicating that the cytoplasmic streaming or organelle transport is suppressed by Ca^{2+}, in particular at the apex of growing pollen tube. The movement of isolated organelles from lily pollen along actin cables in *Nitella* internodal cells is inhibited reversibly by Ca^{2+} at concentrations between 10^{-6} and 10^{-5} M [46, 47], similarly to lily pollen 170-kDa myosin XI. These observations suggest that myosins in pollen tubes possess the Ca-sensitivity in their motile activities, acting as suppressive in the presence of Ca^{2+} higher than 10^{-6} M, and that the Ca-sensitivity in myosins is one of the physiological relevance for the Ca^{2+} regulation of cytoplasmic streaming. The dynamics and the organization of actin filaments in the apical and subapical regions are different from the shank of tubes and are modulated and regulated by Ca^{2+} through the actin binding proteins ([12, 13] and see Chapter). Hence, both "actin-linked" and "myosin-linked" Ca^{2+} regulation may work in concert for regulating the cytoplasmic streaming and organelle transport in pollen tubes.

2.10 Globular Tail Domain

From the similarity to myosin V, the globular tail domain of myosin XI has been deduced to be responsible for the cargo binding [44]. Based on the crystal structure of yeast myosin V, Myo2p, a predicted molecular architecture of MYA1 globular tail domain from its primary sequence is depicted and shows well assemble in that of globular tail in Myo2p [55]; subdomains, GT1 (globular tail 1) and GT2, are present and are associated tightly with each other in MYA1 globular tail domain. The interaction between two subdomains is confirmed in yeast two-hybrid screening and in *Arabidopsis* leaf cells. The live imaging approach, in which the tail region tagged with fluorescent protein is co-expressed with organelle and vesicular marker proteins in higher plant cells, provides the direct evidence that the tail region is actually targeted to the cargo. When the tail region containing α-helical coiled-coil and globular tail domains of several *Arabidopsis* myosin XI isoforms is expressed in the tobacco, onion and *Arabidopsis* cells, it is associated with Golgi and peroxisomes [55, 76]. In some cases, the movement of these organelles and mitochondria is

suppressed or modified dominant negatively by the expression of the tail region of several Myosin XI iosforms ([3, 70, 87] and see Chapter). Each subdomain, GT1 and GT2, of *Arabidopsis* several myosin XI isoforms, can be targeted independently with each other to same or distinct organelles [55]. For instances, GT2 of MYA1 or MYA2 is targeted to peroxisome, while GT1 of MYA1 or MYA2 to preferentially peroxisome and occasionally Golgi or unknown organelles, respectively. These observations extensively support that myosin XI is associated with the cargos through its globular tail domain (Fig. 2.2b), and that the globular tail domain possesses at least two organelle binding sites in GT1 and GT2.

When the construct containing α-helical coiled-coil and globular tail domains of myosin VIII, *Arabidopsis* ATM1 [25] or ATM2 [80], is expressed in *Arabidopsis* and tobacco cells, it is targeted to the plasmodesmata and endosomes. The localization of this myosin class in the plasmodesmata has been also revealed by the immnocytochemical studies using the specific antibodies against this myosin class [7, 75]. These expression analyses further indicate that myosin VIII is also involved in the membrane recycling in plant cells, and that its globular tail domain plays a role in targeting this myosin to the cargo or proper locate in plant cells. The basic residue group at very C-terminus in myosin VIII [45] is probably responsible for the direct binding of this myosin to acidic membrane lipid, like *Chara* myosin XI described below.

2.11 The Mechanism for Cargo Recognition by Higher Plant Myosin XI

Targeting organelles of *Arabidopsis* myosin XI tail region are not completely consistent with binding organelles of its subdomains. MYA1 tail region is targeting to organelles including Golgi, but not to peroxisome to which GT1 and GT2 of MYA1 are targeted. Other myosin XI isoforms showed similar behaviors to that of MYA1, suggesting the presence of a mechanism for selecting cargos of myosin XI in situ. Myosin V in animal and yeast is well known to interact with adaptor/receptor proteins in many cases through its globular tail domain for recruiting to the proper cargos [57]. Together with this evidence, a following model for explaining the selecting mechanism of myosin XI is predicted [55]. Subdomains, GT1 and GT2, take different conformations alternatively in myosin XI tail. Initially the tail domain binds to or interacts weakly with organelle-specific adaptor. This interaction selects one of these conformations, and then the tail domain is locked effectively on the organelle.

Small GTPase Rab proteins of *Arabidopsis*, AtRabD1 and AtRabC2a, are found to bind weakly to the MYA2 globular tail domain in a GTP-dependent manner in vitro [30]. The Rab proteins interchange between the active GTP-bound and inactive GDP-bound forms, which adopt different conformations each other [1]. The GTP-bound form of Rab protein interacts with effector proteins and performs the function. In animal cell and yeast, Rab proteins are one of adaptor/receptor proteins for myosin V targeting to proper cargos and can interact directly or indirectly via

other proteins with myosin V globular tail domain. The fluorescent-protein-tagged AtRabC2a expressed in *Arabidopsis* leaf cells is co-localized with the peroxisome, one of MAY2 targeting organelles ([29], and see Chapter). Hence, the AtRabC2a is indicated to be an adopter/receptor for targeting MYA2 to the peroxisome, and its weak interaction with MYA2 tail domain might be compatible with and support the model described above. But, whether or not the weak interaction of MAY2 with AtRabC2a occurs in situ is obscure.

After the weak interaction of globular tail domain with organelle-specific adaptor, such as AtRabC2a, and subsequent selection of one conformation, this domain should be locked effectively on the organelle surface. At present, this mechanism is not confirmed. In many cases of myosin V, other proteins, in addition to Rab proteins, act as adopter/receptor for connecting this myosin to its targeting cargo [57]. For example, in animal melanocyte, Rab27a localizes to the membrane surface of melanosome and recruits the melanophilin acting as adaptor and binding to myosin V globular tail. Consequently, myosin V is connected to melanosome. Hence, other proteins are conceivable to be implicated in the locking mechanism for higher plant myosin XI. Another possibility cannot be ruled out that the direct binding of the globular tail to membrane lipids is involved in locking the myosin tightly on the surface of organelle membrane, as follows. The globular tail of *Chara* myosin XI is associated directly with membrane lipids probably through its basic amino acid clusters [67]. The clusters are also conserved in higher plant myosin XI [56, 57]. Globular tail of myosin VI interacts with both adaptor/receptor proteins and PIP_2 and is targeted to early endocytic vesicles through this interaction [89].

Both N- and C-terminuses in the MYA2 globular tail domain is necessary for the interaction with AtRabC2a [30]. Similar situation has been shown in the interaction of mammalian myosin Vb globular tail domain with Rab11 family proteins, such as Rab11a, Rab11b and Rab25 [53]. The two interaction sites in the myosin Vb globular tail with these Rab proteins is indicated as follows; one site is LEKNE motif locating in its N-terminus, and the other is LLLD motif in its C-terminus. A similar sequence to the LEKNE motif is not found in the N-terminal region of MYA2 globular tail domain [30]. However, FLLD motif corresponding to LLLD motif is present within 55 amino acids in the C-terminus not only in MYA2 globular tail domain, but also in other *Arabidopsis* myosin XI isoforms and tobacco 175-kDa myosin XI, with exceptions of *Arabidopsis* myosin XI-G and XI-J. It is tempting to deduce that the interaction of other myosin XI isoforms with AtRab proteins through this conserved motif is one of the selecting mechanism for targeting individual myosin XI isoform to proper and certain cargos or sites for exerting its function in situ.

MYA2 also interacts in vitro with the other AtRab protein, AtRabD1, whose targeting organelles or vesicles have not been identified [30]. The subdomain, GT1 and GT2, of MYA2 globular tail, can bind to unidentified organelles and the peroxisome, respectively [55]. An immunocytochemical study also shows the association of MYA2 with other organelles and vesicles that are not peroxisome [29]. It is plausible that the AtRabD1 recruits this myosin XI to these

organelles and vesicles. The identification of organelles associated with this AtRab protein will help elucidating of other function of MYA2 besides of peroxisome transport.

The tail region of myosin VIII ATM1 is targeted to some endosomes marked and decorated with GFP-tagged Ara6 [25], which is a plant Rab5 ortholog and regulates the endocytosis in *Arabidopsis* [102, 103]. Although a direct interaction of ATM1 with Ara6 in vivo or in vitro has not been examined, it is attractive to consider that myosin VIII is also associated with the Ara6 on endosomes.

2.12 Functional Inter-Domain Communication Between α-Helical Coiled-Coil and Globular Tail Domains

As described above, the globular tail domain of the several myosin XI isoforms without the α-helical coiled-coil domain, could not be associated with organelles, while the tail regions including both domains is targeted to organelles [55, 76]. Hence, the α-helical coiled-coil domain is important to adopt a favorable configuration in globular tail domain for binding to organelles, since the α-helical coiled-coil domain alone could not be associated with organelles [55]. Intriguingly, the targeting globular tail domains to organelles is found to stabilize the dimerization between α-helical coiled-coil domains in two tail regions, suggesting the existence of an inter-dependent relationship between dimerization in α-helical coiled-coil domain and organelle binding in globular tail domain within MYA1 [56]. According to this scenario, there is an equilibrium between monomeric and unstable dimeric forms of MYA1, and only targeting to organelles could be arranged into the stable and functional dimer possessing the processivity. However, the isolated tobacco 175-kDa myosin XI molecule is dimer as described above. Although further study is need, it is postulated that dimeric myosin XI is stable and not disassembled easily into monomer once the dimer is formed.

As described above, the dimer formation in myosin VI is induced through its binding to adapter/receptor proteins or PIP_2 on cargos. In this case, two heavy chains of this myosin are connected in their tails, unlike myosin XI. In animal myosin V molecule, dynamic intramolecular conformational change is induced and responsible for the self-regulation of its activity [100]. In the presence of micromolar Ca^{2+}, the myosin V has an extended conformation (active state), whereas it forms a folded shape in the absence of Ca^{2+} (inactive state), in which the globular tail domain is folded back toward and interacts with head region. By Ca^{2+} binding to CaM in the neck region, the globular tail domain dissociates from head region, and the myosin Va activities are restored. An adaptor/receptor proteins for myosin Va, melanophilin, which is associated with Rab27a and recruits this myosin to melanosome, is found to activate the actin-activated ATPase of myosin Va in the Ca^{2+} free condition [58], This finding suggests that binding to cargo induces the activation of myosin V by unfolding inactive folded states.

The folded and inactive conformation in the absence of Ca^{2+} has not been reported in myosin XI thus far.

2.13 Direct Binding of *Chara* Myosin XI to Phospholipid Vesicles

On the contrary to higher plant myosin XI, the *Chara* myosin XI [115] and its recombinant globular tail domain [67] are shown to bind directly to vesicles prepared from acidic pshopholipids, such as phosphatidylserine and PIP_2 but not from neutral phospholipids. When an arginine cluster present in the globular tail is substituted to non-charged amino acids for reducing its positive charge, the binding affinity of globular tail domain decreases drastically, indicating the electrostatic interactions of acid phospholipids with the globular tail domain. The direct binding of myosin tail to acid phospholipids has been well known in animal myosin I [15]. The binding of *Chara* myosin XI to lipid is supposed to be tight and to overcome the shearing force generated during the sliding movement of this myosin heads along actin filament cables [67].

2.14 Cytoplasmic Streaming in Characean Cells

Cytopalsmic streaming is a coordinated and directed bulk flow of cytoplasm carrying smaller organelles and vesicles in plant cells [82]. Its molecular and regulatory mechanism has been extensively investigated by using characean cells, in which the cytoplasm rotates in the same direction stably with so fast velocity at several ten μm/sec. The investigation of plant myosin had begun from attempting to elucidate the molecular mechanism of characeancytoplasmic streaming. The hydrodynamic analysis had proposed that only movement of long carling filamentous [125] or membrane network organelles expanding into the whole cytoplasm [66] could generate the motive force for the cytoplasmic streaming from actin cable to the viscous cytoplasm in characean cells, about 0.7–2 poise compared with 0.01 poise in a water. These organelles have been considered to be ER [38]. Although unambiguous demonstration is lacking, binding of *Chara* myosin XI to ER has been suggested in *Characeae* [67, 116]. Kamitsubo described that transparent structure is running along the actin cables (personal communication to Shimmen in 1978). It was reported that ER-like structure is associated with actin cables [38]. An immunocytochemical study using antibodies against Chara myosin XI shows the localization of this myosin along actin cables as vesicular-like dots [63, 116]. ER strands labeled with fluorescent probes rapidly streams in subcortical cytoplasm [21]. From these observations, an association of *Chara* myosin XI with ER has been suggested.

2.15 The Regulation of Cytoplasmic Streaming in the Characean Cell

In characean cells, the cytoplsamic streaming stops transiently. The necessity of extracellular Ca^{2+} had been indicated in this phenomenon [8]. Electrophysiological studies indicated the involvement of Ca^{2+} channel in generation of action potentials (cited in [86]). The generation of action potential induces the elevation of intracellular Ca^{2+} concentrations up to micromolar, and concomitantly the cytoplasmic streaming ceases transiently [112]. The membrane permeabilized cells, in which the cytoplasmic streaming is restored by the addition of ATP, the streaming is reversibly inhibited by micromolar Ca^{2+} [94]. However, this inhibitory mechanism is remained to be elucidated. Sliding of beads coated with skeletal muscle myosin II along actin cables is completely insensitive to Ca^{2+}, suggesting that characean actin cables are not equipped with Ca-sensitizing mechanism [83]. Therefore, myosin-linked Ca-regulation was suggested for the cytoplasmic streaming in *Characeae*. However, the actin activated ATPase and motile activities of isolated *Chara* myosin XI are affected by neither Ca^{2+} nor Ca^{2+}/CaM [5, 33, 114]. Hence, it is reasonable that Ca^{2+} does not affect directly *Chara* myosin XI activity (indirect myosin-linked Ca^{2+}-regulation). A phosphorylation-dephosphorylation has been suggested to be involved in the regulation of cytoplasmic streaming in characean cells [95]. Vesicle movement on the actin filaments peeling off from the surface of chloroplasts is observed in squeezed endoplasm from *Chara* cells in the presence of ATP, and is inhibited by the addition of phosphatase inhibitor, okadaic acid. On the other hand, it is activated by protein kinase inhibitor, staurosporine [64]. The sliding of F-actin on the glass surface coated with *Chara* myosin XI is similarly inhibited and activated by the treatment with the drugs. The motile activity is also diminished by the treatment with protein kinase C in the extract. The Ca^{2+}-dependent protein kinase, which also phosphorylates the substrate for protein kinase C, has been found to locate on the actin cables and on small organelle- or vesicle-like structures attached to the cables in characean cells [61], thereby making it possible to phosphorylate easily and specifically only *Chara* myosin XI just working on the cytoplasmic streaming. These evidences suggest two mechanisms of phosphorylation leading to dysfunction of *Chara* myosin XI in situ for suppressing the cytoplasmic streaming by Ca^{2+}. First is the inactivation of motile activity in *Chara* myosin XI by the phosphorylation. In animal, the motile and ATPase activities of several classes of myosins, such as ameba myosin I [15] and smooth muscle and non-muscle myosin II [16, 17], are regulated through the phosphorylation of head region or the regulatory light chain, respectively. The phosphorylation occurs in TEDS rule site residing between ATP- and actin-binding sites in motor domain in ameba myosin I and activates its activity. It is well known that the phosphorylation of regulatory light chain in the smooth muscle myosin and non-muscle II activates the ATPase activity and promotes thick filament assembly from the bent and folded monomeric molecule. Conversely, the phosphorylation also exerts the negative effect on the activities of myosin II, depending on the phosphorylation

site(s) in regulatory light chain. Which domain(s) or subunit(s) in *Chara* myosin XI molecule is phosphorylated and affects its motile activity is not clarified. Second is the dissociation of *Chara* myosin XI from the surface of cargos, such as ER. The consensus sequences of the phosphorylation site by the Ca^{2+}-dependent protein kinase and the Ca^{2+}/CaM-dependent protein kinase II are present near the arginine clusters in *Chara* myosin XI globular tail [67]. If this site is phosphorylayed, the positive charge in the cluster is expected to be reduced, and the dissociation of myosin XI from organelles is anticipated. Similarly, myosin V tail has been reported to be dissociated from organelles by its phosphorylation [40]. It is naturally possible that both two inhibitory systems are exerted simultaneously.

2.16 Cytoplasmic Streaming in Higher Plant Cells

The streaming of Green fluorescent proteins expressed in BY-2 cells and diffused in their cytoplasm is found to be dependent on organelle movement [20], suggested that the cytoplasm is dragged by moving organelles. Recently, a reverse genetic approach using *Arabidopsis* mutant strain of myosin XI shows that the streaming ER is a force generating for the cytoplasmic streaming in *Arabidopsis* [104], similarly to characean cell. In *Arabidopsis*, strands of ER actively stream along the long axis of the cell, and the cytoplasmic streaming shows the similar velocity distribution to that of ER streaming. The streaming of ER is impaired, and the morphology and organization of ER is altered in mutants of myosin XI, especially myosin XI-K, indicating this myosin XI isoform primary contributor to ER streaming, which is implicated intimately in the cytoplasmic streaming. An inhibitor of myosin activities, BDM (2,3-butanedione monoxime) that can suppress the motile activity in vitro of lily 170-kDa myosin XI [97] and *Chara* myosin XI [24], also completely suppresses such ER dynamics. These observations further indicate that the sliding movement of myosin XI is involved in maintaining ER morphology. Interestingly, the organization and orientation of actin bundles in cytoplasm is also disturbed concomitantly, and transvacuolar strands disappeared in myosin XI mutant cells. For explaining these co-relations between myosin XI, ER streaming and actin-bundle organization, a following model is proposed. A portion of ER slides along disorderly and randomly oriented actin bundles through ER-associated myosin XI. The following ER streaming arranges the bundles into uniform polarity because of the unidirectional sliding of myosin XI, and forms thicker actin bundles due to aligning the adjacent bundles around ER through myosin XI. Reiteration of these interconnected processes induces a formation of longitudinally and unidirectionally oriented thick actin bundles, which provide tracks for the extensive streaming of ER strands and the structural integrity to transvacuolar strands. According to this model, dynamic interactions between ER streaming by myosin XI and actin bundles define the architecture and movement patterns of ER strands, the polarity and the orientation of actin bundles in cytoplasmic and transvacuolar strands.

However, how and where actin bundles are fixed or anchored within *Arabidopsis* cells are remained obscure. In the characean cells, actin cables are fixed on the immobile chloroplasts, thereby the organelles, maybe ER, could move relative to actin cables. Although chloroplasts are fixed to the gel ectoplasm in *Characeae*, they are trasnlocated in response to various external stimuli in most higher plants [108]. In such plants, for example *Vallisneria* [18], *Arabidopsis* [39] and spinach [51], actin bundles are associated with and surround chloroplasts in circular or basket-like array, which is altered and reorganized depending on the chloroplast movement under the light conditions [18, 51, 78]. Hence, it is improbable to speculate that these actin bundles are involved in translocation of other organelles. One candidate organelle that tethers actin bundles is considered to be the cortical ER. ER network is continues between cortical ER, cytoplasmic ER and outer nuclear envelope [88, 90], and cytoplasmic ER is streaming through the sliding of myosin XI along actin bundles [104, 124]. On the other hand, the cortical ER does stream rarely as a whole, although it takes rapidly shape change and remodeling motions [88]. Actin bundles frequently locate close to or under the cortical ER network associated closely to cell membrane in higher plant cells [9]. This association of ER and cell membrane is relatively tight, overcoming to the centrifugal force [72]. Electron microscopic studies demonstrate that actin bundles attach to the cortical ER network along their several points and appear to be enwrapped with ER membrane [59]. Hence, it is likely that some portions of actin bundles are anchored to cortical region of cells by the cortical ER network.

2.17 Conclusion

Plant specific, three classes of myosins, myosin VIII, myosin XI and myosin XIII are identified and have similar domain structures, with an exception of lacking α-helical coiled-coil domain in myosin XIII. In myosin XI, the head region containing motor and neck domains works in generating motive force and binding to light chain CaM, the α-helical coiled-coil domain in dimerizing two heavy chains, and the globular domains in binding to cargos. In the case of myosin VIII and myosin XIII, the mechanochemical properties in motor head, composition of light chains in neck domain and binding mechanism to the cargo have not yet been shown thus far. Higher plant myosin XI can move processively with 35 nm step size along the actin filament, a favorable property effective in transporting its cargos even by small numbers of myosin molecules, and its motile activity is regulated by CaM light chain. This step size enables the myosin XI to move straight along the actin filament, and the straight motion with its cargos is expected to attenuate the viscous drag of cytosol comparing with the spiral motion. Although it is not defined whether *Chara* myosin XI is a processive motor thus far, multiple and high density of myosin molecule on organelle membrane surface close to actin cables may confer this myosin to transport organelles efficiently at fast velocity. One of these organelles may be ER. The streaming of ER causes the dragging force for cytoplasmic streaming. In *Arabidopsis*, the streaming of ER generated by myosin XI is

implicated not only in the cytoplasmic streaming, but also in sustaining the ER morphology and organizing actin bundles. Hence, it is possible that the orientation and polarity of actin bundles is arranged by sliding and moving of myosin XI associated with ER. The myosin XI via its tail region can bind directly membrane phospholipid of cargo or indirectly to the cargo through the adaptor/receptor proteins such as AtRab proteins. The tail region in myosin VIII is also responsible for the binding and targeting to the cargos, bacterial protein and endosome, and to the proper site, plasmodesmata. Further analyzing the organelle recognition mechanism should provide an insight into understanding the function of individual myosin XI or myosin VIII isoform, for example, total 17 isoforms in *Arabidopsis*, and the regulation of organelle and vesicle transport. With regard to myosin VIII, understanding mechanochemical properties in its motile activity will help further elucidating of its function in situ.

References

1. Ali BR, Seabra MC (2005) Targeting of Rab GTPases to cellular membranes. Biochem Soci Trans 33:652–656
2. Avisar D, Prokhnevsky AI, Dolja VV (2008) Class VIII myosins are required for plasmodesmatal localization of a closterovirus Hsp70 homolog. J Virol 82:2836–2843
3. Avisar D, Prokhnevsky AI, Makarova KS, Koonin EV, Dolja VV (2008) Myosin XI-K is required for rapid trafficking of Golgi stacks, peroxisomes, and mitochondria in leaf cells of *Nicotiana benthamiana*. Plant Physiol 146:1098–1108
4. Avisar D, Abu-Abied M, Belausov E, Sadot E, Hawes C, Sparkes IA (2009) A comparative study on the involvement of 17 *Arabidopsis* myosin family members on the motility of Golgi and other organelles. Plant Physiol 150:700–709
5. Awata J, Saitoh K, Shimada K, Kashiyama T, Yamamoto K (2001) Effects of Ca^{2+} and calmodulin on the motile activity of characean myosin in vitro. Plant Cell Physiol 42:828–834
6. Awata J, Kashiyama T, Ito K, Yamamoto K (2003) Some motile properties of fast characean myosin. J Mol Biol 236:659–663
7. Baluška F, Cvčková F, Kendrick-Jones J, Volkmann D (2001) Sink plasmodesmata as gateways for phloem unloding. Myosin VIII and calreticulin as molecular determinants of sink strength? Plant Physiol 126:39–46
8. Barry WH (1968) Coupling of excitation and cessation of cyclosis in *Nitella*: role of divalent cations. J Cell Physiol 72:153–160
9. Boevink P, Oparka K, Cruz SS, Martin B, Betteridge A, Hawes C (1998) Stacks on tracks: the plant Golgi apparatus traffics on an actin/ER network. Plant J 15:441–447
10. Bookwalter CS, Lord M, Trybus KM (2009) Essential features of the class V myosin from budding yeast for ASH1 mRNA transport. Mol Biol Cell 20:3414–3421
11. Buss F, Kendrick-Jones J (2007) Myosin VI: a multifunctional motor protein. In: Coluccio LM (ed) Myosins: a Superfamily of Molecular Motors. Springer, Berlin, pp 325–352
12. Cai G, Cresti M (2009) Organelle motility in the pollen tube: a tale of 20 years. J Exp Bot 60:495–508
13. Cárdenas L, Lovy-Wheeler A, Kunkel JG, Hepler PK (2008) Pollen tube growth oscillations and intracellular calcium levels are reversibly modulated by actin polymerization. Plant Physiol 146:1611–1621
14. Cheney RE, O'Shea MK, Heuser JE, Coelho MV, Wolenski JS, Espreafico EM, Forscher P, Larson RE, Mooseker MS (1993) Brain myosin-V is a two-headed unconventional myosin with motor activity. Cell 75:13–23

15. Coluccio LM (2007) Myosin I. In: Coluccio LM (ed) Myosins: a Superfamily of Molecular Motors. Springer, Berlin, pp 95–124
16. Conti MA, Kawamoto S, Adelstein RS (2007) Non-muscle myosin II. In: Coluccio LM (ed) Myosins: a Superfamily of Molecular Motors. Springer, Berlin, pp 223–264
17. Cremo CR, Hartshorne DJ (2007) Smooth-muscle myosin II. In: Coluccio LM (ed) Myosins: a Superfamily of Molecular Motors. Springer, Berlin, pp 171–222
18. Dong X-J, Ryu J-H, Takagi S, Nagai R (1996) Dynamic changes in the organization of microfilaments associated with the photocontrolled motility of chloroplasts in epidermal cells of *Vallisneria*. Protoplasma 195:18–24
19. Espindola FS, Suter DM, Partata LBE, Cao T, Wolenski JS, Cheney RE, King SM, Mooseker MS (2000) The light chain composition of chicken brain myosin-Va: calmodulin, myosin-II essential light chains, and 8-kDa dynein light chain/PIN. Cell Motil Cytoskel 47:269–281
20. Esseling-Ozdoba A, Houtman D, Van Lammeren AAM, Eiser E, Emons AMC (2008) Hydrodynamic flow in the cytoplasm of plant cells. J Micro 231:274–283
21. Foissner I, Menzel D, Wasteneys GO (2009) Microtubule-dependent motility and organization of the cortical endoplasmic reticulum in elongating characean internodal cells. Cell Motil Cytoskel 68:142–155
22. Foth BJ, Goedecke MC, Soldati D (2006) New insights into myosin evolution and classification. Proc Nat Acad Sci USA 103:3681–3686
23. Fujita-Becker S, Tsiavaliaris G, Ohkura R, Shimada T, Manstein DJ, Sutoh K (2006) Functional characterization of the N-terminal region of myosin-2. J Biol Chem 281:36102–36109
24. Funaki K, Nagata A, Akimoto Y, Shimada K, Ito K, Yamamoto K (2004) The motility of *Chara corallina* myosin was inhibited reversibly by 2, 3-butanedione monoxime (BDM). Plant Cell Physiol 45:1342–1345
25. Golomb L, Abu-Abied M, Belausov E, Sadot E (2008) Different subcellular localizations and functions of Arabidopsis myosin VIII. BMC Plant Biol 8(3):1–13
26. Gross SP, Vershinin M, Shubeita GT (2007) Cargo transport: two motors are sometimes better than one. Curr Biol 17:R478–R486
27. Hachikubo Y, Ito K, Schiefelbein J, Manstein DJ, Yamamoto K (2007) Enzymatic activity and motility of recombinant *Arabidopsis* myosin XI, MYA1. Plant Cell Physiol 48:886–891
28. Harries PA, Park J-W, Sasaki N, Ballard KD, Maule AJ, Nelson RS (2009) Differing requirements for actin and myosin by plant viruses for sustained intercellular movement. Proc Natl Acad Sci USA 106:17594–17599
29. Hashimoto K, Igarashi H, Mano S, Nishimura M, Shimmen T, Yokota E (2005) Peroxisomal localization of a myosin XI isoform in *Arabidopsis thaliana*. Plant Cell Physiol 46:782–789
30. Hashimoto K, Igarashi H, Mano S, Takenaka C, Shiina T, Yamaguchi M, Demura T, Nishimura M, Shimmen T, Yokota E (2008) An isoform of *Arabidopsis* myosin XI interacts with small GTPases in its C-terminal tail region. J Exp Bot 59:3523–3531
31. Hepler PK, Lovy-Wheeler A, Mckenna ST, Kunkel JG (2006) Ions and pollen tube growth. Plant Cell Monogr 3:47–69
32. Higashi-Fujime S, Nakamura A (2009) Cell and molecular biology of the fastest myosins. Int Rev Cell Mole Biol 276:301–347
33. Higashi-Fujime S, Ishikawa R, Iwasawa H, Kagami O, Kurimoto E, Kohama K, Hozumi T (1995) The fastest actin-based motor protein from the green algae, *Chara*, and its distinct mode of interaction with actin. FEBS Lett 375:151–154
34. Igarashi H, Vidali L, Yokota E, Sonobe S, Hepler PK, Shimmen T (1999) Actin filaments purified from tobacco cultured BY-2 cells can be translocated by plant myosin. Plant Cell Physiol 40:1167–1171
35. Ito K, Kashiyama T, Shimada K, Yamaguchi A, Awata J, Hachikubo Y, Manstein DJ, Yamamoto K (2003) Recombinant motor domain constructs of *Chara corallina* myosin display fast motility and high ATPase activity. Biochem Biophys Res Commun 312:958–964

36. Ito K, Ikebe M, Kashiyama T, Mogami T, Kon T, Yamamoto K (2007) Kinetic mechanism of the fastest motor protein, *Chara* myosin. J Biol Chem 282:19534–19545
37. Jiang SY, Ramachandran S (2004) Identification and molecular characterization of myosin gene family in *Oryza sativa* genome. Plant Cell Physiol 45:590–599
38. Kachar B, Reese TS (1988) The mechanism of cytoplasmic streaming in characean algal cells: sliding of endoplasmic reticulum along actin filaments. J Cell Biol 106:1545–1552
39. Kandasamy MK, Meagher RB (1999) Actin-organelle interaction: association with chloroplast in *Arabidopsis* leaf mesophyll cells. Cell Motil Cytoskel 44:110–118
40. Karcher RL, Roland JT, Zappacosta F, Huddleston MJ, Annan RS, Carr SA, Gelfand VI (2001) Cell cycle regulation of myosin-V by calcium/calmodulin-dependent protein kinase II. Science 293:1317-1320
41. Kashiyama T, Kimura N, Mimura T, Yamamoto K (2000) Cloning and characterization of a myosin from characean alga, the fastest motor protein in the world. J Biochem 127:1065–1070
42. Kersey YM, Hepler PK, Palevitz BA, Wessells NK (1976) Polarity of actin filaments in characean algae. Proc Natl Acad Sci USA 73:165–167
43. Kimura Y, Toyoshima N, Hirakawa N, Okamoto K, Ishijima A (2003) A kinetic mechanism for the fast movement of *Chara* myosin. J Mol Biol 328:939–950
44. Kinkema M, Schiefelbein J (1994) A myosin from a higher plant has structural similarities to class V myosins. J Mol Biol 239:591–597
45. Knight AE, Kendrick-Jones J (1993) A myosin-like protein from a higher plant. J Mol Biol 231:148–154
46. Kohno T, Shimmen T (1988) Accelerated sliding of pollen tube organelles along *Characeae* actin bundles regulated by Ca^{2+}. J Cell Biol 106:1539–1543
47. Kohno T, Shimmen T (1988) Mechanism of Ca^{2+} inhibition of cytoplasmic streaming in lily pollen tubes. J Cell Sci 91:501–509
48. Kohno T, Chaen S, Shimmen T (1990) Characterization of the translocator associated with pollen tube organelles. Protoplasma 154:179–183
49. Korn ED (2000) Coevolution of head, neck, and tail domains of myosin heavy chains. Proc Nat Acad Sci USA 97:12559–12564
50. Krementsov DN, Krementsova EB, Trybus KM (2004) Myosin V: regulation by calcium, calmodulin, and the tail domain. J Cell Biol 164:877–886
51. Kumatani T, Sakurai-Ozato N, Miyawaki N, Yokota E, Shimmen T, Terashima I, Takagi S (2006) Possible association of actin filaments with chloroplasts of spinach mesophyll cells in vivo and in vitro. Protoplasma 229:45–52
52. Lenartowska M, Michalska A (2008) Actin filament organization and polarity in pollen tubes revealed by myosin II subfragment 1 decoration. Planta 228:891–896
53. Lancelle SA, Cresti M, Hepler PK (1987) Ultrastructure of the cytoskeleton in freeze-substituted pollen tubes of *Nicotiana alata*. Protoplasma 140:141–150
54. Lapierre LA, Kumar R, Hales CM, Navarre J, Bhartur SG, Burnette JO, Provance DW Jr, Mercer JA, Bähler M, Goldenring JR (2001) Myosin Vb is associated with plasma membrane recycling systems. Mol Biol Cell 12:1843–1857
55. Li J-F, Nebenführ A (2007) Organelle targeting of myosin XI is mediated by two globular tail subdomains with separate cargo binding sites. J Biol Chem 282:20593–20602
56. Li J-F, Nebenführ A (2008) Inter-dependence of dimerization and organelle binding in myosin XI. Plant J 55:478–490
57. Li J-F, Nebenführ A (2008) The tail that wags the dog: the globular tail domain defines the function of myosin V/XI. Traffic 9:290–298
58. Li X, Ikebe R, Ikebe M (2005) Activation of myosin Va function by melanophilin, a specific docking partner of myosin Va. J Biol Chem 280:17815–17822
59. Lichtscheidl IK, Lancelle SA, Hepler PK (1990) Actin-endoplasmic reticulum complexes in *Drosera*. Their structural relationship with the plasmalemma, nucleus, and organelles in cells prepared by high pressure freezing. Protoplasma 155:116–126
60. Lovy-Wheeler A, Cárdenas L, Kunkel JG, Hepler PK (2007) Differential organelle movement on the actin cytoskeleton in lily pollen tubes. Cell Motil Cytoskel 64:217–232

61. McCurdy DW, Harmon AC (1992) Calcium-dependent protein kinase in the green alga *Chara*. Planta 188:54–61
62. Mooseker MS, Foth BJ (2007) The structural and functional diversity of the myosin family of actin-based molecular motors. In: Coluccio LM (ed) Myosins: a Superfamily of Molecular Motors. Springer, Berlin, pp 1–34
63. Morimatsu M, Nakamura A, Sumiyoshi H, Sakaba N, Taniguchi H, Kohama K, Higashi-Fujime S (2000) The molecular structure of the fastest myosin from green algae, *Chara*. Biochem Biophys Res Commun 270:147–152
64. Morimatsu M, Hasegawa S, Higashi-Fujime S (2002) Protein phosphorylation regulates actomyosin-driven vesicle movement in cell extracts isolated from the green algae, *Chara corallina*. Cell Motil Cytoskel 53:66–76
65. Nascimento AA, Cheney RE, Tauhata SB, Larson RE, Mooseker MS (1996) Enzymatic characterization and functional domain mapping of brain myosin-V. J Biol Chem 271:17561–17569
66. Nothnagel EA, Webb WW (1982) Hydrodynamic models of viscous coupling between motile myosin and endoplasm in characean algae. J Cell Biol 94:444–454
67. Nunokawa S, Anan H, Shimada K, Hachikubo Y, Kashiyama T, Ito K, Yamamoto K (2007) Binding of *Chara* myosin globular tail domain to phospholipid vesicles. Plant Cell Physiol 48:1558–1566
68. Palevitz BA, Ash JF, Hepler PK (1974) Actin in the green alga, *Nitella*. Proc Natl Acad Sci USA 71:363–366
69. Parton RM, Fischer-Parton S, Trewavas AJ, Watahiki MK (2003) Pollen tubes exhibit regular periodic membrane trafficking events in the absence of apical extension. J Cell Sci 116:2707–2719
70. Peremyslov VV, Prokhnevsky AI, Avisar D, Dolja VV (2008) Two class XI myosins function in organelle trafficking and root hair development in Arabidopsis. Plant Physiol 146:1109–1116
71. Phichith D, Travaglia M, Yang Z, Liu X, Zong AB, Safer D (2009) Cargo binding induces dimerization of myosin VI. Proc Natl Acad Sci USA 106:17320–17324
72. Quader H, Hofmann A, Schnepf E (1987) Shape and movement of the endoplasmic reticulum in onion bulb epidermis cells: possible involvement of actin. Eur J Cell Biol 44:17–26
73. Reddy ASN (2001) Molecular motors and their functions in plants. Int Rev Cytol 204:97–178
74. Reddy ASN, Day IS (2001) Analysis of the myosins encoded in the recently completed *Arabidopsis thaliana* genome sequence. Gen Biol 2:0024.1–0024.17
75. Reichelt S, Knight AE, Hodge TP, Baluska F, Samaj J, Volkmann D, Kendrick-Jones J (1999) Characterization of the unconventional myosin VIII in plant cells and its localization at the post-cytokinetic cell wall. Plant J 19:555-567
76. Reisen D, Hanson MR (2007) Association of six YFP-myosin XI-tail fusions with mobile plant cell organelles. BMC Plant Biol 7(6):1–17
77. Romagnoli S, Cai G, Faleri C, Yokota E, Shimmen T, Cresti M (2007) Microtubule- and actin filament-dependent motors are distributed on pollen tube mitochondria and contribute differently to their movement. Plant Cell Physiol 48:345–361
78. Sakai Y, Takagi S (2005) Reorganized actin filaments anchor chloroplasts along the anticlinal walls *Vallisneria* epidermal cells. Planta 221:823–830
79. Sakamoto T, Yildez A, Selvin PR, Sellers JR (2005) Step-size is determined by neck length in myosin V. Biochemistry 44:16203–16210
80. Sattarzadeh A, Franzen R, Schmelzer E (2008) The Arabidopsis class VIII myosin ATM2 is involved in endocytosis. Cell Motil Cytoskel 65:457–468
81. Sellers JR, Weisman LS (2007) Myosin V. In: Coluccio LM (ed) Myosins: a Superfamily of Molecular Motors. Springer, Berlin, pp 289–323
82. Shimmen T (2007) The sliding theory of cytoplasmic streaming: fifty years of progress. SJ Plant Res 120:31–43
83. Shimmen T, Yano M (1985) Ca^{2+} regulation of myosin sliding along *Chara* actin bundles mediated by native tropomyosin. Proc Japan Acad 61:86–89

84. Shimmen T, Yokota E (1994) Physiological and biochemical aspects of cytoplasmic streaming. Int Rev Cytol 155:97–139
85. Shimmen T, Yokota E (2004) Cytoplasmic streaming in plants. Curr Opin Cell Biol 16:68–72
86. Shimmen T, Mimura T, Kikuyama M, Tazawa M (1994) Characean cells as a tool for studying electrophysiological characteristics of plant cells. Cell Struct Funct 19:263–278
87. Sparkes IA, Teanby NA, Hawes C (2008) Truncated myosin XI tail fusions inhibit peroxisome, Golgi and mitochondrial movement in tobacco leaf epidermal cells: a genetic tool for the next generation. J Exp Bot 59:2499–2512
88. Sparkes IA, Frigerio L, Tolley N, Hawes C (2009) The plant endoplasmic reticulum: a cell-wide web. Biochem J 423:145–155
89. Spudich G, Chibalina MV, Au JS-Y, Arden SD, Buss F, Kendrick-Jones J (2007) Myosin VI targeting to clathrin-coated structures and dimerization is mediated by binding to Disabled-2 and PtdIns(4, 5P_2). Nat Cell Biol 9:176–183
90. Staehelin LA (1997) The plant ER: a dynamic organelle composed of a large number of discrete functional domains. Plant J 11:1151–1165
91. Stevens RC, Davis TN (1998) Mlc1p is a light chain for the unconventional myosin Myo2p in *Saccaromyces cerevisiae*. J Cell Biol 142:711–722
92. Sumiyoshi H, Ooguchi M, Ooi A, Okagaki T, Higashi-Fujime S (2007) Insight into the mechanism of fast movement of myosin from *Chara coralline*. Cell Motil Cytoskel 64:131–142
93. Tahara H, Yokota E, Igarashi H, Orii H, Yao M, Sonobe S, Hashimoto H, Hussey PJ, Shimmen T (2007) Clathrin is involved in organization of mitotic spindle and phragmoplast as well as in endocytosis in tobacco cell cultures. Protoplasma 230:1–11
94. Tominaga Y, Shimmen T, Tazawa M (1983) Control of cytoplasmic streaming by extracellular Ca^{2+} in permeabilized *Nitella* cells. Protoplasma 116:75–77
95. Tominaga Y, Wayne R, Tung HYL, Tazawa M (1987) Phosphorylation-dephosphorylation is involved in Ca^{2+}-controlled cytoplasmic streaming of characean cells. Protoplasma 136:161–169
96. Tominaga M, Morita K, Sonobe S, Yokota E, Shimmen T (1997) Microtubules regulate the organization of actin filaments at the cortical region in root hair cells of *Hydrocharis*. Protoplasma 199:83–92
97. Tominaga M, Yokota E, Sonobe S, Shimmen T (2000) Mechanism of inhibition of cytoplasmic streaming by a myosin inhibitor, 2, 3-butanedione monoxime. Protoplasma 213:46–54
98. Tominaga M, Yokota E, Vidali L, Sonobe S, Hepler PK, Shimmen T (2000) The role of plant villin in the organization of the actin cytoskeleton, cytoplasmic streaming and the architecture of the transvacuolar strand in root hair cells of *Hydrocharis*. Planta 210:836–843
99. Tominaga M, Kojima H, Yokota E, Orii H, Nakamori R, Katayama E, Anson M, Shimmen T, Oiwa K (2003) Higher plant myosin XI moves processively on actin with 35 nm steps at high velocity. EMBO J 22:1263–1272
100. Trybus KM (2008) Myosin V from head to tail. Cell Mol Life Sci 65:1378–1389
101. Ueda TQ, Abramson PD, Spudich JA (1996) The neck region of the myosin motor domain acts as a lever arm to generate movement. Proc Natl Acad Sci USA 93:4459–4464
102. Ueda T, Yamaguchi M, Uchimiya H, Nakano A (2001) Ara6, a plant-unique novel type Rab GTPase, functions in the endocytic pathway of *Arabidopsis thaliana*. EMBO J 20:4730–4741
103. Ueda T, Uemura T, Sato MH, Nakano A (2004) Functional differentiation of endosomes in Arabidopsis cells. Plant J 40:783–789
104. Ueda H, Yokota E, Kutsuna N, Shimada T, Tamura K, Shimmen T, Hasezawa S, Dolja VV, Hara-Nishimura I (2010) Myosin-dependent endoplasmic reticulum motility and F-actin organization in plant cells. Proc Natl Acad Sci USA 107:6894-6899
105. Vidali L, Yokota E, Cheung AY, Shimmen T, Hepler PK (1999) The 135 kDa actin-bundling protein from *Lilium longiflorum* pollen is the plant homologue of villin. Protoplasma 209:283–291
106. Volkmann D, Mori T, Tirlapur UK, König K, Fujiwara T, Kendrick-Jones J, Baluška F (2003) Unconventional myosins of the plant-specific class VIII: endocytosis, cytokinesis, plasmodesmata/pit-fields, and cell-to-cell coupling. Cell Biol Int 27:289–291

107. Vugrek O, Sawitzky H, Menzel D (2003) Class XIII myosins from the green alga *Acetabularia*: driving force in organelle transport and tip growth? J Muscle Res Cell Motil 24:87–97
108. Wada M, Kagawa T, Sato Y (2003) Chloroplast movement. Ann Rev Plant Biol 54:455–468
109. Walter N, Holweg CL (2008) Head-neck domain of *Arabidopsis* myosin XI, MYA2, fused with GFP produces F-actin patterns that coincide with fact organelle streaming in different plant cells. BMC Plant Biol 8(74):1–13
110. Wells AL, Lin AW, Chen LQ, Safer D, Cain SM, Hasson T, Carragher BO, Milligan RA, Sweeney HL (1999) Myosin VI is an actin-based motor that move backwards. Nature 401:505–508
111. Williamson RE (1974) Actin in the alga, *Chara corallina*. Nature 248:801-802
112. Williamson RE, Ashley CC (1982) Free Ca^{2+} and cytoplasmic streaming in the alga *Chara*. Nature 296:647–651
113. Yamamoto K (2007) Plant myosins VIII, XI, and XIII. In: Coluccio LM (ed) Myosins: a Superfamily of Molecular Motors. Springer, Berlin, pp 375–390
114. Yamamoto K, Kikuyama M, Sutoh-Yamamoto N, Kamitsubo E (1994) Purification of actin based motor protein from *Chara coralline*. Proc Japan Acad 70:175–180
115. Yamamoto K, Kikuyama M, Sutoh-Yamamoto N, Kamitsubo E, Katayama E (1995) Myosin from alga *Chara*: unique structure revealed by electron microscopy. J Mol Biol 254:109–112
116. Yamamoto K, Shimada K, Ito K, Hamada S, Ishijima A, Tsuchiya T, Tazawa M (2006) *Chara* myosin and the energy of cytoplasmic streaming. Plant Cell Physiol 47:1427–1431
117. Yokota E, Shimmen T (1994) Isolation and characterization of plant myosin from pollen tubes of lily. Protoplasma 177:153–162
118. Yokota E, Shimmen T (1999) The 135-kDa actin-bundling protein from lily pollen tubes arranges F-actin into bundles with uniform polarity. Planta 209:264–266
119. Yokota E, McDonald AR, Liu B, Shimmen T, Palevitz BA (1995) Localization of a 170 kDa myosin heavy chain in plant cells. Protoplasma 185:178–187
120. Yokota E, Takahara K, Shimmen T (1998) Actin-bundling protein isolated from pollen tubes of lily. Biochemical and immunocytochemical characterization. Plant Physiol 116:1421–1429
121. Yokota E, Muto S, Shimmen T (1999) Inhibitory regulation of higher-plant myosin by Ca^{2+} ions. Plant Physiol 119:231–239
122. Yokota E, Yukawa C, Muto S, Sonobe S, Shimmen T (1999) Biochemical and immunocytochemical characterization of two types of myosins in cultured tobacco bright yellow-2 cells. Plant Physiol 121:523–534
123. Yokota E, Vidali L, Tominaga M, Tahara H, Orii H, Morizane Y, Hepler PK, Shimmen T (2003) Plant 115-kDa actin-filament bundling protein, P-115-ABP, is a homologue of plant villin and is widely distributed in cells. Plant Cell Physiol 44:1088–1099
124. Yokota E, Ueda S, Tamura K, Orii H, Uchi S, Sonobe S, Hara-Nishimura I, Shimmen T (2009) An isoform of myosin XI is responsible for the translocation of endoplasmic reticulum in tobacco cultured BY-2 cells. J Exp Bot 60:197–212
125. Yoneda M, Nagai R (1988) Structural basis of cytoplasmic streaming in characean internodal cells. A hydrodynamic analysis. Protoplasma 147:64–76
126. Yu C, Feng W, Wei Z, Miyanoiri Y, Wen W, Zhao Y, Zhang M (2009) Myosin VI undergoes cargo-mediated dimerization. Cell 138:537–548

Chapter 3
Actin-Binding Proteins and Actin Dynamics in Plant Cells

Shanjin Huang, Yun Xiang, and Haiyun Ren

Abbreviations

ABD	Actin binding domain
ABPs	Actin binding proteins
ADF	Actin depolymerizing factor
ADP	Adenonisine disphosphate
Aip1	Actin-interacting protein 1
Arp2/3	Actin-related protein 2 and 3
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BBS	Bud6p-binding site
bp	Base pair
BY-2	Nicotiana tabacum L. cv. Bright Yellow 2
cDNA	Complementary DNA
CDPK	Calmodulin domain-like protein kinase
CP	Capping protein
DAD	Dia-autoregulatory domain
DG	Diacyglycerol
DNase I	Deoxyribonuclease I
E. coli	*Escherichia coli*
F-actin	Filamentous actin
FH1	Formin homology 1
FH2	Formin homology 2
FH3	Formin homology 3
FIP2	Formin interacting protein 2
G-actin	Global actin
GBD	GTPase binding domain
GFP	Green fluourescnt protein
GTPase	Guanosine triphosphatase

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GUS	β-glucuronidase
IP_3	Inositol 1, 4, 5-trisphosphate
kDa	Kilodalton
mRNA	Messenger RNA
nM	Nanomole per liter
PA	Phosphatidic acid
pH	Potential of hydrogen
PLP	Poly-L-Proline
Rho	Ras homologue
RNAi	RNA interference
ROPs	Rho of plants
SCAR	Suppressor of cAMP receptor defects
siRNA	Small interfering RNA
T-DNA	Transferred DNA
TIRFM	Total internal reflection fluorescence microscopy
TM	Trans-membrane domain
UTR	Untranslated region
WASP	Wiskott–Aldrich syndrome protein
WAVE	WASP family verprolin homologous protein

3.1 Introduction

Actin filament is one of the two major cytoskeletal networks found in plant cells. Through its various forms of assembly and its dynamic changes, actin filament provides highly diverse roles that have probably not yet been identified for all of them in different kinds of cells. In interphase plant cells, actin structures mainly include extensive bundles of actin microfilaments that serve as the tracks for cytoplasmic streaming around the nucleus, in transvacuolar strands and in the subcortex, and a dynamic arrays of shorter filaments in the outer cortex of the cells undergoing diffuse growth and in the tip region of the cells undergoing tip growth (for reviews, see [124,138]). In vacuolated plant cells undergoing mitosis, actin is a highly dynamic structure. At the early stage of mitosis, actin forms an actin pre-prophase band co-aligned with the microtubule pre-prophase band [136], which is required for correct narrowing of the pre-prophase band as it develops and for precise positioning of the division site [38,107]. In the mitosis, a spindle-associated actin basket is found in both endosperm cells and walled cells, which could serve as an anchor and thus control lateral spindle movement [58,78]. In late anaphase, during phragmoplast formation, actin filaments have been initially described as a poor defined mass of microfilaments between the incipient daughter nuclei, but soon develop into two antiparallelled arrays at both sides of the division plane, with the barbed ends at the central region [24,72]. Studies using F-actin depolymerizing drugs, such as cytochalasin B or D, have shown that the actin cytoskeleton has a role in directing the

location of cell plate [24,134]. Relatively, the formation, regulation and the functions of the bundled actin filaments are well known than that of its dynamic changes. For the dynamics of the actin cytoskeleton, it is important that actin filaments can rapidly assemble and disassemble, which is regulated by the ionic conditions in the cytoplasm and by a large number of actin-binding proteins, such as monomer-trapping proteins, actin nucleating proteins, filament capping and severing proteins. Here we summarize the recent advances in our knowledge about the candidate proteins or protein complexes as the main players that have evolved in the assembly and maintenance of many actin-based structures in plant cells.

3.2 Profilin

Profilin is a low-molecular-mass (12–15 kDa), cytosolic protein that was first discovered in calf spleen extracts as a globular actin binding protein [18]. Subsequently, profilin was identified in all eukaryotic organisms and cells [28,54,57,85,148,151], even in virus and vaccinia [12]. Profilin can bind G-actin with a K_d in the micromolar range, thereby acts as a G-actin sequestering protein and prevents spontaneous actin nucleation and polymerization. However, profilin can promote the actin assembly via directing the G-actin-ATP to the barbed end of filament. Profilin can also enhance ADP to ATP exchange on actin monomers. Nevertheless, this activity was absent in plant [82,119]. In addition to actin, profilin can bind other ligands, such as phosphoinositides [86], poly-L-proline (PLP) [164] and Arp2/3 complex [111]. Thus, profilin may play a crucial role in the actin dynamics, as well as may provide a link between the signal transduction pathways and actin cytoskeleton rearrangements in the variety of cellular processes.

The first plant profilin was first identified as an allergen from birch pollen [150] and was then proved to be an actin binding protein [151]. Though sharing only 25% amino acid sequence identity with non-plant profilin, plant profilin has the ability to complement profilin-deletion mutations in yeast [23] and *Dictyostelium* [76].The function of some plant profilins binding to PIP_2, PLP and actin has been well characterized [49,82], suggesting they serve the similar function as other eukaryotic counterparts. Plant profilins are encoded by multigene families and have different isoforms. For example, *Arabidopsis* may contain at least 5 profilin isoforms [65], and maize profilin family has six members [49,159]. It has been well demonstrated that these isoforms have distinct patterns of expression in specific cell type, organ, tissue and developmental stage. According to the analyses of the expression levels of mRNA and protein, as well as promoter-GUS gene fusions in transgenic plants, profilins can be divided into two distinct classes in high plant: vegetative and reproductive ones, which shows similar spatial and temporal patterns of expression to actins [65]. In addition, the different profilin isoforms differ in their affinities for actin, Ca^{2+} and PLP in vitro, the vegetative profilins have a relatively high affinity for G-actin and PLP, whereas reproductive profilin are less sensitive to the Ca^{2+}. Such differences have also been confirmed in vivo by coexpressing reproductive

actin and different profilin isovariants in transgenic plant, the phenotype of vegetative actin mutant was only suppressed by simultaneous overproduction of reproductive actin and reproductive profilin, but not reproductive actin and vegetative profilin, the results suggest that class-specific isoforms may play distinct roles in the actin cytoskeleton and plant cell [154].

To investigate the physiological function of profilins, the cellular localization of profilin in living cells from several different plant species has been studied by using immunocytochemical analyses [59,75,155], the microinjection of fluorescently labeled profilin [155] and the expressing the profilins fused with fluorescence protein in transgenic plants [161]. However, there are diverse localization of profilins in different cell types, profilin isoforms and analysis methods. Many results show that profilin is distributed uniformly in vegetative or reproductive cell types of *Arabidopsis* [75], as well as in the pollen tubes of tobacco [75] and *Lilium* [155]. However, profilin is localized to the plasma membrane in microspores of birch pollen [159], and it is also detected close to the site of pollen tube emergence, pollen exine and the tip region of olive pollen tube [109]. In the root hair, another polar-growing cell type, profilin is specifically expressed in the apical region [7,14]. Apart from cytoplasmic localization, profilins were observed in the nuclei of root apical cells of many cell types [7,59,75,153]. Recently, PRF1 and PRF2, two *Arabidopsis* profilin isoforms, were fused with green fluorescent protein and stably expressed in *Arabidopsis*, and then their localization were observed in living cells of transgenic lines. The results show that PRF1-GFP is associated with a filamentous network in different cell types, but PRF2-GFP forms polygonal meshes closely resembling ER structures [161]. Nevertheless, the mechanism of these distinct distributions of pofilin isoforms in different plant cells and developmental stages has not been addressed yet.

The physiological function of profilins in plant cells, however, has not been studies in great detail so far. Utilizing the technique of microinjecting profilins into pollen or *Tradescantia* stamen hair cells, previous researches indicated that profilin disrupts F-actin arrays, causes the nucleus to be displaced from central position, reduces the transvacuolar cytoplasmic strands in stamen hair cells [76,141,152], as well as inhibits the pollen tube growth and cytoplasmic streaming [156]. Whereafter, the transgenic *Arabidopsis* plants that overexpressed or underexpressed profilin 1 show distinct morphological phenotypes, reduction of profilin 1 by expressing anti-sense construct leads to an short hypocotyls, rough epidermal surface, early flowering time and fewer number of leaves as compared to wild types and overexpressing plant. Conversely, increasing the profilin level results in longer roots and roots hair [123]. Incomprehensibly, T-DNA mutant seedlings of profilin 1 showed contrary phenotype to those of the anti-sense lines. The mutant plants have abnormally raised cotyledons, elongated hypocotyls, and elongated cells in the hypocotyl, and some defects in light and circadian responses, such phenotypes can be complemented by wild-type profilin 1 [105]. The reason of these differences between the two studies is not clear yet. To further investigate the function of profilin, whole three profilins were silenced by RNA interference in the Moss *Physcomitrella patens*, which leads to a complete loss of tip growth

and a partial inhibition of cell division, and thus profilin RNAi plants have smaller rounded and fewer cells. In addition, the F-actin network is also disrupted in RNAi plants, where contains a prominent subapical cortical F-actin structure composed of parallel actin cables in moss tip growing cell are disorganized and reduced. Above phenotypes can be fully rescued by expressing any wild-type moss or lily profilin. However, the profilin mutants in actin or polyproline binding sites are unable to entirely complete the normal tip growth, and the effect is more notable in actin sites mutants. These results show that profilin and its binding to actin may play a crucial role in tip growth, and profilin activity also requires a functional polyproline binding site [154]. Similar results are also reported in yeast and pollen. It is demonstrated that the actin binding of profilin is essential for biological function in fission yeast [91] and pollen tube growth [104]. Though PLP has an inhibitory effect, it appears to act independently of porfilin in pollen [104]. However, it is difficult to accurately address the function of different profilin isoforms during the process of plant growth and development due to the presence of multiple profilin isoforms in same plant cells.

Apart from regulating actin dynamics, many clues indicate that profilin may be involved in certain signal transductions in plant cells. It is demonstrated that plant profilin is directly and highly specific interacted with PtdIns(4,5)P_2, and thus inhibits its hydrolysis by phosphoinositede phospholipase C [35]. The turnover of PtdIns(4,5)P_2 can release the profilin as well as the second messengers inositol 1, 4, 5-trisphosphate (IP_3) and diacyglycerol (DG). IP_3 then initiate the Ca^{2+} signal and the released profilin would be available to regulate the actin remodeling. In addition, the actin-sequestering activity of profilin is also affected by Ca^{2+}. Above observations demonstrated that profilin may act at the interface between polyphosphoinositide signaling pathways and reorganization of the actin cytoskeleton (reviewed in [93]). Another evidence for profilin in signaling pathways come from the reports that profilin isoforms can be phosphorylated on serine residue or tyrosine residues and modulate the phosphorylation of pollen proteins [52,89], phosphorylation of profilin regulates its interaction with actin and PLP in vitro [130]. Consequently, it is indicated that profilin may act as a modulator, translating the signals into alteration in the phosphorylation of certain proteins and actin architecture.

3.3 Arp2/3 Complex and Its Regulatory WAVE Complex

Nucleation is the rate-limiting step for de novo actin polymerization, therefore, actin polymerization in vivo requires the intervention of actin nucleation factors. To date, several actin nucleators have been identified in the literature: the Arp2/3 complex, formins, Spire, Cordon-bleu, Leiomodin and JMY [1,21,121,122,166,183]. Among them, Arp2/3 complex and formins are well documented. Arp2/3 complex was reported to be responsible for the formation of branched actin filaments [111]. It was identified originally during the search for profilin interacting proteins [92].

Arp2/3 complex contains seven subunits including Arp2, Arp3, the subunits ARPC1 (p40), ARPC2 (p34), ARPC3 (p21), ARPC4 (p20), and ARPC5 (p16), homologs for all seven subunits of a putative Arp2/3 complex has been revealed by analysis of Arabidopsis genome [144], the subunits ARPC2 and ARP3 have been shown to complement mutants of their yeast homologs [37,87], which indicates that there exists a functional equivalent plant Arp2/3 complex. In non-plant systems, the mutation of Arp2/3 is lethal. The energy of Arp2/3 complex induced actin polymerization is harnessed directly to push the membrane, consequently drive cell motility. It has been shown that actin filaments form dendritic networks at the leading edge of the crawled cell [143]. In budding yeast, mutation of Arp2/3 complex lose their actin patches. And Arp2/3 complex induced actin polymerization was shown to push the endocytic vesicles away from the plasma membrane [73]. RNAi approach was employed to knock down components of the Arp2/3 complex in *Caenorhabditis elegans*, which induces cell migratory defects during the process of ventral enclosure [129]. Small interfering RNA (siRNA) transfection against components or regulators of Arp2/3 complex in *D. melanogaster* failed to form either lamellipodia or filopodia [126]. Drosophila embryos lacking ArpC1 subunit induce spindle fusions and fail to separate adjacent nuclei during cellularization [142,180].

Surprisingly, loss-of-function of Arp2/3 complex only produces very subtle phenotype in plant. No obvious growth phenotypes were detected, the architecture of mutant plant looks quite normal. In addition, the loss-of-function of Arp2/3 does not affect the seed set either, which indicates that Arp2/3 complex is not essential for male and/or female gametophyte development. However, null mutation of Arp2/3 does produce phenotype in several specific cell types including trichomes and leaf epidermal pavement cells. In the null mutant of Arp2/3 complex, the length of seedling hypocotyls and the size of epidermal cells are reduced compare to those of the wild type [100]. In both the hypocotyl and cotyledon epidermal cells, there exists severe cell-cell adhesion defects [37,87,100], the cause of cell adhesion defect in Arp2/3 null mutant is not very clear, but Arp2/3 may affect vesicle trafficking events and subsequently affect cell wall synthesis. Null mutation of Arp2/3 complex has obvious phenotype of trichome morphogenesis, the trichomes become distorted but grow rapidly, the stalks are swollen and twisted and the length of branch is reduced [37,87]. Actin cytoskeleton in Arp2/3 null mutants have been revealed with different approaches, including immuno-labeling and phalloidin labeling in fixed cells and ABD2 (actin binding domain2) fused with GFP labeling in living cell. However, there is discrepancy on the defect of actin distribution in Arp2/3 null mutants, which may be due, to some extent, to the strategies used for actin labeling. The defect of actin cytoskeleton reported for the trichomes of Arp2/3 loss-of-function mutants includes the excessive formation of actin bundles [100,101] and the spatial disorganization of actin bundles that becomes transverse through the cell [37,87].

Recently, orthologs of the Arp2/3 activator WAVE complex have also been identified in plant [10,31,34,36,43,44,88,182]. Mutation of the subunits of WAVE complex phenocopies the mutation of Arp2/3 complex [9,34,36,88,181,182], which indicates the existence of SCAR-Arp2/3 pathway, in particular, mutation of BRICK1 shows the same severe phenotype as the mutation of Arp2/3 [34,88] indicates that

SCAR is the primary regulator of Arp2/3 in plant. In addition, plant SCARs have been shown to promote actin polymerization in vitro by activation of the bovine Arp2/3 complex [10,43], providing direct biochemical evidence of the existence of SCAR-Arp2/3 pathway in plant.

To date, the conclusive subcellular localization of SCAR and Arp2/3 and its interaction is not achieved, though it was reported that Arp3 was localized into a dot-like structures in BY-2 cells and colocalize with actin filaments [94]. The subcellular localization of WAVE-Arp2/3 interaction will provide insights into the question about which population of actin filaments is initiated by Arp2/3. In addition, there is still a gap for us to understand the function of Arp2/3 in plant, given the biochemistry of plant Arp2/3 complex has never been assayed.

3.4 Formin

Formin is a large family of proteins sharing the evolutionarily conserved formin homology domains (FH1) and (FH2) that are present in nearly all eukaryotes [11,19,27]. The crystal structure of FH2 domain of Bni1p has been described and indicates that formin might nucleate actin in a dimer structure [118,169]. FH1 domain is often located N-terminally to the FH2 domain. It is characterized in the high content of prolines. Apart from the two FH domains, several other domains were identified in the N-terminus and C-terminus of different yeast or animals formin proteins, such as FH3 (formin homology 3) domain, GBD (GTPase binding domain), DAD (Dia-autoregulatory domain), BBS (Bud6p-binding site), TM (transmembrane domain) (for review, see [160]). However, the domain composition of plant formin is quite different from its counterparts in other organism, which has no FH3, GBD or DAD for localization and activation. Land plants possess three mutually divergent clades of FH2 proteins, with only two clades (termed Class I and Class II) present in seed plants [27,30]. While most Class I formins carry N-terminal membrane import sequences [26], Class II formins commonly possess a N-terminal domain related to the antioncogene PTEN-like domain, which, however, carries a significant sequence alteration at the presumed catalytic site and might thus mediate association with membranes rather than a catalytic phosphatase activity [27]. Recently, a novel third clade of plant formins, Class III, is described. Class III is present only in lower plants such as algae, mosses and lycophytes, and which carries a presumably catalytically inactive variant of the conserved RhoGAP domain [51].

Formins from yeast and animals nucleates actin filaments by a mechanism independent of the Arp2/3 complex, with which formin form dimers and act as a processive or “leaky” cap at the barbed ends (for a review see [39,81]). In addition, it also binds the side of the actin filament, leading to fragmentation of the filaments [55] and inducing the formation of actin bundles in vitro [56,110]. Studies on *Arabidopsis thiliana* formin homologues (AtFHs) have shown that some of the members are conserved in nucleating, partial capping or bundling activities. Like the counterparts from yeast, animal and fungi, plant formins, the FH1FH2 domain

of AtFH1, AtFH4, AtFH5, AtFH8 can nucleate actin filaments to form unbranched filaments, and the FH2 domain is the functional domain in nucleation [32,69,106,173]. The AtFHs FH1FH2 constructs also associate with the barbed end and change the rate of polymerization and depolymerization in a partial capping mode [69,106,173]. But the capping activity of AtFH1 is a little special in that the FH1FH2 construct works as a "leaky cap", but the FH2 construct is a tight cap that only allows filament elongation in the pointed end [106]. Bundling activity is also identified for AtFH1 [106] and AtFH8 (our unpublished data), and AtFH8 FH1FH2 construct can also sever actin filaments, which is the only one reported in plant formins [173].

Recently, significant progress toward understanding the cellular and molecular functions of class I formins including AtFH1, AtFH3, AtFH4, AtFH5, AtFH6, AtFH8 and a class II formin from *Physcomitrella*-For2. AtFH1 was the first plant formin characterized in vivo. Overexpression of AtFH1 in pollen tubes induces the formation of arrays of actin cables that project into the cytoplasm from the cell membrane. Slight increases in AtFH1 stimulate growth, but its overexpression induces tube broadening, growth depolarization, and growth arrest in transformed pollen tubes. These results suggest that AtFH1-regulated actin polymerization is important for the polarized pollen cell growth process [22]. Ingouff et al. found the loss of AtFH5 function perturbs proper morphogenesis of the endosperm posterior pole and AtFH5-GFP fusion protein accumulates in the cell plate required for cell division [69]. AtFH6 was isolated by Favery et al. using promoter trap strategy. AtFH6 was upregulated during giant cell formation and uniformly distributed to the plasma membrane. What's more, AtFH6 can suppress the budding defect of the *Saccharomyces cerevisiae bnr1Δbni1Δ* mutant, which indicated that AtFH6 regulated polarized growth by controlling the assembly of actin cables [41]. Studies using *AtFH8* overexpressing lines shows high level of AtFH8 can induce abnormal root hair phenotype, including wavy root hair, root hair with swelling tip, different degrees of branched root hairs, two root hairs from one hair-forming site and bulbous structure at root hair basis, all of which are indications of loss of cell polarity. The abnormal root hair phenotype was accompanied by the alternation of actin cytoskeleton distribution in these root hairs, which indicates it is an actin related process regulated by AtFH8 [173]. Other study conducted by Deeks et al. also shows that AtFH8 affect root and root hair development [32]. Very recently, Ye et al. reported Arabidopsis formin AFH3 is required for the assembly of actin cables in pollen tubes, providing the evidence that directly link the formins with the organization of the cytoplasmic actin cables in plants [172]. It is the first time for the formin from class II *Physcomitrella*-For2 being reported by Vidali et al. They found that For2 is an exceptionally rapid actin-elongating factor. By systematically silencing all 9 formin family members, they demonstrate that For2 is critical for polarized cell growth of polarized tip-growing protonemal cells [157]. The overall findings in plant formins suggest that AtFHs also play important roles in polarity, morphogenesis and cell division of plants.

Formins are large multidomain proteins that can interact with a variety of proteins necessary for the regulation of their functions. The ability to interact with small GTPases of the Rho family is apparently a conserved feature of FH2 proteins. Conserved domain architecture of fungal and metazoan formins carrying a

GTPase-binding domain (GBD/FH3), believed to mediate Rho3dependent Rho-dependent activation of the formin through releasing intramolecular inhibition, presents a good example of the ancient connection between Rho and actin organization [27,125]. Since there is no GBD found in plant formins, it seems that they cannot interact with ROPs (Rho of plants), but this has not been demonstrated in experiments. Two-hybrid screen for AtFH1 interactor identified FIP2 (formin interacting protein 2). Using recombinant proteins expressed in *E. coli,* it was demonstrated that FIP2 interacted directly with AtFH1. The amino acid sequence of FIP2 was partially homology to bacterial putative membrane proteins and animal A-type K+ ATPases. AtFH1 may form a membrane anchored complex with FIP2 and involve in the organization of the actin cytoskeleton [8]. The profilin-AtFHs interaction was also observed by in vitro pull down assay and yeast two-hybrid strategy for AtFH8 and AtFH4 [32,173], respectively. So far, the knowledge of the AtFHs interactors is so limit that they are lonely formin family members waiting for scientists to help them find their partners and put them in the proper signal pathway where they inherently locate.

Formins are big multidomain proteins that assemble profilin-actin to the barbed end and form long unbranched actin cables. The cable can be very long because of the capping activity of formins. They were well characterized in vitro by biochemistry experiments and all shown nucleation and capping activities that were variable among different members. Despite of the differences between members in structure and function, the nucleation mechanism seems to the conserved for formins. But efforts should be taken to further clarify this mechanism since the crystal structure of full length formin has not been obtained. Comparing to the knowledge of formins of yeast, animal and fungi, less is known about plant formins, especially for the class II and class III protein. Besides, little is known about the physiological function of the presence of multiple formin subfamilies in a cell. So, many of the following questions need to be resolved. Are there any functional differences between respective members both in vivo and in vitro? How different plant formins are regulated and what is the downstream and upstream of them? Whether different isoforms of formins present in a cell could act as heterodimers that would provide a fine regulatory modulation of actin organization?

3.5 Capping Protein (CP)

One fundamental way to regulate actin dynamics is by capping the end of actin filaments. Capping protein is known to bind to the barbed end of actin filaments, which is a heterodimer consisting of α- and β-subunits, with a molecular weight ranging from 28 to 36 kDa. It was called β-actinin, and was originally purified and characterized from muscle [97–99]. Nonmuscle CP was first purified from *Acanthamoeba* and demonstrated to cap the barbed end of actin filaments [70]. Both α- and β-subunit of CP were shown to be conserved between different organisms [29]. The crystal structure of chicken CapZ was resolved recently [171]. The study reveals that α- and

β-heterodimer forms a compact structure resembling a mushroom with pseudotwo-fold rotational symmetry. CP was reported to be a major actin regulator, e.g., it has been shown to be one of five ABPs that regulate actin polymerization and depolymerization to generate "comet-tail" motility in vitro [90]. CP is essential for several basic cellular processes. Mutation of CP in *Dictyostelium* reduced F-actin level, consequently affected cell motility [68]. Actin filaments are not stable in the mutants of yeast CP [4,5,77]. However, mutation of CP is lethal in *Drosophila* [61].

Two subunits of CP exist in *Arabidopsis* genome and both α- and β-subunit are encoded by single gene. The α-subunit of AtCP (AtCPA) shares 29–35% amino acid sequence identity with α-subunits from amoeba, yeast and vertebrate. The β-subunit of AtCP (AtCPB) shares relative higher amino acid identity (~50%) with β-subunits of *Dictyostelium* and vertebrate. However, the C-terminal is not very conserved comparing AtCP to CPs from other organisms [63]. Arabidopsis capping protein (AtCP) has been purified to homogeneity with the strategy of simultaneous expression of both α- and β-subunit in bacteria cells [63]. The purified recombinant AtCP nucleates actin assembly in vitro. AtCP also caps the barbed end of actin filaments to prevent the addition of profilin-actin complex during a seeded elongation reaction, and it prevents dilution-mediated actin depolymerization as well. The measurement of the binding constant to the barbed end of actin filaments gave a K_d values of 12–24 nM [63], which is higher than that of muscle CapZ [131], but it is not too much different from non-muscle capping protein [33,84,103,128] and recombinant *Schizosaccharomyces pombe* CP [83]. The divergence of the primary amino acid sequence at the C-termini of both subunits may account for these differences in ability to nucleate, and capping [67]. However, it should be noted that the dissociation rate constant of AtCP from barbed end is extremely low, corresponding to a half-time of dissociation about 38 min [64], which is roughly in the same time scale of muscle CapZ [131]. Considering the situation that actin is buffered by almost equal molar of profilin in plant cells [48,135,155], the higher affinity binding of AtCP to the barbed end of actin filaments will prevent the addition of profilin-actin complex into the barbed end of actin filaments, consequently leads to the phenomenon of low F-actin level in plant cells [48,135,162]. Indeed, in the presence of CP, the addition of profilin-actin into the barbed end is prevented [63]. AtCP was shown to bind to and be regulated by PtdIns(4,5)P_2 and phosphatidic acid (PA) [63,64], which may be relevant to the motility of organelles or the plasma membrane, given the inactivation of CP by the phospholipids is predicted to lead to the polymerization of actin filaments near the surface of organelles or the plasma membrane. However, the in vivo function of CP in plant still needs to be determined.

3.6 Actin Depolymerizing Factors (ADFs)

ADF is another major actin regulator in plants, which binds to both G-actin and F-actin with a small mass (15–22 kDa). ADFs have been shown to be stimulus responsive proteins, whose activity is regulated by pH, phospholipids and reversible

phosphorylation. It was shown very recently that AtADF4 was specifically required for resistance triggered by the effector AvrPphB, conferring its role in regulating actin dynamics mediating the activation of resistance response in Arabidopsis following inoculation with the phytopathogenic bacterium Pseudomonas syringae [147]. And also ADF2-mediated actin dynamics was shown to be essential for nematode infection of Arabidopsis [25]. ADFs were first identified in plants during search for pollen specific transcripts in *Lilium longiflorum* [79], and are present as a small multigene family in maize and Arabidopsis. Plant ADFs can be separated into two phylogenetic classes: vegetative ADFs and reproductive ADFs [74,127]. Plant ADFs belong to a relative big gene family. Twelve ADF isoforms are encoded in *Arabidopsis* genome, among which *Arabidopsis* ADF1 [13,17] is well studied biochemically. The deduced amino acid sequences of plant ADFs share only 28–35% identity with vertebrate ADF sequences. However, residues for putative actin binding and a presumed phosphorylation residue share greatest similarity with the corresponding site of mammalian ADFs [108].

AtADF1 is one of the best documented vegetative plant ADFs. Recombinant AtADF1 binds G-actin in a 1:1 complex, but with 100-fold preference for ADP-bound actin versus ATP-bound actin [17]. AtADF1 also binds to F-actin, with a preference for the ADP-bound form. However, AtADF1 interacts with F-actin with less pH-dependent manner compared with nonplant ADFs [17]. AtADF1 increased the initial rate of actin polymerization. This could be due to the combination of nucleation activity and severing activity. Indeed, it was reported that several nonplant ADFs have nucleation activity at their higher concentration and sever actin filaments efficiently [6]. Direct visualization of the effect of AtADF1 on actin dynamics with TIRFM will provide insight into the detailed action mechanism of AtADF1. In particular, it was shown very recently that the dynamics of plant cortical actin filaments is dominated by severing activity [140]. It will be important to find out if ADF is a major player here. In addition, the in vitro kinetic actin depolymerization assay showed that AtADF1 increases actin depolymerization rate by 22-fold at pointed end [17], which indicates that the binding of AtADF1 to F-actin may alter the association between two adjacent actin monomers, consequently increase the rate of actin monomer dissociation from pointed end. ZmADF3 is another well characterized vegetative ADF. It exhibits a pH-sensitive activity [53]. ZmADF3 binds F-actin at pH below 7.0 and it depolymerizes actin filaments at pH 8.0. The activity of ZmADF3 is specifically inhibited by the phosphoinositides PIP and PIP2 [53]. It was also shown that ZmADF3 can be phosphorylated on Ser-6 by a calmodulin domain-like protein kinase (CDPK) and that can regulate its activity [3,133]. ZmADF3 localized uniformly in initiating root hairs with no specific colocalizes with actin cables, and it was relocated to the tip of elongating root hairs [71]. It was proposed that ZmADF3 is essential for active tip growth. However, considering the higher calcium concentration in this region and phosphorylation of ADF by CDPK and, it is unlikely active in there. The precise in vivo role of ZmADF3 needs to be explored further in the future.

Reproductive ADFs share great identity with each other (70–94% identity) than with the vegetative ADFs (54–60% identity), suggesting generative ADFs may

conduct a conserved role in pollen development and function. The well characterized reproductive ADF is LiADF1. Like vegetative ADF, LiADF1 bound G-actin and F-actin, but it has different effect on actin dynamics. The severing activity of LiADF1 was shown to only have 20% of the activity of ZmADF3. And it was also shown that the activity of LiADF1 to activate actin disassembly at the pointed ends is only 10% of that of ZmADF3. The biochemical data suggested that LiADF1 is an inefficient actin depolymerizing factor [132]. This may be the reason to explain why pollen ADF decorates actin filaments in pollen grains and pollen tubes [132]. However, more biochemical and intracellular localization studies are needed to extend this conclusion to other pollen specific ADF isoforms.

The activity of ADF is highly dependent on the presence of other actin binding proteins, e.g., the presence of AtVLN1 suppresses the depolymerization activity of AtADF1 [66]. Whereas Aip1, another actin-interacting protein, containing 7–10 WD repeats, enhances the depolymerization activity of ADF in vitro. In the absence of ADF/cofilin, Aip1 interacts with actin filaments weakly; whereas in the presence of ADF/cofilin, Aip1 caps filament barbed ends and binds weakly along the sides of filaments [113–116]. Although recombinant AtAip1-1 enhances the weak depolymerizing activity of lily ADF1 in vitro [2], the precise molecular mechanism underlying the enhancement of ADF severing activity by Aip1 needs further analysis.

3.7 Villin/Gelsolin/Fragmin Superfamily Proteins

Villin/gelsolin/fragmin superfamily proteins, belonging to actin binding proteins, are identified by sharing three (fragmin, capG) or six (gelsolin, villin) 15 kDa gelsolin-like repeat domains [165]. All members in this family have the conserved gelsolin domains, and some of them have special amino acids sequences in N-(e.g. flightless I) or C-(e.g. Villin) terminals, the complicated protein structure leads to the diverse function in some extents. Villin/gelsolin/fragmin superfamily members can sever, cap, nucleate and bundle actin in a Ca^{2+} and/or PIP_2-regulated manners. Gelsolin, the founding member of the family with a molecular weight about 82–84 kDa, is composed of six gelsolin domains. It was first identified from macrophage extracts, and then another gelsolin is discovered in blood plasma [20] which is similar with those from cytoplasm [95,174]. Gelsolin had the Ca^{2+}-dependent abilities to severe, cap and nucleate actin filaments. While villin is originally isolated from the microfilament bundles of intestine epidermal microvillus [15,102], which has a special fragment called headpiece in C-terminal of its six gelsolin domains that makes the function of villin quite different from gelsolin [45,145]. Apart from bundling F-actin, villin could also sever actin and cap the barbed end of F-actin in the presence of millimolar Ca^{2+} [50,60,96]. However, villin is not simply formed by adding headpiece to gelsolin, because the gelsolin fused headpiece to C-terminal cannot generate villus as villin does, but villin without sequence of headpiece will destroy filament stress [42,46].

Severin/fragmin/CapG contain the former three domains of villin/gelsolin/fragmin superfamily (G1–G3), and their molecular mass are probably 40 kDa. Among the family, Severin was first detected in *Dictyostelium* [16]. It can sever actin, but this activity is inhibited by PPI. Fragmin is currently discovered in *Physarum* [47], which can sever, cap and nucleate actin. CapG is the only known member of the family that localized in the nucleus [117,120]. It can bind and cap the actin in vitro, but lack of severing activity [137].

The research of villin/gelsolin/fragmin superfamily in plant is lagged behind that in animal. P-135-ABP is the first plant villin purified from germinated pollen proteins [176], and the full-length cDNA encoding P-135-ABP is screened from *lilium* pollen cDNA expressing library by utilizing the specific antibody, subsequently [158]. This plant villin shows the ability to bind and bundle actin filaments in vitro [176,177] and in vivo [149]. In addition, this bundling activity is inhibited while there are coexisted Ca^{2+} and calmodulin with the micromolar concentration, but neither element alone has such an effect [175]. Recent research demonstrated that P-135-ABP also could bind to G-actin, nucleate the actin filament, accelerate the F-actin depolymerization and inhibit actin polymerization from barbed end in the presence of micromolar Ca^{2+} in vitro [178]. The analysis of *Arabidopsis* and the rice genome indicated that plant villin was encoded by multi-gene families, which is similar to other plant ABPs. So far as we know, *Arabidopsis* genome has five villins, named *AtVLN 1-AtVLN 5* [80,139]. The recombinant protein of AtVLN-GFP and AtVLN headpiece-GFP can bind to actin in vivo [80]. Surprisingly, unlike other reported plant villins, recombinant AtVLN1 are lack of the Ca^{2+}-dependent severing, capping, and nucleating activities in vitro. In addition, AtVLN1 only has the function of binding to actin and bundling F-actin in a Ca^{2+}-independent manner. AtVLN1 also could inhibit actin depolymerization by ADF/cofilin in vitro [66]. Our recent work on AtVLN4 shows that it promotes punctate actin foci formation in the pollen tube treated by Ca^{2+} ionophore A23187 (Fig. 3.1).

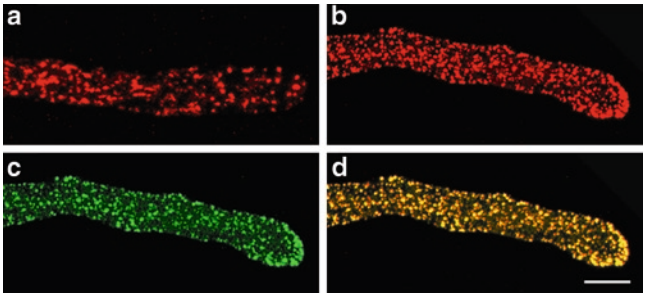

Fig. 3.1 AtVLN4 promotes punctate actin foci formation in tobacco pollens. Actin organization was imaged using rhodamine-phalloidin staining. (**a**) Actin organization in the control pollen tube; (**b**) Actin organization in the AtVLN4-GFP transformed tobacco pollen treated with 10 μM A23187 in the presence of 200 μM Ca^{2+}; (**c**) AtVLN4-GFP; (**d**) Merged image of (**b**) and (**c**); Bar = 10 μm

Bioinformatics analysis shows that there are no genes solely encoding gelsolin/fragmin family members in the genome data-base of *Arabidopsis* and *Oryza sativa* L., which are encoded by individual genes in animal cells instead. Nevertheless, utilizing the method of immunoblotting, gelsolin has been detected in maize and *lilium* pollen [146,167]. The first plant gelsolin-like protein named PrABP80 is also purified from poppy pollen by affinity chromatography on DNase I-Sepharose, and mass spectrum analysis indicates that it is highly similar to P-135-ABP and villins in *Arabidopsis*. Moreover, PrABP80 is capable to sever, cap and nucleate actin filament, as well as accelerate actin depolymerization mediated by profilin in vitro, these activities are all regulated by Ca^{2+} [62]. Furthermore, there are also some reports about fragmin-like proteins in plant, for example, a fragmin-like protein was detected in *mimosa* with a molecular weight about 42 kDa, and it can accelerate actin polymerization in vitro [170]. LdABP41, isolated from *lilium* pollen, is highly homologous to G1–G3 domains of P-135-ABP [40]. Biochemical analysis shows that it could sever, cap and nucleate actin in a Ca^{2+}-regulated manner. From above and other results, it is well accepted that gelsolin/fragmin family members are generated by mRNA alternative splicing from plant villin [40,62,139]. Recently, this hypothesis is confirmed by the discovery of ABP29 in lily pollen. In their assay, Xiang et al. (168) has cloned a 1,006-bp full-length cDNA sequence from *lilium* pollen, apart from the 16 bp sequence starting with GT before the stop codon TAA and the whole 3'UTR, which is absolutely identical to P-135-ABP. The sequence encodes a 29-kDa protein (ABP29) that merely contains G1 and G2 domains, which is the smallest member in villin/gelsolin/fragmin superfamily. ABP29 can sever, nucleate, cap F-actin in vitro and these activities are all Ca^{2+} and PIP_2 regulated. Further study shows that the specific sequences of ABP29 are derived from the intron of the gene encoding P-135-ABP. In addition, GT at acceptor (5') splice site is a conserved sequence for the majority of introns, together strongly suggests that ABP29 is a mRNA alternative splicing product of a pre-ended transcription from plant villin. Lately, the expression pattern of the villin/gelosolin/framin superfamily proteins during lily pollen tube development was detected using the gel blot analysis. Due to the high homology among this superfamily, the purified anti-LdABP41 antibody can recognize ABP29 [168], LdABP41 [40], ABP80 [62], ABP115 [112,179] and ABP135 [176] from dehydrated, hydrated or germinating lily pollen grains, however, their expressed levels changed greatly in the different stages. It is showed that LdABP41 is abundant in the ungerminated pollen, however, the amount of LdABP41 decreases dramatically, and ABP80, ABP-115 and ABP135 merely turn up after the pollen germinated, but ABP29 levels remain almost constant. Furthermore, the specific expression patterns of different members correlate highly with actin architecture corresponding to different pollen stages [163,168]. This assay also provided us with strong evidence that gelsolin/fragmin members are not the degradation product from villins. Thus, plant villin may generate diverse gelsolin/fragmin members via mRNA alternative splicing in precise patterns to regulate the actin dynamics, which is an important mechanism for them to perform the distinct physiological functions.

In plant, many studies demonstrate that villin/gelsolin/fragmin superfamily members not only have disparate activities in vitro, but also differ in cellular localizations and functions in vivo. According to the analysis of promoter-GUS fusions transgenic plant, AtVLN genes of Arabidopsis villin are expressed in all organs, and they show different expression levels in certain type of cells [80]. Moreover, the result of western blotting utilizing the anti-villin antibody reveals that AtVLNs widely exist in numerous plant tissues. These results indicate that plant villin does not possess special functions and distributions, whereas mammalian villins are expressed only in the microvilli of brush border cells. P-135-ABP is colocalized with the actin cytoskeleton in lily pollen tube [158,176] and root hair cells [149]. Microinjection of the P-135-ABP antibody into the root hair cells leads to the microfilament become thinner, and affect the orientation of cytoplastic streaming [149]. This investigation demonstrates that P-135-ABP may participate in forming actin bundles in the shank of pollen tube [178]. Gelsolin is localized in lily pollen and the tip of pollen tube; this character hints that it may take part in controlling pollen tube germinating [146]. PrABP80 is possible as a Ca^{2+}sensor to regulate actin dynamic during self-incompatibility response in poppy [62]. Furthermore, microinjecting the antibody against LdABP41 into lily pollen could inhibit pollen tube growth, and increase its width [40]. Further microinjection assays indicate that LdABP41 cause the fragment of actin filaments in vivo, inhibit the velocity of cytoplasmic streaming and shorten the length of clear zone in the microinjected pollen tubes [163]. Immunofluorescence localization assay also shows that LdABP41 is expressed in the cytoplasm and especially localized in the tip of the pollen tube. Above studies suggest that LdABP41 may be concerned with dynamic short actin structure in the apical region of pollen tube, and play a crucial role in pollen tube growth [40]. Increasing the amount of ABP29 will destroy the actin architecture in stamen hair cell and pollen tube, as well as inhibit the pollen germination and pollen tube growth. All consequences indicate that villin/gelsolin/fragmin superfamily members may be crucial in pollen tube polar growth. In addition, abundant evidences revealing that the activities of the family members, such as LlABP29, LdABP41, PrABP80 and 135-ABP, are regulated by Ca^{2+} and/or PIP_2 in vitro. Hence, we presume that villin/gelsolin/fragmin superfamily proteins might be the downstream substrate of these signal factors, and participate in certain signal pathways. However, it is not clear how villin/gelsolin/fragmin superfamily members localize and play their roles in cells at present.

3.8 Conclusions

In recent years, many roles of the actin cytoskeleton in plant cells are found revolve around its “dynamics”. However, how it functions is still poorly understood. Although advances in the characterization of actin-binding proteins from plants reveal unique features of them compared to mammalian or yeast proteins, little is known about the connection of these insights with an understanding of the dynamic

properties and the exact functions of actin filament structures in plant cells. The dynamics of cortical actin network and its regulation are well studied in protrusion of migrating animal cells, which can generate forces that are necessary for the cell movement. There also exist some processes that are likely to depend on actin-based force generation by the dynamic actin cytoskeleton in the plant cell, such as secretory/endocytic vesicle traffic, polarized cell expansion and vesicle transportation during cytokinesis though plant cell does not move, while the force generation by the dynamic actin cytoskeleton in plant cells has not been well studied. It would therefore be of considerable interest for researchers in the field to understand the function underlying cellular actin dynamics and how the different actin binding proteins cooperatively contribute to build up the dynamic actin structures.

References

1 Ahuja R, Pinyol R, Reichenbach N, Custer L, Klingensmith J, Kessels MM, Qualmann B (2007) Cordon-bleu is an actin nucleation factor and controls neuronal morphology. Cell 131:337–350
2 Allwood EG, Anthony RG, Smertenko AP, Reichelt S, Drobak BK, Doonan JH, Weeds AG, Hussey PJ (2002) Regulation of the pollen-specific actin-depolymerizing factor LlADF1. Plant Cell 14:2915–2927
3 Allwood EG, Smertenko AP, Hussey PJ (2001) Phosphorylation of plant actin-depolymerising factor by calmodulin-like domain protein kinase. FEBS Lett 499:97–100
4 Amatruda JF, Gattermeir DJ, Karpova TS, Cooper JA (1992) Effects of null mutations and overexpression of capping protein on mor phogenesis, actin distribution and polarized secretion in yeast. J Cell Biol 119:1151–1162
5 Amatruda JF, Cannon JF, Tatchell K, Hug C, Cooper JA (1990) Disruption of the actin cytoskeleton in yeast capping protein mutants. Nature 344:352–354
6 Andrianantoandro E, Pollard TD (2006) Mechanism of actin filament turnover by severing and nucleation at different concentrations of ADF/cofilin. Mol Cell 24:13–23
7 Baluska F, von Witsch M, Peters M, Hlavacka A, Volkmann D (2001) Mastoparan alters subcellular distribution of profilin and remodels F-actin cytoskeleton in cells of maize root apices. Plant Cell Physiol 42:912–922
8 Banno H, Chua NH (2000) Characterization of the arabidopsis formin-like protein AFH1 and its interacting protein. Plant Cell Physiol 41: 617–626
9 Basu D, El-Assal Sel D, Le J, Mallery EL, Szymanski DB (2004) Interchangeable functions of Arabidopsis PIROGI and the human WAVE complex subunit SRA1 during leaf epidermal development. Development 131:4345–4355
10 Basu D, Le J, El-Essal Sel D, Huang S, Zhang C, Mallery EL, Koliantz G, Staiger CJ, Szymanski DB (2005) DISTORTED3/SCAR2 is a putative arabidopsis WAVE complex subunit that activates the Arp2/3 complex and is required for epidermal morphogenesis. Plant Cell 17:502–524
11 Blanchoin L, Staiger CJ (2008) Plant formins: Diverse isoforms and unique molecular mechanism. Biochim Biophys Acta. 1803:201–206
12 Blasco R, Cole NB, Moss B (1991) Sequence analysis, expression, and deletion of a vaccinia virus gene encoding a homolog of profilin, a eukaryotic actin-binding protein. J Virol 65: 4598–4608
13 Bowman GD, Nodelman IM, Hong Y, Chua NH, Lindberg U, Schutt CE (2000) A comparative structural analysis of the ADF/cofilin family. Proteins 41:374–384

14 Braun M, Baluska F, von Witsch M, Menzel D (1999) Redistribution of actin, profilin and phosphatidylinositol-4, 5-bisphosphate in growing and maturing root hairs. Planta 209: 435–443
15 Bretscher A, Weber K (1979) Villin: the major microfilament-associated protein of the intestinal microvillus. Proc Natl Acad Sci USA 76:2321–2325
16 Brown SS, Yamamoto K, Spudich JA (1982) A 40,000-dalton protein from Dictyostelium discoideum affects assembly properties of actin in a Ca^{2+}-dependent manner. J Cell Biol 93: 205–210
17 Carlier MF, Laurent V, Santolini J, Melki R, Didry D, Xia GX, Hong Y, Chua NH, Pantaloni D (1997) Actin depolymerizing factor (ADF/cofilin) enhances the rate of filament turnover: implication in actin-based motility. J Cell Biol 136:1307–1322
18 Carlsson L, Nystrom LE, Sundkvist I, Markey F, Lindberg U (1977) Actin polymerizability is influenced by profilin, a low molecular weight protein in non-muscle cells. J Mol Biol 115: 465–483
19 Chalkia D, Nikolaidis N, Makalowski W, Klein J, Nei M (2008) Origins and evolution of the formin multigene family that is involved in the formation of actin filaments. Mol Biol Evol 25:2717–2733
20 Chaponnier C, Borgia R, Rungger-Brandle E, Weil R, Gabbiani G. (1979) An actin-destabilizing factor is present in human plasma. Experientia 35:1039–1041
21 Chereau D, Boczkowska M, Skwarek-Maruszewska A, Fujiwara I, Hayes DB, Rebowski G, Lappalainen P, Pollard TD, Dominguez R (2008) Leiomodin is an actin filament nucleator in muscle cells. Science 320:239–243
22 Cheung AY, Wu HM (2004) Overexpression of an arabidopsis formin stimulates supernumerary actin cable formation from pollen tube cell membrane. Plant Cell 16:257–269
23 Christensen HE, Ramachandran S, Tan CT, Surana U, Dong CH, Chua NH (1996) Arabidopsis profilins are functionally similar to yeast profilins: identification of a vascular bundle-specific profilin and a pollen-specific profilin. Plant J 10:269–279
24 Cleary AL, Gunning BES, Wasteneys GO and Hepler PK (1992) Microtubule and F-actin dynamics at the division site in living Tradescantia stamen hair cells. J Cell Sci 103: 977–988
25 Clement M, Ketelaar T, Rodiuc N, Banora MY, Smertenko A, Engler G, Abad P, Hussey PJ, de Almeida EJ (2009) Actin-depolymerizing factor2-mediated actin dynamics are essential for root-knot nematode infection of arabidopsis. Plant Cell 21:2963–2973
26 Cvrčková F (2000) Are plant formins integral membrane proteins? Genome Biol 1:1–7
27 Cvrčková F, Novotný M, Pícková D, Zárský V (2004) Formin homology 2 domains occur in multiple contexts in angiosperms. BMC Genomics (5):1471–2164
28 Cooley L, Verheyen E, Ayers K (1992) chickadee encodes a profilin required for intercellular cytoplasm transport during Drosophila oogenesis. Cell 69:173–184
29 Cooper JA, Sept D (2008) New insights into mechanism and regulation of actin capping protein. Int Rev Cell Mol Biol 267:183–206
30 Deeks MJ, Hussey PJ, Davies B (2002) Formins: intermediates in signal-transduction cascades that affect cytoskeletal reorganization. Trends Plant Sci 7:492–498
31 Deeks MJ, Kaloriti D, Davies B, Malho R, Hussey PJ (2004) Arabidopsis NAP1 is essential for Arp2/3-dependent trichome morphogenesis. Curr Biol 14:1410–1414
32 Deeks MJ, Cvrcková F, Machesky LM, Mikitová V, Ketelaar T, Zársky V, Davies B, Hussey PJ (2005) *Arabidopsis* group Ie formins localize to specific cell membrane domains, interact with actinbinding proteins and cause defects in cell expansion upon aberrant expression. New Phytologist 168:529–540
33 DiNubile MJ, Cassimeris L, Joyce M, Zigmond SH (1995) Actin filament barbed-end capping activity in neutrophil lysates: the role of capping protein-beta 2. Mol Biol Cell 6:1659–1671
34 Djakovic S, Dyachok J, Burke M, Frank MJ, Smith LG (2006) BRICK1/HSPC300 functions with SCAR and the ARP2/3 complex to regulate epidermal cell shape in Arabidopsis. Development 133:1091–1100

35 Drøbak BK, Watkins PAC, Valenta R, Dove SK, Lloyd CW, Staiger CJ (1994) Inhibition of plant plasma membrane phosphoinositide phospholipase C by the actin-binding protein, profilin. Plant J 6:389–400
36 El-Assal Sel D, Le J, Basu D, Mallery EL, Szymanski DB (2004) Arabidopsis GNARLED encodes a NAP125 homolog that positively regulates ARP2/3. Curr Biol 14:1405–1409
37 El-Din El-Assal S, Le J, Basu D, Mallery EL, Szymanski DB (2004) DISTORTED2 encodes an ARPC2 subunit of the putative Arabidopsis ARP2/3 complex. Plant J 38:526–538
38 Eleftheriou EP, Palevitz BA (1992) The effect of cytochalasin D on preprophase band organization in root tip cells of Allium. J Cell Sci 103:989–998
39 Faix J, Grosse R (2006) Staying in shape with formins. Dev Cell 10:693–706
40 Fan X, Hou J, Chen X, Chaudhry F, Staiger CJ, Ren, H (2004) Identification and characterization of a Ca^{2+}-dependent actin filament-severing protein from lily pollen. Plant Physiol 136:3979–3989
41 Favery B, Chelysheva LA, Lebris M, Jammes F, Marmagne A, De Almeida-Engler J, Lecomte P, Vaury C, Arkowitz RA, Abad P (2004) Arabidopsis formin AtFH6 is a plasma membrane–associated protein upregulated in giant cells induced by parasitic nematodes. Plant Cell 16:2529–2540
42 Finidori J, Friederich E, Kwiatkowski DJ, Louvard D (1992) In vivo analysis of functional domains from villin and gelsolin. J Cell Biol 116:1145–1155
43 Frank M, Egile C, Dyachok J, Djakovic S, Nolasco M, Li R, Smith LG (2004) Activation of Arp2/3 complex-dependent actin polymerization by plant proteins distantly related to Scar/WAVE. Proc Natl Acad Sci USA 101:16379–16384
44 Frank MJ, Cartwright HN, Smith LG (2003) Three Brick genes have distinct functions in a common pathway promoting polarized cell division and cell morphogenesis in the maize leaf epidermis. Development 130:753–762
45 Friederich E, Louvard D (1999) Villin. In: Kreis T, Vale R (eds) Guidebook to the cytoskeletal and motor proteins. Oxford University New York pp:175–179
46 Friederich E, Pringault E, Arpin M, Louvard D (1990) From the structure to the function of villin, an actin-binding protein of the brush border. Bioessays 12: 403–408
47 Furuhashi K, Hatano S (1989) A fragmin-like protein from plasmodium of Physarum polycephalum that severs F-actin and caps the barbed end of F-actin in a Ca^{2+}-sensitive way. J Biochem 106:311–318
48 Gibbon BC, Kovar DR, Staiger CJ (1999) Latrunculin B has different effects on pollen germination and tube growth. Plant Cell 11:2349–2363
49 Gibbon BC, Zonia LE, Kovar DR, Hussey PJ, Staiger CJ (1998) Pollen profilin function depends on interaction with proline-rich motifs. Plant Cell 10: 981–993
50 Glenney JR, Bretscher A, Weber K (1980) Calcium control of the intestinal microvillus cytoskeleton: its implications for the regulation of microfilament organizations. Proc Natl Acad Sci USA 77:6458–6462
51 Grunt M, Zárský V, Cvrčková F (2008) Roots of angiosperm formins: the evolutionary history of plant FH2 domain3containing proteins. BMC Evol Biol 8:1319
52 Guillen G., Valdes-Lopez V, Noguez R, Olivares J, Rodriguez-Zapata LC, Perez H, Vidali L, Villanueva MA, Sanchez F (1999) Profilin in Phaseolus vulgaris is encoded by two genes (only one expressed in root nodules) but multiple isoforms are generated in vivo by phosphorylation on tyrosine residues. Plant J 19:497–508
53 Gungabissoon RA, Jiang CJ, Drobak BJ, Maciver SK, Hussey PJ (1998) Interaction of maize actin-depolymerizing factor with F-actin and phosphoinositides and its inhibition of plant phospholipase C. Plant J 16:689–696
54 Haarer BK, Petzold AS, Brown SS (1993) Mutational analysis of yeast profilin. Mol Cell Biol 13:7864–7873
55 Harris ES, Li F, Higgs HN (2004) The mouse formin, FRLa, slows actin filament barbed end elongation, competes with capping protein, accelerates polymerization from monomers, and severs filaments. J Biol Chem 279:20076–20087

56 Harris ES, Rouiller I, Hanein D, Higgs HN (2006) Mechanistic differences in actin bundling activity of two mammalian formins, FRL1 and mDia2. J Biol Chem 281:14383–14392
57 Haugwitz M, Noegel AA, Rieger D, Lottspeich F, Schleicher M (1991) Dictyostelium discoideum contains two profilin isoforms that differ in structure and function. J Cell Sci 100: 481–489
58 Hepler PK, Cleary AL, Gunning BES, Wadsworth P, Wasteneys GO, Zhang DH (1993) Cytoskeletal dynamics in living plant cells. Cell Biol Int 17:127–142
59 Hess MW, Valenenta R (1997) Profilin revealed in pollen nuclei: immuno-electron microscopy of high-pressure frozen Ledebouria socialis Roth (Hyacinthaceae). Sex Plant Report 10: 283–287
60 Hesterberg LK, Weber K (1983) Demonstration of three distinct calcium-binding sites in villin, a modulator of actin assembly. J Biol Chem 258:365–369
61 Hopmann R, Cooper JA, Miller KG (1996) Actin organization, bristle morphology, and viability are affected by actin capping protein mutations in Drosophila. J Cell Biol 133:1293–1305
62 Huang S, Blanchoin L, Chaudhry F, Franklin-Tong VE, Staiger CJ (2004) A gelsolin-like protein from Papaver rhoeas pollen (PrABP80) stimulates calcium-regulated severing and depolymerization of actin filaments. J Biol Chem 279:23364–23375
63 Huang S, Blanchoin L, Kovar DR, Staiger CJ (2003) Arabidopsis capping protein (AtCP) is a heterodimer that regulates assembly at the barbed ends of actin filaments. J Biol Chem 278: 44832–44842
64 Huang S, Gao L, Blanchoin L, Staiger CJ (2006) Heterodimeric capping protein from Arabidopsis is regulated by phosphatidic acid. Mol Biol Cell 17:1946–1958
65 Huang S, McDowell JM, Weise MJ, Meagher RB (1996) The Arabidopsis profilin gene family. Evidence for an ancient split between constitutive and pollen-specific profilin genes. Plant Physiol 111:115–126
66 Huang S, Robinson RC, Gao LY, Matsumoto T, Brunet A, Blanchoin L, Staiger CJ (2005) Arabidopsis VILLIN1 generates actin filament cables that are resistant to depolymerization. Plant Cell 17:486–501
67 Hug C, Jay PY, Reddy I, McNally JG, Bridgman PC, Elson EL, Cooper JA (1995) Capping protein levels influence actin assembly and cell motility in dictyostelium. Cell 81:591–600
68 Hug C, Miller TM, Torres MA, Casella JF, Cooper JA (1992) Identification and characterization of an actin-binding site of CapZ. J Cell Biol 116:923–931
69 Ingouff M, Gerald JNF, Guérin C, Robert H, Sorensen MB, Van Damme D, Geelen D, Blanchoin L, Berger F (2005) Plant formin AtFH5 is an evolutionarily conserved actin nucleator involved in cytokinesis. Nat Cell Biol 7:374–380
70 Isenberg G, Aebi U, Pollard TD (1980) An actin-binding protein from Acanthamoeba regulates actin filament polymerization and interactions. Nature 288:455–459
71 Jiang CJ, Weeds AG, Hussey PJ (1997) The maize actin-depolymerizing factor, ZmADF3, redistributes to the growing tip of elongating root hairs and can be induced to translocate into the nucleus with actin. Plant J 12:1035–1043
72 Kakimoto T, Shibaoka H (1988) Cytoskeletal ultrastructure of phragmoplast-nuclei complexes isolated from cultured tobacco cells. Protoplasma (suppl) 2:95–103
73 Kaksonen M, Sun Y, Drubin DG (2003) A pathway for association of receptors, adaptors, and actin during endocytic internalization. Cell 115:475–487
74 Kandasamy MK, Burgos-Rivera B, McKinney EC, Ruzicka DR, Meagher RB (2007) Classspecific interaction of profilin and ADF isovariants with actin in the regulation of plant development. Plant Cell 19:3111–3126
75 Kandasamy MK, McKinney EC, Meagher RB (2002) Plant profilin isovariants are distinctly regulated in vegetative and reproductive tissues. Cell Motil Cytoskeleton 52:22–32
76 Karakesisoglou I, Schleicher M, Gibbon BC, Staiger CJ (1996) Plant profilins rescue the aberrant phenotype of profilin-deficient Dictyostelium cells. Cell Motil Cytoskeleton 34: 36–47

77 Karpova TS, Tatchell K, Cooper JA (1995) Actin filaments in yeast are unstable in the absence of capping protein or fimbrin. J Cell Biol 131:1483–1493
78 Kengen HMP, Eygensteyn J, van Amstel TNM (1995) F-actin in mitotic spindles of synchronized suspension culture cells of tobacco visualized by confocal laser scanning microscopy. Cell Biol Int 19:585–592
79 Kim SR, Kim Y, An G (1993) Molecular cloning and characterization of anther-preferential cDNA encoding a putative actin-depolymerizing factor. Plant Mol Biol 21:39–45
80 Klahre U, Friederich E, Kost B, Louvard D, Chua NH (2000) Villin-like actin-binding proteins are expressed ubiquitously in Arabidopsis. Plant Physiol 122:35–48
81 Kovar DR (2006) Molecular details of formin-mediated actin assembly. Curr Opin in Cell Biol 18:11–17
82 Kovar DR, Drobak BK, Staiger CJ (2000) Maize profilin isoforms are functionally distinct. Plant Cell 12:583–598
83 Kovar DR, Wu JQ, Pollard TD (2005) Profilin-mediated competition between capping protein and formin Cdc12p during cytokinesis in fission yeast. Mol Biol Cell 16:2313–2324
84 Kuhlman PA, Fowler VM (1997) Purification and characterization of an alpha 1 beta 2 isoform of CapZ from human erythrocytes: cytosolic location and inability to bind to Mg2+ ghosts suggest that erythrocyte actin filaments are capped by adducin. Biochemistry 36:13461–13472
85 Kwiatkowski DJ, Bruns GA (1988) Human profilin. Molecular cloning, sequence comparison, and chromosomal analysis. J Biol Chem 263:5910–5915
86 Lassing I, Lindberg U (1985) Specific interaction between phosphatidylinositol 4,5-bisphosphate and profilactin. Nature 314:472–474
87 Le J, El-Assal Sel D, Basu D, Saad ME, Szymanski DB (2003) Requirements for Arabidopsis ATARP2 and ATARP3 during epidermal development. Curr Biol 13:1341–1347
88 Le J, Mallery EL, Zhang C, Brankle S, Szymanski DB (2006) Arabidopsis BRICK1/HSPC300 is an essential WAVE-complex subunit that selectively stabilizes the Arp2/3 activator SCAR2. Curr Biol 16:895–901
89 Limmongkon A, Giuliani C, Valenta R, Mittermann I, Heberle-Bors E, Wilson C (2004) MAP kinase phosphorylation of plant profilin. Biochem Biophys Res Commun 324: 382–386
90 Loisel TP, Boujemaa R, Pantaloni D, Carlier MF (1999) Reconstitution of actin-based motility of Listeria and Shigella using pure proteins. Nature 401:613–616
91 Lu J, Pollard TD (2001) Profilin binding to poly-L-proline and actin monomers along with ability to catalyze actin nucleotide exchange is required for viability of fission yeast. Mol Biol Cell 12:1161–1175
92 Machesky LM, Atkinson SJ, Ampe C, Vandekerckhove J, Pollard TD (1994) Purification of a cortical complex containing two unconventional actins from Acanthamoeba by affinity chromatography on profilin-agarose. J Cell Biol 127:107–115
93 Machesky LM, Poland TD (1993) Profilin as a potential mediator of membrane-cytoskeleton communication. Trends Cell Biol 3:381–385
94 Maisch J, Fiserova J, Fischer L, Nick P (2009) Tobacco Arp3 is localized to actin-nucleating sites in vivo. J Exp Bot 60:603–614
95 Markey F, Larsson H, Weber K, Lindberg U (1982) Nucleation of actin polymerization from profilactin. Opposite effects of different nuclei. Biochim Biophys Acta 704:43–51
96 Markus MA, Nakayama T, Matsudaira P, Wagner G (1994) Solution structure of villin 14T, a domain conserved among actin-severing proteins. Protein Sci 3:70–81
97 Maruyama K (1965) A new protein-factor hindering network formation of F-actin in solution. Biochim Biophys Acta 94:208–225
98 Maruyama K (1966) Effect of beta-actinin on the particle length of F-actin. Biochim Biophys Acta 126:389–398
99 Maruyama K, Kimura S, Ishi T, Kuroda M, Ohashi K (1977) beta-actinin, a regulatory protein of muscle. Purification, characterization and function. J Biochem 81:215–232

100 Mathur J, Mathur N, Kernebeck B, Hulskamp M (2003) Mutations in actin-related proteins 2 and 3 affect cell shape development in Arabidopsis. Plant Cell 15:1632–1645
101 Mathur J, Mathur N, Kirik V, Kernebeck B, Srinivas BP, Hulskamp M (2003) Arabidopsis CROOKED encodes for the smallest subunit of the ARP2/3 complex and controls cell shape by region specific fine F-actin formation. Development 130:3137–3146
102 Matsudaira PT, Burgess DR (1979) Identification and organization of the components in the isolated microvillus cytoskeleton. J Cell Biol 83:667–673
103 Maun NA, Speicher DW, DiNubile MJ, Southwick FS (1996) Purification and properties of a Ca(2+)-independent barbed-end actin filament capping protein, CapZ, from human polymorphonuclear leukocytes. Biochemistry 35:3518–3524
104 McKenna ST, Vidali L, Hepler PK (2004) Profilin inhibits pollen tube growth through actin-binding, but not poly-L-proline-binding. Planta 218:906–915
105 McKinney EC, Kandasamy MK, Meagher RB (2001) Small changes in the regulation of one Arabidopsis profilin isovariant, PRF1, alter seedling development. Plant Cell 13: 1179–1191
106 Michelot A, Guérin C, Huang SJ, Ingouff M, Richard S, Rodiuc N, Staiger CJ, Blanchoin L (2005) The formin homology 1 domain modulates the actin nucleation and bundling activity of *Arabidopsis* FORMIN1. Plant Cell 17:2296–2313
107 Mineyuki Y, Palevitz BA (1990) Relationship between preprophase band organization, F-actin and the division site in Allium. J Cell Sci 97:283–295
108 Moon A, Drubin DG (1995) The ADF/cofilin proteins: stimulus-responsive modulators of actin dynamics. Mol Biol Cell 6:1423–1431
109 Morales S, Jimenez-Lopez JC, Castro AJ, Rodriguez-Garcia MI, Alche JD (2008) Olive pollen profilin (Ole e 2 allergen) co-localizes with highly active areas of the actin cytoskeleton and is released to the culture medium during in vitro pollen germination. J Microsc 231: 332–341
110 Moseley JB, Goode BL (2005) Differential activities and regulation of S. cerevisiae formins Bni1 and Bnr1 by Bud6. J Biol Chem 280(30):28023–28033
111 Mullins RD, Heuser JA, Pollard TD (1998) The interaction of Arp2/3 complex with actin: nucleation, high affinity pointed end capping, and formation of branching networks of filaments. Proc Natl Acad Sci USA 95:6181–6186
112 Nakayasu T, Yokota E, Shimmen T (1998) Purification of an actin-binding protein composed of 115-kDa polypeptide from pollen tubes of lily. Biochem Biophys Res Commun 249: 61–65
113 Okada K, Blanchoin L, Abe H, Chen H, Pollard TD, Bamburg JR (2002) Xenopus actin-interacting protein 1 (XAip1) enhances cofilin fragmentation of filaments by capping filament ends. J Biol Chem 277:43011–43016
114 Okada K, Obinata T, Abe H (1999) XAIP1: a Xenopus homologue of yeast actin interacting protein 1 (AIP1), which induces disassembly of actin filaments cooperatively with ADF/cofilin family proteins. J Cell Sci 112(pt 10):1553–1565
115 Okada K, Ravi H, Smith EM, Goode BL (2006) Aip1 and cofilin promote rapid turnover of yeast actin patches and cables: a coordinated mechanism for severing and capping filaments. Mol Biol Cell 17:2855–2868
116 Ono S, Mohri K, Ono K (2004) Microscopic evidence that actin-interacting protein 1 actively disassembles actin-depolymerizing factor/Cofilin-bound actin filaments. J Biol Chem 279:14207–14212
117 Onoda K, Yu FX, Yin HL (1993) gCap39 is a nuclear and cytoplasmic protein. Cell Motil Cytoskeleton 26:227–238
118 Otomo T, Otomo C, Tomchick DR, Machius M, Rosen MK (2005) Structural basis of Rho GTPase-mediated activation of the formin mDia1. Mol Cell 18:273–281
119 Perelroizen I, Didry D, Christensen H, Chua NH, Carlier MF (1996) Role of nucleotide exchange and hydrolysis in the function of profilin in action assembly. J Biol Chem 271: 12302–12309

120 Prendergast GC, Ziff EB (1991) Mbh 1: a novel gelsolin/severin-related protein which binds actin in vitro and exhibits nuclear localization in vivo. EMBO J 10:757–766
121 Pruyne D, Evangelista M, Yang C, Bi E, Zigmond S, Bretscher A, Boone C (2002) Role of formins in actin assembly: nucleation and barbed-end association. Science 297:612–615
122 Quinlan ME, Heuser JE, Kerkhoff E, Mullins RD (2005) Drosophila Spire is an actin nucleation factor. Nature 433:382–388
123 Ramachandran S, Christensen HE, Ishimaru Y, Dong CH, Chao-Ming W, Cleary AL, Chua NH (2000) Profilin plays a role in cell elongation, cell shape maintenance, and flowering in Arabidopsis. Plant Physiol 124:1637–1647
124 Ren H, Xiang Y (2007) The function of actin-binding proteins in pollen tube growth. Protoplasma 230:171–182
125 Rivero F, Muramoto T, Meyer AK, Urushihara H, Uyeda TQ, Kitayama C (2005) A comparative sequence analysis reveals a common GBD/FH3-FH1-FH2-DAD architecture in formins from Dictyostelium, fungi and metazoa. BMC Genomics 6: 28
126 Rogers SL, Wiedemann U, Stuurman N, Vale RD (2003) Molecular requirements for actin-based lamella formation in Drosophila S2 cells. J Cell Biol 162:1079–1088
127 Ruzicka DR, Kandasamy MK, McKinney EC, Burgos-Rivera B, Meagher RB (2007) The ancient subclasses of Arabidopsis actin depolymerizing factor genes exhibit novel and differential expression. Plant J 52:460–472
128 Sauterer RA, Eddy RJ, Hall AL, Condeelis JS (1991) Purification and characterization of aginactin, a newly identified agonist-regulated actin-capping protein from Dictyostelium amoebae. J Biol Chem 266:24533–24539
129 Sawa M, Suetsugu S, Sugimoto A, Miki H, Yamamoto M, Takenawa T (2003) Essential role of the C. elegans Arp2/3 complex in cell migration during ventral enclosure. J Cell Sci 116:1505–1518
130 Sathish K, Padma B, Munugalavadla V, Bhargavi V, Radhika KV, Wasia R, Sairam M, Singh SS (2004) Phosphorylation of profilin regulates its interaction with actin and poly (L-proline). Cell Signal 16:589–596
131 Schafer DA, Jennings PB, Cooper JA (1996) Dynamics of capping protein and actin assembly in vitro: uncapping barbed ends by polyphosphoinositides. J Cell Biol 135:169–179
132 Smertenko AP, Allwood EG, Khan S, Jiang CJ, Maciver SK, Weeds AG, Hussey PJ (2001) Interaction of pollen-specific actin-depolymerizing factor with actin. Plant J 25:203–212
133 Smertenko AP, Jiang CJ, Simmons NJ, Weeds AG, Davies DR, Hussey PJ (1998) Ser6 in the maize actin-depolymerizing factor, ZmADF3, is phosphorylated by a calcium-stimulated protein kinase and is essential for the control of functional activity. Plant J 14: 187–193
134 Smith LG (1999) Divide and conquer: cytokinesis in plant cells. Curr Opin Plant Biol 2: 447–453
135 Snowman BN, Kovar DR, Shevchenko G, Franklin-Tong VE, Staiger CJ (2002) Signalmediated depolymerization of actin in pollen during the self-incompatibility response. Plant Cell 14:2613–2626
136 Sonobe S, Shibaoka H (1989) Cortical fine actin filaments in higher plants cells visualized by rhodamine-phalloidin after pretreatment with m-maleinmidobenzoyl N-hydroxysuccinimide ester. Protoplasma 148:80–86
137 Southwick FS, DiNubile MJ (1986) Rabbit alveolar macrophages contain a Ca2+-sensitive, 41,000-dalton protein which reversibly blocks the "barbed" ends of actin filaments but does not sever them. J Biol Chem 261: 14191–14195
138 Staiger CJ, Blanchoin L (2006) Actin dynamics: old friends with new stories. Curr Opin Plant Biol 9:554–562
139 Staiger CJ, Hussey PJ (2004) The plant cytoskeleton in cell differentiation and development. In The Plant Cytoskeleton in Cell Differentiation and Development, P.J. Hussey, ed (Oxford: Blackwell Publishers), pp:32–80
140 Staiger CJ, Sheahan MB, Khurana P, Wang X, McCurdy DW, Blanchoin L (2009) Actin filament dynamics are dominated by rapid growth and severing activity in the Arabidopsis cortical array. J Cell Biol 184:269–280

141 Staiger CJ, Yuan M, Valenta R, Shaw PJ, Warn RM, Lloyd CW (1994) Microinjected profilin affects cytoplasmic streaming in plant cells by rapidly depolymerizing actin microfilaments. Curr Biol 4:215–219
142 Stevenson V, Hudson A, Cooley L, Theurkauf WE (2002) Arp2/3-dependent pseudocleavage [correction of psuedocleavage] furrow assembly in syncytial Drosophila embryos. Curr Biol 12:705–711
143 Svitkina TM, Borisy GG (1999) Arp2/3 complex and actin depolymerizing factor/cofilin in dendritic organization and treadmilling of actin filament array in lamellipodia. J Cell Biol 145:1009–1026
144 Szymanski DB (2005) Breaking the WAVE complex: the point of Arabidopsis trichomes. Curr Opin Plant Biol 8:103–112
145 Sun HQ, Yamamoto M, Mejillano M, Yin HL (1999) Gelsolin, a multifunctional actin regulatory protein. J Biol Chem 274:33179–33182
146 Tao Z, Ren H (2003) Regulation of gelsolin to plant actin filaments and its distribution on pollen. Sci China 46:379–388
147 Tian M, Chaudhry F, Ruzicka DR, Meagher RB, Staiger CJ, Day B (2009) Arabidopsis actindepolymerizing factor AtADF4 mediates defense signal transduction triggered by the Pseudomonas syringae effector AvrPphB. Plant Physiol 150:815–824
148 Tilney LG., Bonder EM, Coluccio LM, Mooseker MS (1983) Actin from Thyone sperm assembles on only one end of an actin filament: a behavior regulated by profilin. J Cell Biol 97:112–124
149 Tominaga M, Yokota E, Vidali L, Sonobe S, Hepler PK, Shimmen T (2000) The role of plant villin in the organization of the actin cytoskeleton, cytoplasmic streaming and the architecture of the transvacuolar strand in root hair cells of Hydrocharis. Planta 210:836–843
150 Valenta R, Duchene M, Pettenburger K, Sillaber C, Valent P, Bettelheim P, Breitenbach M, Rumpold H, Kraft D, Scheiner O (1991) Identification of profilin as a novel pollen allergen; IgE autoreactivity in sensitized individuals. Science 253:557–560
151 Valenta R, Ferreira F, Grote M, Swoboda I, Vrtala S, Duchene M, Deviller P, Meagher RB, McKinney E, Heberle-Bors E (1993) Identification of profilin as an actin-binding protein in higher plants. J Biol Chem 268:22777–22781
152 Valster AH, Pierson ES, Valenta R, Hepler PK, Emons A (1997) Probing the Plant Actin Cytoskeleton during Cytokinesis and Interphase by Profilin Microinjection. Plant Cell 9: 1815–1824
153 Valster AH, Vidali L, Hepler PK (2003) Nuclear localization of profilin during the cell cycle in Tradescantia virginiana stamen hair cells. Protoplasma 222:85–95
154 Vidali L, Augustine RC, Kleinman KP, Bezanilla M (2007) Profilin is essential for tip growth in the moss Physcomitrella patens. Plant Cell 19:3705–3722
155 Vidali L, Hepler PK (1997) Characterization and localization of profilin in pollen grains and tubes of Lilium longiflorum. Cell Motil Cytoskeleton 36:323–338
156 Vidali L, McKenna ST, Hepler PK (2001) Actin polymerization is essential for pollen tube growth. Mol Biol Cell 12:2534–2545
157 Vidali L, van Gisbergena PAC, Guerin C, Francoa P, Li M, Burkarta GM, Augustinea RC, Blanchoin L, Bezanilla M (2009) Rapid formin-mediated actin-filament elongation is essential for polarized plant cell growth. Proc Natl Acad Sci USA 106(32):13341–13346
158 Vidali L, Yokota E, Cheung AY, Shimmen T, Hepler PK (1999) The 135 kDa actin-bundling protein from Lilium longiflorum pollen is the plant homologue of villin. Protoplasma 209: 283–291
159 von Witsch M, Baluska F, Staiger CJ, Volkmann D (1998) Profilin is associated with the plasma membrane in microspores and pollen. Eur J Cell Biol 77:303–312
160 Wallar BJ, Alberts AS (2003) The formins: active scaffolds that remodel the cytoskeleton. Trends in Cell Bio 13:435–446
161 Wang F, Jing Y, Wang Z, Mao T, Samaj J, Yuan M, Ren H (2009) Arabidopsis profilin isoforms, PRF1 and PRF2 show distinctive binding activities and subcellular distributions. J Integr Plant Biol 51:113–121

162 Wang HY, Yu Y, Chen ZL, Xia GX (2005) Functional characterization of Gossypium hirsutum profilin 1 gene (GhPFN1) in tobacco suspension cells. Characterization of in vivo functions of a cotton profilin gene. Planta 222:594–603
163 Wang T, Xiang Y, Hou J, Ren HY (2008) ABP41 is involved in the pollen tube development via fragmenting actin filaments. Mol Plant 1:1048–1055
164 Wasserman S (1998) FH proteins as cytoskeletal organizers. Trends Cell Biol 8: 111–115
165 Way M, Weeds A (1988) Nucleotide sequence of pig plasma gelsolin. Comparison of protein sequence with human gelsolin and other actin-severing proteins shows strong homologies and evidence for large internal repeats. J Mol Biol 203:1127–1133
166 Welch MD, Iwamatsu A, Mitchison TJ (1997) Actin polymerization is induced by Arp2/3 protein complex at the surface of Listeria monocytogenes. Nature 385: 265–269
167 Wu W, Yan LF (1997) Identification of gelsolin by western blotting in maize pollen. Chinese Sci Bulletin 42:1784–1786
168 Xiang Y, Huang X, Wang T, Zhang Y, Liu Q, Hussey PJ, Ren H (2007) ACTIN BINDING PROTEIN 29 from Lilium pollen plays an important role in dynamic actin remodeling. Plant Cell 19:1930–1946
169 Xu Y, Moseley JB, Sagot I, Poy F, Pellman D, Goode BL, Eck MJ (2004) Crystal structures of a formin homology-2 domain reveal a tethered dimer architecture. Cell 116: 711–723
170 Yamashiro S, Kameyama K, Kanzawa N, Tamiya T, Mabuchi I, Tsuchiya T (2001) The gelsolin/fragmin family protein identified in the higher plant Mimosa pudica. J Biochem 130:243–249
171 Yamashita A, Maeda K, Maeda Y (2003) Crystal structure of CapZ: structural basis for actin filament barbed end capping. EMBO J 22:1529–1538
172 Ye J, Zheng Y, Yan A, Chen N, Wang Z, Huang S, and Yang Z (2009) Arabidopsis Formin3 Directs the Formation of Actin Cables and Polarized Growth in Pollen Tubes, The Plant Cell, 21:3868–3884
173 Yi KX, Guo CQ, Chen D, Zhao B, Yang B, Ren H (2005) Cloning and functional characterization of a formin-like protein (AtFH8) from Arabidopsis. Plant Physiol 2005(138):1071–1082
174 Yin HL, Kwiatkowski DJ, Mole JE, Cole FS (1984) Structure and biosynthesis of cytoplasmic and secreted variants of gelsolin. J Biol Chem 259:5271–5276
175 Yokota E, Muto S, Shimmen T (2000) Calcium-calmodulin suppresses the filamentous actin-binding activity of a 135-kilodalton actin-bundling protein isolated from lily pollen tubes. Plant Physiol 123:645–654
176 Yokota E, Shimmen Ki TT (1998) Actin-bundling protein isolated from pollen tubes of lily. Biochemical and immunocytochemical characterization. Plant Physiol 116:1421–1429
177 Yokota E, Shimmen T (1999) The 135-kDa actin-bundling protein from lily pollen tubes arranges F-actin into bundles with uniform polarity. Planta 209:264–266
178 Yokota E, Tominaga M, Mabuchi I, Tsuji Y, Staiger CJ, Oiwa K, Shimmen T (2005) Plant villin, lily P-135-ABP, possesses G-actin binding activity and accelerates the polymerization and depolymerization of actin in a Ca^{2+}-sensitive manner. Plant Cell Physiol 46: 1690–1703
179 Yokota E, Vidali L, Tominaga M, Tahara H, Orii H, Morizane Y, Hepler PK, Shimmen T (2003) Plant 115-kDa actin-filament bundling protein, P-115-ABP, is a homologue of plant villin and is widely distributed in cells. Plant Cell Physiol 44:1088–1099
180 Zallen JA, Cohen Y, Hudson AM, Cooley L, Wieschaus E, Schejter ED (2002) SCAR is a primary regulator of Arp2/3-dependent morphological events in Drosophila. J Cell Biol 156:689–701
181 Zhang C, Mallery EL, Schlueter J, Huang S, Fan Y, Brankle S, Staiger CJ, Szymanski DB (2008) Arabidopsis SCARs function interchangeably to meet actin-related protein 2/3 activation thresholds during morphogenesis. Plant Cell 20:995–1011
182 Zhang X, Dyachok J, Krishnakumar S, Smith LG, Oppenheimer DG (2005) IRREGULAR TRICHOME BRANCH1 in Arabidopsis encodes a plant homolog of the actin-related protein2/3 complex activator Scar/WAVE that regulates actin and microtubule organization. Plant Cell 17:2314–2326
183 Zuchero JB, Coutts AS, Quinlan ME, Thangue NB, Mullins RD (2009) p53-cofactor JMY is a multifunctional actin nucleation factor. Nat Cell Biol 11:451–459

Chapter 4
Microtubule Nucleation and Organization in Plant Cells

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4.1 Introduction

Microtubules are tube-shaped hollow polymers consisting of α- and β-tubulin heterodimers. As in the case of DNA synthesis [1], polymerization of a tubulin heterodimer requires an existing microtubule. A free heterodimer binds to the end of a microtubule (Fig. 4.1, right) or a tubulin oligomer before forming a microtubule as a seed (Fig. 4.1, middle) [10]. Seed formation, which is the first step of microtubule formation, is called "nucleation" (Fig. 4.1) [25]. Purified tubulin heterodimers spontaneously form tubulin oligomers above a critical concentration in vitro [66]. However, self-oligomerization of tubulin in living cells has not been demonstrated, although a protein complex functions to form seeds in living cells. The protein complex is a γ-tubulin complex, which contains γ-tubulin and other proteins [75]. Localization and activity of the complexes regulate microtubule nucleation and subsequent organization in cells [34].

The γ-tubulin complexes of metazoan cells are mainly localized in the centrosome [74], the organelle that fans out many microtubules in both interphase and mitosis. A unique property of land plant cells is the lack of a centrosome [17, 42]. Arrays of microtubules organize during progression of cell cycles without the influence of the centrosome [68]. This review will discuss how microtubule nucleation is regulated in land plant cells, and how it influences microtubule organization.

4.2 Microtubule Nucleation Sites in Land Plant Cells

4.2.1 Patterns of Microtubules in the Cell Cycle of Land Plants

Microtubules in land plant cells organize during progression of the cell cycle (Fig. 4.2). In interphase (Fig. 4.2a), microtubules orient mostly in the same direction along the plasma membrane to form a cortical array [68]. Arrayed microtubules regulate

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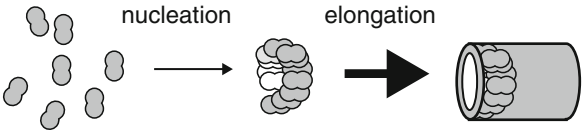

Fig. 4.1 Microtubule nucleation. Tubulin heterodimers (*left*) are assembled into oligomers, and the oligomers incorporate free tubulin (*right*)

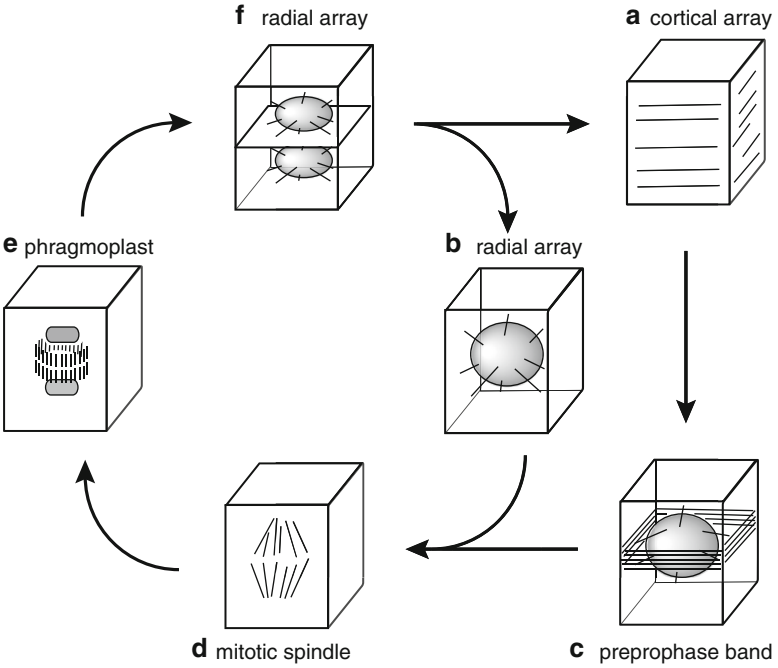

Fig. 4.2 Changes in microtubule organization during progression of the cell cycle in land plant cells. (**a**) Cortical array. (**b**) Radial array. (**c**) Preprophase band. (**d**) Mitotic spindle. (**e**) Phragmoplast. (**f**) Radial array at the transition from M phase to G1 phase. Note that development of the radial array depends on the cell type. Most of the vegetative cells form the cortical array and preprophase band

orientation of newly synthesized cellulose microfibrils on the plasma membrane with guiding cellulose assembly complexes [46]. As well as the cortical array, microtubules radiate from the nuclear envelope to the cortical cytoplasm and form a radial array [29,37]. Development of the arrays depends on cell types and stages of the cell cycle. In endosperm cells of *Haemanthus katharinae*, the radial array is prominent throughout interphase and the cortical array does not develop (Fig. 4.2b) [4]. On the other hand, in suspension culture cells of *Nicotiana tabacum*, the cortical array instead of the radial array dominantly develops during most of the G1 phase, and both radial and cortical arrays develop at the S and G2 phases [20]. The cortical array in most cell types changes in organization during G2 phase: when the cells approach prophase, cortical microtubules concentrate to form the preprophase band (Fig. 4.2c) [49]. The preprophase band is localized at the future site of cell plate

adhesion, and is involved in determination of the division site, in which the cortical cytoplasm is specialized to attract a growing cell plate at cytokinesis [19]. The mitotic spindle, which divides chromosomes into two daughter cells, develops at mitosis (Fig. 4.2d). In cytokinesis, a cell plate is assembled in the phragmoplast (Fig. 4.2e), which contains microtubules, actin, and membranes [59]. The phragmoplast microtubules function to transport vesicles that contain molecules for cell plate formation, although the molecular mechanism of vesicle transport along phragmoplast microtubules is still unclear [26, 30]. The following sections discuss microtubule nucleation sites in these microtubule arrays.

4.2.2 Microtubule Nucleation in Interphase Cells

4.2.2.1 Nucleation Sites for the Cortical Array

Candidate nucleation sites of the cortical array have been proposed to be localized in the cortical cytoplasm and on the nuclear surface [9]. In the latter case, microtubules are assumed to elongate into the cortical cytoplasm [29].

Microtubules appear in the cortical cytoplasm without any connection to the nucleus during the recovery of microtubules after artificial depolymerization [8, 15, 21, 35, 69]. Live imaging of fluorescent protein-tagged tubulin confirmed that microtubules are nucleated in the cortical cytoplasm [52]. Live imaging techniques also demonstrated the position and structure for microtubule nucleation. Unexpectedly, preexisting microtubules were found to be the major nucleation sites of microtubules [40]. γ-Tubulin complexes on the existing microtubules likely nucleate microtubule formation, because depletion of γ-tubulin inhibits microtubule branching in vitro and γ-tubulin is localized at the branching points. The nascent microtubules elongate as branches at an angle of approximately 40° to the existing microtubules (Fig. 4.3a) [40] and reorient by formation of parallel bundles upon encountering existing microtubules [12, 52]. Detailed quantification of microtubule nucleation patterns using a plus-end marker (EB1) and a minus-end marker (NEDD1) indicated that elongation of the nascent microtubules is toward the plus-ends [7]. The plus-end directed and angled elongation is consistent with the tree-like microtubule clusters formed when microtubules recover after artificial depolymerization [69]. Chan et al. [7] also found that a fraction of the nascent microtubules elongate parallel to the existing microtubules at the time of nucleation (Fig. 4.3b). These authors proposed that bundle formation upon nucleation plays a role in maintenance of ordered microtubules in microtubule turnover [7].

Shaw et al. [51] reported that approximately half of the microtubules are nucleated *de novo* in the absence of microtubules (Fig. 4.3c). However, *de novo* nucleation was not observed in more detailed analyses using plus- and minus-end markers [7]. These observations suggest that the frequency of microtubule-independent nucleation reported by [52] was overestimated by counting the microtubules nucleated in a microtubule-dependent manner, which lost the mother microtubules through depolymerization [40].

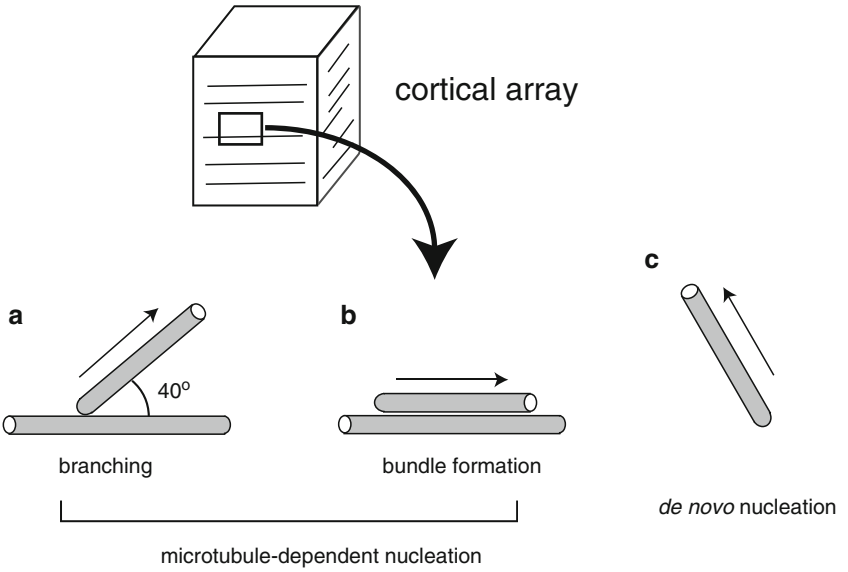

Fig. 4.3 Patterns of microtubule nucleation in the cortical cytoplasm of interphase plant cells. The nascent microtubules elongate (**a**) at 40° to the original microtubules or (**b**) at 0° in bundle formation. Microtubules may form independently from existing microtubules ((**c**); *de novo* nucleation)

4.2.2.2 Nucleation Sites for the Radial Array

At interphase, microtubules radiate from the nuclear envelope to form the radial array (Fig. 4.2b). The involvement of microtubule-dependent microtubule nucleation in radial array formation is unclear. There is a great deal of evidence for *de novo* microtubule nucleation by the nuclear envelope (Fig. 4.4) [29], although live imaging of microtubule nucleation around interphase nuclei has not been reported. The most convincing evidence of the *de novo* nucleation at the nuclear envelope is microtubule nucleation activity of isolated nuclei in vitro [14, 60]. Purified brain tubulin polymerizes on the surface of isolated nuclei of maize and tobacco suspension cells without any additional proteins. The absence of microtubules on isolated nuclei was demonstrated by electron microscopy, indicating that microtubules nucleate on the nuclear envelope *de novo* [60]. The involvement of γ-tubulin complexes in this assembly system has been demonstrated [14].

4.2.3 Microtubule Nucleation for Preprophase Band Development

The preprophase band of microtubules develops in the cortical cytoplasm, where a cell plate fuses with the parental cell wall in cytokinesis [36, 57]. The microtubule

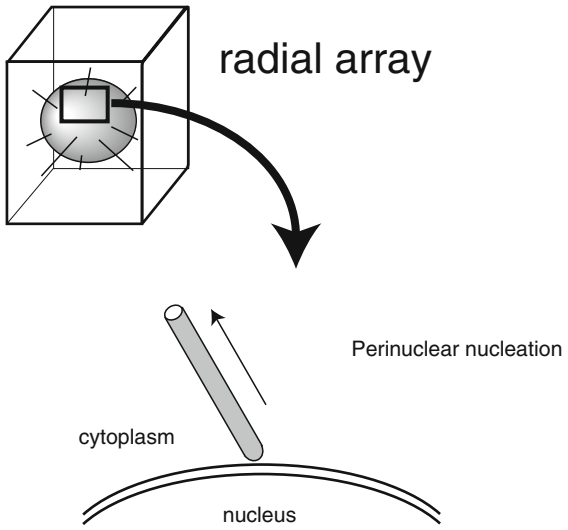

Fig. 4.4 Microtubule nucleation from the nuclear envelope. Microtubules nucleate on and elongate from the nuclear envelope, forming radial arrays

array of the preprophase band regulates the cell division plane and is important for development in land plants. However, localization of microtubule nucleation for the array is still unclear. Live imaging of individual microtubules is difficult because of their high density [11]. We speculate that microtubules nucleate in the array because γ-tubulin is localized in the preprophase band [3, 32]. In addition to microtubule-dependent microtubule nucleation, we cannot exclude the possibility of *de novo* nucleation because microtubules of the preprophase band can reform after complete depolymerization [43].

4.2.4 Microtubule Nucleation in Mitosis

4.2.4.1 Three Pathways in Metazoan Cells

There are three pathways of microtubule nucleation for spindle formation in metazoan cells: the centrosome pathway, the microtubule-dependent pathway, and the chromosome pathway [16, 27, 67]. In the centrosome pathway, microtubules nucleate with γ-tubulin complexes on the centrosome. In the microtubule-dependent pathway, microtubules nucleate on existing spindle microtubules on which augmin complexes recruit γ-tubulin complexes. In the chromosome pathway, microtubules nucleate around the chromosomes, independent of either centrosomes or existing microtubules. The target protein for Xklp2 (TPX2), characterized in a cell-free spindle formation system using *Xenopus* oocyte extract, is involved in this pathway.

The involvement of γ-tubulin in this pathway has not been proven [18]. With a combination of these three pathways, microtubules in the spindles are organized by the interactions of microtubules. This process is mediated through microtubule-dependent motor proteins [34, 67].

4.2.4.2 Origin of Spindle Microtubules at Prophase

The first step of spindle formation is microtubule formation around the nucleus at prophase [1]. In metazoan cells, the duplicated centrosomes move to the spindle poles and radiate microtubules. As cells of land plants lack centrosomes, the origin of the initial microtubules is unclear.

In flowering plants, microtubules accumulate around the nuclear envelope at prophase [70]. TPX2, a factor involved in the chromosome pathway in metazoan cells, plays a role in microtubule nucleation around the nuclear envelope before its breakdown [65]. GFP-tagged TPX2 is localized to the nucleus of *Arabidopsis thaliana* during interphase and is exported to the perinuclear region at prophase. Inhibition of TPX2 function by antibody injection in tobacco cells causes arrest at prophase, suggesting its important role for microtubule assembly on the nuclear envelope. γ-Tubulin is also likely involved in microtubule nucleation on the nuclear envelope because it accumulates on the nuclear envelope at prophase [32]. However, the role of the perinuclear γ-tubulin for spindle formation has not been analyzed in detail. Prophase-specific inhibition of γ-tubulin function, such as antibody injection at prophase, will clarify the role of perinuclear γ-tubulin at prophase.

In the monoplastidic cell division of basal land plants, including mitosis and meiosis of hornworts, and meiosis of mosses and some liverworts, the pattern of spindle formation is different from mitosis of flowering plants [6]. Microtubules radiate from a chloroplast at the spindle pole, instead of the nuclear envelope. Microtubule nucleation activity of isolated chloroplasts and localization of γ-tubulin on the chloroplasts have been demonstrated in the liverwort *Dumortiera hirsuta* [53]. The chloroplast pathway appears to function before prometaphase because microtubules do not associate with chloroplasts after nuclear envelope breakdown.

4.2.4.3 Microtubule Nucleation Sites During Spindle Development

Development of the mitotic spindle has been characterized extensively in endosperm cells of *H. katharinae* [54, 56]. At interphase and early prophase, microtubules radiate from the nuclear envelope. Foci of many microtubules, called microtubule converging centers, are formed on the nucleus-radiated microtubules and are considered important structures for microtubule assembly [54]. More recently, we pointed out that the converging center may not be formed by conversion of existing microtubules but by nucleation of new microtubules mediated by γ-tubulin complexes on an existing microtubule (Murata and Hasebe [41]). It is unclear whether augmin complexes mediate the localization of γ-tubulin on spindle microtubules.

In endosperm cells of *H. katharinae*, microtubule converging centers point to the nuclear envelope before prophase. However, during spindle formation at prophase, orientation of the microtubule converging centers reverses to the cytoplasm and a microtubule array encages the nucleus [55]; these then gather to form spindle poles by the onset of prometaphase. The mechanisms underlying this assembly are unknown, but microtubule sliding caused by motor proteins appears to be involved [56]. At metaphase, microtubule fir trees, which are composed of many microtubule converging centers, develop on kinetochore microtubules [55].

Microtubules increase in density and the fully developed spindle forms after breakdown of the nuclear envelope [71]. In the case of metazoan cells, the chromosome pathway is involved in microtubule nucleation at this stage, but the contribution of the chromosome pathway in land plants is unclear. The only evidence for the existence of the pathway is microtubule formation around chromosomes upon recovery after artificial microtubule depolymerization [15]. Injection of anti-TPX2 antibody at metaphase does not induce a significant delay in the cell cycle [65]. Further studies are required to determine both the existence and molecular mechanisms of the chromosome pathway in land plants.

4.2.5 Microtubule Nucleation in Cytokinesis

Uptake of externally added tubulin into the phragmoplast of detergent-lysed cells was reported in the 1990s [2, 63]. These studies clearly demonstrated the incorporation of tubulin near the cell plate, and the incorporation pattern corresponded with localization of the plus-ends of microtubules near the cell plate. In addition to incorporation onto the existing ends, de novo assembly of microtubules was detected near the cell plate using electron microscopy [63]. However, γ-tubulin does not concentrate near the cell plate [32], and TPX2 signals were not detected in cytokinesis [65]. Localization of tubulin uptake in the lysed cells should be considered with care until the results are confirmed in analyses of factors for microtubule nucleation or live imaging of microtubules.

4.3 Proteins Involved in Microtubule Nucleation

4.3.1 γ-Tubulin Complexes

γ-Tubulin functions in microtubule nucleation, and its orthologs are found in all eukaryotes. The protein is a component of a γ-tubulin complex, which consists of GCP2 (γ-tubulin complex protein 2), GCP3, GCP4, GCP5, GCP6, and NEDD1 as well as γ-tubulin [72]. GCP2, GCP3, and γ-tubulin form a subcomplex called the γ-tubulin small complex. Members of the small complex are conserved in almost all eukaryotes,

but the other members have been lost in some organisms, such as budding yeasts [23]. The roles of the latter proteins vary among organisms. NEDD1 is a targeting factor of γ-tubulin large complexes in *Homo sapiens* [33], but γ-tubulin small complexes can localize without other members of the large complex, including NEDD1, in *Drosophila melanogaster* [64]. Both the complete and small complexes can nucleate microtubules, although the activity of the large complexes is higher in *D. melanogaster* [45].

All components of the γ-tubulin complex are found in the genomes of all major lineages of land plants, including *Arabidopsis thaliana*, *Oryza sativa*, *Selaginella moellendorffii*, and *Physcomitrella patens* [42, 72]. Biochemical analyses have shown that γ-tubulin in land plant cells forms protein complexes of a size similar to metazoan γ-tubulin complexes [61]. Gel-filtration fractionation showed that the fraction equivalent to the complete complex has activity of microtubule nucleation, but that to the small complex does not [13]. Binding of GCP2 and GCP3 to tagged γ-tubulin was demonstrated by tandem affinity purification [51]. Among the members of the complexes, GCP2 and NEDD1 have been genetically characterized in *A. thaliana*. A point mutant of *AtGCP2* with a substitution of Gly305 was named *spiral3* because of its twisted roots [44]; the angles of microtubule branches in cortical arrays are changed in the mutant, suggesting that GCP2 protein is involved in binding of γ-tubulin complexes onto the sides of microtubules. The plant GCP2 is essential for gametophyte development, because the *gcp2* T-DNA null mutant is gametophytic lethal [44]. For NEDD1, a T-DNA insertion mutant is also lethal. The phenotype in microtubule organization is analyzed in male gametophytes, which have only the mutant gene in the haploid genome, generated by meiosis of heterozygous plants. The mutant has a defect in asymmetric division at the first mitosis of pollen development [73]. Organization of spindle and phragmoplast microtubules is abnormal in the *nedd1* mutant. Lethality because of a defect in cell division is also reported in null mutants of γ-tubulin [47]. These results confirm that components other than the small γ-tubulin subcomplex are necessary for microtubule nucleation in *A. thaliana*, which is similar to *Homo sapiens* and different from *D. melanogaster* [33].

4.3.2 Other Centrosomal Proteins

The molecular mechanisms underlying the recognition of targeting sites in plant cells by γ-tubulin complexes are mostly unknown. The centrosome is a major site of γ-tubulin complex localization in metazoan cells, and characterization of the orthologs of animal centrosomal proteins may provide insight into those in plants. NIMA kinase localized in centrosomes is involved in microtubule organization around centrosomes in mammalian cells [50]. A mutant of one of the plant NIMA kinases has been named *ibo1,* and the mutant phenotype and localization of GFP fusion protein have been characterized [39]. GFP-IBO1 is localized on microtubules of the interphase cortical array, and IBO1 appears to be involved in recruitment of γ-tubulin complexes.

A loss-of-function mutant of *TONNEU1* (*TON1*) shows defective development of the preprophase band of microtubules [62]. The preprophase band is a microtubule array that develops in the cortical cytoplasm of expected division sites before

prophase. The mutant also has a defect in orientation of the interphase cortical array. The sister genes *TON1a* and *TON1b* are tandemly encoded in the genome of *A. thaliana*, and the *ton1* mutant has defects in both genes [3]. TON1 proteins have similar amino acid sequences to FOP and OFD1, which are centrosomal proteins of vertebrates. TON1 protein can bind to the *A. thaliana* ortholog of centrin [3], the centrosomal protein conserved in eukaryotes. As centrin is localized to the centrioles of metazoan cells, and the centrioles have been lost in land plants, the binding of centrin with TON1 protein implies recruitment of the centrin functional site from the centrioles to preprophase bands. Although GFP-TON1 is localized to the cortical arrays and preprophase bands, it is still unclear whether TON1 is involved in microtubule nucleation in cooperation with γ-tubulin complexes.

4.3.3 Is There γ-Tubulin-Independent Nucleation?

Job et al. [24] suggested a γ-tubulin-independent pathway for microtubule nucleation [24]. In fertilized eggs of the nematode *Caenorhabditis elegans*, microtubules are formed through either a γ-tubulin-dependent pathway or an aurora-A kinase-dependent pathway [38]. In *A. thaliana*, male gametophytes with a mutant γ-tubulin gene, formed in heterozygous γ-tubulin null plants, retain microtubules [47], and the microtubules were speculated to be formed independently from γ-tubulin. However, we cannot exclude the possibility that γ-tubulin is carried over to the mutant gametophytes from heterozygous pollen mother cells [47]. It is still quite difficult to clarify whether microtubules of the mutants nucleate through a γ-tubulin-independent pathway or γ-tubulin derived from the pollen mother cells.

In spindle formation, TPX2 plays a role in microtubule nucleation. It is still unclear whether TPX2 nucleates microtubules independently from γ-tubulin in animal and plant cells [18, 65]. The relationship between TPX2 and γ-tubulin in the TPX2-mediated chromosome pathway will be clarified by characterizing downstream proteins of TPX2.

Mizuno and coworkers reported that histone H1 has activity for microtubule nucleation on isolated nuclei in vitro [22]. However, it is unlikely that the protein actually nucleates microtubules in interphase cells because histone proteins are localized inside the nuclear envelope. It is possible is that histone H1 nucleates microtubules after breakdown of the nuclear envelope at mitosis. Further studies are required to evaluate the function of histone H1 in living cells.

4.4 Role of Microtubule Nucleation in Microtubule Organization

Microtubules in plant cells are organized as ordered arrays, such as cortical arrays in interphase, preprophase bands before entering mitosis, mitotic spindles in mitosis, and phragmoplasts in cytokinesis. Here, we discuss how microtubule nucleation contributes to the organization of these arrays.

One approach to evaluate the contribution of microtubule nucleation to array organization is to inhibit the expression of genes that are essential for microtubule nucleation. RNAi and null mutant analyses of γ-tubulin have been reported [5, 28, 47]. The *A. thaliana* genome contains two γ-tubulin genes with similar amino acid sequences and 98% identity [31]. Single null mutants in each gene grow normally, but the double mutant formed by meiosis of the heterozygous mutants becomes lethal during pollen development [47]. Simultaneous RNAi silencing of the two genes causes severe growth inhibition of seedlings. At the cellular level, the density of microtubules is decreased in the epidermal cells of γ-tubulin RNAi plants [5], indicating that microtubule nucleation is the limiting factor for the amount of microtubules in epidermal cells.

Orientation of microtubule arrays has been suggested to be coupled with directional microtubule nucleation [48]. In the branching pathway, newly formed microtubules elongate obliquely relative to existing microtubules; therefore, branching potentially changes the orientation of the microtubule arrays. The activity of microtubule branching may be regulated during reorientation of microtubule arrays, although only expression of γ-tubulin complex genes has been analyzed to date. Hypergravity stimuli, which cause reorientation of the microtubule cortical array, increase the levels of expression of each γ-tubulin and GCP3 transcript [58]. To test whether increases in expression drive microtubule reorientation under hypergravity, expression of γ-tubulin and GCP3 can be artificially controlled during reorientation. In addition, mutant plants with modified angles of microtubule branching seem to be useful for analysis of the role of microtubule branching for reorientation of cortical arrays. Kong et al. [28] reported that the angle of microtubule branching is significantly reduced by gene silencing of GCP4 in leaf epidermal cells of *A. thaliana*, although the molecular basis of the angle change is unknown.

4.5 Concluding Remarks

Knowledge regarding microtubule nucleation in plant cells has increased over the last few years. Nucleation sites were identified using live imaging, and orthologs of genes that play major roles in microtubule nucleation in animal cells were characterized. However, analyses of individual microtubule nucleation sites and those of microtubule organization have not yet been adequately linked. For example, we do not know how microtubule nucleation contributes to reorientation of the cortical arrays. As microtubules show complicated behavior after nucleation (elongation, shrinkage, movement, stabilization, and severing), and all of these behaviors influence microtubule organization, the observation of microtubule nucleation under a fluorescence microscope is not sufficient for linking changes of microtubule nucleation with array reorganization. It is necessary to develop methods to quantify changes in microtubule nucleation and all other steps that influence microtubule organization, and to evaluate the effects of changes in microtubule nucleation on microtubule organization. The development of these methods will facilitate the linking of the functions of proteins

that regulate microtubule nucleation with organization of microtubules (e.g., reorientation of cortical arrays, development of mitotic spindles).

References

1. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P (2008) Molecular biology of the cell. Garland Science, New York
2. Asada T, Sonobe S, Shibaoka H (1991) Microtubule translocation in the cytokinetic apparatus of cultured tobacco cells. Nature 350:238–241
3. Azimzadeh J, Nacry P, Christodoulidou A, Drevensek S, Camilleri C, Amiour N, Parcy F, Pastuglia M, Bouchez D (2008) Arabidopsis TONNEAU1 proteins are essential for preprophase band formation and interact with centrin. Plant Cell 20:2146–2159
4. Bajer AS, Mole-Bajer J (1986) Reorganization of microtubules in endosperm cells and cell fragments of the higher plant *Haemanthus* in vivo. J Cell Biol 102:263–281
5. Binarova P, Cenklova V, Prochazkova J, Doskocilova A, Volc J, Vrlik M, Bogre L (2006) γ-Tubulin is essential for acentrosomal microtubule nucleation and coordination of late mitotic events in *Arabidopsis*. Plant Cell 18:1199–1212
6. Brown RC, Lemmon BE (1997) The quadripolar microtubule system in lower land plants. J Plant Res 110:93–106
7. Chan J, Sambade A, Calder G, Lloyd C (2009) Arabidopsis cortical microtubules are initiated along, as well as branching from, existing microtubules. Plant Cell 21:2298–2306
8. Cleary AL, Hardham AR (1988) Depolymerization of microtubule arrays in root-tip cells by oryzalin and their recovery with modified nucleation patterns. Can J Bot 66:2353–2366
9. Cyr RJ, Palevitz BA (1995) Organization of cortical microtubules in plant cells. Curr Opin Cell Biol 7:65–71
10. Desai A, Mitchison TJ (1997) Microtubule polymerization dynamics. Ann Rev Cell Dev Biol 13:83–117
11. Dhonukshe P, Gadella TW Jr (2003) Alteration of microtubule dynamic instability during preprophase band formation revealed by yellow fluorescent protein-CLIP170 microtubule plus-end labeling. Plant Cell 15:597–611
12. Dixit R, Cyr R (2004) Encounters between dynamic cortical microtubules promote ordering of the cortical array through angle-dependent modifications of microtubule behavior. Plant Cell 16:3274–3284
13. Drykova D, Cenklova V, Sulimenko V, Volc J, Draber P, Binarova P (2003) Plant γ-tubulin interacts with αβ-tubulin dimers and forms membrane-associated complexes. Plant Cell 15:465–480
14. Erhardt M, Stoppin-Mellet V, Campagne S, Canaday J, Mutterer J, Fabian T, Sauter M, Muller T, Peter C, Lambert AM, Schmit AC (2002) The plant Spc98p homologue colocalizes with γ-tubulin at microtubule nucleation sites and is required for microtubule nucleation. J Cell Sci 115:2423–2431
15. Falconer MM, Donaldson G, Seagull RW (1988) MTOCs in higher-plant cells – an immunofluorescent study of microtubule assembly sites following depolymerization by APM. Protoplasma 144:46–55
16. Goshima G, Kimura A (2010) New look inside the spindle: microtubule-dependent microtubule generation within the spindle. Curr Opin Cell Biol 22:44–49
17. Graham LE, Kaneko Y (1991) Subcellular structures of relevance to the origin of land plants (embryophytes) from green-algae. Crit Rev Plant Sci 10:323–342
18. Groen AC, Maresca TJ, Gatlin JC, Salmon ED, Mitchison TJ (2009) Functional overlap of microtubule assembly factors in chromatin-promoted spindle assembly. Mol Biol Cell 20:2766–2773
19. Gunning BES (1982) The cytokinetic apparatus: Its development and spatial regulation. In: Lloyd CW (ed) The cytoskeleton in plant growth and development. Academic, London, pp 229–292

20. Hasezawa S, Marc J, Palevitz BA (1991) Microtubule reorganization during the cell-cycle in synchronized BY-2 tobacco suspensions. Cell Motil Cytoskeleton 18:94–106
21. Hogetsu T (1986) Re-formation and ordering of wall microtubules in *Spirogyra* cells. Plant Cell Physiol 28:875–883
22. Hotta T, Haraguchi T, Mizuno K (2007) A novel function of plant histone H1: microtubule nucleation and continuous plus end association. Cell Struct Funct 32:79–87
23. Jaspersen SL, Winey M (2004) The budding yeast spindle pole body: structure, duplication, and function. Annu Rev Cell Dev Biol 20:1–28
24. Job D, Valiron O, Oakley B (2003) Microtubule nucleation. Curr Opin Cell Biol 15:111–117
25. Johnson KA, Borisy GG (1977) Kinetic analysis of microtubule self-assembly in vitro. J Mol Biol 117:1–31
26. Jurgens G (2005) Cytokinesis in higher plants. Annu Rev Plant Biol 56:281–299
27. Karsenti E, Vernos I (2001) The mitotic spindle: a self-made machine. Science 294:543–547
28. Kong Z, Hotta T, Lee J, Horio T, Liu B (2010) The γ-tubulin complex protein GCP4 is required for organizing functional microtubule arrays in Arabidopsis thaliana. Plant Cell 22:191–204
29. Lambert AM (1993) Microtubule-organizing centers in higher plants. Curr Opin Cell Biol 5:116–122
30. Lee YR, Giang HM, Liu B (2001) A novel plant kinesin-related protein specifically associates with the phragmoplast organelles. Plant Cell 13:2427–2439
31. Liu B, Joshi HC, Wilson TJ, Silflow CD, Palevitz BA, Snustad DP (1994) γ-Tubulin in Arabidopsis: gene sequence, immunoblot, and immunofluorescence studies. Plant Cell 6:303–314
32. Liu B, Marc J, Joshi HC, Palevitz BA (1993) A γ-tubulin-related protein associated with the microtubule arrays of higher plants in a cell cycle-dependent manner. J Cell Sci 104:1217–1228
33. Luders J, Patel UK, Stearns T (2006) GCP-WD is a γ-tubulin targeting factor required for centrosomal and chromatin-mediated microtubule nucleation. Nat Cell Biol 8:137–147
34. Luders J, Stearns T (2007) Microtubule-organizing centres: a re-evaluation. Nat Rev Mol Cell Biol 8:161–167
35. Marc J, Mineyuki Y, Palevitz BA (1989) A planar microtubule organizing zone in guard cells of *Allium* – Experimental depolymerization and reassembly of microtubules. Planta 179:530–540
36. Mineyuki Y (1999) The preprophase band of microtubules: Its function as a cytokinetic apparatus in higher plants. Int Rev Cytol 187(187):1–49
37. Mineyuki Y (2007) Plant microtubule studies: past and present. J Plant Res 120:45–51
38. Motegi F, Velarde NV, Piano F, Sugimoto A (2006) Two phases of astral microtubule activity during cytokinesis in *C. elegans* embryos. Dev Cell 10:509–520
39. Motose H, Tominaga R, Wada T, Sugiyama M, Watanabe Y (2008) A NIMA-related protein kinase suppresses ectopic outgrowth of epidermal cells through its kinase activity and the association with microtubules. Plant J 54:829–844
40. Murata T, Sonobe S, Baskin TI, Hyodo S, Hasezawa S, Nagata T, Horio T, Hasebe M (2005) Microtubule-dependent microtubule nucleation based on recruitment of γ-tubulin in higher plants. Nat Cell Biol 7:961–968
41. Murata T, Hasebe M (2007) Microtubule-dependent microtubule nucleation in plant cells. J Plant Res 120:73-78
42. Murata T, Tanahashi T, Nishiyama T, Yamaguchi K, Hasebe M (2007) How do plants organize microtubules without a centrosome? J Integr Plant Biol 49:1154–1163
43. Murata T, Wada M (1991) Re-formation of the preprophase band after cold-Induced depolymerization of microtubules in *Adiantum* protonemata. Plant Cell Physiol 32:1145–1151
44. Nakamura M, Hashimoto T (2009) A mutation in the Arabidopsis gamma-tubulin-containing complex causes helical growth and abnormal microtubule branching. J Cell Sci 122:2208–2217
45. Oegema K, Wiese C, Martin OC, Milligan RA, Iwamatsu A, Mitchison TJ, Zheng Y (1999) Characterization of two related Drosophila gamma-tubulin complexes that differ in their ability to nucleate microtubules. J Cell Biol 144:721–733

46. Paredez AR, Somerville CR, Ehrhardt DW (2006) Visualization of cellulose synthase demonstrates functional association with microtubules. Science 312:1491–1495
47. Pastuglia M, Azimzadeh J, Goussot M, Camilleri C, Belcram K, Evrard JL, Schmit AC, Guerche P, Bouchez D (2006) Gamma-tubulin is essential for microtubule organization and development in Arabidopsis. Plant Cell 18:1412–1425
48. Pickett-Heaps JD (1969) The evolution of the mitotic apparatus: an attempt at comparative ultrastructural cytology in dividing plant cells. Cytobios 3:257–280
49. Pickett-Heaps JD, Northcote DH (1966) Organization of microtubules and endoplasmic reticulum during mitosis and cytokinesis in wheat meristems. J Cell Sci 1:109–120
50. Roig J, Groen A, Caldwell J, Avruch J (2005) Active Nercc1 protein kinase concentrates at centrosomes early in mitosis and is necessary for proper spindle assembly. Mol Biol Cell 16:4827–4840
51. Seltzer V, Janski N, Canaday J, Herzog E, Erhardt M, Evrard JL, Schmit AC (2007) Arabidopsis GCP2 and GCP3 are part of a soluble gamma-tubulin complex and have nuclear envelope targeting domains. Plant J 52:322–331
52. Shaw SL, Kamyar R, Ehrhardt DW (2003) Sustained microtubule treadmilling in *Arabidopsis* cortical arrays. Science 300:1715–1718
53. Shimamura M, Brown RC, Lemmon BE, Akashi T, Mizuno K, Nishihara N, Tomizawa K, Yoshimoto K, Deguchi H, Hosoya H, Horio T, Mineyuki Y (2004) Gamma-tubulin in basal land plants: characterization, localization, and implication in the evolution of acentriolar microtubule organizing centers. Plant Cell 16:45–59
54. Smirnova EA, Bajer AS (1992) Spindle poles in higher plant mitosis. Cell Motil Cytoskeleton 23:1–7
55. Smirnova EA, Bajer AS (1994) Microtubule converging centers and reorganization of the interphase cytoskeleton and the mitotic spindle in higher plant *Haemanthus*. Cell Motil Cytoskeleton 27:219–233
56. Smirnova EA, Bajer AS (1998) Early stages of spindle formation and independence of chromosome and microtubule cycles in *Haemanthus* endosperm. Cell Motil Cytoskeleton 40:22–37
57. Smith LG (2001) Plant cell division: building walls in the right places. Nat Rev Mol Cell Biol 2:33–39
58. Soga K, Kotake T, Wakabayashi K, Kamisaka S, Hoson T (2008) Transient increase in the transcript levels of gamma-tubulin complex genes during reorientation of cortical microtubules by gravity in azuki bean (*Vigna angularis*) epicotyls. J Plant Res 121:493–498
59. Staehelin LA, Hepler PK (1996) Cytokinesis in higher plants. Cell 84:821–824
60. Stoppin V, Vantard M, Schmit AC, Lambert AM (1994) Isolated plant nuclei nucleate microtubule assembly – the nuclear-surface in higher-plants has centrosome-like activity. Plant Cell 6:1099–1106
61. Stoppin-Mellet V, Peter C, Lambert AM (2000) Distribution of gamma-tubulin in higher plant cells: Cytosolic gamma-tubulin is part of high molecular weight complexes. Plant Biol 2:290–296
62. Traas J, Bellini C, Nacry P, Kronenberger J, Bouchez D, Caboche M (1995) Normal differentiation patterns in plants lacking microtubular preprophase bands. Nature 375:676–677
63. Vantard M, Levilliers N, Hill AM, Adoutte A, Lambert AM (1990) Incorporation of *Paramecium* axonemal tubulin into higher plant cells reveals functional sites of microtubule assembly. Proc Natl Acad Sci USA 87:8825–8829
64. Verollet C, Colombie N, Daubon T, Bourbon HM, Wright M, Raynaud-Messina B (2006) *Drosophila melanogaster* γ-TuRC is dispensable for targeting γ-tubulin to the centrosome and microtubule nucleation. J Cell Biol 172:517–528
65. Vos JW, Pieuchot L, Evrard JL, Janski N, Bergdoll M, de Ronde D, Perez LH, Sardon T, Vernos I, Schmit AC (2008) The plant TPX2 protein regulates prospindle assembly before nuclear envelope breakdown. Plant Cell 20:2783–2797
66. Voter WA, Erickson HP (1984) The kinetics of microtubule assembly. Evidence for a two-stage nucleation mechanism. J Biol Chem 259:10430–10438
67. Wadsworth P, Khodjakov A (2004) E pluribus unum: towards a universal mechanism for spindle assembly. Trends Cell Biol 14:413–419

68. Wasteneys GO (2002) Microtubule organization in the green kingdom: chaos or self-order? J Cell Sci 115:1345–1354
69. Wasteneys GO, Williamson RE (1989) Reassembly of microtubules in *Nitella tasmanica* – assembly of cortical microtubules in branching clusters and its relevance to steady-state microtubule assembly. J Cell Sci 93:705–714
70. Wick SM, Duniec J (1983) Immunofluorescence microscopy of tubulin and microtubule arrays in plant cells. I. Preprophase band development and concomitant appearance of nuclear envelope-associated tubulin. J Cell Biol 97:235–243
71. Wick SM, Seagull RW, Osborn M, Weber K, Gunning BE (1981) Immunofluorescence microscopy of organized microtubule arrays in structurally stabilized meristematic plant cells. J Cell Biol 89:685–690
72. Wiese C, Zheng Y (2006) Microtubule nucleation: γ-tubulin and beyond. J Cell Sci 119:4143–4153
73. Zeng CJT, Lee YRJ, Liu B (2009) The WD40 Repeat Protein NEDD1 Functions in Microtubule Organization during Cell Division in *Arabidopsis thaliana*. Plant Cell 21:1129–1140
74. Zheng Y, Jung MK, Oakley BR (1991) Gamma-tubulin is present in *Drosophila melanogaster* and *Homo sapiens* and is associated with the centrosome. Cell 65:817–823
75. Zheng Y, Wong ML, Alberts B, Mitchison T (1995) Nucleation of microtubule assembly by a γ-tubulin-containing ring complex. Nature 378:578–583

Chapter 5
Microtubule Plus End-Tracking Proteins and Their Activities in Plants

Robin E. Young and Sherryl R. Bisgrove

5.1 Introduction

Microtubules have intrigued plant cell biologists since the time of their discovery in the early 1960s [71]. These long tubule-shaped filaments are integral components of the cell division apparatus and they play important roles in cell division, elongation, and morphogenesis. Microtubules are also found in different arrangements in cells, depending on the cell type and the stage of the cell cycle (see Wasteneys [138] for a summary of the microtubule arrays found in plant cells). In dividing plant cells microtubules reorganize from a cortical interphase array into preprophase bands, mitotic spindles and phragmoplasts as cells progress into and through mitosis. Interphase cells are characterized by cortical microtubules organized into parallel arrays that are tightly associated with the cytoplasmic face of the plasma membrane. In rapidly elongating cells, cortical microtubules are aligned perpendicular to the direction of cell expansion. The preprophase band is a circular bundle of microtubules, actin filaments, and other proteins that forms in the cortex of cells just before they enter mitosis. It encircles the nucleus and defines a site in the cortex where the growing cell plate will fuse at the end of cytokinesis. It is also thought to play a role in establishing the bipolar spindle [6]. The preprophase band disappears as the spindle forms and the nuclear envelope breaks down. Towards the end of mitosis, during telophase, the phragmoplast assembles between the two daughter nuclei. It consists of two opposing sets of parallel microtubules arranged into cylindrically shaped arrays. Golgi-derived vesicles are transported along the phragmoplast microtubules to the midzone where they fuse to the growing cell plate. As cytokinesis proceeds the phragmoplast and cell plate expand towards the cortex of the cell and when they reach the plasma membrane fusion occurs at the site marked previously by the preprophase band. Although mitotic spindles

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are common to dividing cells across eukaryotic lineages, preprophase bands, phragmoplasts, and the cortical microtubule arrays in interphase cells are found only in the green plant lineage.

In addition to a unique set of microtubule arrays, plant cells also lack discrete microtubule organizing centers such as the centrosomes that are found in many animal cells and the spindle pole bodies present in fungal cells. The presence or absence of an organizing center can profoundly affect how microtubules are arranged and how they function [13]. For example, interphase animal cells usually have organizing centers located in the interior of the cell, on or near the nuclear envelope. Microtubules are anchored in the organizing center and they extend outwards through the cytoplasm and into the peripheral regions of the animal cell where they often make contacts with proteins embedded in the cortical actin cytoskeleton or associated with the plasma membrane. Because they traverse the cytoplasm these microtubules often serve as tracks for the motor-driven transport of organelles and other material between the inner regions of the cell and the plasma membrane. Microtubules emanating from the organizing center also connect the nucleus with sites in the cortex of the cell and they have roles in positioning the nucleus within the cell and aligning the mitotic apparatus during cell division in polarized cells (for review see [47]). In contrast, plants do not have the equivalent of a centrosome or spindle pole body [12, 109]. During interphase plant microtubules are nucleated from dispersed sites in the cortex; they are distributed around the periphery of the cell tightly linked to the plasma membrane [139]. These cortical microtubules play key roles in cell wall deposition and their effects on the wall influence its mechanical properties and determine how and where cells expand. Despite the absence of discrete microtubule nucleating centers, plants are able to assemble bipolar spindles and properly position their nuclei and mitotic arrays within the cell [6, 93].

The unique nature of the microtubule arrays found in plants raises questions about their organization and function. Have plants adopted different ways of regulating and utilizing their microtubules? Or, do they rely on conserved mechanisms modified to fit the needs of the plant? The data that is emerging indicates the answer to both of these questions is yes. Microtubules in plants appear to function in ways that are a combination of conserved and derived mechanisms. A case in point is the proteins that bind to and regulate microtubule activities in cells. These microtubule associated proteins, or MAPs, play key roles in almost all microtubule-based activities in eukaryotic cells. In plant lineages, there have been modifications to both the repertoire of MAPs that are present as well as the functions of some of the conserved proteins. In this chapter we discuss a specialized group of MAPs known as microtubule plus-end tracking proteins or +TIPs. These MAPs preferentially localize to the more active plus ends of microtubules and they have key roles in many microtubule dependent processes in eukaryotic cells. We begin with a general description of microtubules and +TIP activities in cells and follow with a discussion of the +TIP families that have been studied in plants (Table 5.1).

Table 5.1 List of known +TIPs in plants and their homologs in yeast and animals

+TIP family	Arabidopsis	Yeast	Animal	References for plant homologs
EB1	EB1a (At3g47690)	Bim1p (budding)	EB1, EB2, EB3 (human)	[1, 20, 21, 25, 26, 38, 63, 83, 129, 130]
	EB1b (At5g62500)	Mal1p (fission)		
	EB1c (At5g67270)			
TOG domain proteins	MOR1 (At2g35630)	Stu2p	XMAP215 (*Xenopus laevis*) TOGp (human)	[59, 60, 128, 142, 145]
	CLASP (At2g20190)	Stu1p	CLASP	[8, 9]
Kinesins	ATK5 (At4g05190)	Unknown	Unknown	[5, 7]
Plant specific	SPIRAL1 (At2g03680)	Not found	Not found	[94, 95, 112]
	SPIRAL1-LIKE (At1g26355)			
	SPIRAL1-LIKE2 (At1g69230)			
	SPIRAL1-LIKE3 (At3g02180)			
	SPIRAL1-LIKE4 (At5g15600)			
	SPIRAL1-LIKE5 (At4g23496)			

5.2 Microtubule Structure and Dynamics

Microtubules are intrinsically dynamic structures. In cells they are usually found in states of either growth or shrinkage and they exhibit rapid transitions between the two phases. This feature, termed dynamic instability, underlies many aspects of microtubule function including the ability to rearrange into different arrays [55, 131]. Microtubules grow and shrink by the addition and loss of subunits to and from their ends. The subunits are dimers of α- and β-tubulin; they are arranged head to tail into 13 protofilaments that are aligned longitudinally in the tubule wall. This arrangement endows the microtubule with an intrinsic polarity; one end of the microtubule terminates with α-tubulin and the other with β-tubulin. The two ends also differ with respect to the rates at which they grow and shrink. Subunit addition and loss occurs more rapidly at the end that terminates with β-tubulin and this end of the microtubule is known as the plus end because it is more dynamic than the other end [113]. At growing plus ends tubulin subunits assemble into protofilaments that are linked together laterally to form a curved sheet. As the microtubule grows, the edges of the sheet are zippered together behind the growing tip to form the tubule-shaped filament ([27], and reviewed in [30, 55, 115]). Tubulin dimers are GTP-binding proteins; they are recruited to the growing plus end in a GTP-bound form. After subunit incorporation, β-tubulin slowly hydrolyzes its bound GTP to GDP, and these GDP-bound subunits make up most of the tubule wall. Microtubules

containing GDP-bound β-tubulin are less stable and more prone to depolymerization than microtubules made of GTP-bound tubulin. One hypothesis proposes that the cap of GTP-bound tubulin present at the growing end could stabilize the microtubule against depolymerization [90]. Loss of the GTP cap can cause microtubules to immediately and rapidly depolymerize in an event termed a catastrophe [33, 36]. In addition to GTP hydrolysis, there is also a conformational change in the protofilaments when the tubulin sheet closes up. Sheet closure causes the curved protofilaments to straighten and it has been proposed that this conformational change could make the curved sheet more stable than the cylindrical wall [28, 30].

In cells, microtubules function together with a fleet of microtubule-associated proteins known as MAPs. When bound these proteins influence the ability of microtubules to grow and shrink. They also mediate microtubule interactions with other proteins or structures in the cell [24]. Some MAPs bind along the lengths of the tubule wall while others preferentially associate with the minus or the plus ends of the microtubule. MAPs that accumulate at the more active plus ends are known as microtubule plus end-tracking proteins, or +TIPs. These proteins form a specialized group that have affinities for the more active ends of microtubules. There are a few +TIPs that bind the plus ends of depolymerizing or stable microtubules (for recent reviews see [4, 118]). However, most +TIPs that have been identified so far associate with growing microtubule plus ends. In living cells, GFP-labelled versions appear to track the ends in a characteristic comet-shaped pattern as the microtubules grow through the cell. Although +TIPs appear to move through the cytoplasm with growing microtubule ends, this is most likely an optical illusion since most of them do not translocate along the microtubule. Instead they bind to the end and are released as the end grows out and they become situated on the tubule wall, a process that is referred to as treadmilling (Fig. 5.1; [4]). While bound to microtubule ends, +TIPs modulate microtubule dynamics and mediate interactions of the plus end within the cell.

5.3 Holding onto a Moving Target: How +TIPs Find, Recognize and Maintain Contact with the Plus End

Recruitment of proteins to the microtubule plus end is both highly dynamic and supported at least in part by the cytoplasmic pool of soluble +TIPs [132]. Cytoplasmic proteins with an affinity for microtubule plus ends, such as EB1, probably encounter the ends stochastically as the microtubule grows and shrinks through the cytoplasm [132]. Other +TIPs can bind to free tubulin dimers and they appear to be incorporated into the microtubule at the growing end with the subunits that they are bound to (Fig. 5.1). CLIP-170 is an example of a +TIP that appears to be recruited to microtubule ends in this manner [41]. In some cases proteins are recruited to the microtubule and then translocated to the plus end, either by lateral diffusion or by motor-driven transport. This appears to be the case for several proteins, including XMAP215 and Adenomatous polyposis coli (APC) [22, 87].

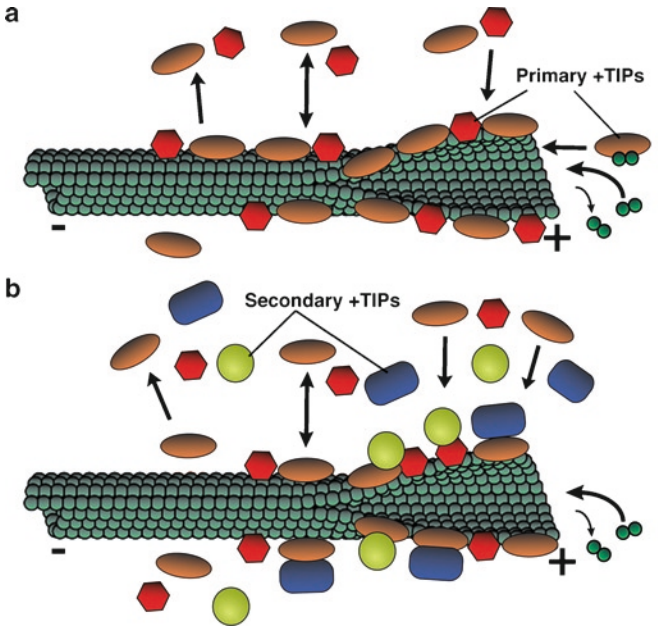

Fig. 5.1 +TIP complex formation on microtubule ends. (**a**) Primary +TIPs (hexagons and ovals) associate directly with the growing microtubule end. They can reach the microtubule by diffusing through the cytoplasm or by binding tubulin dimers (small circles) and assembling with them as they are incorporated into the growing microtubule. Some +TIPs may also associate with the tubule wall behind the tip and move along the microtubule lattice towards the end by diffusion or motor-driven transport (not shown). Primary +TIPs recognize structural and/or chemical features unique to the plus end. They bind to the end but do not translocate forward with it as it grows through the cytoplasm. Instead they are released from the microtubule when they become associated with the tubule wall. (**b**) Primary +TIPs (hexagons and ovals) such as EB1 are proposed to function as diffusional sinks for the recruitment of secondary +TIPs to microtubule ends (large circles and boxes). Secondary +TIPs comprise a large and diverse group of proteins that include signalling factors, regulators of the actin cytoskeleton, and proteins that mediate interactions with other structures in the cell. Together these +TIP families form a dynamic network of proteins that can be modified and changed depending on the needs of the cell. For simplicity only two secondary +TIPs are depicted here

Primary plus-end binding proteins interact directly with some unique feature of the microtubule plus end [118], although the underlying mechanisms are not entirely clear (Fig. 5.1a). The binding of +TIPs directly to microtubule ends, as opposed to other parts of the microtubule, is thought to be mediated by an affinity for structural or chemical features that are unique to the plus end [17, 108]. Growing plus ends are curved sheets that are zipped together into cylindrically-shaped tubules behind the tip (Fig. 5.1 and [27, 115]). Thus possible +TIP recognition sites may include morphological features such as open portions of the curved sheet or the edges that form the seam in the tubule wall, a feature that the fission yeast EB1 homolog Mal3p appears to recognize [4, 32, 108]. Another feature that could serve as a +TIP recognition site is the cap of GTP-bound tubulin located at

growing microtubule ends [132]. GTP hydrolysis appears to be required for plus end localization by mammalian and plant isoforms of EB1, since the proteins bind along the tubule walls when GTP hydrolysis is inhibited [37, 57, 146]. Secondary +TIPs are those that require other proteins to associate effectively with the microtubule plus end (Fig. 5.1b). They accumulate on microtubule ends by interacting with treadmilling primary +TIPs. These "hitchhikers" also form transient associations with the microtubule end, dissociating from the more mature regions of the microtubule wall along with the treadmilling +TIP that they are partnered with.

5.4 +TIPs and Their Role in the Cell

Many +TIPs are able to influence microtubule growth and shrinkage, a feature that is not surprising given their location at the more active end of the microtubule. Dynamic microtubules that rapidly grow, shrink, and then re-grow in a new direction are more efficient at exploring the cytoplasm than less active or stabilized microtubules. As they search the cytoplasm, these microtubules encounter different kinds of target sites. Some sites may interact with signalling or regulatory components of the +TIP complex as the microtubule end passes by while other sites could contain molecules that capture and stabilize the microtubule. Thus, microtubule searching and +TIP interactions can influence signal transduction pathways or promote microtubule attachments to structural entities such as chromosome kinetochores during mitosis or defined sites in the cell cortex [3, 68]. These activities are thought to be important components of the mechanisms that underlie cellular processes such as alignment of the mitotic apparatus in budding yeast [65, 74], attachment of chromosomes to the mitotic spindle in dividing cells [63, 80, 123, 124] and choosing sites of polarity in fission yeast or of tip growth in elongating root hairs and pollen tubes [16, 61, 76, 89, 114].

Participation in such a wide spectrum of cellular processes requires a great deal of flexibility in terms of the protein components associated with the microtubule end. As a group +TIPs are structurally and functionally unrelated; most of them share only an affinity for microtubule plus ends [4, 118]. Current models suggest that these proteins form a dynamically changing network that is modulated based on the needs of the cell [4, 97, 118, 132]. Thus, the compliment of proteins associated with microtubule ends is likely to differ between organisms as well as with time, location, and cell type.

5.5 +TIPs in Plants

Plants represent a unique case amongst eukaryotic phyla with respect to +TIP function since their microtubule arrays are organized and function differently from the arrays in other cell types. Plant microtubule arrays include preprophase bands,

phragmoplasts, and cortical interphase arrays, arrangements not seen in other organisms. In addition, plant cells lack discrete microtubule organizing centers, a feature that impacts the mechanisms used to organize microtubules into different arrays [12, 13]. For example, the alignment and ordering of microtubules in the preprophase band and interphase arrays involve interactions of growing plus ends with other microtubules and/or proteins associated with the plasma membrane [5, 7, 34, 35, 38, 39, 113, 135]. Plant microtubules also function in ways that are intimately linked to the presence of the cell wall, a structure not found in animal cells. Cortical microtubules in expanding cells are involved in the oriented deposition of cellulose microfibrils [31, 143]. These microtubules line the plasma membrane and are attached to it along their lengths, in contrast to the end-on interactions that occur between microtubules and the cell cortex in interphase animal cells. Plant cortical microtubules also appear to target the delivery and removal of vesicles bearing cell wall enzymes to and from specific sites in the plasma membrane, implying that their plus ends may be interacting with specific sites in the plasma membrane [31, 104, 107]. Given the unique aspects of microtubule organization and function in plants, it is not surprising that plants have a modified network of MAPs, including those associated with the plus end (Table 5.1).

Functional studies are revealing that the +TIP family in plants is composed of conserved proteins with known +TIP activity in other organisms as well as plant-specific proteins that are not found in other cell types (Table 5.1). In addition, some of the proteins with +TIP activity in other organisms do not show an obvious preference for microtubule ends in plant cells. Finally, there are +TIPs that are conspicuously absent from plant genomes. These include the CAP-Gly domain-containing proteins CLIP-170 and p150^{Glued} [19, 49]. In animal and fungal cells these proteins recruit dynein/dynactin, a large protein complex that has motor activity, to microtubules [111, 120]. Dynein motors also appear to be absent from several sequenced plant genomes, an observation that suggests this complex has also been lost from higher plant lineages [18, 70]. Whether the apparent absence of CLIP-170 and p150^{Glued} is related to the loss of the dynein/dynactin complex is not known, although the observation highlights a unique aspect of +TIP networks in plants. Thus, the +TIP family in plants appears to be composed of a distinct combination of conserved and novel proteins.

In this chapter we focus on plus-end binding proteins that have been studied in plants. Our discussion includes proteins that have been identified as +TIPs in other organisms and are also found in plants as well as proteins that appear to be present only in plant lineages (Table 5.1). Kinesin motors as well as proteins that function in the phragmoplast have entire chapters dedicated to them in other sections of this book and we do not cover them in detail here. First we discuss the EB1 family, proteins that are conserved in plants and are thought to be “master regulators” of the plus end. Then we look at the TOG domain proteins MOR1 and CLASP. These proteins act as +TIPs in other organisms, although their functions may not be confined to microtubule plus ends in plants. Next we will look at ATK5, a unique plant kinesin that appears to localize to microtubule ends using a mechanism that does not depend on its motor domain function. We also discuss possible roles for +TIP

complexes in mediating crosstalk between microtubules and actin in plant cells. Finally we will look at the SPIRAL1 family, a group of +TIPs with no known homologs in other organisms. The chapter ends with a discussion of some of the unanswered questions in the field and future avenues of research.

5.6 EB1, the Quintessential +TIP

The EB1 (*E*nd *B*inding 1) family of +TIPs are emerging as key components of the protein network associated with microtubule plus ends [4, 118, 133]. They were first identified as proteins that interact with a key tumour suppressor in mammalian cells known as *A*denomatous *P*olyposis *C*oli or APC [4, 10, 118, 122]. Subsequent studies revealed that EB1 proteins are +TIPs and that their interaction with APC is responsible for recruiting APC to microtubule plus ends [88, 92, 133]. Although the relevance of the EB1-APC interaction with respect to tumour formation is not yet resolved, the identification of EB1 as a +TIP launched an extensive set of analyses aimed at understanding its function in cells. This work revealed that the animal and fungal proteins bind directly to microtubule plus ends, modify microtubule dynamics, and interact with a diverse array of additional proteins. The emergence of the EB1 family as proteins capable of tracking and recruiting other proteins to microtubule ends led to the proposal that they are central regulators of +TIP complex formation in cells (see [4, 68, 118, 133] for reviews).

EB1 family members are present in the genomes of angiosperms, gymnosperms, and moss as well as green and red algae [19, 49, 84, 98]. In higher plants experimental analyses have focused on the Arabidopsis isoforms and these studies are beginning to reveal similarities between the plant proteins and their animal and fungal counterparts. The plant homologues have the conserved protein domains found in all family members and they preferentially localize to microtubule plus ends where they can influence microtubule dynamics [20]. Presumably the plant proteins also mediate complex formation at microtubule ends, although this is still an open question. In addition to these conserved properties, studies with the Arabidopsis homologues are also unveiling some unique features that may be correlated with the regulation of vascular plant microtubule arrays.

5.7 Structural Conservation Amongst EB1 Family Members

The Arabidopsis genome encodes three EB1 family members designated AtEB1a, AtEB1b, and AtEB1c. All three proteins are predicted to have the structural domains that define the EB1 family. There is a calponin homology domain at the amino terminus followed by a variable linker region, an α-helical coiled-coil, and a flexible tail segment at the carboxy terminus. The conserved EB1 domain, one of the identifying features of the family, is located towards the carboxy terminus partially

overlapping the coiled-coil region [4, 20, 50, 51, 53, 118, 119]. Microtubule binding has been mapped to the calponin homology domain while amino acids in the α-helical coiled-coil and flexible tail have been shown to interact with several binding partners found in animal and fungal cells [23, 50, 51, 53, 63, 85, 119].

Biochemical analyses and X-ray crystallography studies indicate that EB1 proteins form dimers in which the two subunits are arranged in parallel and α-helices from the coiled-coil domain of each monomer wrap around one another [23]. The conserved residues of the EB1 domain form surface patches on the dimers that contain essential contact points for some binding partners [51–53, 63, 99, 119]. These amino acids are also present in the Arabidopsis family members [19] and they presumably mediate interactions with other proteins, although the relevant EB1 binding partners in plant cells are largely unknown.

Although all EB1 family members share these conserved structural domains, there are functionally relevant differences between isoforms. The three Arabidopsis proteins fall into two groups based on sequences in their C-terminal tails [38, 63]. EB1a and EB1b have acidic tails, a feature also found in animal and fungal homologues. In contrast, the EB1c tail is more basic in nature and it contains amino acids that target it to the nucleus. These differences have functional consequences. Since EB1c is sequestered in the nucleus during interphase, it is not available to bind microtubules in the cortical array although it is in an ideal location to play a role early in mitotic spindle formation [38, 63, 129]. One idea that has been put forth suggests that the EB1c isoform may have evolved in vascular plants to enable spindle formation as the preprophase band dissolves and the nuclear envelope breaks down [63]. In addition to directing EB1c to the nucleus, sequence differences in the C-terminal tail are also likely to influence interactions of the proteins with potential binding partners (see below for further discussion).

5.8 EB1 Localization Patterns in Plant Cells

All three of the Arabidopsis EB1 homologues accumulate on growing microtubule ends. In interphase cells, green fluorescent protein (GFP) fusions to EB1a and EB1b label the growing plus ends of microtubules in the characteristic comet shapes seen in animal and fungal cells [25, 26, 63, 83, 129]. During mitosis, all three of the Arabidopsis proteins accumulate in regions where the plus ends of microtubules are more concentrated such as the midzones of mitotic spindles and phragmoplasts [20, 26, 38, 63, 83, 129].

In addition to microtubule plus ends, the Arabidopsis proteins also label other sites in the cell. EB1a-GFP fusions mark the less dynamic minus ends of microtubules in the cortical array as well as sites on the nuclear envelope from which microtubule polymerization occurs during preprophase and prophase [25, 26, 35, 129]. This is not a unique feature of the plant isoforms as EB1 proteins are found in association with microtubule minus ends at spindle poles and centrosomes in several organisms [11, 15, 78, 92, 102, 105, 121, 125, 126]. It is unknown how EB1 might

function at minus ends but it has been suggested that the proteins could either anchor the minus ends in place or form a reservoir for binding to the plus ends of microtubules shortly after their nucleation [25, 102]. EB1b-GFP fusions also label the membranes surrounding internal organelles including the endoplasmic reticulum (ER), chloroplasts, mitochondria, and nuclei [25, 26, 83]. An interaction between EB1 and Stromal Interaction Molecule 1 (STIM1), a protein that resides in the ER membrane, has been reported in mammalian cells [48]. In these cells, the binding of EB1 to STIM1 mediates contact of polymerizing microtubule ends with the ER and is thought to play a role in microtubule dependent ER tubule extensions that occur during ER remodeling. However, the significance of the labeling patterns in plants are unknown, since many of the analyses were done using GFP fusions placed under the regulation of 35S, a promoter known to drive high levels of gene expression. Both overexpression and the presence of GFP tags are known to alter localization patterns of EB1 [38, 116], and it is therefore possible that the patterns observed with these constructs do not accurately reflect endogenous EB1 in plant cells.

As noted above, EB1c bears sequences in its C-terminal tail that target it to the nucleus during interphase. Proteins that have the EB1c nuclear targeting sequence are encoded in several sequenced vascular plant genomes but are not found in the genomes of *Physcomitrella patens* (a moss) or algae, yeasts, and animals, indicating that this may be a plant-specific feature [63]. Why EB1c is found in the nucleus is unknown, although cells compromised for *EB1c* expression have mitotic defects indicative of roles in spindle formation and positioning as well as microtubule attachment to chromosome kinetochores. Based on these observations it has been suggested that the EB1c isoforms may have evolved in vascular plants to preferentially regulate microtubules during the early stages of spindle formation [63].

5.9 EB1 and the Regulation of Microtubule Dynamics

The effects of EB1 on microtubule dynamics have been measured in vitro as well as in several cellular systems in which EB1 proteins were absent, depleted, or overexpressed. When assayed in vitro with purified tubulin, EB1 generally promotes microtubule growth, although the reported results are contradictory with respect to the precise effects on the individual parameters associated with microtubule dynamics (reviewed in [30]). For example, in some studies human and fission yeast homologs promoted growth [32, 108, 118, 134]. However others have reported that human EB1 either had no effect on microtubule dynamics [37] or suppressed shortening without affecting growth [82]. These differences may reflect changes in experimental conditions that alter the extent or pattern of EB1 binding to microtubules such as buffer composition or the concentrations of EB1 and tubulin used [30]. The Arabidopsis family members have been analyzed in vitro, and these studies indicate that all three isoforms promote microtubule assembly [63].

How EB1 promotes microtubule growth is not known. One hypothesis suggests that the proteins induce sheet formation and stimulate closure of the sheet into a tubule

by enhancing lateral interactions between protofilaments in the microtubule lattice [64, 134]. Visualization of the fission yeast homologue Mal3p by high resolution electron microscopy suggest that the proteins act at the edges of the flattened sheet and the groove between protofilaments [32, 108]. In addition, measurements made on high-resolution electron micrographs of growing microtubules suggest that human EB1 induces sheet formation and stimulates closure of the sheet into a tubule [134].

In several EB1 homologues, the carboxy-terminal tail region alters the effects of the protein on microtubule growth. The tail usually has an inhibitory effect; recombinant proteins that are missing their tails assemble microtubules more efficiently than full-length versions [51, 53, 63, 77, 96, 119]. In addition, some EB1 binding partners are able to relieve the autoinhibitory effects of the tail, suggesting that there is cooperation between EB1 and other +TIPs with respect to the regulation of microtubule dynamics. Of the Arabidopsis homologues, the AtEB1b tail appears to have an autoinhibitory effect on microtubule polymerization while the AtEB1c tail does not [63]. Microtubule growth has been measured in cells overexpressing GFP fusions to AtEB1a and AtEB1b. In these experiments, EB1a-GFP stimulated microtubule growth while EB1b-GFP did not [130]. Whether this difference reflects an in vivo autoinhibitory role for the EB1b tail is not known.

5.10 EB1 and +TIP Complex Assembly

As central regulators of +TIP complex formation EB1 family members interact with and recruit an array of structurally and functionally diverse proteins to microtubule ends. Yeast two hybrid analyses, protein pull-down, co-localization and other biochemical assays have identified many proteins that EB1 interacts with, mainly in animal and fungal cells (reviewed in [4]). Plant versions of EB1 are also capable of interacting with and recruiting proteins to microtubules. The *M*ovement *P*rotein (MP) of Tobacco Mosaic Virus has been shown to interact with one of the Arabidopsis EB1 family members (AtEB1a) both in vitro and in vivo [21]. Assays to identify endogenous plant versions of EB1 interactors have not yet been published, although the relevant screens are underway [63].

How EB1 is able to specifically interact with so many different proteins is an active area of investigation. Interactions between EB1 and its binding partners are under analysis both biochemically and by X-ray crystallography. Molecular details are now available for two types of EB1-interactors. One group consists of proteins that contain Cytoskeleton-Associated Protein-Glycine-rich (CAP-Gly) domains. These include CLIP-170 and the dynactin p150 subunit, proteins that appear to missing from plant genomes [19, 120]. Structural analysis of EB1-CAP-Gly complexes revealed two sites of interaction, one with the conserved EB1 domain and the other with three residues (EEY) found at the carboxy-terminus of the flexible tail [51, 54, 118]. This EEY motif is conserved in several of the animal and fungal versions of EB1, but it is missing from the plant isoforms, organisms that do not have the appropriate CAP-Gly domain partners [4, 63].

The second set of EB1-interactors that have been analyzed include a group of structurally diverse proteins defined by a four amino acid SxIP motif that is embedded within a short stretch of basic and proline/serine-rich amino acids [52]. These proteins bind to a site in the conserved EB1 domain and the interaction includes intermolecular contacts between several amino acids in the EB1 domain and the SxIP residues.

Both the EB1 domain and residues that make contact with the SxIP motif are conserved in all three of the Arabidopsis homologues, suggesting that they may have the ability to interact with SxIP-motif containing proteins. Searches of the Arabidopsis genome for proteins that contain an SxIP motif identify over 3,000 sequences, indicating that there is a large pool of plant proteins that bear this motif (S. Squires, unpublished results). Whether any of these proteins represent *bona fide* EB1 interactors is not known. Screens for EB1 binding partners by protein pull-down or yeast two hybrid assays would yield information about whether plant EB1-binding proteins also contain SxIP motifs.

Although endogenous plant proteins that interact with EB1 have not yet been identified, the Arabidopsis genome encodes several proteins that are known to bind EB1 in other organisms. Included in this list are XMAP215, CLASP, MCAK kinesins, and several proteins involved in modulating the actin cytoskeleton [14, 52, 62, 66, 86, 89]. These as well as some +TIPs that appear to function only in plants are discussed below.

5.11 Proteins Bearing TOG Domains: MOR1 and CLASP

XMAP215 and CLASP (*C*LIP-*A*ssociated *P*rotein) represent two MAPs that have been identified as +TIPs in animal cells. Both proteins belong to a superfamily that is characterized by the presence of conserved TOG (*T*umour *O*verexpressed *G*ene) domains, within which are several HEAT repeats made up of α-helix-turn-α-helix motifs, arrayed in a linear fashion [117]. XMAP215 was originally characterized as a MAP that preferentially promoted the elongation of microtubule plus ends [43, 44]. Subsequent studies have shown that the protein exhibits primary plus end tracking activity in vitro; it is thought to accumulate at the plus end by a facilitated diffusion mechanism that involves its ability to processively bind and catalyze the addition of tubulin subunits to the microtubule end [22]. MOR 1 (MICROTUBULE ORGANIZATION 1) is the lone representative of the XMAP215 family in Arabidopsis [142]. In plants it is an essential protein [128, 142]. Studies with temperature-sensitive mutant alleles have shown that the protein functions during the organization of mitotic, cytokinetic, and cortical microtubule arrays in plant cells [59, 142]. Like XMAP215, MOR1 promotes rapid microtubule assembly [60]. However, the plant proteins do not appear to be enriched at microtubule plus ends. Instead they associate with the full length of microtubules [59]. Like the XMAP215 homologs, MOR1 promotes rapid microtubule growth and shrinkage, but the molecular details underlying these activities are not known [60].

In addition to an association with microtubule plus ends in animals, XMAP215 interacts with EB1 as part of a large complex that is involved in the differential

modulation of microtubule dynamics at various stages of the cell cycle [97]. Possible interactions between EB1 and MOR1 have not been tested directly in plants, although observations using EB1b-GFP in the temperature-sensitive *mor1-1* mutant show that the localization pattern of EB1b-GFP is changed when the plants are observed at restrictive temperature compared to those at non-restrictive temperature [60]. At the restrictive temperature the association of EB1b-GFP with microtubule ends is reduced and binding to tubule walls increases, suggesting that MOR1 somehow affects the affinity of EB1 for microtubule ends. However, it is unknown whether MOR1 affects EB1 localization through a direct interaction with EB1 or by a more indirect mechanism such as altering structural features of the microtubule end [60].

CLASP belongs to the second group of TOG-domain containing proteins that have +TIP activity in animal cells. Family members have one N-terminal TOG domain and several HEAT repeats [117]. The proteins were discovered based on their ability to interact with CLIP 170, a +TIP that appears to be missing from plant genomes. They also interact with the conserved +TIP EB1 [2, 19, 49, 86]. In animal cells CLASPs stabilize microtubules and mediate interactions between microtubule ends and the cell cortex [2, 67, 86, 144]. One CLASP homologue is encoded in the Arabidopsis genome [19, 45]. Analyses of T-DNA insertional mutants as well as plants expressing GFP-tagged proteins indicate that Arabidopsis CLASP stabilizes microtubules and has functions in preprophase bands, mitotic spindles, phragmoplasts, and cortical microtubule arrays in expanding cells [8]. Arabidopsis GFP-CLASP binds the sidewalls of microtubules with a slight enrichment at plus-ends instead of the more typical comet-shaped pattern seen for other +TIPs [8, 9]. This enhanced binding to sidewalls plays a role in microtubule attachment to the cell cortex, since loss of *clasp-1* activity in mutants results in the partial detachment of cortical microtubules [9]. Microtubule contacts with the cortex will, in turn, affect the positioning of microtubules within the cortical array [139]. Although CLASP homologs in animal cells often mediate end-on interactions of the microtubule rather than lateral associations [86], enhanced CLASP binding to sidewalls is seen in a subset of microtubules in migrating animal epithelial cells. CLASPs track microtubule plus ends in the bodies of these cells, but they also label along the microtubule sidewalls in leading edge lamella [144]. The spatiotemporal regulation of sidewall labelling is mediated by the phosphorylation status of CLASP and it is also thought to have a role in microtubule stabilization and associations with the cell cortex [67]. Whether microtubule binding of plant CLASPs might be regulated in a similar fashion is not known.

5.12 Plant Kinesins and ATK5

An interesting and diverse group of MT-binding proteins are the kinesins. These proteins are molecular motors that use the energy of ATP hydrolysis to move along microtubules towards either the plus or the minus end, depending on the type of kinesin.

Kinesins are found ubiquitously across eukaryotic phyla; they are characterized by a highly conserved motor domain where the ATPase and microtubule binding activities are located. The motor domains can be located at the amino-terminus, internally, or at the carboxy-terminus. Outside of the motor domain, there is a short neck region that aids the motor in generating movement, a coiled-coil region involved in dimerization, and a tail that is thought to bind cargo.

The conserved motor domain has been used to classify eukaryotic kinesins into 14 families [69]. Flowering plants have a large number of kinesins compared to sequenced animal genomes [103]. For example, the Arabidopsis genome codes for 61 kinesins [101]. Despite this, certain subgroups within the kinesin superfamily are not represented, while others are expanded significantly [101, 103]. Many of the plant kinesins fall into the 14 known families based on sequences in their motor domains [101]. However, they are highly divergent outside of the motor domain, making them proteins that are essentially unique to plants [75]. This includes several with actin and MT-binding domains in their non-motor regions, with roles in cross-linking MTs to either other MTs or to actin filaments, bundling and mitotic spindle formation [75, 101] thus defining them as them MAPs not only based on their motor domains, but their non-motor domains as well. Most notable in the Arabidopsis genome is the increased number of minus-end directed kinesins, with 21 of the 61 putative kinesin genes falling into this category [101]. Within this group of minus-end motors is ATK5 [7] a Kinesin-14 family member that is thus far considered to be the only kinesin to have MT plus-end binding activity that is separate from its motor domain [7]. Live-cell imaging studies have shown that ATK5 is involved in the early stages of spindle formation, by aiding in the co-alignment of microtubules at the midzone [5]. Removal of the C-terminal motor domain does not disrupt plus-end localization, suggesting that its accumulation at microtubule plus ends is independent of the motor domain [7]. Whether ATK5 binds to microtubule plus ends directly or indirectly, by interacting with another +TIP, is not known, although it does have an SXIP motif near its N-terminus (S. Squires, unpublished results) raising the possibility that it might interact with EB1.

Another kinesin of note in the world of +TIPs is the mammalian protein, MCAK (Mitotic Centromere-Associated Kinesin). In mammalian cells, MCAK has been shown to localize to the +TIP of MTs in the mitotic spindle [91] with the aid of EB1 and another +TIP known as TIP150 [58, 73]. Its function is unique amongst kinesins; instead of moving along microtubules it binds the plus ends and depolymerizes them (see [56, 136] and references therein). This depolymerizing activity is important during mitotic spindle assembly and for the poleward movement of chromosomes in anaphase [81, 137]. In plants two MCAK homologs have been identified, AtKinesin-13A and AtKinesin-13B [75]. These proteins share a high level of sequence similarity with MCAK in their motor domains only, there appears to be little similarity elsewhere in the genes. Domains considered to be crucial to MCAK function, such as the Lys-rich neck motif thought to be important for the depolymerizing function of MCAK, are not present [75]. Functional analysis of AtKinesin-13A shows that this protein decorates Golgi stacks and vesicles, putting it in an ideal location to be involved in movement of these organelles within the cell [79, 140].

It does not appear to have a role in the mitotic spindle, unlike its mammalian counterpart [79]. Though much less is known about AtKinesin-13B, sequence analysis indicates that it also lacks the neck domain and bears little resemblance to other kinesins in the family [75, 101]. The lack of a neck domain indicates that it is also likely to function in ways that are different from animal MCAK, though what this function might be remains unclear.

5.13 Actin-Related Proteins

+TIPs are thought to play prominent roles in the cross-talk that coordinates the actin and microtubule cytoskeletons [100]. Some +TIPs, CLASPs for example, can bind to actin filaments [127]. Other +TIPs interact with proteins that regulate the actin cytoskeleton such as formins or other regulatory molecules [14, 46, 89, 100, 141]. The +TIP EB1 interacts with a Rho-type guanine nucleotide exchange factor (RhoGEF) that activates a Rho GTPase involved in actomyosin-mediated contractile shape changes in *Drosophila* cells [106]. Interactions between microtubule plus ends and actin filaments are involved in several processes in animal and fungal cells including regulation of the actin cytoskeleton during cell migration, proper alignment of the mitotic apparatus during cell division, and the establishment of sites of polarity in cells [72, 89, 110].

Interactions between actin and microtubules also occur in plant cells [100]. One example includes root hairs and pollen tubes, cells that elongate by a tip growth mechanism in which cell expansion is confined to the end of the elongated cell. In tip growing cells microtubules are oriented parallel with the long axis of the cell and their plus ends extend into an area behind the apex where F-actin is located. These microtubules are thought to provide a scaffold for the formation of the apical actin arrays and to provide directionality to root hair growth [16, 40, 61, 114]. Interactions between actin and microtubules also occur in elongating cells in the root. Studies have shown that chemical disruption of microtubules results in changes to the actin network in the root elongation zone [29]. Candidates do exist for EB1-interactors in the Arabidopsis genome, since several actin-related proteins carry SXIP motifs. These include myosins, some subunits of the Arp2/3-activating WAVE complex, the nucleolar-localized Arp4 and a homolog of a subunit of the F-actin capping complex, CapZ.

5.14 Plant-Specific +TIPs

In addition to proteins that are conserved across eukaryotic lineages, plants also contain +TIPs that are not found in other genomes. The *SPIRAL1* family is comprised of *SPIRAL1* (SPR1) and six *SPIRAL1-LIKE* (*SPR1L*) homologs that appear to be unique to plants [95, 112]. *SPIRAL1* was initially identified by the presence

of a right handed twisting phenotype in the epidermal cell files of Arabidopsis plants bearing mutations in the gene [42]. Disruption of additional family members resulted in randomly oriented MT arrays, as well as isotropic expansion of epidermal cells [95]. Studies with GFP-tagged versions of *SPR1* showed a comet shaped accumulation of SPR1 protein at the plus-end of MTs, similar to that of EB1, indicating that the protein is a +TIP. However, pulldown assays using polymerized tubulin were not able to identify SPR1, implying that the protein might bind microtubules indirectly [112]. SPIRAL1 is predicted to be a rod-like protein with a repeated motif at the N and C-terminus, suggesting that it might act as an intermolecular linker protein of some kind [112]. All SPR1 and SPR1L proteins have conserved sequences in their N and C-terminal regions, while the central regions of the proteins are variable [95]. The family members have overlapping expression patterns and overexpression of *SPR1L* genes rescues the *spr1* mutant phenotype, suggesting that they may share similar functions [95].

5.15 Conclusions and Unanswered Questions

Analyses of +TIPs in plants are well underway. Studies on several protein families have been published and these analyses are revealing that plants have a somewhat modified network of proteins on their plus ends. This network includes the conserved +TIP EB1, thought to be a key regulator of complex formation on microtubule ends. It also includes plant-specific proteins such as the SPR1 protein family and the kinesin ATK5. In addition, some +TIPs (CAP-Gly containing proteins) are missing from plant complexes while others are not restricted to the plus end but instead label along the microtubule lattice (MOR1 the plant XMAP215 homolog and CLASP). Some plant +TIPs also appear to function in ways that are different from their animal and fungal counterparts. EB1c, for example, appears to be a plant-specific EB1 isoform that localizes to the nucleus during interphase and appears to have a role in the early stages of mitotic spindle formation. The story that is unfolding suggests that plants have a +TIP network that has been adapted to suit their needs.

The discovery of proteins capable of tracking microtubule ends in plants opens the door for the identification of additional components of plant +TIP complexes. In particular, the identification of plant proteins that interact with EB1 would be particularly informative because EB1 is thought to be the central player in the +TIP network. In addition, EB1 interactors could include proteins involved in a wide range of cellular processes that are dependant on microtubule plus-end interactions.

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References

1. Abe T, Hashimoto T (2005) Altered microtubule dynamics by expression of modified α-tubulin protein causes right-handed helical growth in transgenic *Arabidopsis* plants. Plant J 43:191–204
2. Akhmanova A, Hoogenraad CC, Drabek K, Stepanova T, Dortland B, Verkerk T, De Zeeuw CI, Grosveld F, Galjart N (2001) CLASPs are CLIP-115 and -170 associating proteins involved in the regional regulation of microtubule dynamics in motile fibroblasts. Cell 104:923–935
3. Akhmanova A, Stehbens SJ, Yap AS (2009) Touch, grasp, deliver and control: functional cross-talk between microtubules and cell adhesions. Traffic 10:268–274
4. Akhmanova A, Steinmetz MO (2008) Tracking the ends: a dynamic protein network controls the fate of microtubule tips. Nat Rev Mol Cell Biol 9:309–322
5. Ambrose JC, Cyr R (2007) The kinesin ATK5 functions in early spindle assembly in Arabidopsis. Plant Cell 19:226–236
6. Ambrose JC, Cyr R (2008) Mitotic spindle organization by the preprophase band. Mol Plant 1:950–960
7. Ambrose JC, Li W, Marcus A, Ma H, Cyr R (2005) A minus-end-directed kinesin with plus-end tracking protein activity is Iivolved in spindle morphogenesis. Mol Biol Cell 16:1584–1592
8. Ambrose JC, Shoji T, Kotzer AM, Pighin JA, Wasteneys GO (2007) The Arabidopsis CLASP gene encodes a microtubule-associated protein involved in cell expansion and division. Plant Cell 19:2763–2775
9. Ambrose JC, Wasteneys GO (2008) CLASP modulates microtubule-cortex interaction during self-organization of acentrosomal microtubules. Mol Biol Cell 19:4730–4737
10. Aoki K, Taketo MM (2007) Adenomatous polyposis coli (APC): a multi-functional tumor suppressor gene. J Cell Sci 120:3327–3335
11. Askham JM, Vaughan KT, Goodson HV, Morrison EE (2002) Evidence that an interaction between EB1 and $p150^{Glued}$ is required for the formation and maintenance of a radial microtubule array anchored at the centrosome. Mol Biol Cell 13:3627–3645
12. Bannigan A, Lizotte-Waniewski M, Riley M, Baskin TI (2008) Emerging molecular mechanisms that power and regulate the anastral mitotic spindle of flowering plants. Plant Cell Physiol 65:1–11
13. Bartolini F, Gundersen GG (2006) Generation of noncentrosomal microtubule arrays. J Cell Sci 119:4155–4163
14. Bartolini F, Moseley JB, Schmoranzer J, Cassimeris L, Goode BL, Gundersen GG (2008) The formin mDia2 stabilizes microtubules independently of its actin nucleation activity. J Cell Biol 181:523–536
15. Berrueta L, Kraeft S-K, Tirnauer JS, Schuyler SC, Chen LB, Hill DE, Pellman D, Bierer BE (1998) The adenomatous polyposis coli-binding protein EB1 is associated with cytoplasmic and spindle microtubules. Proc Natl Acad Sci U S A 95:10596–10601
16. Bibikova TN, Blancaflor EB, Gilroy S (1999) Microtubules regulate tip growth orientation in root hairs of Arabidopsis thaliana. Plant J 17:657–665
17. Bieling P, Laan L, Schek H, Munteanu EL, Sandblad L, Dogterom M, Brunner D, Surrey T (2007) Reconstitution of a microtubule plus-end tracking system in vitro. Nature 450:1100–1105
18. Bill Wickstead KG (2007) Dyneins across eukaryotes: a comparative genomic analysis. Traffic 8:1708–1721
19. Bisgrove SR, Hable WE, Kropf DL (2004) +TIPs and microtubule regulation. The beginning of the plus end in plants. Plant Physiol 136:3855–3863
20. Bisgrove SR, Lee YR, Liu B, Peters NT, Kropf DL (2008) The microtubule plus-end binding protein EB1 functions in root responses to touch and gravity signals in Arabidopsis. Plant Cell 20:396–410
21. Brandner K, Sambade A, Boutant E, Didier P, Mely Y, Ritzenthaler C, Heinlein M (2008) Tobacco mosaic virus movement protein interacts with green fluorescent protein-tagged microtubule end-binding protein 1. Plant Physiol 147:611–623

22. Brouhard GJ, Stear JH, Noetzel TL, Al-Bassam J, Kinoshita K, Harrison SC, Howard J, Hyman AA (2008) XMAP215 is a processive microtubule polymerase. Cell 132:79–88
23. Bu W, Su L-K (2003) Characterization of functional domains of human EB1 family proteins. J Biol Chem 278:49721–49731
24. Cai G (2010) Assembly and disassembly of plant microtubules: tubulin modifications and binding to MAPs. J Exp Bot 61:623–626
25. Chan J, Calder G, Fox S, Lloyd C (2005) Localization of the microtubule end binding protein EB1 reveals alternative pathways of spindle development in Arabidopsis suspension cells. Plant Cell 17:1737–1748
26. Chan J, Calder GM, Doonan JH, Lloyd CW (2003) EB1 reveals mobile microtubule nucleation sites in *Arabidopsis*. Nat Cell Biol 5:967–971
27. Chretien D, Fuller SD, Karsenti E (1995) Structure of growing microtubule ends: two dimensional sheets close into tubes at variable rates. J Cell Biol 129:1311–1328
28. Chretien D, Janosi I, Taveau J-C, Flyvbjerg H (1999) Microtubule's conformational cap. Cell Struct Funct 24:299–303
29. Collings DA, Lill AW, Himmelspach R, Wasteneys GO (2006) Hypersensitivity to cytoskeletal antagonists demonstrates microtubule-microfilament cross-talk in the control of root elongation in Arabidopsis thaliana. New Phytol 170:275–290
30. Coquelle FM, Vitre B, Arnal I (2009) Structural basis of EB1 effects on microtubule dynamics. Biochem Soc Trans 37:997–1001
31. Crowell EF, Bischoff V, Desprez T, Rolland A, Stierhof Y-D, Schumacher K, Gonneau M, Hofte H, Vernhettes S (2009) Pausing of Golgi bodies on microtubules regulates secretion of cellulose synthase complexes in Arabidopsis. Plant Cell 21:1141–1154
32. des Georges A, Katsuki M, Drummond DR, Osei M, Cross RA, Amos LA (2008) Mal3, the Schizosaccharomyces pombe homolog of EB1, changes the microtubule lattice. Nat Struct Mol Biol 15:1102–1108
33. Desai A, Mitchison TJ (1997) Microtubule polymerization dynamics. Ann Rev Cell Dev Biol 13:83–117
34. Dhonukshe P, Gadella TWJJ (2003) Alteration of microtubule dynamic instability during preprophase band formation revealed by yellow fluorescent protein-CLIP170 microtubule plus-end labeling. Plant Cell 15:596–611
35. Dhonukshe P, Mathur J, Hulskamp M, Gadella TJ (2005) Microtubule plus-ends reveal essential links between intracellular polarization and localized modulation of endocytosis during division-plane establishment in plant cells. BMC Biol 3:11–25
36. Dimitrov A, Quesnoit M, Moutel S, Cantaloube I, Pous C, Perez F (2008) Detection of GTP-tubulin conformation in vivo reveals a role for GTP remnants in microtubule rescues. Science 322:1353–1356
37. Dixit R, Barnett J, Lazarus JE, Tokito M, Goldman YE, Holzbaur ELF (2009) Microtubule plus-end tracking by CLIP-170 requires EB1. Proc Natl Acad Sci U S A 106:492–497
38. Dixit R, Chang E, Cyr R (2006) Establishment of polarity during organization of the acentrosomal plant cortical microtubule array. Mol Biol Cell 17:1298–1305
39. Dixit R, Cyr R (2004) Encounters between dynamic cortical microtubules promote ordering of the cortical array through angle-dependent modifications of microtubule behavior. Plant Cell 16:3274–3284
40. Emons AM, Keelaar T (eds) (2009) Intracellular organization: a prerequisite for root hair elongation and cell wall deposition. Plant cell monograph, vol 12. Springer, Berlin, pp 27–44
41. Folker ES, Baker BM, Goodson HV (2005) Interactions between CLIP-170, tubulin, and microtubules: implications for the mechanism of CLIP-170 plus-end tracking behavior. Mol Biol Cell 16:5373–5384
42. Furutani I, Watanabe Y, Prieto R, Masukawa M, Suzuki K, Naoi K, Thitamadee S, Shikanai T, Hashimoto T (2000) The *SPIRAL* genes are required for directional control of cell elongation in Aarabidopsis thaliana. Development 127:4443–4453
43. Gard DL, Becker BE, Josh Romney S (2004) MAPping the eukaryotic tree of life: structure, function, and evolution of the MAP215/Dis1 family of microtubule-associated proteins. Int Rev Cytol 239:179–272

44. Gard DL, Kirschner MW (1987) A microtubule-associated protein from *Xenopus* eggs that specifically promotes assembly at the plus-end. J Cell Biol 105:2203–2215
45. Gardiner J, Marc J (2003) Putative microtubule-associated proteins from the Arabidopsis genome. Protoplasma 222:61–74
46. Geraldo S, Khanzada UK, Parsons M, Chilton JK, Gordon-Weeks PR (2008) Targeting of the F-actin-binding protein drebrin by the microtubule plus-tip protein EB3 is required for neuritogenesis. Nat Cell Biol 10:1181–1189
47. Goode BL, Drubin DG, Barnes G (2000) Functional cooperation between the microtubule and actin cytoskeletons. Curr Opin Cell Biol 12:63–71
48. Grigoriev I, Gouveia SM, van der Vaart B, Demmers J, Smyth JT, Honnappa S, Splinter D, Steinmetz MO, Putney JW Jr, Hoogenraad CC, Akhmanova A (2008) STIM1 is a MT-plus-end-tracking protein involved in remodeling of the ER. Curr Biol 18:177–182
49. Guo L, Ho C-HK, Kong Z, Lee Y-RJ, Qian Q, Liu B (2009) Evaluating the microtubule cytoskeleton and its interacting proteins in monocots by mining the rice genome. Ann Bot 103:387–402
50. Hayashi I, Ikura M (2003) Crystal structure of the amino-terminal microtubule-binding domain of end-binding protein 1 (EB1). J Biol Chem 278:36430–36434
51. Hayashi I, Wilde A, Mal TK, Ikura M (2005) Structural basis for the activation of microtubule assembly by the EB1 and $p150^{Glued}$ complex. Mol Cell 19:449–460
52. Honnappa S, Gouveia SM, Weisbrich A, Damberger FF, Bhavesh NS, Jawhari H, Grigoriev I, van Rijssel FJA, Buey RM, Lawera A, Jelesarov I, Winkler FK, Wüthrich K, Akhmanova A, Steinmetz MO (2009) An EB1-binding motif acts as a microtubule tip localization signal. Cell 138:366–376
53. Honnappa S, John CM, Kostrewa D, Winkler FK, Steinmetz MO (2005) Structural insights into the EB1-APC interaction. EMBO J 24:261–269
54. Honnappa S, Okhrimenko O, Jaussi R, Jawhari H, Jelesarov I, Winkler FK, Steinmetz MO (2006) Key interaction modes of dynamic +TIP networks. Mol Cell 23:663–671
55. Howard J, Hyman AA (2009) Growth, fluctuation and switching at microtubule plus ends. Nat Rev Mol Cell Biol 10:569–574
56. Hunter AW, Caplow M, Coy DL, Hancock WO, Diez S, Wordeman L, Howard J (2003) The kinesin-related protein MCAK is a microtubule depolymerase that forms an ATP-hydrolyzing complex at microtubule ends. Mol Cell 11:445–457
57. Ishida T, Kaneko Y, Iwano M, Hashimoto T (2007) Helical microtubule arrays in a collection of twisting tubulin mutants of Arabidopsis thaliana. Proc Natl Acad Sci U S A 104:8544–8549
58. Jiang K, Wang J, Liu J, Ward T, Wordeman L, Davidson A, Wang F, Yao X (2009) TIP150 interacts with and targets MCAK at the microtubule plus ends. EMBO 10:857–865
59. Kawamura E, Himmelspach R, Rashbrooke MC, Whittington AT, Gale KR, Collings DA, Wasteneys GO (2006) *MICROTUBULE ORGANIZATION 1* regulates structure and function of microtubule arrays during mitosis and cytokinesis in the *Arabidopsis* root. Plant Physiol 140:102–114
60. Kawamura E, Wasteneys GO (2008) MOR1, the Arabidopsis thaliana homologue of Xenopus MAP215, promotes rapid growth and shrinkage, and suppresses the pausing of microtubules in vivo. J Cell Sci 121:4114–4123
61. Ketelaar T, de Ruijter NC, Emons AM (2003) Unstable F-actin specifies the area and microtubule direction of cell expansion in Arabidopsis root hairs. Plant Cell 15:285–292
62. King JS, Veltman DM, Georgiou M, Baum B, Insall RH (2010) SCAR/WAVE is activated at mitosis and drives myosin-independent cytokinesis. J Cell Sci 123:2246–2255
63. Komaki S, Abe T, Coutuer S, Inze D, Russinova E, Hashimoto T (2010) Nuclear-localized subtype of end-binding 1 protein regulates spindle organization in Arabidopsis. J Cell Sci 123:451–459
64. Komarova Y, De Groot CO, Grigoriev I, Gouveia SM, Munteanu EL, Schober JM, Honnappa S, Buey RM, Hoogenraad CC, Dogterom M, Borisy GG, Steinmetz MO, Akhmanova A (2009) Mammalian end binding proteins control persistent microtubule growth. J Cell Biol 184:691–706
65. Korinek WS, Copeland MJ, Chaudhuri A, Chant J (2000) Molecular linkage underlying microtubule orientation toward cortical sites in yeast. Science 287:2257–2259

66. Kronja I, Kruljac-Letunic A, Caudron-Herger M, Bieling P, Karsenti E (2009) XMAP215-EB1 interaction is required for proper spindle assembly and chromosome segregation in *Xenopus* egg extract. Mol Biol Cell 20:2684–2696
67. Kumar P, Lyle KS, Gierke S, Matov A, Danuser G, Wittmann T (2009) GSK3b phosphorylation modulates CLASP-microtubule association and lamella microtubule attachment. J Cell Biol 184:895–908
68. Lansbergen G, Akhmanova A (2006) Microtubule plus end: a hub of cellular activities. Traffic 7:499–507
69. Lawrence CJ, Dawe RK, Christie KR, Cleveland DW, Dawson SC, Endow SA, Goldstein LSB, Goodson HV, Hirokawa N, Howard J, Malmberg RL, McIntosh JR, Miki H, Mitchison TJ, Okada Y, Reddy ASN, Saxton WM, Schliwa M, Scholey JM, Vale RD, Walczak CE, Wordeman L (2004) A standardized kinesin nomenclature. J Cell Biol 167:19–22
70. Lawrence CJ, Morris NR, Meagher RB, Dawe RK (2001) Dyneins have run their course in plant lineage. Traffic 2:362–363
71. Ledbetter MC, Porter KR (1963) A "microtubule" in plant cell fine structure. J Cell Biol 19:239–250
72. Lee L, Tirnauer JS, Li J, Schuyler SC, Liu JY, Pellman D (2000) Positioning of the mitotic spindle by a cortical microtubule capture mechanism. Science 287:2260–2262
73. Lee T, Langford KJ, Askham JM, Bruning-Richardson A, Morrison EE (2008) MCAK associates with EB1. Oncogene 27:2494–2500
74. Lee W-L, Bezanilla M, Pollard TD (2000) Fission yeast myosin-I, Myo1p, stimulates actin assembly by Arp2/3 complex and shares functions with WASp. J Cell Biol 151:789–799
75. Lee Y-RJ, Liu B (2004) Cytoskeletal motors in Arabidopsis. Sixty-one kinesins and seventeen myosins. Plant Physiol 136:3877–3883
76. Lee YJ, Yang Z (2008) Tip growth: signaling in the apical dome. Curr Opin Plant Biol 11:662–671
77. Ligon LA, Shelly SS, Tokito M, Holzbaur ELF (2003) The microtubule plus-end proteins EB1 and dynactin have differential effects on microtubule polymerization. Mol Biol Cell 14:1405–1417
78. Louie RK, Bahmanyar S, Siemers KA, Votin V, Chang P, Stearns T, Nelson WJ, Barth AIM (2004) Adenomatous polyposis coli and EB1 localize in close proximity of the mother centriole and EB1 is a functional component of centrosomes. J Cell Sci 117:1117–1128
79. Lu L, Lee Y-RJ, Pan R, Maloof JN, Liu B (2005) An internal motor kinesin is associated with the Golgi apparatus and plays a role in trichome morphogenesis in Arabidopsis. Mol Biol Cell 16:811–823
80. Maiato H, Fairley EAL, Rieder CL, Swedlow JR, Sunkel CE, Earnshaw WC (2003) Human CLASP1 is an outer kinetochore component that regulates spindle microtubule dynamics. Cell 113:891–904
81. Maney T, Hunter AW, Wagenbach M, Wordeman L (1998) Mitotic centromere-associated kinesin is important for anaphase chromosome segregation. J Cell Biol 142:787–801
82. Manna T, Honnappa S, Steinmetz MO, Wilson L (2008) Suppression of microtubule dynamic instability by the +TIP protein EB1 and its modulation by the CAP-Gly domain of $p150^{Glued}$. Biochemistry 47:779–786
83. Mathur J, Mathur N, Kernebeck B, Srinivas BP, Hulskamp M (2003) A novel localization pattern for an EB1-like protein links microtubule dynamics to endomembrane organization. Curr Biol 13:1991–1997
84. Matsuzaki M, Misumi O, Shin-i T, Maruyama S, Takahara M, Miyagishima SY, Mori T, Nishida K, Yagisawa F, Nishida K, Yoshida Y, Nishimura Y, Nakao S, Kobayashi T, Momoyama Y, Higashiyama T, Minoda A, Sano M, Nomoto H, Oishi K, Hayashi H, Ohta F, Nishizaka S, Haga S, Miura S, Morishita T, Kabeya Y, Terasawa K, Suzuki Y, Ishii Y, Asakawa S, Takano H, Ohta N, Kuroiwa H, Tanaka K, Shimizu N, Sugano S, Sato N, Nozaki H, Ogasawara N, Kohara Y, Kuroiwa T (2004) Genome sequence of the ultrasmall unicellular red alga *Cyanidioschyzon merolae* 10D. Nature 428:653–657

85. Miller RK, Cheng S-C, Rose MD (2000) Bim1p/Yeb1p mediates the Kar9p-dependent cortical attachment of cytoplasmic microtubules. Mol Biol Cell 11:2949–2959
86. Mimori-Kiyosue Y, Grigoriev I, Lansbergen G, Sasaki H, Matsui C, Severin F, Galjart N, Grosveld F, Vorobjev I, Tsukita S, Akhmanova A (2005) CLASP1 and CLASP2 bind to EB1 and regulate microtubule plus-end dynamics at the cell cortex. J Cell Biol 168:141–153
87. Mimori-Kiyosue Y, Shiina N, Tsukita S (2000) Adenomatous Polyposis Coli (APC) protein moves along microtubules and concentrates at their growing ends in epithelial cells. J Cell Biol 148:505–518
88. Mimori-Kiyosue Y, Shiina N, Tsukita S (2000) The dynamic behavior of the APC-binding protein EB1 on the distal ends of microtubules. Curr Biol 10:865–868
89. Minc N, Bratman SV, Basu R, Chang F (2009) Establishing new sites of polarization by microtubules. Curr Biol 19:83–94
90. Mitchison T, Kirschner M (1984) Dynamic instability of microtubule growth. Nature 312:237–242
91. Moore AT, Rankin KE, von Dassow G, Peris L, Wagenbach M, Ovechkina Y, Andrieux A, Job D, Wordeman L (2005) MCAK associates with the tips of polymerizing microtubules. J Cell Biol 169:391–397
92. Morrison EE, Wardleworth BN, Askham JM, Markham AF, Meredith DM (1998) EB1, a protein which interacts with the APC tumour suppressor, is associated with the microtubule cytoskeleton throughout the cell cycle. Oncogene 17:3471–3477
93. Müller S, Wright AJ, Smith LG (2009) Division plane control in plants: new players in the band. Trends Cell Biol 19:180–188
94. Nakajima K, Furutani I, Tachimoto H, Matsubara H, Hashimoto T (2004) *SPIRAL1* encodes a plant-specific microtubule-localized protein required for directional control of rapidly expanding Arabidopsis cells. Plant Cell 16:1178–1190
95. Nakajima K, Kawamura T, Hashimoto T (2006) Role of the SPIRAL1 gene family in anisotropic growth of Arabidopsis thaliana. Plant Cell Physiol 47:513–522
96. Nakamura M, Zhou XZ, Lu KP (2001) Critical role for the EB1 and APC interaction in the regulation of microtubule polymerization. Curr Biol 11:1062–1067
97. Niethammer P, Kronja I, Kandels-Lewis S, Rybina S, Bastiaens P, Karsenti E (2007) Discrete states of a protein interaction network govern interphase and mitotic microtubule dynamics. PLoS Biol 5:190–202
98. Pedersen LB, Geimer S, Sloboda RD, Rosenbaum JL (2003) The microtubule plus end-tracking protein EB1 is localized to the flagellar tip and basal bodies in *Chlamydomonas reinhardtii*. Curr Biol 13:1969–1974
99. Pedersen LB, Miller MS, Geimer S, Leitch JM, Rosenbaum JL, Cole DG (2005) *Chlamydomonas* IFT172 is encoded by *FLA11*, interacts with CrEB1, and regulates IFT at the flagellar tip. Curr Biol 15:262–266
100. Petrásek J, Schwarzerová K (2009) Actin and microtubule cytoskeleton interactions. Curr Opin Plant Biol 12:728–734
101. Reddy AS, Day IS (2001) Kinesins in the Arabidopsis genome: a comparative analysis among eukaryotes. BMC Genomics 2:2
102. Rehberg M, Graf R (2002) *Dictyostelium* EB1 is a genuine centrosomal component required for proper spindle formation. Mol Biol Cell 13:2301–2310
103. Richardson DE, Simmons MP, Reddy ASN (2006) Comprehensive comparative analysis of kinesins in photosynthetic eukaryotes. BMC Genomics 7:18
104. Robert S, Bichet A, Grandjean O, Kierzkowski D, Satiat-Jeunemaitre B, Pelletier S, Hauser M-T, Hofte H, Vernhettes S (2005) An Arabidopsis endo-1, 4-β-D-glucanase involved in cellulose synthesis undergoes regulated intracellular cycling. Plant Cell 17:3378–3389
105. Rogers SL, Rogers GC, Sharp DJ, Vale RD (2002) *Drosophila* EB1 is important for proper assembly, dynamics, and positioning of the mitotic spindle. J Cell Biol 158:873–884
106. Rogers SL, Wiedemann U, Hacker U, Turck C, Vale RD (2004) *Drosophila* RhoGEF2 associates with microtubule plus ends in an EB1-dependent manner. Curr Biol 14:1827–1833

107. Roudier F, Fernandez AG, Fujita M, Himmelspach R, Borner GHH, Schindelman G, Song S, Baskin TI, Dupree P, Wasteneys GO, Benfey PN (2005) COBRA, an *Arabidopsis* extracellular glycosyl-phosphatidyl inositol-anchored protein, specifically controls highly anisotropic expansion through its involvement in cellulose microfibril orientation. Plant Cell 17:1749–1763
108. Sandblad L, Busch KE, Tittmann P, Gross H, Brunner D, Hoenger A (2006) The *Schizosaccharomyces pombe* EB1 homolog Mal3p binds and stabilizes the microtubule lattice seam. Cell 127:1415–1424
109. Schmit A-C, Nick P (2008) Microtubules and the evolution of mitosis. In: Nick P (ed) Plant cell monograph, vol 11. Springer, Berlin, pp 234–266
110. Schober JM, Cain JM, Komarova YA, Borisy GG (2009) Migration and actin protrusion in melanoma cells are regulated by EB1 protein. Cancer Lett 284:30–36
111. Schroer TA (2004) Dynactin. Annu Rev Cell Dev Biol 20:759–779
112. Sedbrook JC, Ehrhardt DW, Fisher SE, Scheible WR, Somerville CR (2004) The Arabidopsis sku6/spiral1 gene encodes a plus end-localized microtubule-interacting protein involved in directional cell expansion. Plant Cell 16:1506–1520
113. Shaw SL, Kamyar R, Ehrhardt DW (2003) Sustained microtubule treadmilling in *Arabidopsis* cortical arrays. Science 300:1715–1718
114. Sieberer BJ, Timmers CJ (2009) Microtubules in plant root hairs and their role in cell polarity and tip growth. In: Emons AM, Ketelaar T (eds) Plant cell monograph, vol 12. Springer, Berlin, pp 233–248
115. Simon JR, Salmon ED (1990) The structure of microtubule ends during the elongation and shortening phases of dynamic instability examined by negative-stain electron microscopy. J Cell Sci 96:571–582
116. Skube SB, Chaverri JM, Goodson HV (2009) Effect of GFP tags on the localization of EB1 and EB1 fragments in vivo. Cell Motil Cytoskeleton 67:1–12
117. Slep KC (2009) The role of TOG domains in microtubule plus end dynamics. Biochem Soc Trans 37:1002–1006
118. Slep KC (2010) Structural and mechanistic insights into microtubule end-binding proteins. Curr Opin Cell Biol 22:88–95
119. Slep KC, Rogers SL, Elliot SL, Okhura H, Kolodziej PA, Vale RD (2005) Structural determinants for EB1-mediated recruitment of APC and spectraplakins to the microtubule plus end. J Cell Biol 168:587–598
120. Steinmetz MO, Akhmanova A (2008) Capturing protein tails by CAP-Gly domains. Trends Biochem Sci 33:535–545
121. Straube A, Brill M, Oakley BR, Horio T, Steinberg G (2003) Microtubule organization requires cell cycle-dependent nucleation at dispersed cytoplasmic sites: polar and perinuclear microtubule organizing centers in the plant pathogen *Ustilago maydis*. Mol Biol Cell 14:642–657
122. Su L-K, Burrell M, Hill DE, Gyuris J, Brent R, Wiltshire R, Trent J, Vogelstein B, Kinzler KW (1995) APC binds to the novel protein EB1. Cancer Res 55:2972–2977
123. Tanaka K, Mukae N, Dewar H, van Breugel M, James EK, Prescott AR, Antony C, Tanaka TU (2005) Molecular mechanisms of kinetochore capture by spindle microtubules. Nature 434:987–994
124. Tirnauer JS, Canman JC, Salmon ED, Mitchison T (2002) EB1 targets to kinetochores with attached polymerizing microtubules. Mol Biol Cell 13:4308–4316
125. Tirnauer JS, Grego S, Salmon ED, Mitchison T (2002) EB1-microtubule interactions in *Xenopus* egg extracts: role of EB1 in microtubule stabilization and mechanisms of targeting to microtubules. Mol Biol Cell 13:3614–3626
126. Tirnauer JS, O'Toole E, Berrueta L, Bierer BE, Pellman D (1999) Yeast Bim1p promotes the G1-specific dynamics of microtubules. J Cell Biol 145:993–1007
127. Tsvetkov AS, Samsonov A, Akhmanova A, Galjart N, Popov SV (2007) Microtubule-binding proteins CLASP1 and CLASP2 interact with actin filaments. Cell Motil Cytoskeleton 64:519–530

128. Twell D, Park SK, Hawkins TJ, Schubert D, Schmidt R, Smertenko A, Hussey PJ (2002) MOR1/GEM1 has an essential role in the plant-specific cytokinetic phragmoplast. Nat Cell Biol 4:711–714
129. Van Damme D, Bouget F-Y, Van Poucke K, Inze D, Geelen D (2004) Molecular dissection of plant cytokinesis and phragmoplast structure: a survey of GFP-tagged proteins. Plant J 40:386–398
130. Van Damme D, Van Poucke K, Boutant E, Ritzenthaler C, Inze D, Geelen D (2004) In vivo dynamics and differential microtubule-binding activities of MAP65 proteins. Plant Physiol 136:3956–3967
131. van der Vaart B, Akhmanova A, Straube A (2009) Regulation of microtubule dynamic instability. Biochem Soc Trans 37:1007–1013
132. Vaughan KT (2004) Surfing, regulating and capturing: are all microtubule-tip-tracking proteins created equal? Trends Cell Biol 14:491–496
133. Vaughan KT (2005) TIP maker and TIP marker; EB1 as a master controller of microtubule plus ends. J Cell Biol 171:197–200
134. Vitre B, Coquelle FM, Heichette C, Garnier C, Chretien D, Arnal I (2008) EB1 regulates microtubule dynamics and tubulin sheet closure in vitro. Nat Cell Biol 10:415–421
135. Vos JW, Dogterom M, Emons AMC (2004) Microtubules become more dynamic but not shorter during preprophase band formation: a possible "search and capture" mechanism for microtubule translocation. Cell Motil Cytoskeleton 57:246–258
136. Walczak CE (2003) The Kin I kinesins are microtubule end-stimulated ATPases. Mol Cell 11:286–288
137. Walczak CE, Mitchison T, Desai A (1996) XKCM1: a *Xenopus* kinesin-related protein that regulates microtubule dynamics during mitotic spindle assembly. Cell 84:37–47
138. Wasteneys GO (2002) Microtubule organization in the green kingdom: chaos or self-order? J. Cell Sci 115:1345–1354
139. Wasteneys GO, Ambrose JC (2009) Spatial organization of plant cortical microtubules: close encounters of the 2D kind. Trends Cell Biol 19:62–71
140. Wei L, Zhang W, Liu Z, Li Y (2009) AtKinesin-13A is located on Golgi-associated vesicle and involved in vesicle formation/budding in Arabidopsis root-cap peripheral cells. BMC Plant Biol 9:138
141. Wen Y, Eng CH, Schmoranzer J, Cabrera-Poch N, Morris EJS, Chen M, Wallar BJ, Alberts AS, Gundersen GG (2004) EB1 and APC bind to mDia to stabilize microtubules downstream of Rho and promote cell migration. Nat Cell Biol 6:820–830
142. Whittington AT, Vugrek O, Wei KJ, Hasenbein NG, Sugimoto K, Rashbrooke MC, Wasteneys GO (2001) MOR1 is essential for organizing cortical microtubules in plants. Nature 411:610–613
143. Wightman R, Turner SR (2008) The roles of the cytoskeleton during cellulose deposition at the secondary cell wall. Plant J 54:794–805
144. Wittmann T, Waterman-Storer CM (2005) Spatial regulation of CLASP affinity for microtubules by Rac1 and GSK3β in migrating epithelial cells. J Cell Biol 169:929–939
145. Yasuhara H, Muraoka M, Shogaki H, Mori H, Sonobe S (2002) TMBP200, a microtubule bundling polypeptide isolated from telophase tobacco BY-2 cells is a MOR1 homologue. Plant Cell Physiol 43:595–603
146. Zanic M, Stear JH, Hyman AA, Howard J (2009) EB1 recognizes the nucleotide state of tubulin in the microtubule lattice. PLoS One 4:e7585

Chapter 6
Microtubule Motor Proteins in the Eukaryotic Green Lineage: Functions and Regulation

A.S.N. Reddy and Irene S. Day

6.1 Introduction

Microtubules (MTs), one of the three cytoskeletal structures in all eukaryotes, play key roles in many fundamental cellular and developmental processes such as cell growth, cell shape, cell division, cell differentiation and intracellular transport. MTs are cylindrical polar polymers made up of repeating α- and β-tubulin. MTs undergo alternating phases of polymerization and depolymerization. Like individual MTs, arrays of MTs are highly dynamic in that they can form, breakdown and reform. In plants interphase MTs are a mainly parallel array found beneath the plasma membrane and function in cell shape and growth processes. MTs also function in intracellular transport and separation of chromosomes in cell division. During cytokinesis, MTs are part of the phragmoplast that is responsible for the formation of a new cell plate between daughter cells. MTs together with actin filaments/microfilaments (MFs) form two distinct cytoskeletal networks that are independent but can coordinate activity in such processes as mitotic/meiotic spindle formation, cytokinesis and movement of cargo. Molecular motors use the MTs and MFs in regulating numerous cellular and developmental processes. There are three families of motors in eukaryotes: the myosins, the dyneins, and the kinesins. All three motors use energy released from ATP hydrolysis to move along either the MTs or MFs. The MT-associated motors are dyneins and kinesins. Dynein is a microtubule-dependent, minus end-directed motor that is required for intraflagellar transport, is involved in organizing the Golgi apparatus and spindle poles, and has roles in moving nuclei, vesicles, pigment granules, and chromosomes [21, 27]. A comparative analysis of 24 diverse eukaryotic genomes confirmed that dyneins are not represented in higher plant genomes [77]. This chapter focuses on various aspects of kinesins in photosynthetic eukaryotes. Myosins, the actin-based motor proteins, are discussed in detail in Sect. 1, Chap. 3.

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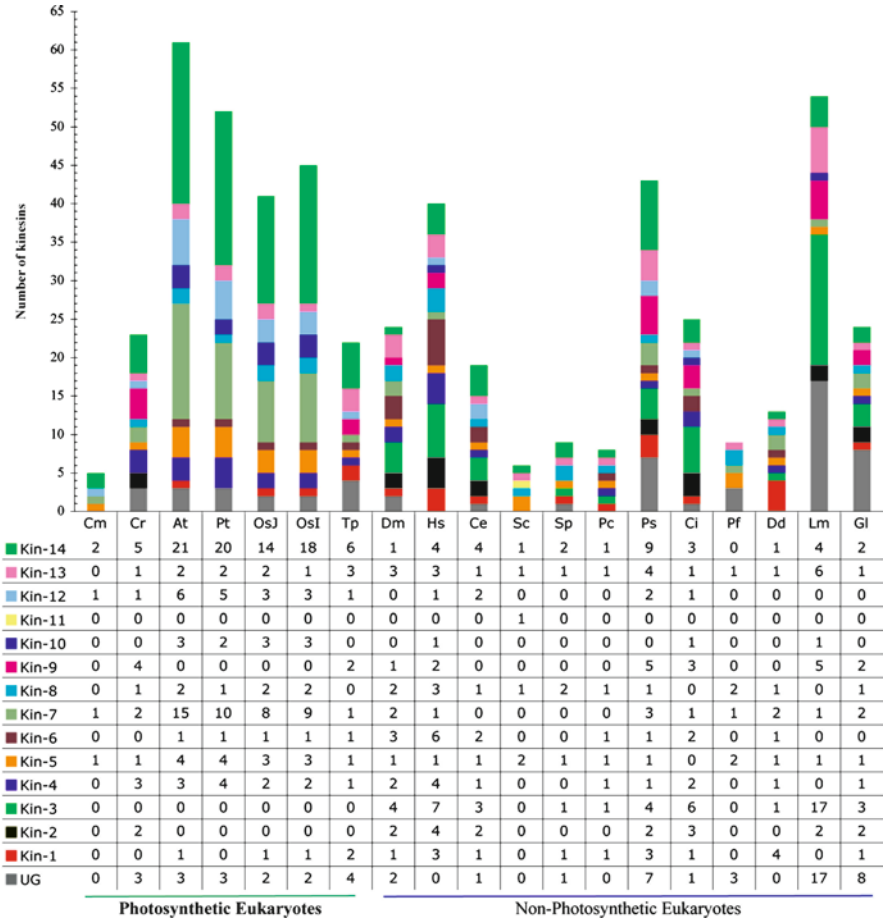
6.2 Kinesins

Kinesins, a super family of microtubule-based motors, are present in plants and animals [30, 48, 55]. Members of the kinesin family have a highly conserved catalytic region of about 350 residues constituting the motor domain, which contains the ATP- and MT-binding sites. Most kinesins have a stalk region that forms an alpha-helical coiled-coil region and a variable tail, which is thought to interact with a specific cargo.

6.3 Kinesin Types and Evolutionary Relationships

Fourteen families of kinesins have been recognized in eukaryotes (Kinesin-1 to -14) [26]. The kinesin motor domain was used to identify kinesins in the completed genome sequences of 19 species, including both photosynthetic and non-photosynthetic eukaryotes [55]. Figure 6.1 shows the species used and a distribution of the kinesins in each species. A total of 529 kinesins was used to perform comprehensive analysis of kinesins and to construct gene trees using the Bayesian and parsimony approaches. The previously recognized 14 families of kinesins were resolved as distinct lineages in their inferred gene tree (Fig. 6.2). Structurally these 14 families can be grouped into N-type kinesins with the MD at or near to the N-terminus (Kinesin-1 to 12), M-type kinesins with the MD flanked on both sides by other domains (Kinesin-13), and C-type kinesins with the MD close to the C-terminus (Kinesin-14) (Fig. 6.3a). This structure-based classification corresponds to a functional classification, plus-end directed motors (N-type), minus-end directed motors (C-type), and non-motor kinesins (M-type). Eleven percent of the kinesins were not resolved into the 14 families including a group of kinesins that is plant specific (see Fig. 6.2). Four of the 14 recognized families were not represented in flowering plants. These included Kinesin-2s that are present in ciliated and flagellated cells and function in intraflagellar transport, Kinesin-3 whose members are involved in organelle transport, Kinesin-9 of unknown function, and Kinesin-11 whose members function in signal transduction (Miki et al. 2005). The green alga, *Chlamydomonas reinhardtii* has two Kinesin-2 family members, reflecting the presence of flagella in this organism and it also has four Kinesin-9 family members. The Kinesin-1 family, also involved in cargo transporting, is underrepresented in plants. Arabidopsis has one possible Kinesin-1 as does rice. Plant Kinesin-1s form a subfamily in the Kinesin-1 family and do not have a light chain binding domain found in animal Kinesin-1 (Miki et al. 2006).

Two families (Kinesin-7 and Kinesin-14) have been greatly expanded in flowering plants [49, 55]. The Kinesin-14 family is the largest family of kinesins in flowering plants (Fig. 6.4). Analysis of the expanded family indicates duplication events that took place prior to the divergence of the red and green algae, before the divergence of the monocots and dicots and within the dicot or monocots [55]. Several of the



	Cm	Cr	At	Pt	OsJ	OsI	Tp	Dm	Hs	Ce	Sc	Sp	Pc	Ps	Ci	Pf	Dd	Lm	Gl
Kin-14	2	5	21	20	14	18	6	1	4	4	1	2	1	9	3	0	1	4	2
Kin-13	0	1	2	2	2	1	3	3	3	1	1	1	1	4	1	1	1	6	1
Kin-12	1	1	6	5	3	3	1	0	1	2	0	0	0	2	1	0	0	0	0
Kin-11	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Kin-10	0	0	3	2	3	3	0	0	1	0	0	0	0	0	1	0	0	1	0
Kin-9	0	4	0	0	0	0	2	1	2	0	0	0	0	5	3	0	0	5	2
Kin-8	0	1	2	1	2	2	0	2	3	1	1	2	1	1	0	2	1	0	1
Kin-7	1	2	15	10	8	9	1	2	1	0	0	0	0	3	1	1	2	1	2
Kin-6	0	0	1	1	1	1	1	3	6	2	0	0	1	1	2	0	1	0	0
Kin-5	1	1	4	4	3	3	1	1	1	1	2	1	1	1	0	2	1	1	1
Kin-4	0	3	3	4	2	2	1	2	4	1	0	0	1	1	2	0	1	0	1
Kin-3	0	0	0	0	0	0	0	4	7	3	0	1	1	4	6	0	1	17	3
Kin-2	0	2	0	0	0	0	0	2	4	2	0	0	0	2	3	0	0	2	2
Kin-1	0	0	1	0	1	1	2	1	3	1	0	1	1	3	1	0	4	0	1
UG	0	3	3	3	2	2	4	2	0	1	0	1	0	7	1	3	0	17	8

Fig. 6.1 Number and distribution of kinesins. Tabular and graphical representation of the number of kinesins found in completely sequenced genomes. The number of kinesins is shown on the y-axis with the 19 species displayed across the *x*-axis. Different colors represent the distribution of kinesins into specific families. The data table below the chart details the specific number of kinesins in each family per species. *Cm, Cyanidoschyzon merolae; Cr, Chlamydomonas reinhardtii; At, Arabidopsis thaliana; Pt, Populus trichocarpa; OsJ, Oryza sativa ssp. Japonica; OsI, Oryza sativa ssp. Indica; Tp, Thallassiosira pseudonana; Dm, Drosophila melanogaster; Hs, Homo sapiens; Ce, Caenorhabditis elegans; Sc, Saccharomyces cerevisiae; Sp, Schizosaccharomyces pombe; pc, Phaenerochaete chryosporium; Ps, Phytopthora sojae, Ci, Ciona intestinalis; Pf; Plasmodiumfalciparum; Dd, Dictyostelium discoideum; Lm, Leishmania major; Gl, Giardia lamblia*. Reproduced from Richardson et al. [55]

plant Kinesin-14s have an M-type or N-type motor rather than the canonical C-type of Kinesin-14 family members. The C-type motors have been shown in several cases to be minus-end motors but the direction of the M- and N-type Kinesin-14 members has not been determined. There are several plant-specific subfamilies

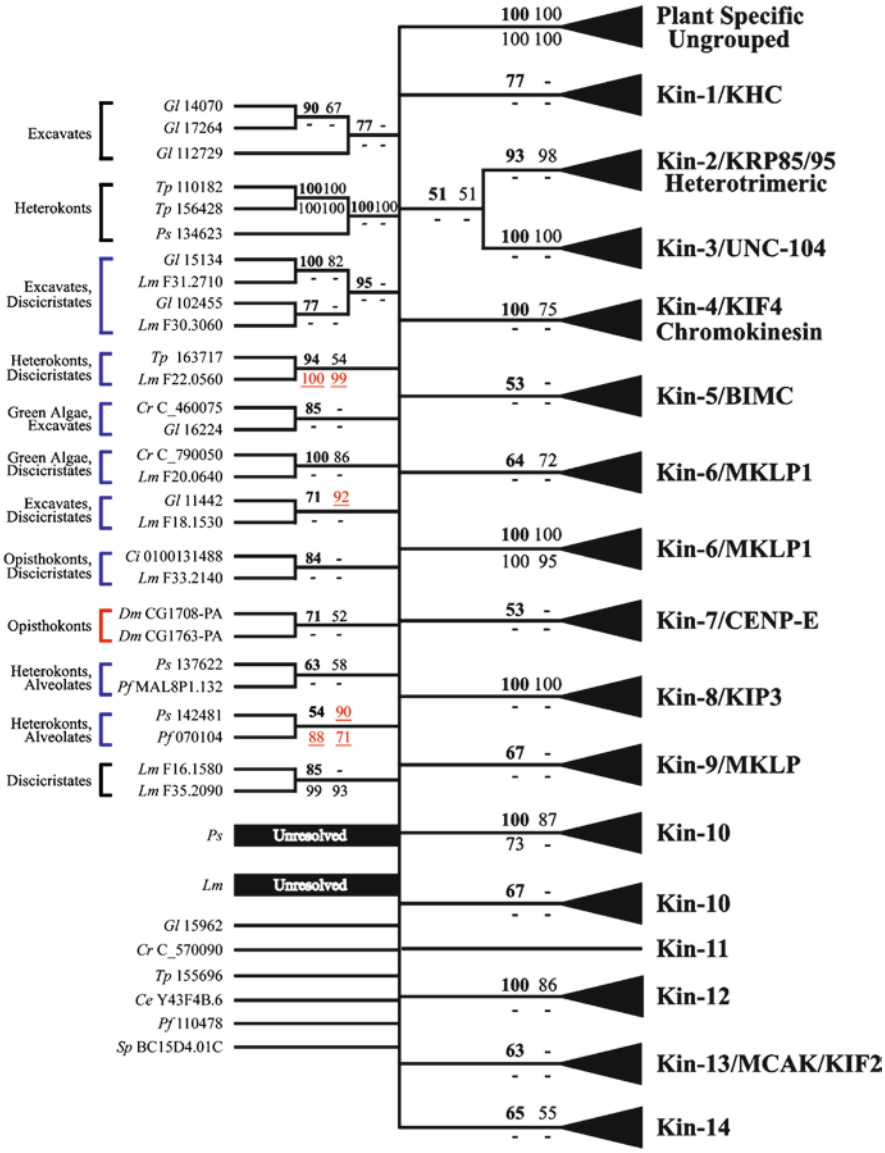


Fig. 6.2 Unrooted Bayesian tree of 529 kinesins based on their motor domain amino-acid-plus-gap-characters. Support values from each parsimony and Bayesian analysis are presented adjacent to the nodes. Support values above each branch correspond to Bayesian posterior probabilities whereas values below each branch correspond to parsimony jackknife support. In both cases the leftmost values are for the amino-acid-plus-gap-characters analyses and the rightmost values are for amino-acid-characters analyses. Bayesian posterior probabilities for the amino-acid-plus-gap-characters are also shown in bold. If a branch was unresolved in one of the other three analyses, it is indicated by "-" at the respective node. If a branch was contradicted in one of these other three analyses, it is indicated by underlined *red* font at the respective node with the single highest posterior probability or jackknife support value for the contradicting clade(s) shown. Ungrouped

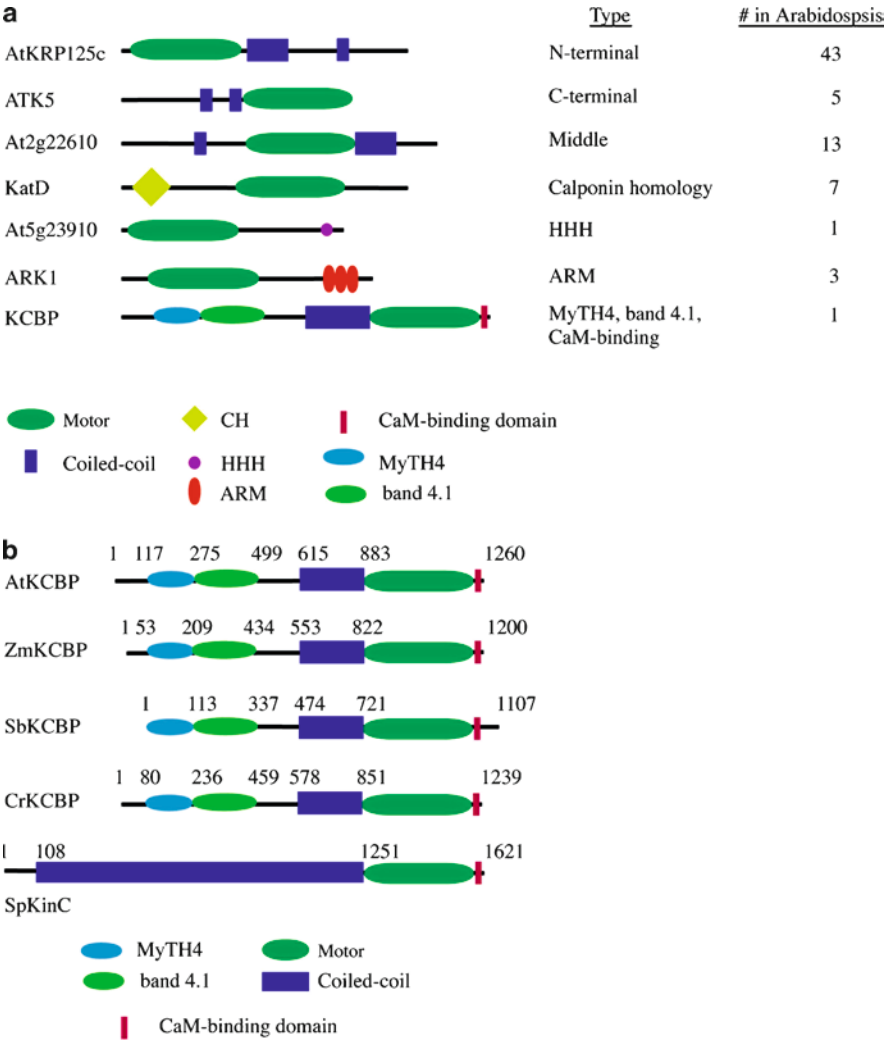


Fig. 6.3 Schematic diagrams of representative kinesins. (**a**) AtKRP125c is an N-terminal kinesin. ATK5 is a C-terminal kinesin and At2g22610 is an internal kinesin. Kat D has a calponin homology domain, At5g23910 has a HHH domain and ARK1 has three ARM domains. KCBP has two unique N-terminal domains, MyTH4 and band 4.1 and a C-terminal CaM-binding domain. (**b**) Schematic diagram of representative KCBPs and proteins with motors most similar to the KCBP motor. Domains are as shown in the key. Amino acid numbers are given at each domain transition

Fig. 6.2 (continued) kinesins are presented on the *left* side of the main polytomy, with the exception of the plant-specific ungrouped family that is shown on the *upper right* of the tree. The *Ps* and *Lm* unresolved blocks each contain 4 and 9 sequences, respectively. Brackets denote the major eukaryotic groupings. *Blue brackets* indicate taxa that are from multiple groups, *black brackets* indicate protozoan species and *red brackets* are reserved for opisthokonts. For full names of species see Fig. 6.1 legend. Reproduced from Richardson et al. [55]

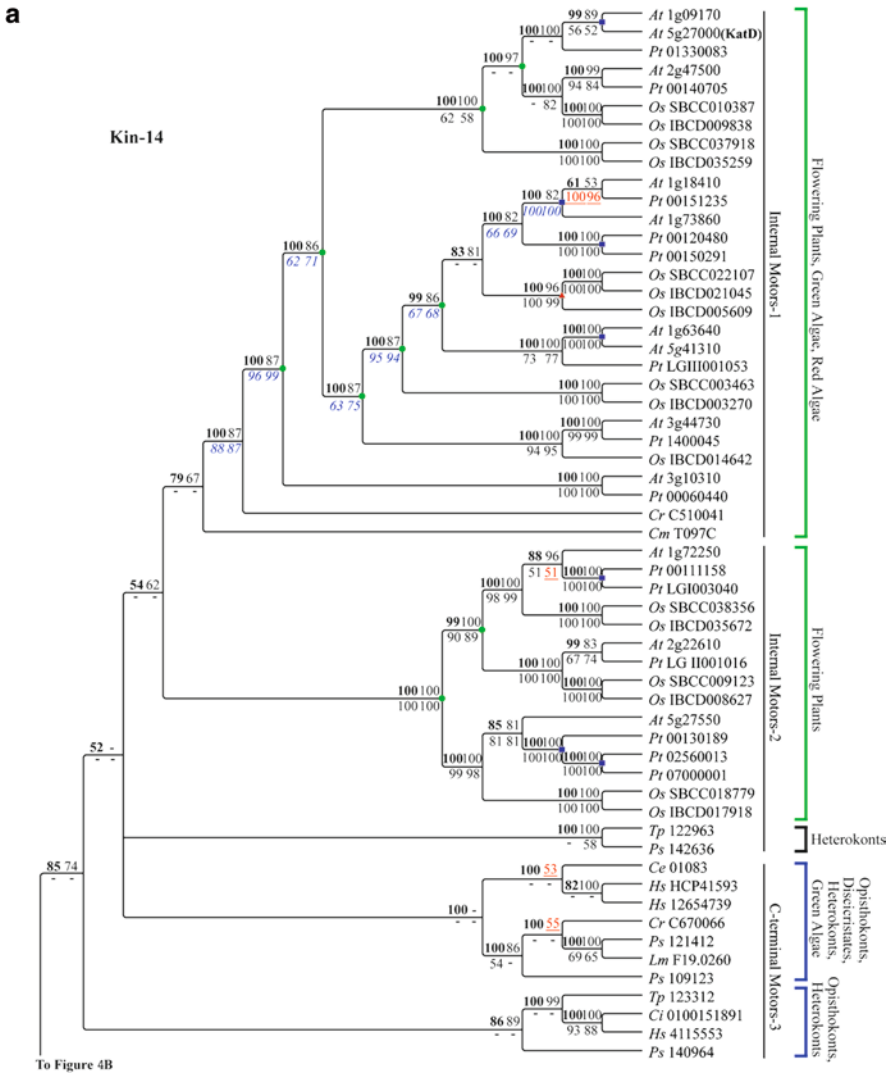


Fig. 6.4 Kinesin-14 family. Motor domain localizations are indicated adjacent to taxon labels. Experimentally studied Arabidopsis kinesins are indicated in parenthesis with its published name in *bold*. *Green brackets* indicate plant-specific groups, mixed clades are shown in *blue brackets*, *black brackets* indicate protozoan species and *red brackets* indicate groupings composed of opisthokonts. *Green circles*, *blue squares* and a *red triangle* indicate gene duplications in flowering plants, dicots and monocots, respectively. See Fig. 6.1 for full name of species and Fig. 6.2 for explanation of values. Reproduced from Richardson et al. [55]

including those with M-type and N-type motors while several other of the plant Kinesin-14s are in a sub-family with the true C-terminal motors in plants, diatoms, animals and protozoans. The functions of several of the Kinesin-14s in plants have been studied and will be discussed below. This family appears to play an important

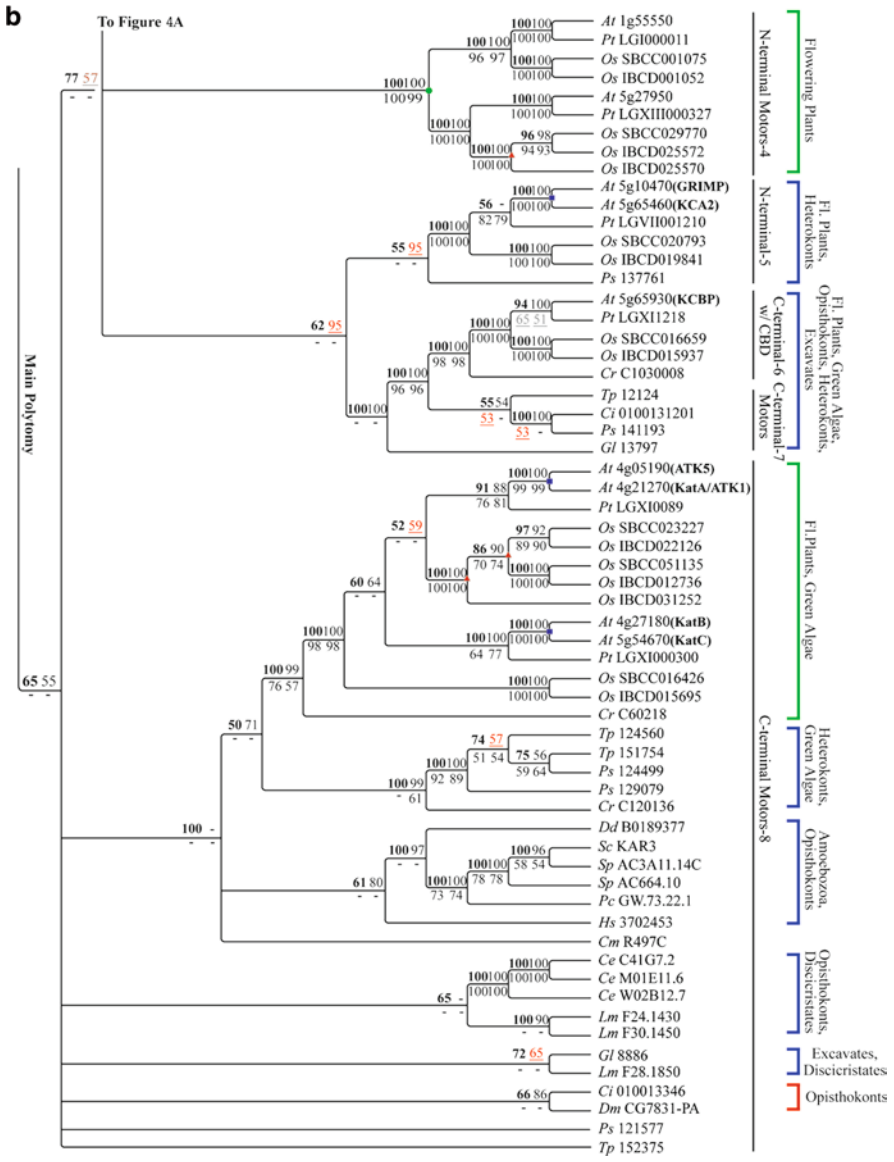


Fig. 6.4 (continued)

role in cell division including mitosis, meiosis and cytokinesis and may be involved in cargo transport. Plants have unique MT arrays such as the preprophase band and phragmoplast and lack centrosomes to organize a bipolar spindle, suggesting a requirement for unique kinesins to function with these arrays and to organize the spindle. Kinesin-7 is the second largest kinesin family in plants, one sub-family of which is specific to flowering plants (Fig. 6.5). This family has also been expanded

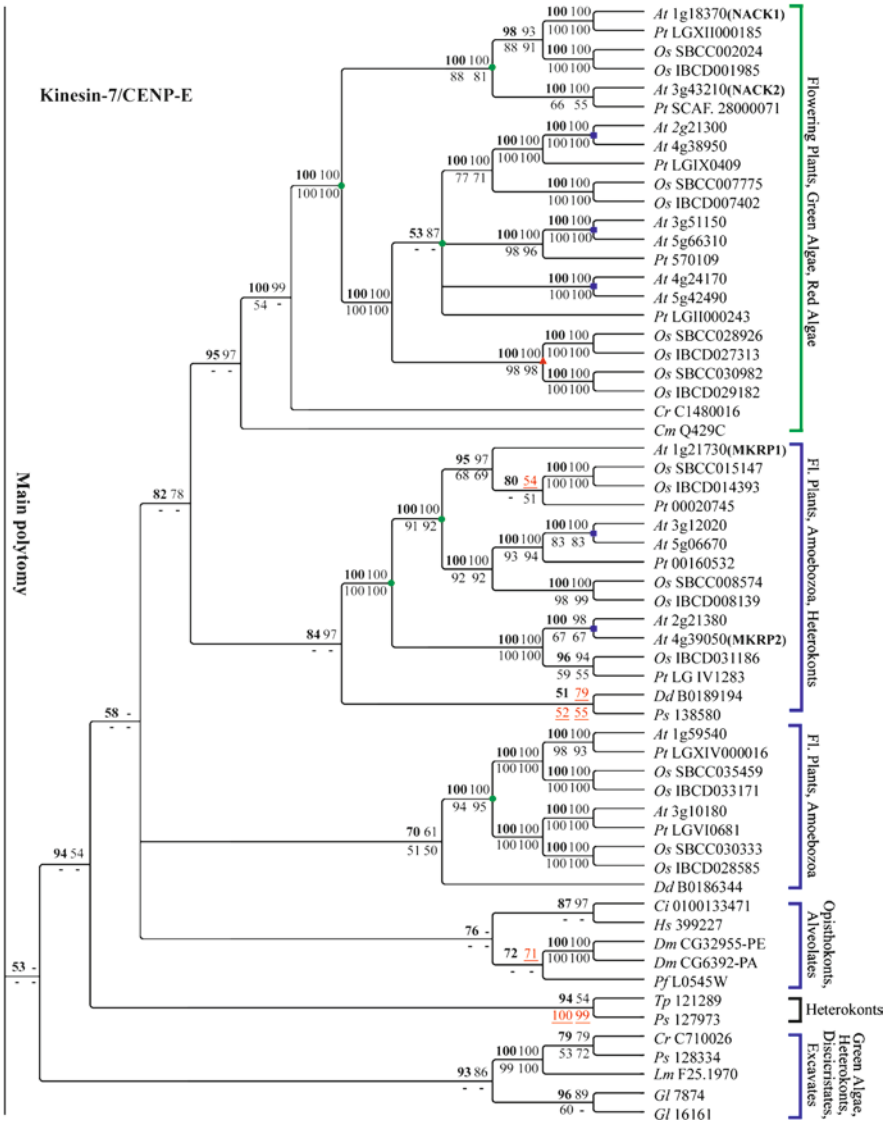


Fig. 6.5 Kinesin-7 family. Experimentally studied Arabidopsis kinesins are indicated in parenthesis with its published name in *bold*. *Green brackets* indicate plant-specific groups, mixed clades are shown in *blue brackets*, *black brackets* indicate protozoan species. *Green circles*, *blue squares* and a *red triangle* indicate gene duplications in flowering plants, dicots and monocots, respectively. See Fig. 6.1 for full name of species and Fig. 6.2 for explanation of values. Reproduced from Richardson et al. [55]

by duplications prior to the divergence of the monocots and dicots and within the dicots and monocots. Some plant members of this family appear to be involved in cytokinesis while others are targeted to mitochondria [18, 65].

The flowering plant families in Kinesin-4 and -5 form a separate clade as a sister group to *Chlamydomonas* [55]. There are two to four Kinesin-4s in flowering plants, one of which is involved with cell wall deposition [84]. A plant Kinesin-5 has been shown to be involved in spindle formation, as are other Kinesin-5 family members [4, 37]. Kinesin-6 family members are involved in cytokinesis in non-plant organisms, but as the mechanism of cytokinesis is different in plants, the one Kinesin-6 family member found in plants may have a different function [37, 55]. The functions of plant members of the Kinesin-8 and Kinesin-10 family are unknown; non-plant Kinesin-8s have been reported to function in nuclear migration and mitochondrial transport while Kinesin-10s have been reported to function in cell division and especially in chromosomal movements [37, 55]. The four flowering plant Kinesin-8 and -10 family members are in two different clades in both cases. Plant members of the Kinesin-12 family have been shown to be involved in phragmoplast and preprophase band functions while animal Kinesin-12s function in organelle transport [28, 37, 38, 74]. There are multiple members of Kinesin-12s in flowering plants in two distinct clades. The presence of a red algae kinesin as a sister to both clades and a *Chlamydomonas* kinesin to one suggests a duplication after the divergence of the red algae and another one prior to the divergence of the green algae and from flowering plants [55]. Plant kinesins in the Kinesin-13 group form a distinct clade with a sister *Chlamydomonas* kinesin. They, as do most Kinesin-13s, have M-type motors that are distinct from the Kinesin-14s with M-type motors. In animals, Kinesin-13s have been shown to transport vesicles and have MT depolymerizing activities while a plant Kinesin-13 has been shown to localize to Golgi stacks [33, 55, 76].

6.4 Domains in Kinesins

In addition to the motor domain, most kinesins contained a coiled-coil domain. Other domains have been identified in some of the kinesins, some of which are specific to photosynthetic eukaryotes and others that are specific to non-photosynthetic eukaryotes (Fig. 6.6) [49, 55]. The only domain found in both plants and animals is the helix-hairpin-helix (HHH) DNA binding motif found in the Kinesin-10 family (e.g. At5g23910, see Fig. 6.3a). KID, a HHH kinesin found in humans, was found to bind chromosomes and function in chromosome segregation [66]. MyTH4 (myosin tail homology) and band 4.1 domains are found only in a Kinesin-14 family member that is present only in land plants and green algae, KCBP [1, 2]. These domains are that is present in some animal proteins including some myosins but not in any other non-plant/green algal kinesin [55]. The MyTH4 and band 4.1 domains of KCBP (Fig. 6.3a) have been shown to bind microtubules suggesting that they may be involved in cross-linking and/or bundling MTs [40]. Myosins with these domains also bind MTs and, therefore, may function in cross-linking the actin and MT cytoskeleton [75]. A subfamily of plant-specific, but ungrouped kinesins, have an Armadillo repeat motif (ARM, Fig. 6.3a) [49, 55]. ARMs form a superhelix of

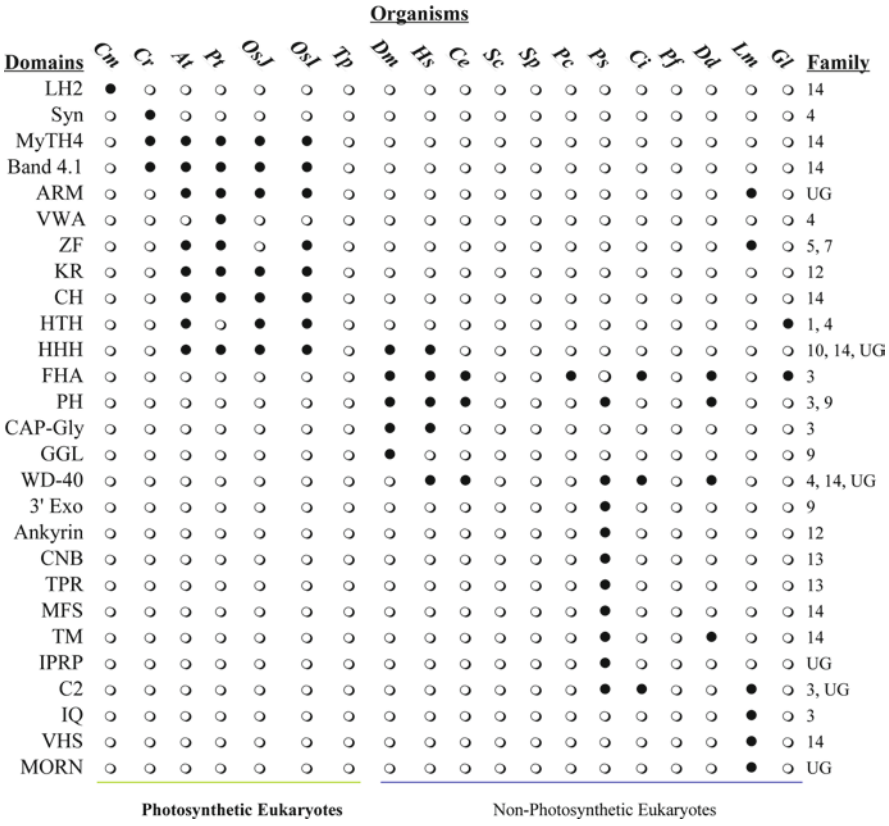


Fig. 6.6 Distribution of domains found within kinesins of 19 species. Schematic showing the presence or absence of particular domains and which families these domains are found in across the 19 species sampled. Species are listed horizontally across the top of the figure with the various domains listed vertically. Presence of a domain is indicated by a solid circle and its absence by a hollow circle. *LH2* lipoxygenase; *Syn* N-terminal syntaxin; *MyTH4* myosin tail homology 4; *ARM* armadillo; *VWA* von willebrand factor, type A; *ZF* zinc finger; *KR* kinesin-related; *CH* calponin homology; *HTH* helix-turn-helix; *HHH* helix-hairpin-helix; *FHA* fork head associated; *PH* pleckstrin homology; *CAP-Gly* glycine rich domain found in cytoskeleton associated proteins (CAPs); *GGL* G-protein gamma motif like; *WD-40* A 40 amino acid repeat motif with W and D dipeptides at the terminus; *3′ Exo* 3′ exoribonuclease; *CNB* cyclic nucleotide binding; *TPR* tetratricopeptide repeat; *MFS* major facilitator superfamily; *TM* transmembrane; *IPRP* inositol polyphospate related phosphatase; *C2* protein kinase C conserved region 2; *VHS* domain present in Vps-27, Hrs, STAM; *MORN* membrane occupation and recognition nexus. In *T. pseudonana*, most kinesins are short which could be due to poor quality of the gene models. Hence, it is possible that they contain additional domains. For full names of species see Fig. 6.1 legend. Reproduced from Richardson et al. [55]

helices and function in intracellular signaling and cytoskeletal regulation. No animal kinesins have an ARM but a Kinesin-2 family-associated protein called KAP3 contains the ARM repeat [81]. Some Kinesin-14 members have a calponin-homology (CH, see Fig. 6.3a) domain [49, 55]. CH domains are modules of approximately 100 amino acids. Animal proteins with pairs of CH domains, cross-link F-actin,

bundle actin or connect intermediate filaments to cytoskeleton while proteins with one CH domain are involved in signal transduction [6, 31].

The phylogenetic analysis of the 19 eukaryotic species from protists to animals and plants [55] and a similar analysis more heavily featuring animal (largely mammal) kinesins [37] both show an overall emergence of new kinesins and loss of others in different lineages reflecting the emergence of new structures or cargos that led to a new means of transport or loss of some structures or cargos resulting in loss of some motors. For instance, the Kinesin-2 loss in higher plants coincides with the absence of ciliated or flagellated cells in higher plants. No kinesins have been identified in lower plants having flagellated sperm, so it remains to be seen if this family has been retained in lower plants. The Kinesin-3 family involved in organelle transport is expanded in animals while absent in plants. The protozoan lineages in both analyses showed the most divergence with many of their kinesins not being resolved into any recognized family.

6.5 Known Functions of Kinesins in Plants

Here we have briefly discussed the functions of plant kinesins as they are covered in more detail in other chapters (Sect. 2, Chaps. 2 and 4). KCBP is a Kinesin-14 family member isolated in two diverse screens. A protein-protein interaction screen using Ca^{2+}/calmodulin (CaM) as a probe, identified KCBP as CaM-binding protein [53]. A screen for trichome mutants identified *ZWICHEL* (KCBP) as a protein involved in trichome morphogenesis [43]. Besides the motor, coiled-coil stalk and tail domain found in most kinesins, KCBP has three other unique domains, a C-terminal calmodulin-binding domain following the motor domain and N-terminal MyTH4 and band 4.1 domains. The KCBP motor domain binds microtubules in an ATP-dependent manner and a second region in the N terminus binds microtubules in an ATP-independent manner [40]. Localization of KCBP in Arabidopsis and tobacco revealed that KCBP localizes to the preprophase band, the mitotic spindle and the phragmoplast, suggesting its involvement in spindle formation and cytokinesis (Fig. 6.7) [7]. Localization of KCBP in *Haemanthus* endosperm revealed its mitotic distribution, first in association with the prophase spindle, then decorating microtubules of kinetochore-fibers through metaphase, redistributing and accumulating at the spindle polar regions by mid-anaphase and relocating toward the phragmoplast in telophase [60]. Furthermore, when an antibody to the CaM-binding domain of KCBP was injected into *Tradescantia virginiana* stamen hair cells in late prophase, rendering the KCBP constitutively active, breakdown of the nuclear membrane was induced in late prophase cells and the cells did not progress into anaphase [73]. Injection of the antibody later in mitosis resulted in aberrant phragmoplast formation and delayed cytokinesis. KCBP also has functions in cell growth and morphogenesis. KCBP mutants (*zwi*) in Arabidopsis have aberrant trichomes with fewer branches and shorter, thicker stalks suggesting a role in cell expansion and branching during trichome development [17, 43, 47]. KCBP may be involved in reorientation of microtubules and thus direction of the deposition of

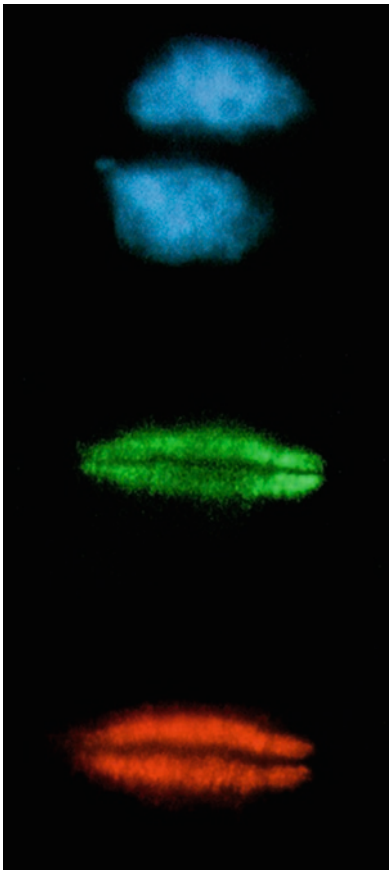


Fig. 6.7 Localization of KCBP to phragmoplast. Triple localization of the nuclei (stained with DAPI (*top*), microtubules of the phragmoplast localized with monoclonal antibody to β-tubulin (*middle*) or KCBP localized with affinity purified polyclonal antibody to KCBP (*bottom*)

cellulose microfibrils (Reddy and Day 200). In support of this, it is known that KCBP has two microtubule binding sites and has the ability to bundle microtubules [20, 40]. Double mutants *zwi*/*suz1* (*suz1* is a suppressor of *zwi* that shows no phenotype singly) had a defect in pollen tube germination and growth indicating a role for KCBP in other growth processes [23]. A model is presented in Fig. 6.8 showing KCBP function in cell division and development. Cotton KCBP (GhKCBP) was isolated from a cotton fiber-specific cDNA library and using immunofluorescence, GhKCBP was detected as punctate along cortical microtubules in fiber cells of different developmental stages suggesting a role in interphase cell growth [45].

Several kinesins have been shown to function during cell division (both mitosis and meiosis). The Kinesin-5,-7,-12 and -14 families have members whose involvement in cell division has been reported in the literature. Analysis of transcript profiling revealed that twenty-three kinesins are up-regulated during mitosis [67].

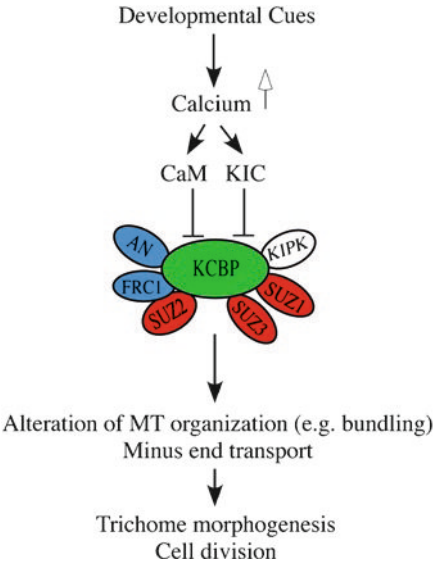


Fig. 6.8 Regulation of KCBP function. A model illustrating how changes in Ca^{2+} levels regulate trichome morphogenesis and cell division through Ca^{2+}-sensors, calmodulin (CaM) and KIC, and an MT-based motor, KCBP, in Arabidopsis. Based on the magnitude of changes in Ca^{2+} levels in trichome cells in response to developmental cues, CaM or KIC can be activated which in turn negatively regulate the microtubule-based motor, KCBP in the trichome morphogenic complex or during cell division. The positive and negative regulation of intermediate components in the pathway is shown with an arrow and a line ending with solid-bar, respectively. Some of the known positive and negative regulators of trichome morphogenesis that interact with KCBP are shown in green and red, respectively. Biochemical and/or genetic analysis show interaction between positive (KCBP and AN/FRC1/KIPK) or between positive and negative (KCBP/SUZ) regulators [12, 23, 34, 50]. *KIC* KCBP interacting Ca^{2+}-binding protein; *KCBP* kinesin-like CaM-binding protein; *KIPK* kinesin-interacting protein kinase; *AN* angustifolia; *FRC1* furca 1; *SUZ* suppressor of *zwi*3. Adapted from Reddy et al. [50]

Some Kinesin-14s are associated with spindle MTs and are required for focusing MTs at the poles. ATK1 (AtKin14a/KatA/At4g21270) and ATK5 (AtKin14b/At4g05190) are paralogs involved in mitotic and meiotic spindle function. *atk1* mutants lack microtubule accumulation at the predicted spindle poles during prophase and have reduced spindle bipolarity during prometaphase [35, 36]. The mutant mitotic spindles are slightly broader than normal, but the mutant has no gross morphological defect. Spindles of *atk5-1* plants become abnormally elongated, are frequently bent, and have splayed poles by prometaphase [3]. In meiotic cells the spindle phenotype is more pronounced and is more severe in male meiotic cells; the spindle in *atkin14a/atkin14a* (ATK1) male meiocytes is broad, unfocused and multi-axial at metaphase I, with abnormal chromosome segregation at anaphase I [8, 36, 46]. Double mutants *atkin14a/atkin14a atkin14b/atkin14b* were lethal due to the abnormal male meiosis. In mitosis, ATK1 and ATK5 have overlapping functions and either one of them is necessary and sufficient for gametophyte

development but their functions are somewhat diverged in male meiosis, where ATK1 is required. Two other Arabidopsis Kinesin-14s (GRIMP/KCA1; At5g10480 and KCA2; At5g65460) were isolated in yeast two-hybrid screens with a cyclin-dependent kinase (*CDKA;1*) and also KCA1 with the geminivirus protein AL1 [11, 22, 68]. KCA1 and KCA2 have putative CDKA phosphorylation sites that when mutated resulted in reduced interaction with CDKA. Using immunolocalization studies KCA1 was located at the spindle poles as well as on the phragmoplast, indicating a function in cell division. KCA1 was also localized to the cytoplasm and may have a role in moving virus proteins through the plasmodesmata.

Kinesin-5 members in animals are antagonistic to the Kinesin-14s, they cross-link and slide antiparallel MTs of the spindle midzone [59]. AtKRP125c (At2g28620) is one of four kinesin-5 motor proteins in Arabidopsis. It decorates microtubules throughout the cell cycle and appears to function in both interphase and mitosis. At the restrictive temperature, a temperature-sensitive mutant had disorganized interphase cortical microtubules and the mitotic spindles were massively disrupted consistent with a defect in the stabilization of anti-parallel microtubules in the spindle midzone [5].

Kinesins are also involved in cytokinesis in both mitotic and meiotic cell division (Also see Sect. 2, Chap. 4) Kinesins in tobacco, Nucleus- and phragmoplast-localized Protein Kinase1 (NPK1)-Activating Kinesin1 (NACK1) and NACK2, activate the NACK-PQR (NPK1, NQK1, NRK1) pathway through a sequence of phosphorylation of MAP kinase cascade components positively regulating cell plate expansion [41, 63]. The NACK-PQR pathway consists of NACK1 and NACK2, NPK1, the MAPK kinase (MAPKK) NQK1/NtMEK1 and the MAPK NRK1/NTF6. The orthologs of NACK in Arabidopsis are HINKEL/AtNACK1 (HIN, At1g18370) and TETRASPORE/AtNACK2 (TET, At3g43210) [41, 65]. The NACKs are members of the Kinesin-7 family. TET mutants show male-meiosis specific cytokinesis defects and HIN mutants are embryo lethal; however, HIN and TES have been shown to have functionally redundant roles during cellularization of the embryo sac and in pollen mitosis I [42, 62, 65, 83]. As with AtK1 and AtK5, the two Arabidopsis NACKs function redundantly in some cytokinesis events and specifically in others. Two Kinesin-12 family members, PAKRP1/Kinesin-12A (At4g13150) and PAKRP1L/Kinesin-12B (At3g23670) function in the organization of phragmoplast microtubules. They exhibit a cell cycle–dependent localization pattern and specifically decorate the plus ends of phragmoplast microtubules [29, 44]. Studies of their function in male meiotic cytokinesis show that the two kinesins appear to be redundant as single mutants did not show a noticeable defect [28]. However, in double mutants, dividing microspores were often disorganized following chromatid segregation and they did not form an antiparallel microtubule array resulting in abolishment of the first postmeiotic cytokinesis and no cell plate formation. Two moss (*Physcomitrella patens*) kinesins, KINID1a and KINID1b belong to the same Kinesin-12 clad as PAKRP1/Kinesin-12A&B [16]. They were localized to the putative interdigitated part of antiparallel microtubules of the phragmoplast and in double mutants, the interdigitation disappeared and the cell plate did not reach the plasma membrane in twenty percent of the mutants.

Two other Kinesin-12 members, POK1 (PHRAGMOPLAST ORIENTING KINESIN1, At3g17360) and POK2 (At3g19050) were isolated in a yeast two-hybrid screen for proteins that interact with Tangled (Tan), an Arabidopsis ortholog of a maize microtubule binding protein TANGLED1 (TAN1), a key player in the determination of division planes [38]. Double mutants *pok1:pok2* resembled the maize *tan1* mutants with misoriented mitotic cytoskeletal arrays and misplaced cell walls. POK1 and POK2 are required for the recruitment of AtTAN to the preprophase band where TAN remains throughout mitosis and cytokinesis [74].

Two cotton kinesins also in the Kinesin-14 family have a CH domain, GhKCH1 and GhKCH2 [45, 79, 80]. An in vitro actin-binding assay showed that GhKCH1's N-terminal region including the CH domain interacted directly with actin microfilaments. Immunofluorescence studies using an antibody to KCH protein revealed that in cotton fibers, GhKCH1 decorated cortical microtubules in a punctate manner and was found to be associated with transverse-cortical actin microfilaments, but never with axial actin cables in cotton fibers [45]. GhKCH2 was also found to be distributed along microtubes and microfilaments in a punctate manner [79]. A rice Kinesin-14, CH-containing protein (OsKCH1) also was shown to bind both microtubules and actin filaments [13]. YFP-constructs of full-length OsKCH1 co-localized with both a microtubule marker, MBD-MAP4 (microtubule-binding domain of microtubule-associated protein 4)–DsRed, and an actin marker, FABD2 (fimbrin actin-binding domain 2). A subclone of OsKCH1 containing the kinesin motor co-localized only with the MBD-MAP4–DsRed and the OsKCH1 subclone containing the CH domain co-localized with only the actin filament marker FABD2 [13]. These data suggest that kinesins containing a CH domain are involved in interactions between microtubules and actin filaments. KatD (At5g27000) is a CH domain-containing kinesin (see Fig. 6.3a) in Arabidopsis that is flower specific but whose function is not known [64].

AtKinesin-13A (At3g16630) has been shown to localize to Golgi stacks in plant cells [33]. Immunofluorescent labeling and confocal microscopic observation revealed that it co-localizes with Golgi stacks in Arabidopsis root tip cells while immunoelectron microscopy indicated that AtKinesin-13A is primarily localized on Golgi associated vesicles in Arabidopsis root-cap cells [76]. In a knockout mutant of *AtKinesin-13A*, there was a sharp decrease in size and number of Golgi vesicles in root-cap peripheral cells with a concomitant vacuolation in comparison to the corresponding cells of the wild type. These results suggest that AtKinesin-13A may be involved in regulating the formation of Golgi vesicles in the root-cap peripheral cells in Arabidopsis [76]. Three plant-specific but ungrouped kinesins, ARK1 (Armadillo repeat kinesen1), ARK2 and ARK3 (At3g54870, At1g01950, At1g12430) have an Armadillo repeat motif. ARK1 was first identified in a transcriptome comparison of wild-type and a root hair mutant, *mrh2*; the *mrh2* mutants had wavy root hairs [19]. ARK1 was again identified as an enhancer for GTPases that have been shown to cause the disruption of root hair polarity [82]. In the *mrh2* (ARK1) mutant root hairs, the cortical microtubules were fragmented and displayed random orientation. ARK1 was isolated a third time in a screen for root hair mutants verifying its involvement in root-hair morphogenesis [58].

6.6 Structure and Regulation of Kinesins

The first atomic structures of kinesin motor domains by X-ray crystallography were of KIF5 and Ncd in the ADP-bound state [24, 57]. The motor domain appears as an asymmetrical arrowhead with a central β-strand and 6 α-helices [57]. Kinesin "switches" are structural elements that respond to different nucleotide states of the motor [71]. The nucleotide sensing elements of the kinesin motor are the switch I loop L9 and the switch II cluster comprised of helices α4, α5 and loops L11 and L12. The main microtubule-binding site on the surface of the kinesin motor is the Loop L11, helix α4, and loop L12 of switch II. Kinesin motors have a neck region consisting of a neck linker that attaches to the catalytic core and a coiled coil region that is responsible for dimerization. The crystal structures of several kinesins in different subfamilies have been solved, revealing that they have common structures of the catalytic core [14]. Crystal studies of the C-terminal motor of the plant kinesin KCBP have revealed its structure [71, 72]. The motor core has the typical β -strands and α-helices found in animal kinesins; however, there is a C-terminal extension of the motor domain that includes the CaM-binding domain and two irregular segments. One irregular segment that connects the motor domain to the CaM-binding domain is in the position of the neck in N-terminal kinesin and has sequence similarity to this neck domain and so has been termed the "neck mimic" domain while the other irregular segment follows the CaM-binding domain and is negatively charged [72].

Kinesin activity requires ATP usage and so to prevent futile ATP hydrolysis, regulation of kinesins is important. Recent reviews have presented on the mechanisms involved in regulation of various animal and yeast kinesins [14, 15, 69]. One method of regulation present in several kinesins is autoinhibition in the absence of cargo. Several kinesins have been shown to have two forms, a folded form and an extended form with the folded form corresponding to the inactive state. The folded conformation allows non-motor regions to contact the motor domain and block microtubule binding and ATPase activity. In some kinesins the tail contacts the Switch I helix in the motor domain and prevents ADP release and, therefore, activity of the kinesin while others use internal segments for autoinhibition. Cargo binding is one element of release of autoinhibition; however, in some cases other processes such as phosphorylation are necessary for activation. Release of cargo can be regulated by signaling pathways including MAPK pathways and Ca^{2+}/CaM regulation. Kinesins involved in mitosis are regulated in several different ways. These include regulation of protein level, nuclear or cytoplasmic sequestration, activation by small GTPase, recruitment to specific spindle locations by binding to partner proteins and phosphorylation of kinesins.

Regulation of plant kinesins has been studied in just a few cases. *AtKP1*, a Kinesin-14 family member, was shown to be expressed in the vasculature of young organs and young leaf trichomes [32]. A 43-bp upstream element termed KPRE was found to be responsible for full and partial repression of promoter activity in the cotyledons and roots of Arabidopsis seedlings [25]. Two members of the whirly transcription factor family, AtWHY1 and AtWHY3, bind to this element and

mediate the transcriptional repression of AtKP1 [78]. A yeast two-hybrid screen using ARK1 (a kinesin with armadillo repeats) isolated a NIMA (never in mitosis, gene A)-related protein kinase (NekA) gene [58]. Further experiments with a NEKA mutant revealed a role in epidermal-cell morphogenesis and suggested involvement in cell morphogenesis in Arabidopsis via microtubule functions associated with ARKs. AtKCA1 (At5g10470) and AtKCA2 (At5g65460) are kinesins isolated in a yeast two-hybrid screen with the cell cycle-dependent protein kinase CDKA;1 [68]. AtKCA1 and AtKCA2 have consensus phosphorylation sites for CDKA;1. Interaction of these kinesins with CDKA;1 was verified in vitro and in vivo. Mutation of phosphorylation sites on the kinesins resulted in loss of dimerization of the kinesins and impeded folding suggesting the requirement of phosphorylation for folding and dimerization of these kinesins. The animal kinesin BIMC is regulated by phosphorylation of a conserved "BIMC Box" threonine residue and three members of the Arabidopsis TKRP-like proteins have the conserved "BIMC Box" suggesting that they might also be regulated by phosphorylation [4]. Phosphorylation may also be involved in regulation of KCBP as it has been found to interact with a plant-specific kinase, KIPK [9]. Expression studies using differenent length upstream regions of *KCBP* showed developmental and cell-specific regulation of *KCBP* expression in dividing cells and cells that exhibit polarized growth such as root hairs, pollen and trichomes [52]. Interestingly, the regulatory region included coding and non-coding regions of the upstream gene *CHY1*.

KCBP, found in flowering plants and *Chlamydomonas,* is unique in having a CaM-binding domain; only one other kinesin (kinesin-C, found in sea urchin, see Fig. 6.3b) is known to have a CaM-binding domain [1, 2, 56]. CaM is a ubiquitous calcium-binding protein that is highly conserved in eukaryotes and regulates many diverse cellular functions. CaM acts mainly by modulating the activity of the proteins that interact with it. It is activated via a conformational change upon binding calcium. Several molecular/biochemical studies have confirmed the regulation of KCBP by Ca^{2+}/CaM interaction. KCBP binding of Ca^{2+}/CaM was confirmed using several CaM-binding assays and three different Arabidopsis CaMs [53, 54]. KCBP was shown to bind MTs in the absence of Ca^{2+}/CaM but not in the presence of Ca^{2+}/CaM resulting in a decrease in the MT-stimulated ATPase activity of KCBP [10, 20, 39, 40, 61]. Furthermore, when the CaM-binding domain of KCBP was fused to two non-Ca^{2+}/CaM regulated kinesins, it functioned to inhibit the interaction of the kinesins with MTs and their MT-stimulated ATPase activity in a Ca^{2+}/CaM dependent manner [51]. The recently solved structure of KCBP suggests a mechanism for Ca^{2+}/CaM regulation of KCBP [71, 72]. The neck mimic links the regulatory subdomains of KCBP to the motor with the calmodulin-binding helix positioned in the vicinity of the KCBP MT-binding face in the ATP-like state when interactions between the MTs and KCBP are strong. The docking of the neck mimic along the motor core is important for the proper orientation of the calmodulin binding helix. The motor is fully active but with rising concentration of Ca^{2+}, calmodulin is activated and binds KCBP. The calmodulin-binding helix is accessible while KCBP is bound to the MTs, which allows activated calmodulin to bind and block the motility of a working motor. Modeling of bound Ca^{2+}/CaM to KCBP shows interference with

loop L8 of the KCBP motor core, disabling one of the specific contacts between the motor and the MTs. When Ca^{2+}/CaM binds to KCBP, the negative coil is stabilized, which blocks the microtubule-binding sites of KCBP. The negative coil mimics the negatively charged C-terminus of tubulin that binds to the kinesin motors. The negative coil is only able to compete with the more extensive, negatively charged surface of the MT when Ca^{2+}/CaM binds to KCBP. When the Ca^{2+} concentration decreases, CaM would disassociate from KCBP that is then free to bind to MTs.

A second Ca^{2+}-binding protein, KIC (KCBP-interacting Ca^{2+}-binding protein) was found to regulate KCBP [50]. A single EF-hand Ca^{2+}-binding protein, KIC was isolated as a protein that interacts with the C-terminus of KCBP. It was shown to bind Ca^{2+} and the interaction with KCBP was Ca^{2+}-dependent. Like CaM, Ca^{2+}/KIC/KCBP interaction negatively regulates the binding of KCBP to microtubules and the microtubule-dependent ATPase activity of KCBP. Neither centrin, a KIC-like protein (At4g37010), nor two proteins showing homology to KIC (At4g27280 and At5g54490) interacted with or regulated KCBP. The structure of the Ca^{2+}/KIC/KCBP interaction has also been solved [70]. Ca^{2+}/KIC binds the CaM-binding domain that lies between the neck and the negatively charged region of the C-terminus of KCBP (Fig. 6.9). Binding of Ca^{2+}/KIC to the motor functions as an allosteric trap where the neck mimic of KCBP associates with KIC rather than itself

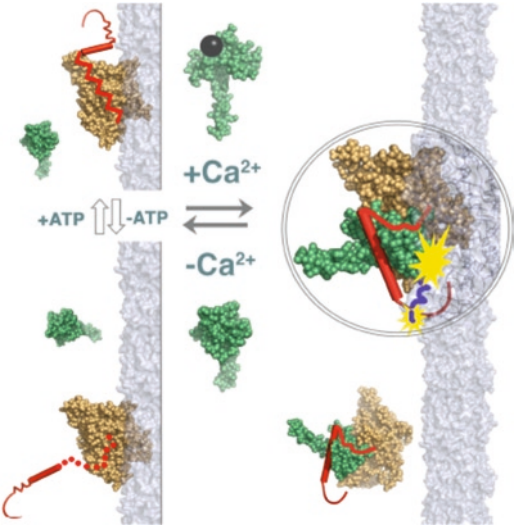


Fig. 6.9 Structural model for KIC regulation of KCBP. At resting Ca^{2+} levels (*left side*), KIC (*green*) does not bind KCBP (*beige*) in either the ATP or ADP form. The regulatory domain of KCBP is shown in red with the calmodulin-binding helix presented as a cylinder. The neck linker is shown as a *red zigzag line* in the ATP state when it is zipped (*upper left*), and as a dotted line in the ADP or no-nucleotide state (*lower left*). Microtubles are shown in *gray*. When the Ca^{2+} concentration is elevated (*right side*), Ca^{2+} binds KIC (*black*) which can then bind KCBP and the neck linker associates with KIC instead of itself resulting in stabilization in the ADP form and release from the microtubules. The negatively charged C terminus of α-tubulin is *purple*. Clashes between KIC and microtubules are highlighted in *yellow*. Reproduced from Vinogradnova et al. [70]

resulting in stabilization of the motor in the ADP conformation. The motor is then arrested in a state that has weak affinity for microtubules. The interactions of motor with microtubules are further destabilized by steric hindrance and electrostatic repulsion between the negative coil of KCBP and the negatively charged C-terminus of tubulin. Interestingly, the structure of KIC reveals that there is another EF-hand of nearly identical conformation but because of its amino acid composition, the loop of this EF-hand does not bind metal ions. When KIC interacts with KCBP, the canonical hand is loaded with Ca^{2+} but not the non-canonical EF-hand.

6.7 Concluding Remarks

Kinesin motors play important roles in fundamental cellular and developmental processes in eukaryotes. A large number of kinesins have been identified in photosynthetic organisms. There is a lack of information as to the numbers and types of kinesins in non-flowering plants. As the genomes of representatives of these groups of plants become available, identification of kinesins in these organisms and phylogenetic analysis can give more insight into the evolution of kinesins. The number of kinesins in flowering plants is larger than in other eukaryotes, e.g. Arabidopsis has 61 kinesins, suggesting that there are a large number of processes in which kinesins are involved. Some of the kinesins found in plants are plant specific suggesting their role in plant-specific MT processes such as preprophase band, phragmoplast and cellulose deposition. Furthermore, some of the functions that are performed by dyneins in non-plant systems may have been taken over by kinesins as plants do not contain dyneins. The function of a few of the kinesins found in plants have been elucidated. However, the functions of the majority of the predicted kinesins have not been determined. Regulation of the kinesins has also only been studied in a few of the kinesins. The challenge of future work on kinesins will be to identify the function and regulation of these kinesins.

References

1. Abdel-Ghany SE, Day IS, Simmons MP, Kugrens P, Reddy AS (2005) Origin and evolution of Kinesin-like calmodulin-binding protein. Plant Physiol 138:1711–1722
2. Abdel-Ghany SE, Reddy ASN (2000) A novel calcium/calmodulin-regulated kinesin-like protein is highly conserved between monocots and dicots. DNA Cell Biol 19:567–578
3. Ambrose JC, Cyr R (2007) The kinesin ATK5 functions in early spindle assembly in Arabidopsis. Plant Cell 19:226–236
4. Asada T, Kuriyama R, Shibaoka H (1997) TKRP125, a kinesin-related protein involved in the centrosome-independent organization of the cytokinetic apparatus in tobacco BY-2 cells. J Cell Sci 110:179–189
5. Bannigan A, Scheible WR, Lukowitz W, Fagerstrom C, Wadsworth P, Somerville C, Baskin TI (2007) A conserved role for kinesin-5 in plant mitosis. J Cell Sci 120:2819–2827
6. Banuelos S, Saraste M, Carugo KD (1998) Structural comparisons of calponin homology domains: implications for actin binding. Structure 6:1419–1431

7. Bowser J, Reddy ASN (1997) Localization of a kinesin-like calmodulin-binding protein in dividing cells of *Arabidopsis* and tobacco. Plant J 12:1429–1438
8. Chen C, Marcus A, Li W, Hu Y, Calzada JP, Grossniklaus U, Cyr RJ, Ma H (2002) The Arabidopsis ATK1 gene is required for spindle morphogenesis in male meiosis. Development 129:2401–2409
9. Day IS, Miller C, Golovkin M, Reddy ASN (2000) Interaction of a kinesin-like calmodulin-binding protein with a protein kinase. J Biol Chem 275:13737–13745
10. Deavours BE, Reddy ASN, Walker RA (1998) Ca^{2+}/calmodulin regulation of the Arabidopsis kinesin-like calmodulin- binding protein. Cell Motil Cytoskeleton 40:408–416
11. DeVeylder L, Segers G, Glab N, Van Montagu M, Inze D (1997) Identification of proteins interacting with the Arabidopsis Cdc2aAt protein. J Exp Bot 48:2114–2114
12. Folkers U, Berger J, Hulskamp M (1997) Cell morphogenesis of trichomes in Arabidopsis: differential control of primary and secondary branching by branch initiation regulators and cell growth. Development 124:3779–3786
13. Frey N, Klotz J, Nick P (2009) Dynamic bridges–a calponin-domain kinesin from rice links actin filaments and microtubules in both cycling and non-cycling cells. Plant Cell Physiol 50:1493–1506
14. Hirokawa N, Noda Y (2008) Intracellular transport and kinesin superfamily proteins, KIFs: structure, function, and dynamics. Physiol Rev 88:1089–1118
15. Hirokawa N, Noda Y, Tanaka Y, Niwa S (2009) Kinesin superfamily motor proteins and intracellular transport. Nat Rev Mol Cell Biol 10:682–696
16. Hiwatashi Y, Obara M, Sato Y, Fujita T, Murata T, Hasebe M (2008) Kinesins are indispensable for interdigitation of phragmoplast microtubules in the moss Physcomitrella patens. Plant Cell 20:3094–3106
17. Hulskamp M (2000) How plants split hairs. Curr Biol 10:R308–R310
18. Itoh R, Fujiwara M, Yoshida S (2001) Kinesin-related proteins with a mitochondrial targeting signal. Plant Physiol 127:724–726
19. Jones MA, Raymond MJ, Smirnoff N (2006) Analysis of the root-hair morphogenesis transcriptome reveals the molecular identity of six genes with roles in root-hair development in Arabidopsis. Plant J 45:83–100
20. Kao Y-L, Deavours BE, Phelps KK, Walker R, Reddy ASN (2000) Bundling of microtubules by motor and tail domains of a kinesin-like calmodulin-binding protein from Arabidopsis: regulation by Ca^{2+}/calmodulin. Biochem Biophys Res Commun 267:201–207
21. King SM (2000) The dynein microtubule motor. Biochem Biophys Acta 1496:60–75
22. Kong L-J, Hanley-Bowdoin L (2002) A geminivirus replication protein interacts with a protein kinase and a motor protein that display different expression patterns during plant development and infecion. Plant Cell 14:1817–1832
23. Krishnakumar S, Oppenheimer DG (1999) Extragenic suppressors of the Arabidopsis zwi-3 mutation identify new genes that function in trichome branch formation and pollen tube growth. Development 126:3079–3088
24. Kull FJ, Sablin EP, Lau R, Fletterick RJ, Vale RD (1996) Crystal structure of the kinesin motor domain reveals a structural similarity to myosin. Nature 380:550–555
25. Lai C, Xiong J, Li X, Qin X (2009) A 43-bp A/T-rich element upstream of the kinesin gene AtKP1 promoter functions as a silencer in Arabidopsis. Plant Cell Rep 28:851–860
26. Lawrence CJ, Dawe RK, Christie KR, Cleveland DW, Dawson SC, Endow SA, Goldstein LSB, Goodson HV, Hirokawa N, Howard J, Malmerg RL, McIntosh JR, Miki H, Mitchison TJ, Okada Y, Reddy ASN, Saxton WM, Schliwa M, Scholey JM, Vale RD, Walczak CE, Wordeman L (2004) A standardized kinesin nomenclature. J Cell Biol 167:19–22
27. Lawrence CJ, Morrie NR, Meagher RB, Dawe RK (2001) Dyneins have run their course in plant lineage. Traffic 2:362–363
28. Lee YR, Li Y, Liu B (2007) Two Arabidopsis phragmoplast-associated kinesins play a critical role in cytokinesis during male gametogenesis. Plant Cell 19:2595–2605
29. Lee Y-RJ, Liu B (2000) Identification of a phragmoplast-associated kinesin-related protein in higher plants. Curr Biol 10:797–800

30. Lee YR, Liu B (2004) Cytoskeletal motors in Arabidopsis. Sixty-one kinesins and seventeen myosins. Plant Physiol 136:3877–3883
31. Leinweber BD, Leavis PC, Grabarek Z, Wang CL, Morgan KG (1999) Extracellular regulated kinase (ERK) interaction with actin and the calponin homology (CH) domain of actin-binding proteins. Biochem J 344:117–123
32. Li XY, Wang HQ, Xu T, Cao QH, Ren DT, Liu GQ (2007) Molecular cloning, expression and biochemical property analysis of AtKP1, a kinesin gene from *Arabidopsis thaliana*. Chin Sci Bull 52:1339–1346
33. Lu L, Lee YR, Pan R, Maloof JN, Liu B (2005) An internal motor kinesin is associated with the Golgi apparatus and plays a role in trichome morphogenesis in Arabidopsis. Mol Biol Cell 16:811–823
34. Luo D, Oppenheimer DG (1999) Genetic control of trichome branch number in Arabidopsis: the roles of FURCA loci. Development 126:5547–5557
35. Marcus AI, Ambrose JC, Blickley L, Hancock WO, Cyr RJ (2002) Arabidopsis thaliana protein, ATK1, is a minus-end directed kinesin that exhibits non-processive movement. Cell Motil Cytoskeleton 52:144–150
36. Marcus AI, Li W, Ma H, Cyr RJ (2003) A kinesin mutant with an atypical bipolar spindle undergoes normal mitosis. Mol Biol Cell 14:1717–1726
37. Miki H, Okada Y, Hirokawa N (2005) Analysis of the kinesin superfamily: insights into structure and function. Trends Cell Biol 15:467–476
38. Muller S, Han S, Smith LG (2006) Two kinesins are involved in the spatial control of cytokinesis in Arabidopsis thaliana. Curr Biol 16:888–894
39. Narasimhulu SB, Kao Y-L, Reddy ASN (1997) Interaction of Arabidopsis kinesin-like calmodulin-binding protein with tubulin subunits: modulation by Ca^{2+}-calmodulin. Plant J 12:1139–1149
40. Narasimhulu SB, Reddy ASN (1998) Characterization of microtubule binding domains in the Arabidopsis kinesin-like calmodulin-binding protein. Plant Cell 10:957–965
41. Nishihama R, Soyano T, Ishikawa M, Araki S, Tanaka H, Asada T, Irie K, Ito M, Terada M, Banno H, Yamazaki Y, Machida Y (2002) Expansion of the cell plate in plant cytokinesis requires a kinesin-like protein/MAPKKK complex. Cell 109:87–99
42. Oh SA, Bourdon V, Das 'Pal M, Dickinson H, Twell D (2008) Arabidopsis kinesins HINKEL and TETRASPORE act redundantly to control cell plate expansion during cytokinesis in the male gametophyte. Mol Plant 1:794–799
43. Oppenheimer DG, Pollock MA, Vacik J, Szymanski DB, Ericson B, Feldmann K, Marks D (1997) Essential role of a kinesin-like protein in Arabidopsis trichome morphogenesis. Proc Natl Acad Sci USA 94:6261–6266
44. Pan R, Lee YR, Liu B (2004) Localization of two homologous Arabidopsis kinesin-related proteins in the phragmoplast. Planta 220:156–164
45. Preuss ML, Kovar DR, Lee YR, Staiger CJ, Delmer DP, Liu B (2004) A plant-specific kinesin binds to actin microfilaments and interacts with cortical microtubules in cotton fibers. Plant Physiol 136:3945–3955
46. Quan L, Xiao R, Li W, Oh SA, Kong H, Ambrose JC, Malcos JL, Cyr R, Twell D, Ma H (2008) Functional divergence of the duplicated AtKIN14a and AtKIN14b genes: critical roles in Arabidopsis meiosis and gametophyte development. Plant J 53: 1013–1026
47. Reddy ASN, Day IS (2000) The role of the cytoskeleton and a molecular motor in trichome morphogenesis. Trends Plant Sci 5:503–505
48. Reddy AS, Day IS (2001) Analysis of the myosins encoded in the recently completed Arabidopsis thaliana genome sequence. Genome Biol 2:Research0024.0021–0024.0017
49. Reddy ASN, Day IS (2001) Kinesins in the Arabidopsis genome: a comparative analysis among eukaryotes. BMC Genomics 2:2
50. Reddy VS, Day IS, Thomas T, Reddy AS (2004) KIC, a novel Ca^{2+} binding protein with one EF-hand motif, interacts with a microtubule motor protein and regulates trichome morphogenesis. Plant Cell 16:185–200

51. Reddy VS, Reddy AS (2002) The calmodulin-binding domain from a plant kinesin functions as a modular domain in conferring Ca^{2+}-CaM regulation to animal plus- and minus-end kinesins. J Biol Chem 277(50):48058–48065
52. Reddy VS, Reddy AS (2004) Developmental and cell-specific expression of ZWICHEL is regulated by the intron and exon sequences of its gene. Plant Mol Biol 54:273–293
53. Reddy ASN, Safadi F, Narasimhulu SB, Golovkin M, Hu X (1996) A novel plant calmodulin-binding protein with a kinesin heavy chain motor domain. J Biol Chem 271:7052–7060
54. Reddy V, Safadi F, Zielinski RE, Reddy ASN (1999) Interaction of a kinesin-like protein with calmodulin isoforms from Arabidopsis. J Biol Chem 274:31727–31733
55. Richardson DN, Simmons MP, Reddy AS (2006) Comprehensive comparative analysis of kinesins in photosynthetic eukaryotes. BMC Genomics 7:18
56. Rogers GC, Hart CL, Wedman KP, Scholey JM (1999) Identification of kinesin-C, a calmodulin-binding carboxy-terminal kinesin in animal (*Strongylocentrotus purpuratus*) cells. J Mol Biol 294:1–8
57. Sablin EP, Kull FJ, Cooke R, Vale RD, Fletterick RJ (1996) Crystal structure of the motor domain of the kinesin-related motor ncd. Nature 380:555–559
58. Sakai T, van der Honing H, Nishioka M, Uehara Y, Takahashi M, Fujisawa N, Saji K, Seki M, Shinozaki K, Jones MA, Smirnoff N, Okada K, Wasteneys GO (2008) Armadillo repeat-containing kinesins and a NIMA-related kinase are required for epidermal-cell morphogenesis in Arabidopsis. Plant J 53:157–171
59. Sharp DJ (2000) Microtubule motors in mitosis. Nature 407:41–47
60. Smirnova E, Reddy ASN, Bowser J, Bajer AS (1997) Distribution of kinesin-like minus end-directed protein during mitosis in endosperm of higher plant *Haemanthus*. Mol Biol Cell 8:378a
61. Song H, Golovkin M, Reddy ASN, Endow SA (1997) In vitro motility of AtKCBP, a calmodulin-binding kinesin-like protein of Arabidopsis. Proc Natl Acad Sci USA 94:322–327
62. Strompen G, El Kasmi F, Richter S, Lukowitz W, Assaad FF, Jurgens G, Mayer U (2002) The Arabidopsis HINKEL gene encodes a kinesin-related protein involved in cytokinesis and is expressed in a cell cycle-dependent manner. Curr Biol 12:153–158
63. Takahashi Y, Soyano T, Sasabe M, Machida Y (2004) A MAP kinase cascade that controls plant cytokinesis. J Biochem 136:127–132
64. Tamura K, Nakatani K, Mitsui H, Ohashi Y, Takahashi H (1999) Characterization of katD, a kinesin-like protein gene specifically expressed in floral tissues of Arabidopsis thaliana. Gene 230:23–32
65. Tanaka H, Ishikawa M, Kitamura S, Takahashi Y, Soyano T, Machida C, Machida Y (2004) The AtNACK1/HINKEL and STUD/TETRASPORE/AtNACK2 genes, which encode functionally redundant kinesins, are essential for cytokinesis in Arabidopsis. Genes Cells 9:1199–1211
66. Tokai N, Fujimoto-Nishiyama A, Toyoshima Y, Yonemure S, Tsukita S, Inoue J, Yamamoto T (1996) Kid, a novel kinesin-like DNA binding protein, is localized to chromosomes and the mitotic spindle. EMBO J 15:457–467
67. Vanstraelen M, Inze D, Geelen D (2006) Mitosis-specific kinesins in Arabidopsis. Trends Plant Sci 11:167–175
68. Vanstraelen M, Torres Acosta JA, DeVeylder L, Inze D, Greelen D (2004) A plant-specific subclass of C-terminal kinesins contains a conserved a-type cyclin-dependent kinase site implicated in folding and dimerization. Plant Physiol 135:141–1429
69. Verhey KJ, Hammond JW (2009) Traffic control: regulation of kinesin motors. Nat Rev Mol Cell Biol 10:765–777
70. Vinogradova MV, Malanina GG, Reddy AS, Fletterick RJ (2009) Structure of the complex of a mitotic kinesin with its calcium binding regulator. Proc Natl Acad Sci USA 106:8175–8179
71. Vinogradova MV, Malanina GG, Reddy VS, Reddy AS, Fletterick RJ (2008) Structural dynamics of the microtubule binding and regulatory elements in the kinesin-like calmodulin binding protein. J Struct Biol 163:76–83

72. Vinogradova MV, Reddy VS, Reddy AS, Sablin EP, Fletterick RJ (2004) Crystal structure of kinesin regulated by Ca(2+)-calmodulin. J Biol Chem 279:23504–23509
73. Voss JW, Safadi F, Reddy ASN, Hepler PK (2000) The kinesin-like calmodulin binding protein is differentially involved in cell division. Plant Cell 12:979–990
74. Walker KL, Muller S, Moss D, Ehrhardt DW, Smith LG (2007) Arabidopsis TANGLED identifies the division plane throughout mitosis and cytokinesis. Curr Biol 17:1827–1836
75. Weber KL, Sokac AM, Berg JS, Cheney RE, Bement WM (2004) A microtubule-binding myosin required for nuclear anchoring and spindle assembly. Nature 431:325–329
76. Wei L, Zhang W, Liu Z, Li Y (2009) AtKinesin-13A is located on Golgi-associated vesicle and involved in vesicle formation/budding in Arabidopsis root-cap peripheral cells. BMC Plant Biol 9:138
77. Wickstead B, Gull K (2007) Dyneins across eukaryotes: a comparative genomic analysis. Traffic 8:1708–1721
78. Xiong JY, Lai CX, Qu Z, Yang XY, Qin XH, Liu GQ (2009) Recruitment of AtWHY1 and AtWHY3 by a distal element upstream of the kinesin gene AtKP1 to mediate transcriptional repression. Plant Mol Biol 71:437–449
79. Xu T, Qu Z, Yang X, Qin X, Xiong J, Wang Y, Ren D, Liu G (2009) A cotton kinesin GhKCH2 interacts with both microtubules and microfilaments. Biochem J 421:171–180
80. Xu T, Sun X, Jiang S, Ren D, Liu G (2007) Cotton GhKCH2, a plant-specific kinesin, is low-affinitive and nucleotide-independent as binding to microtubule. J Biochem Mol Biol 40:723–730
81. Yamazaki H, Nakata T, Okada Y, Hirokawa N (1996) Cloning and characterization of KAP3: a novel kinesin superfamily- associated protein of KIF3A/3B. Proc Natl Acad Sci USA 93:8443–8448
82. Yang G, Gao P, Zhang H, Huang S, Zheng ZL (2007) A mutation in MRH2 kinesin enhances the root hair tip growth defect caused by constitutively activated ROP2 small GTPase in Arabidopsis. PLoS One 2:e1074
83. Yang CY, Spielman M, Coles JP, Li Y, Ghelani S, Bourdon V, Brown RC, Lemmon BE, Scott RJ, Dickinson HG (2003) TETRASPORE encodes a kinesin required for male meiotic cytokinesis in Arabidopsis. Plant J 34:229–240
84. Zhong R, Burk DH, Morrison WH 3rd, Ye ZH (2002) A kinesin-like protein is essential for oriented deposition of cellulose microfibrils and cell wall strength. Plant Cell 14:3101–3117

Part II
Cytoskeletal Reorganization in Plant Cell Division

Chapter 7
The Preprophase Band and Division Site Determination in Land Plants

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7.1 Introduction

7.1.1 Focus of this Chapter

In complex eukaryotes, there is undoubtedly more to multicellular development than mere addition of individual cell activities, and plant development provides a number of evidences for a supra-cellular level of developmental control [78]. Nevertheless, temporal and spatial organization of cell division and growth remains a key component of morphogenesis in all multicellular organisms, including plants.

As multicellularity evolved independently in plants and animals, many basic cellular processes underlying multicellular development differ markedly between plants and metazoans: first, not all parts of the organism contribute equally to growth and development, but instead plants rely on a few specialized tissues, the meristems, which remain juvenile throughout their lifetime, and are the active sites of cell division. In addition, the presence of a pecto-cellulosic cell wall around each cell of a land plant has profound implications in terms of development, tissue organization and cellular activities: cells are embedded within a semi-rigid cell wall which glues them together, preventing any migration, restricting their growth, and implying specific modes of elongation and division. As a consequence, three-dimensional cellular organization of tissues can be viewed as a direct result of cell division, since new cells are deposited in place by cell division with no possibility for subsequent relocation: therefore tight control of both the timing and orientation of cell division is especially important to establish the body plan of multicellular land plants.

Although it is convenient to distinguish between the temporal control of cell division, mainly relying on the cell cycle machinery, and the spatial regulation of the orientation of cell division, the two processes are obviously intimately coordinated,

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although the nature of this cross-talk is currently very poorly understood. In this chapter, we focus on the cellular and molecular aspects of the spatial control of cell division in land plants (Embryophytes) with an emphasis on the preprophase band of microtubules (PPB), thought to play a major role in this process. We also develop on the possible connections with the cell cycle which are emerging from recent work.

7.1.2 What is a PPB?

The preprophase band (PPB) was initially discovered more than 40 years ago in wheat cells [135], as a dense ring of cortical microtubules (MTs) that forms at the onset of mitosis in plant cells. The PPB first develops into a broad band of MTs that progressively narrows down into a dense, tight ring encircling the nucleus, before disappearing concomitantly with nuclear envelope breakdown (NEB) at prophase. The most fascinating feature of the PPB is that it precisely predicts, within a micron, the position of the future cell plate between the two daughter nuclei, which is established at telophase by another plant-specific cytoskeletal apparatus, the phragmoplast. Temporally speaking, the PPB might be seen as part of the early mitotic apparatus. 40 years of observations and research have clearly established the PPB as part of the cytokinetic apparatus of plant cells, and as an early cytological mark of division plane establishment in cells committed to mitosis.

A minimal interpretation of this conspicuous premitotic cytological mark is that contrarily to many eucaryotes, division plane determination in plant cells takes place before entry into M phase per se. Indeed, the PPB forms during the G2 phase in cells that have duplicated their genetic material, but there seems quite some variation in the timing of appearance of this structure, depending on the species and cell type considered (reviewed in [110]). The original term “preprophase” is somewhat inappropriate in this respect, because it seems to refer to a specific stage of the cell cycle, i.e. early prophase. Strictly speaking, preprophase must be understood here as the stage where a G2 cell becomes committed to divide and shows visible activity related to division plane establishment [110].

The PPB, together with a few other cytological modifications, is one of the first signs of commitment to divide in a plant cell. Its strict spatial correlation with the future cell plate at the end of mitosis, and many years of pharmalogical, cytological or genetic manipulation converge to identify the PPB as a key player in the set up of the cortical division site (CDS), defined as the site of the cell cortex where the centrifugally growing cell plate finally attaches during cytokinesis (see Section 7.5).

The formation of the PPB and its precise role in CDS definition is still poorly understood, although recent years have seen a number of advances in the field, thanks to progresses in microscopy and imaging techniques, as well as functional genomics and mutant analysis. One of the most intriguing questions is the nature of the information deposited at the cell cortex that is recognized at later stages for cell plate insertion. It is generally assumed that the PPB is involved in leaving this (presumably molecular) mark, but formal demonstration of this hypothesis and analysis of the molecular networks and protein partners involved is just starting at present.

7.1.3 To Have or Not to Have a PPB

Do all plant cells divide with a PPB? To date, the generalization proposed by previous reviews on the PPB [110, 174] still holds true: *PPBs are formed before mitosis in all types of vegetative land plant cells where karyokinesis is immediately followed by cytokinesis.* This holds from the first division of the zygote on, and whether divisions are symmetrical or asymmetrical, occurs in large vacuolated cells or small meristematic ones, etc. There are few exceptions to this rule, and most of them would need to be revisited using current imaging techniques. One well established and notable exception, however, is the filamentous stage of moss protonemata, where divisions are devoid of PPBs [50]. However, when buds emerge from the protonema to form the moss leafy shoot (the gametophore), all divisions occur with prior PPB formation. This switch is classically interpreted as a transition from 2-D growth to 3-D growth phase, where tight control of division plane positioning is required.

In cells where karyokinesis and cytokinesis are disconnected in time, like during development of the syncitial endosperm, there is no formation of a PPB. Likewise, PPBs are not formed during meiosis and sporogenesis, including male and female gametophyte development. Instead, an alternative "radial microtubule system" is present in all reproductive phases of land plants and organizes the cytoplasm into domains which determine placement of new cell walls by so-called "adventitious" phragmoplasts [21].

7.1.4 The PPB in Evolution

The conquest of land by green plants about 500 My ago is one of the most important evolutionary transitions in the history of life on earth. Land plants constitute a monophyletic group which evolved from a common ancestor related to modern charophycean green algae. The sister group of land plants, Charophyte green algae, exhibits preadaptative cellular features that may represent early stages in transformation series toward the conquest of land (wall composition, plasmodesmatas…) [62]. It is particularly interesting to note, that the transition from an "algal" type of cytokinesis (involving an animal-like actin-driven cleavage furrow) to a "land plant" type (involving de novo construction of a cell plate eventually connecting to the cell cortex), precedes terrestrialization of plants, as a phragmoplast is found in the most advanced forms of charales like *Coleochaete* and *Chara*, considered to be related to ancestors of land lands [134].

However, the PPB constitutes an autapomorphy of embryophytes, i.e. a derived character specific of this monophyletic group, not known to occur in any other group of extent organisms. "Introduction of the PPB into the cytokinetic apparatus is a topic of extreme interest in the evolution of land plants" [20]: this innovation of land plants may well represent an adaptation to life within the aerial/terrestrial environment, facilitating the coordination of cell division and the 3-D organization of plant tissues, in order to produce large exchange surfaces in a desiccating and gravity-prone environment.

7.2 The PPB and the Cell Cycle

7.2.1 A Coupling Between Cell Cycle and PPB Formation?

The role of the PPB in premitotic definition of the cytokinetic plane of division, as well as the spatio-temporal coincidence between PPB formation, nuclear positioning and entry into prophase strongly suggest a link, or at least a cross-talk between the cell cycle and PPB formation. The nature of this link is not well understood, but a general view can be assembled from both recent and older studies: (1) the cell cycle and the MT cycle are clearly not fully coupled: formation of a functional PPB is not required for cell cycle progression, and entry into mitosis can proceed apparently normally in cells genetically impaired for PPB formation (e.g. *ton1* [11] and *fass* [107] mutants), not to mention generative cell divisions, or moss protonemal divisions that do not involve a PPB. Plant cells do not have a general "checkpoint" mechanism that prevent entry into mitosis if a PPB does not form. (2) On the other hand, the PPB is a specific feature of cycling cells, and forms at a defined stage of the cell cycle, in cells committed to divide. This implies that the cell cycle controls the timing of appearance of the PPB. The recruitment of several key players of the core cell cycle machinery to the PPB (see below) is an additional indication for a strong link between the nuclear cycle and PPB formation and/or function. Accordingly, when cells are blocked in S phase by high doses of aphidicolin, PPB formation is suppressed. This suppression is reversible upon drug removal [80, 169].

What drives the coupling – partial or complete – between PPB formation and the cell cycle is still largely unknown, and more work is needed to address this important question. CDKA, the central regulator of the cell cycle and entry into mitosis, was reported several years ago to localize on mature PPBs, and recent studies have identified a number of other cell cycle components which localize to the PPB (Table 7.1 and references herein). The significance of this recruitment remains to be established, but these cell-cycle proteins might be involved in the aforementioned cross-talk between the PPB, NEB, prophase spindle establishment and PPB disassembly.

7.2.2 The Cell Cycle and G2/M Transition

A variety of proteins determines the temporal sequence of events in the cell cycle, establishing regulatory cascades which control transitions and progressions in the different stages. G1/S and G2/M transitions are considered as major control points of cell growth and division. In all eukaryotes, these transitions are controlled by a special class of serine-threonine protein kinases, the cyclin-dependent kinases (CDKs). CDKs require binding of a cyclin for activity. CDK complex activity is modulated at least by four classes of regulatory proteins: this includes positive effectors (cyclins), negative regulators (CDK inhibitor), as well as activating or inhibitory kinases (CAK and WEE1, respectively). In addition, targeted degradation plays an important role in the regulation of CDK activity.

Table 7.1 Localization of cell cycle proteins at the PPB

Protein (full name)	Species	Techniques used for localization studies	Localization during cell cycle				References
			Interphase	G2/M	Metaphase/ anaphase	Telophase	
CDKA;1 (cyclin-dependent kinase A;1)	Onion, wheat, maize, barley, pea	Immunolocalization using an anti-PSTAIR antibody	Cytoplasm	Cytoplasm, PPB	Cytoplasm	Cytoplasm	[111, 113]
	Maize	Immunolocalization using anti-ZmCDKA and anti-PSTAIRE antibodies	Nucleus	Mature PPB, nucleus	Diffuse staining in spindle region	Newly formed nuclei, diffuse staining in cytoplasm	[36, 109]
	Alfalfa	Immunolocalization using anti-AtCDKA;1 and anti-PSTAIRE antibodies	Nucleus, cytoplasm	Mature PPB, nucleus, cytoplasm	Spindle and anaphase chromosomes	Phragmoplast	[151]
	BY-2 cells	Confocal imaging of MsCDKA;2 driven by a tetracyclin inducible promoter	Nucleus, cytoplasmic strands	Mature PPB, nucleus, cytoplasm	Spindle	Newly formed nuclei, phragmoplast	[172]
	Arabidopsis BY-2 cells	Confocal imaging of p35S driven CDKA;1-GFP and pCDKA;1:CDKA; 1-YFP in a *cdka;1* mutant background	Nucleus, cytoplasm	PPB, condensing chromatin	Spindle, at the midzone in anaphase spindle	Newly formed nuclei, phragmoplast	[16]
CDKB2;1 (cyclin-dependent kinase B2;1)	Alfalfa	Immunolocalization using an anti MsCDKB2;1 antibody	ND	PPB	Spindle	Phragmoplast	[108]
	BY-2 cells	Confocal imaging of CDKB2; 1-GFP driven by an estrogen inducible promoter	Nucleus, cytoplasm	Nucleus, cytoplasm, PPB	Spindle, chromosomes	Phragmoplast, newly formed nuclei	[92]

(continued)

Table 7.1 (continued)

Protein (full name)	Species	Techniques used for localization studies	Localization during cell cycle				References
			Interphase	G2/M	Metaphase/ anaphase	Telophase	
CYCA1;1 (cyclin A1)	*Arabidopsis* BY-2 cells	Confocal imaging of p35S driven CYCA1;1-GFP	Nucleus, cytoplasm	PPB, nucleus	Spindle, absent after anaphase		[16]
	Maize	Immunolocalization using an anti ZmCYCA1;1 antibody	Nucleus, cortical MTs, cytoplasm	PPB, nucleus	Spindle	Phragmoplast	[109]
Cyclin B1 (CYCB1;2)	Maize	Immunolocalization using an anti ZmCYCB1;2 antibody	Cytoplasm	Mature PPB, nuclear envelope	Spindle region, chromosomes		[109]
CKS2 (CDK subunit 2)	*Arabidopsis* BY-2 cells	Confocal imaging of p35S driven CKS2-GFP	Nucleus, cytoplasm	PPB, nucleus	Chromosomes		[16]
KRP4 (Kip-related protein 4)	*Arabidopsis* BY-2 cells	Confocal imaging of p35S driven KRP4-GFP	Nucleus, cytoplasm	PPB, nucleus, cytoplasm	Chromosomes		[16]
CCS52A2 (cell cycle switch)	*Arabidopsis* BY-2 cells	Confocal imaging of p35S driven GFP-CCS52A2	Nucleus, punctate in cytoplasm	PPB, nucleus, punctate in cytoplasm	Spindle region	Newly formed nuclei	[16]
RAE1 (ribonucleic acid export)	BY-2 cells	Immunolocalization using an anti-HA antibody on BY-2 cells expressing p35:HA-RAE1	Nuclear envelope, nucleoplasm, cytoplasm	PPB	Spindle	Phragmoplast	[93]

This table describes the localization of PPB-associated core cell cycle proteins during the cell cycle. Localization discrepancies between studies are likely due to the use of heterogenous techniques or to variations between species examined. ND: not determined

Here, we give a brief overview of the main cell cycle regulators identified or suspected in plants to be involved in G2/M transition, in order to discuss their potential implication in PPB establishment and function(s).

7.2.2.1 Cyclin-Dependent Kinases

Several CDKs have been characterized in plants. Plant CDK-related genes have been classified into seven classes, from A to G [158]. To date, only the A, B, D and F classes have been shown to be involved in cell cycle regulation. Plant A-type CDK (CDKA) is required both for G1/S and G2/M transition [72]. CDKA is considered to be the *bona fide* plant CDK because of the presence of the consensus PSTAIRE motif also found in yeast cdc2 and animal CDK1, and of its ability to complement yeast *cdc2* mutant [55]. The *Arabidopsis* genome encodes only one A-type CDK, which is essential for viability [73, 124]. B-type CDKs (CDKB) are specific to the plant kingdom; they possess an altered PSTAIRE motif, and are proposed to play a critical role at the G2/M transition [17, 72]. The *Arabidopsis* genome encodes four B-type CDKs. Plant D- and F-type CDK are involved in phosphorylation-dependent activation of other CDK complexes during the cell cycle, and must therefore be considered as CDK-activating kinases (CAKs).

7.2.2.2 Cyclins

A large family of cyclins has been described in plants [72]. Cyclins are positive cofactors of CDK complexes, and their binding to CDK induce a conformational change which liberates the CDK catalytic core. The *Arabidopsis* genome encodes at least 32 cyclins with a putative role in cell cycle progression [72]. A-type cyclins (CYCA) generally appear at the beginning of S phase, are involved in S-phase progression, and are destroyed around the G2/M transition. B-type cyclins (CYCB) appear during G2, control G2/M and mitotic transitions, and are destroyed as cells enter anaphase. D-type cyclins (CYCD), although previously thought to regulate the G1/S transition, appear to also have a function in G2/M transition (reviewed in [72]). The level of many cell cycle players is determined both through highly regulated transcription, and by specific protein-turnover mechanisms. A- and B-type cyclins possess a destruction box that targets their timely degradation via ubiquitination through the anaphase-promoting complex during early-to-mid mitosis [72]. In animal and yeast, activation and substrate specificity of the anaphase-promoting complex are controlled by two proteins, Cdc20 and Cdh1. *Arabidopsis* contains five Cdc20 genes and three Cdh1-related proteins, one of which (CCS52B) is expressed from G2/M to M [57].

7.2.2.3 CDK Inhibitors

CDK inhibitors can bind CDK complexes and inhibit their activity. The first CDK inhibitor was discovered as an interactant of plant CDKA;1 [170]. Plant CDK inhibitors

were thereafter named ICK/KRP (*I*nteractor of *CDK*A and *K*ip *R*elated *P*rotein) as they share homology with the animal CDK inhibitors Kip proteins. Apart from two motifs at their C-terminus, plant ICK/KRP diverge greatly among themselves and from their animal counterparts. All of them can interact with D-type cyclins, and most of them with CDKA [171]. Even if ICK/KRP proteins are considered to mainly act at the G1/S transition, a role in G2/M transition or in mitosis is not excluded.

7.2.2.4 CKS Proteins

Another level of regulation of CDK complexes is the binding of CDK subunit (CKS) proteins that can act as inhibitors and activators of CDK activity and appear to have a role in scaffolding the interaction of the CDK complex with substrates [41].

7.2.2.5 Regulation of CDK Complexes by Phosphorylation

Activity of CDK complexes is also regulated by phosphorylation/dephosphorylation events on specific residues. CDK-activating kinases (CAKs) activate CDK complexes by phosphorylation of a canonical threonin residue (Thr161) in the T-loop region of CDK. This phosphorylation event is crucial for full kinase activity of the cyclin/CDK complex, since it stabilizes the opening of the T-loop, thus allowing proper recognition and binding of CDK substrates [45]. However, involvement of this CAK-mediated phosphorylation in G2/M transition in plants has not been clearly established [153]. Another target of regulation of animal and fungal CDKs is the P-loop ATP-binding domain. In animals, CDK activity can be negatively regulated by phosphorylation on two inhibitory residues, Threonin 14 and Tyrosin 15. Wee1 is a tyrosine kinase that phosphorylates Tyr15, and Myt1 is a dual-specificity kinase that can phosphorylate both sites, with a propensity toward the Thr14. These inhibitory phosphorylations are removed by Cdc25 phosphatases [125]. Orthologs of the WEE1 kinase that can phosphorylate CDKA;1 on Tyr15 has been described in plants, but their in vivo role in cell cycle progression is not well defined, since knock-out mutants have no developmental phenotype [38]. In addition, to date, no clear CDC25 homologue have been identified in genomes of higher plants [18]. One possibility might be that tyrosine dephosphorylation in plants is achieved by a phosphatase unrelated to CDC25. Alternatively, Boudolf et al. proposed that in plants, Tyr15 phosphorylation might not be relevant for G2/M transition, that would rather rely on activation of plant specific B-type CDKs [18].

7.2.3 Core Cell Cycle Proteins Identified at the PPB

CDKA was the first protein reported to localize at the PPB [36, 113]. Ever since, this localization has been repeatedly described in a number of species (see references

in Table 7.1). Moreover, CDKA seems to localize specifically to mature (narrow) PPBs [36, 109, 172], and this localization is dependent on PPB MTs [36]. Interestingly, the localization of a B-type cyclin, CYCB1;2, coincides with CDKA;1, since the association of CYCB1;2 with the PPB occurs shortly before PPB disassembly [109]. Moreover, in *Trandescantia* stamen hair cells, microinjection of an active CDKA;1/Cyclin B-type complex leads to rapid depolymerization of the PPB and premature NEB [69]. In contrast, treatment with roscovitine, a specific inhibitor of CDKs, leads to an arrest of cells in G2 phase and to PPB stabilization in *Vicia faba* root tip cells [14], and to PPB loosening in onion root tip cells [122]. Taken together, these results suggest a role for CDK in PPB disassembly and NEB. CDK recruitment at the PPB could be required for regulating MT associated proteins (MAP) and/or MT destabilizing factors localized at the PPB. For example, phosphorylation of *Arabidopsis* MAP65-1 during mitosis reduces its affinity for MTs and affects its localization [146]. Alternatively, CDK recruitment at the PPB could participate in depleting CDK complexes from the nucleus and the cytoplasm in order to prevent premature entry into prophase, before proper cortical division site definition. Another cyclin, CYCA1;1, has been consistently located at the PPB in *Arabidopsis*, maize and tobacco BY-2 cells [16, 109]. In vivo interaction between CYCA1;1 and CDKA;1 has been shown recently in tobacco leaf cells [16]. Since both are present at the PPB, this result suggests that a CDKA;1/CYCA1;1 complex could be assembled at the PPB during G2/M transition. A B-type CDK isoform, CDKB2;1, whose expression is specific to G2/M phase transition, has also been localized to the PPB [108] (Table 7.1). These results strongly support the presence of active cyclin-CDK complexes at the PPB.

Several other regulatory cell cycle proteins have been described on the PPB (Table 7.1). This includes KRP4, a CDK inhibitor from the KRP family, that has been shown to interact with *Arabidopsis* CDKA;1 and several A-type and D-type cyclins [16, 163, 184]. KRP4 could be a key regulator of CDK complexes at the PPB, by keeping the cyclin/CDKA complexes inactive until required. CKS2, the CDK complex scaffold protein, also localizes at the PPB in *Arabidopsis* and BY-2 cells [16] (Table 7.1), and is able to interact *in planta* with both CDKA;1 and KRP4 [16]. Finally, two components of the anaphase-promoting complex localize to the PPB [16, 93]. This includes the Cdh1-related protein, CCS52A2, which is able to interact with several cyclins in *Arabidopsis* [57] and RAE1, which has been shown to form a complex with Cdh1-activated anaphase promoting complex and to prevent premature separation of sister chromatids in mice [75]. This suggests that targeted protein degradation might participate in the temporal regulation of the cell cycle proteins associated with the PPB.

These results suggest a tight coordination between PPB function and the cell cycle, as expected from previous observations. Although not an absolute requirement for cell cycle progression and entry into mitosis, recruitment of several key components of the cell cycle machinery to the PPB might be necessary for the temporal and spatial interplay between nuclear events (e.g. prophase spindle establishment, NEB,…) and cortical division site determination. It appears that several CDK complexes are potentially assembled at the PPB, together with key regulators of their activity. Such play-

ers have presumably no ability to bind MTs by themselves, and recruitment of CDK complexes to the PPB probably requires other linker partners. Co-purification of *Arabidopsis* TON1 and CDKA from TAP-tag experiments might be relevant in this respect [163]. The time course of interaction networks at the PPB, as well as the precise function of cell cycle proteins at the PPB are by now unknown, and is a major challenge awaiting cell biologists for a better understanding of division plane establishment, PPB function, and cell cycle regulation in plant cells.

7.3 The PPB and the Pre-Mitotic Cell

The PPB is not the only structure that predicts the division plane in a cell engaging in mitosis. Depending on the cell type and division, other cytological events precede or accompany formation of the PPB.

7.3.1 The Nucleus

In plant cells, nuclear position is an indicator of the future division plane, and the PPB exhibits close spatio-temporal relationships with the nucleus, the ring-shaped cortical PPB being always ultimately co-aligned with the nuclear equator in early prophase, under normal conditions. To achieve such co-positioning, premitotic nuclear migration is required in vacuolated cells, as well as in some cases of asymmetric divisions. This is not the case in small and densely cytoplasmic cells (e.g. meristematic ones), where the nucleus occupies a comparatively larger volume in the cell and remains in a central position. In vacuolated cells, during interphase, the nucleus is confined to the cortical cytoplasm by the large central vacuole. Upon preparation for mitosis and prior to formation of the PPB, the nucleus moves from the periphery to the centre of the cell, "suspended" by transvacuolar cytoplasmic strands [144]. The mechanism that governs this movement is most probably driven by the cytoskeleton. Although only actin is found in cytoplasmic strands during interphase, both MTs and microfilaments (MFs) are present at the premitotic stage [56, 80]. Contrary to the case of interphase cells where only actin seems required for nuclear positioning [32, 81, 85], the relative contribution of MTs and MFs in premitotic nuclear migration, as assessed by drug studies (where it is not easy to distinguish direct from indirect effects), is not clear and may depend on the cell system under study. In BY-2 cells, two independent studies conclude to a role of MTs, without excluding a role for actin in the process [44, 80]. After migration, the nucleus becomes anchored in place, its position being more resistant to centrifugation or cytoskeletal-disrupting drugs [80, 129, 165].

Given the sequence of events in vacuolated cells and the spatial relationship between the nucleus and the PPB, the question arises whether the position of the nucleus determines the site of PPB formation. In a series of experiments using protonemata cells of the fern *Adiantum* [118], displacement of the nucleus by

centrifugation induced formation of a PPB around the displaced nucleus, or of a second PPB when the nucleus was displaced after initiation of a first PPB. However, in asymmetrically dividing stomatal cells, PPB formation precedes nuclear migration and is independent from nucleus position (reviewed in [59]). Taken together, these observations support the conclusion that although the nucleus can have a direct influence on PPB formation and localization, proximity of the nucleus is not an absolute requirement for building a PPB, and other factors and spatial cues contribute to PPB formation and positioning as well. The exact nature (chemical and/or mechanical) of the determinant(s) of PPB positioning and their origin (intercellular and/or intrinsic) remain to be determined.

7.3.2 Vacuoles and the Phragmosome

Like those of all eucaryotes, plant cells are highly compartmentalized into a variety of membrane-bound organelles which play a diversity of functions in the interphase cell [98]. During division, the cell must secure proper distribution of the cytoplasm and organelles between daughter cells, but organelles also contribute to mitosis, especially when the new cell plate is constructed during cytokinesis.

Vacuoles are the largest organelles in mature plant cells; they are highly dynamic and display a variety of function, shape and size [104]. During mitosis, the vacuolar compartment displays specific configurations as the cell progresses through the cell cycle both in cultured BY-2 cells [90, 91] and in meristematic cells of *Arabidopsis* [141] and horseradish [104]. The mitotic configuration of vacuoles includes fragmentation, exclusion from the division plane region, and projection of tubular extensions which encircle the spindle and connect parts of the vacuolar system through the phragmosome.

In cells possessing a large vacuole, during preparation for mitosis, the progressive exclusion of vacuoles from the center of the cell defines a "phragmosome," a continuous, disk-shaped region of cytoplasm within which karyokinesis and cytokinesis occur. Phragmosome formation is tightly coupled with premitotic nuclear migration. During this stage, the nucleus is suspended in the cell by transvacuolar strands containing MT and MF, which link the nucleus to the cortex and pull it into the centre of the vacuole [97]. As the cell proceeds to division, transvacuolar strands radiating from the nucleus start to realign and coalesce into an equatorial disc-shaped domain of cytoplasm surrounding the nucleus, which prefigures the site of PPB formation [165]. The phragmosome is another marker of the future division plane in such large vacuolated cells, and defines the cytoplasmic region where mitosis occurs. It is not observed in several cell types, such as densely cytoplasmic meristematic cells.

The phragmosome confines the vacuolar compartment to the polar regions. The two parts of the vacuole are connected by tubular extensions across the phragmosome until telophase [91, 141]. In *Coleus* pith cells, the division plane is marked even earlier by a cortical cytoplasmic ring, rich in actin filaments and endoplasmic reticulum, which is formed in interphase before appearance of the phragmosome [130].

7.3.3 Golgi Apparatus and Endocytosis

The activity of the Golgi apparatus is directly required for cell plate formation at telophase, and Golgi stacks are prominently associated with the phragmoplast and the growing cell plate [141]. In addition to Golgi-derived vesicles, the plasma membrane also contributes to the growing cell plate by endocytosis [42]. During interphase, Golgi stacks are evenly distributed in the cytoplasm [120]. They double in number at G2 in meristematic *Arabidopsis* cells [141]. During early mitotic stages in BY-2 cells, most Golgi stacks accumulate in the phragmosome region concomitantly with PPB formation and narrowing [44, 120], forming a "Golgi belt" that fully develops at metaphase. At metaphase, a large number of Golgi stacks also accumulates around the spindle and spindle poles [120]. The role of Golgi accumulation at the PPB site is unclear, and Golgi secretion does not appear to be essential for PPB function at the cortex [47].

The endoplasmic reticulum undergoes drastic reorganization during mitosis as well [67] and is closely associated with the spindle and phragmoplast microtubules [64]. The ER is reorganized during the preprophase/prophase stage, and a dense accumulation of ER tubules were detected at the PPB site in *Pinus* root-tip cells, forming a cortical ring together with the PPB [183].

The PPB region is also an active site of endocytosis, and endocytic vesicles form a cortical belt loosely colocalizing with the microtubular PPB [44], with an accumulation of clathrin-coated pits and vesicle in the PPB region [79]. The strong endocytic activity at the PPB site could be involved in the selective removal of proteins from the plasma membrane (e.g. KCA1, [164]), contributing to establishing the cortical division site and preparing the plasma membrane for phragmoplast guidance and ultimate fusion with the expanding cell plate.

7.4 Structure and Dynamics of the PPB

Ever since the first description of the PPB by Pickett-Heaps and Northcote 40 years ago [135], a number of cytological studies have been published describing this structure in many lower and higher land plant species, and in a variety of cell types. This rich literature is discussed in a comprehensive and major review published 10 years ago [110]. Here, we will discuss recent results connected to the different constituents of the PPB, mainly MTs, actin, and the growing class of proteins localized at the PPB and their proposed functions.

7.4.1 Microtubules

At the onset of mitosis, the cortical MT array undergoes a complete and major reorganization, leading to formation of the PPB and to depolymerization of CMTs outside the PPB. Recent years have seen the identification of several mechanisms that may contribute to cortical array formation in plant cells. Fine regulation of

these different activities via proteins that have the ability to nucleate, anchor, bundle, severe and modulate MT ends dynamics can lead to dramatic rearrangements in CMTs organization [52] and are likely to play a role during PPB formation.

7.4.1.1 MT Dynamics at the Cortex

In interphase plant cells, MTs are mostly found at the cortex, defined as the thin layer of cytoplasm underneath the plasma membrane. In actively elongating cells, they are organized in parallel hoop-like arrays transverse to the cell elongation axis, although sparse MTs traversing the cytoplasm can also be observed. CMTs serve as a spatial template for cellulose microfibril extrusion and cell wall deposition, and drive organization of cellulose microfibrils by guiding the trajectories of cellulose synthase complexes in the plasma membrane, thus orienting nascent microfibrils [37, 65, 132]. The emergence of organized arrays from a pool of individual MTs rely on various cellular activities that modify intrinsic properties of MTs and drive formation of higher-order structures [133]:

1. Individual MTs are highly dynamic and polar polymers: they constantly alternate between phases of polymerization and depolymerization, especially at their (+) end [52, 142]. Eukaryotic cells possess an arsenal of protein activities that can modify parameters of MT dynamic instability, from the rate of polymerization/depolymerization to the frequency of transitions (catastrophy and rescue rates). The local concentration of tubulin dimers can also vary significantly within a cell and exert a profound influence on MT dynamics parameters [162]. In addition, gradients of tubulin concentration have been shown in vitro to contribute to MT self-organization [131].
2. Microtubule initiation is a templated and regulated process. Recruitment of cytoplasmic γ-tubulin nucleation complexes onto existing cortical microtubules, and initiation of new MTs at fixed angle (40°) from these sites has been described [117]. MT-independent nucleation and nucleation parallel to existing MTs may also contribute to populate the CMT pool [2].
3. Collision-dependent catastrophe, bundling or severing. MTs which colonize the cortex undergo frequent encounters, crossings and collisions. Depending on the angle of collision, the fate of the incoming MT may change drastically: lateral association (bundling) with the encountered MT (when the angle is shallow), catastrophe (steep angle) [46, 48]. Collision-induced severing has also been reported [176].
4. Tip and side-association of MTs with cellular structures and organelles: examples are MT-MT lateral association (bundling) [29] and anchoring of MTs to the plasma membrane [8, 40, 43].

7.4.1.2 Origin and Dynamics of PPB Microtubules

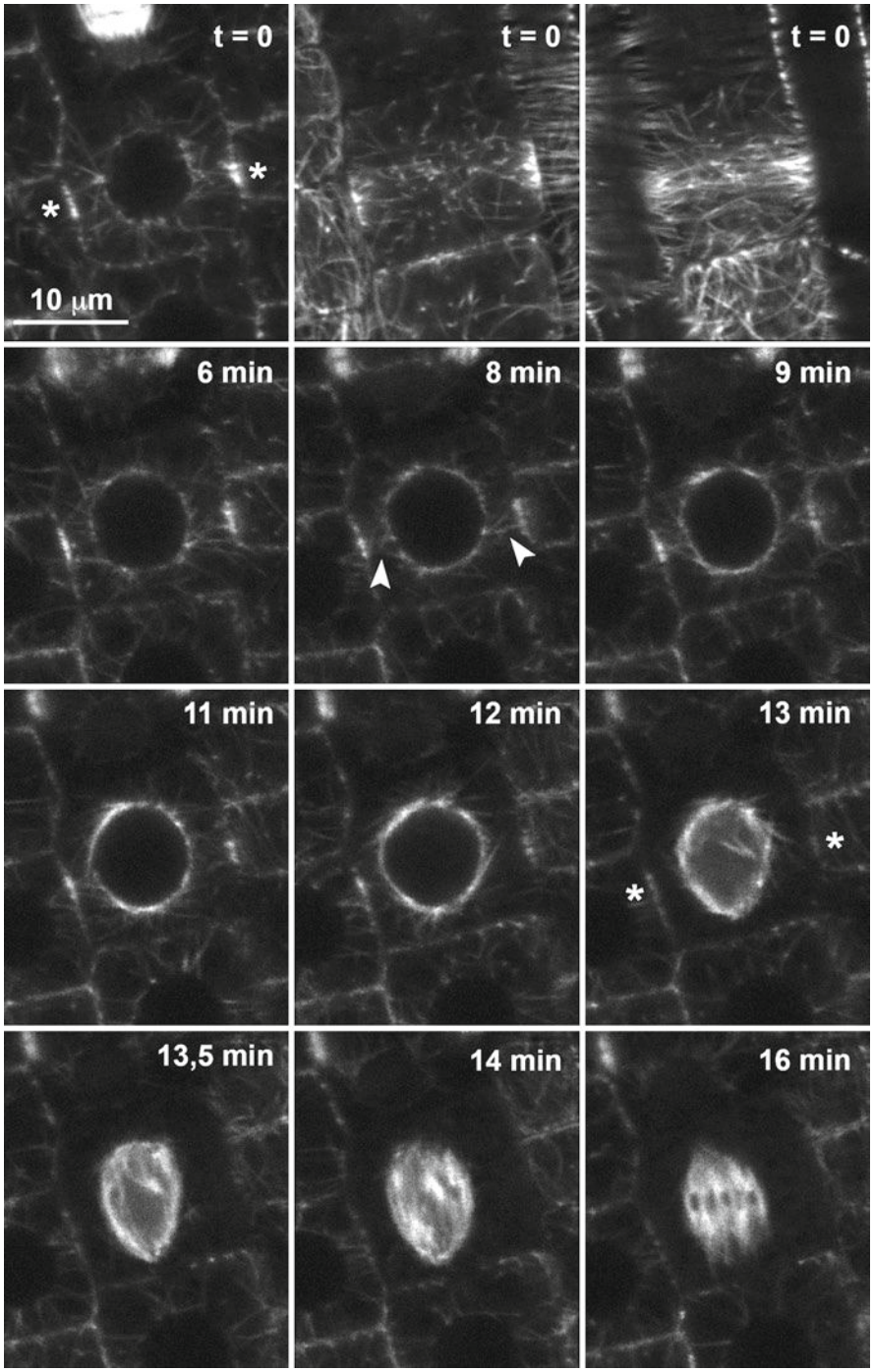
The origin of MTs contributing to PPB formation has been questioned. PPBs still form in cells where protein synthesis are inhibited, indicating that de novo tubulin

synthesis is not required for PPB formation [123]. It is likely that progressive depolymerization of interphase CMT generates high cytoplasmic tubulin pools which can be recycled for PPB MT polymerization. Colocalization of γ-tubulin with PPB MTs strongly suggests that MT nucleation occurs within the PPB, and can partly account for PPB formation [77, 95, 96] (see Table 7.2). Motor-based translocation of CMTs towards the site of PPB formation has been proposed earlier to participate in formation of this array [110]. However, up to now, significant lateral translocation of MTs has never been observed neither during interphase nor during PPB formation [142, 166]. In contrast, modulation of dynamic instability appears to be essential for PPB formation. At the onset of mitosis, dynamic instability of MTs increases significantly outside the PPB, where MTs become more dynamic and longer. This change in MT dynamics has been proposed to contribute to PPB formation via a "search and capture" mechanism [166], with highly dynamic MT (+) ends probing the cortex, and then being selectively stabilized and bundled to other MTs once they reach the PPB site [166]. Contrary to non-PPB MTs which become more dynamic, FRAP analysis showed that PPB MTs, do not differ significantly from interphase CMTs [70]. However, the activities which trigger selective stabilization of MTs at the future division site where PPB is forming, and which increases MT dynamics outside this narrow band are still to be identified.

7.4.1.3 Non PPB Microtubules During the G2/M Transition

At the same time as the PPB forms, highly dynamic cytoplasmic MTs are polymerized, which connect the nucleus with the cell cortex [44]. This MT population could originate both from the PPB and from the nuclear envelope, a well established site of MT nucleation in plant cells [152]. γ-Tubulin is present at both sites [54, 77, 95, 96]. As PPB matures, two classes of cytoplasmic MTs can be distinguished (Fig. 7.1): (1) unidirectional MTs radiating from the nucleus through transvacuolar strands and connecting cortical sites outside the PPB, their (+) end oriented towards the cortex, likely contributing to maintain the mitotic apparatus at the center of the cell

Fig. 7.1 Time series showing PPB formation and disassembly in *Arabidopsis* root epidermal cell. Confocal imaging of root tip cells expressing the MBD-GFP MT marker expressed under the control of the 35S promoter [25]. The same cell is imaged in every panels. *Upper* panels represent different focal planes of the same cell at the same time. At $t=0$, the cortical PPB of MTs encircles the nucleus (*asterisk* in *upper left* panel). Note cytoplasmic MTs bridging the nucleus to the cell cortex in every directions (*upper left* panel, and *dots* in *upper middle* panel corresponding to transverse sections of cytoplasmic bundled MTs). As cell progresses into mitosis, the PPB narrows down (compare PPB width at $t=0$ and 11 min) and cytoplasmic MTs connecting the PPB to the nucleus are more prominent (*arrowheads* at $t=8$ min). At the same time, MTs accumulate on the nuclear envelope with higher density at the future poles of division (compare $t=0$–12 min) hence forming the prophase spindle ($t=12$ min). When nuclear envelope breaks down, PBB is still visible (*asterisk* at $t=13$ min). Soon after, PPB is completely disassembled ($t=14$ min), and a typical metaphase spindle is set up ($t=16$ min). Each image is a single optical section. Time when the scans were performed is indicated in each panel. Bar = 10 μm in every panel


t = 0
t = 0
t = 0
10 μm
6 min
8 min
9 min
11 min
12 min
13 min
13,5 min
14 min
16 min

[44]. (2) bidirectional MTs that connect the PPB to nucleus, with their (+) end growing either from and out of the nucleus [44]. These cytoplasmic MTs are thought to contribute to position and maintain the nucleus at the centre of the PPB.

It has also been proposed that these MTs provide spatial cues for establishing the axis of the prophase spindle perpendicularly to the plane defined by the PPB [4] (see also Sect. 7.5.3). Indeed, as cell progresses in prophase, PPB starts to break down, first slowly, then progressively faster. At the same time, the MT density around the nucleus dramatically increases, forming the prophase spindle (Fig. 7.1) [166]. At the end of prophase, the nuclear envelope breaks down and is reformed around daughter nuclei after separation of the chromosomes. In animal cells, NEB results from microtubule-dependent tearing of the nuclear envelope. Microtubules could play such role in plant cells as well (reviewed in [5]). NEB always precedes PPB disappearance by a few minutes (Fig. 7.1) [49]. Furthermore, the rate of PPB disappearance correlates with the proximity of the nucleus to the PPB site, which suggests that diffusible nuclear factor(s) released from the nucleus upon NEB might trigger disassembly of the PPB [49].

7.4.2 Actin

The presence of actin at the cortex during mitosis is still a debated question and contradictory results regarding the presence of actin in the PPB, and later on at the cortex, have been published [127]. This uncertainty could result either from variations in actin distribution between cell types and species, or from technical problems related to fixation or imaging methods used to visualize actin [127].

When actin is seen at the PPB, actin microfilaments are aligned in the cell cortex, parallel to MTs and co-localized with the PPB, forming a broad band during preprophase that narrows as prophase progresses [34, 126, 156]. Cytochalasin, an anti-actin drug, does not affect formation of a broad PPB of MTs but prevents its narrowing [53, 112]. After PPB disassembly, several observations suggest that actin is present at the cortex but is excluded from the CDS, thus defining a actin-depleted zone (ADZ), considered as a negative marker of the CDS (reviewed in [110]). In BY-2 cells, cortical actin, as revealed by the expression of the actin-binding domain of fimbrin fused to GFP, is present as so-called twin peaks, two high density bands of actin flanking a zone of actin low density corresponding to the CDS [137]. This suggests that the ADZ would be a region of low density of actin, rather than a zone completely devoid of actin. Hence, it seems that the presence at the cortex of actin, a notoriously vulnerable structure, should be reexamined carefully during mitosis to get a clearer view of the function of this structure.

Another feature of actin organization during mitosis is the absence of thick intra-cytoplasmic actin cables which, during interphase, run across the cytoplasm and are involved in intense cytoplasmic streaming and organelle movement [127]. This arrest of cytoplasmic streaming during mitosis has been suggested to induce stability around the CDS thus avoiding disturbances around the site of cell plate insertion at the CDS [127].

7.4.3 Proteins Localized to the PPB: A Critical Review

Genetic and cytological analysis have led to the identification of a number of proteins potentially involved in cytoskeletal organization that appear to localize at the PPB. Table 7.2 lists such proteins localized at the PPB, together with informations as to their other localizations in the cell.

7.4.3.1 Preliminary Remarks

Depending on the technique used, several proteins show inconsistent localization between studies. This is the case for the SPR2 protein, which locates to all MT arrays when SPR2-GFP expression is driven by the 35S promoter, whereas SPR2-GFP only labels interphase CMT arrays when expressed under the control of the *SPR2* promoter. In such instance, complementation of the mutant phenotype by GFP fusions does not provide a definite validation of the observed localization, since even the p35S-driven SPR2 construct complements the *spr2* phenotype. Similarly, the localization of EB1 proteins (three isoforms in *Arabidopsis*) is somehow inconsistent between studies. In contrast to p35S driven EB1-GFP fusion proteins that label the whole length of MTs and even MT (−) ends in spindle poles [27, 28, 105, 160], fine-tuned expression of EB1a and b GFP fusions labels MT (+) ends in all MT arrays, making EB1a (and b) ideal MT (+) end markers [46]. Other examples of such discrepancies between studies are found in Table 7.2. This obviously indicates that localization studies must be interpreted cautiously, and that careful attention must be paid to techniques used to localize proteins. In several cases, more experiments are needed to definitively establish localization of certain proteins at the PPB (or elsewhere). The potential functions of such players at the PPB are discussed below, with respect to the formation and role of the PPB in determining the cortical division site.

7.4.3.2 Proteins Potentially Involved in PPB Formation

A specific role in PPB formation and function can be anticipated for a number of proteins that locate at the PPB (Table 7.2), and/or whose mutation leads to defects in PPB structure and/or function. This obviously includes *Arabidopsis* TON1 and FASS/TON2 whose mutation leads to complete absence of PPB, as well as DCD1 and ADD1, the FASS maize homologues, whose simultaneous depletion leads to absent or malformed PPB. Interestingly, both TON1 and FASS proteins share similarities with proteins located at the centrosome in mammals. TON1 proteins share several motifs with FOP and OFD1 from human and interacts with Centrin, another protein whose animal, fungal and algal homologues localize to Microtubule Organizing Centers (MTOCs) [11]. In addition, TON1 was shown recently to co-purify with CDKA;1 in *Arabidopsis* cells [163], which provides a long-sought link between the cell cycle machinery and the cortical cytoskeleton. CDKA;1 is known from several studies to transiently associate with the PPB in plant cells (see Table 7.1).

Table 7.2 Proteins localized at the PPB

Protein (abbreviation)	Species	Protein function	Mutant phenotype	Techniques used for localization studies (reliability)	Localization	References
TONNEAU1 (TON1a and TON1b)	Arabidopsis	N-terminal part similar to two human centrosomal proteins, FOP and OFD1; two proteins with redundant function 29 kDa	See Table 7.3	Protein fusions (pTON1:GFP-TON1 and p35S:GFP-TON1) expressed in Arabidopsis stable lines. Partial complementation of the *ton1* phenotype	Interphase CMT arrays as punctate labelling, PPB	[11]
DISCORDIA1 (DCD1)	Maize	Putative PP2A regulatory B″ subunit; similar to FASS/TON2 55 kDa	See Table 7.3	Immunolocalization using an anti-peptide directed against both DCD1 and ADD1 proteins	Decorates PPB, persists at the CDS at least until metaphase	[178]
ALTERNATIVE DISCORDIA1 (ADD1)	Maize	Nearly identical to DCD1 55 kDa	See Table 7.3	Immunolocalization using an anti-peptide directed against both DCD1 and ADD1 proteins	Decorates PPB, persists at the CDS at least until metaphase	[178]
TANGLED1 (TAN1)	Maize	Highly basic protein with MT binding activity. Weak similarity with basic regions of vertebrate APC proteins. Interacts with POK1 and POK2 kinesins 41 kDa	See Table 7.3	Immunolocalization using a anti-TAN1 antibody that may recognize maize TAN1-related proteins	All MT arrays using an anti-TAN1 antibody	[148]
				Heterologous localization in Arabidopsis expressing the p35S:TAN1-GFP construct	PPB	[168]
TANGLED (TAN)	Arabidopsis	Similar to the maize TAN1 protein 53 kDa	See Table 7.3	pTAN1:TAN-YFP and p35S:TAN-YFP	Localization at PPB dependent on MTs and POK1 and POK2. At the CDS throughout mitosis and cytokinesis	[168]

MICROTUBULE ORGANISATION1 (MOR1)	Arabidopsis, tobacco	MAP215/Dis1 family of MAPs; promotes rapid growth and shrinkage and suppresses the pausing of MTs in vivo 217 kDa	See Table 7.3	Immunlocalization using an antibody directed against a C-terminal polypeptide Immunlocalization using an antibody directed against residues 235–249 of MOR1	CMT interphase arrays as punctate staining, PPB, spindle and midline of the phragmoplast All arrays on the full length of MTs	[66, 157] [83]
CLASP	Arabidopsis	Similar to the + end tracking protein CLASP from animal and fungi Interacts with the C-terminus of POK1 158 kDa	See Table 7.3	Protein fusions (pCLASP: YFP-CLASP and p35S: YFP-CLASP) expressed in Arabidopsis stable lines. Full complementation of the *clasp* mutant phenotype	Prominent labelling of CMT, both interphase and PPB, as discrete spots along interphase MTs. Faint labelling of spindle and phragmoplast	[86]
				Protein fusion (p35S:GFP-CLASP) expressed in Arabidopsis (full rescue of the *clasp* mutant phenotype) and BY-2 cells	Interphase CMTs with MT (+) end enrichment (Arabidopsis). PPB, spindle and phramoplast in BY-2 cells	[7]
RanGAP1	Arabidopsis	Ran GTPase activating protein AtRanGAP1 interacts with the C-terminus of POK1 69 kDa	Gametophytic lethality of *rg1 rg2* double mutant. RNAi depletion induces oblique division planes and presence of cell wall stubs	Immunolocalization using an anti-AtRanGAP1 antibody. No signal in a RanGAP1 null mutant For real-time imaging, use of a p35S driven RanGAP1-GFP fusion in Arabidopsis dividing root cells	NE during interphase. PPB. Persists at the CDS throughout mitosis. Kinetochore region of chromosomes in metaphase, midline of the phragmoplast later on. Persistence at the PPB and CDS localization independent of MTs. Persistence at the CDS requires POK1 and POK2	[76, 180]

(continued)

Table 7.2 (continued)

Protein (abbreviation)	Species	Protein function	Mutant phenotype	Techniques used for localization studies (reliability)	Localization	References
*A*UXIN-*I*NDUCED in *R*OOT cultures (AIR9)	Arabidopsis	Unknown function. Isolated in a proteomic screen for MT-associated proteins 187 kDa		Confocal imaging of tobacco BY-2 cells expressing a p35S driven GFP-AIR9 fusion protein	CMT, PPB, very weak staining of the spindle and phragmoplast. At the CDS when phragmoplast contacts cortex, then labels cross walls	[22]
SPIRAL1/SKU6 (SPR1)	Arabidopsis	Plant-specific protein. N- and C-terminal region sufficient for MT localization 12 kDa	Right-handed helical growth phenotype	Confocal imaging of pSPR1:SPR1-GFP expressing plants (rescue the spr1 phenotype). Immunolocalization using an anti-SPR1 antibody	Interphase CMTs	[119]
				Confocal imaging of pSPR1:SPR1-GFP expressing plants (rescue the spr1 phenotype)	Concentrated at MT (+) ends in interphase CMT, PPB, spindle and phramoplast	[140]
SPIRAL2/ TORTIFOLIA1 (SPR2/TOR1) and SPIRAL2-like SP2L	Arabidopsis	MT-associated proteins. Promotes MT stabilization. Suppresses the pause state at MT ends and thus enhances MT growth 94 kDa	Right-handed helical growth phenotype Allelic to *tortifolia1* [23]	Confocal imaging of p35S driven SPR2-GFP fusion protein expressed in Arabidopsis (rescue of the *spr2* phenotype) or tobacco BY-2 cells	Interphase CMT arrays, PPB, spindle and phragmoplast	[23, 143]
				Confocal imaging of SPR2 and SP2L fusions to tandem GFP expressed under the control of SPR2 and SP2L native regulatory elements	Interphase CMT arrays. Punctate pattern along MTs; weak association with MT (+) ends	[182]

RUNKEL (RUK)	Arabidopsis	Contains a putative ser/thr kinase domain (with no activity in vitro) and a large MT binding domain 152 kDa	Seedling lethal. Abnormal phragmoplast and arrested cell plate expansion	Immunolocalization on transgenic Arabidopsis expressing the pRUK:6x HA-RUK construct that recues the *runkel* mutant phenotype	Localizes at the PPB, spindle, on the phragmoplast, but not the interphase CMT arrays	[89]
ATK5	Arabidopsis	Kinesin of the Arabidopsis Kinesin-14 family. (−) end directed motors	No developmental phenotype but abnormally broadened spindle in *atk5-1* and *atk5-2*	Confocal imaging of p35S driven YFP-ATK5 fusion protein expressed in Arabidopsis (rescue of the *atk5-1* spindle phenotype) or tobacco BY-2 cells	Interphase CMT arrays where it behaves as a MT (+) end tracking protein. PPB, prophase spindle, spindle and phragmoplast	[3, 6]
Kinesin-like calmodulin binding protein (KCBP)	Arabidopsis Tobacco Cotton	MT (−) end directed motor 140 kDa	Essential for trichome morphogenesis in Arabidopsis	Immunolocalization using an anti-KCPB antibody (anti-peptide in Arabidopsis and anti-polypeptide 561–867 residues in cotton)	Interphase CMT arrays as a punctate staining, PPB, spindle and phragmoplast	[1, 19, 136]
TKRP125 and DcKRP120-1	Tobacco BY-2 cells, carrot	BimC-type kinesin-related protein 125 kDa		Immunolocalization using an anti-TKRP125 antibody in carrot and tobacco BY-2 cells	Interphase CMT arrays, PPB, prophase spindle, spindle, phragmoplast	[9, 13]
KRP120-2	Carrot	BimC-type kinesin-related protein 125 kDa		Immunolocalization using an anti-DcKRP120 antibody in carrot cells	Faintly labels Interphase CMT arrays and PPB and strongly phragmoplast and its midzone	[13]
MPK6	Arabidopsis	Mitogen-activated protein kinase 45 kDa	See Table 7.3	Immunolocalization using an anti-MPK6 antibody on root tip cells	Spot-like structures on PPB and phragmoplast MTs and on cell plate. To the plasma membrane and the trans golgi network	[114]

(continued)

Table 7.2 (continued)

Protein (abbreviation)	Species	Protein function	Mutant phenotype	Techniques used for localization studies (reliability)	Localization	References
End-binding1 (EB1a, EB1b, EB1c)	Arabidopsis, BY-2 cells	Similar to yeast and animal EB1 MT (+) end tracking protein 31 kDa	Defects in response to gravity/touch stimuli	Confocal imaging of p35S driven EB1-GFP fusion proteins in BY-2 or Arabidopsis culture cells	AtEB1a and b: CMT arrays, spindle poles EB1c: nuclear, faint labelling of PPB, spindle poles	[27, 105, 160]
				Confocal imaging of pHSP18 driven EB1-GFP fusion proteins (low expression) and pEB1b driven EB1-GFP in BY-2 cells or Arabidopsis seedlings	EB1a and b: MT (+) end. Interphase CMT. Perinuclear MTs and PPB. Spindle midzone and the phragmoplast EB1C:nuclear	[46]
				Immunolocalization using an enriched anti-EB1c antibody	Spindle and phragmoplast midzones	[15]
AtMAP65. Sequence similarity with PRC1/ Ase1p from mammals and yeast	Arabidopsis	AtMAP65-1 promotes bundling of anti-parallel MTs		Confocal imaging of p35S driven MAP65-1-GFP in BY-2 cells	CMT, PPB, aster-like MTs, spindle midzone at anaphase, phragmoplast	[30, 58, 99, 147]
		AtMAP65-2 stabilizes MTs		Confocal imaging of p35S driven MAP65-2-GFP proteins in Arabidopsis cells	All MT arrays	[94]
		AtMAP65-3	Defective cytokinesis in roots, abnormal spindles, distorted phragmoplasts in *pleiad* mutant	Immunolocalization using a specific anti-MAP65-3 antibody	PPB, phragmoplast midline	[116]
				Confocal imaging of pAtMAP65-3 driven MAP65-3-GFP proteins in Arabidopsis	All MT arrays. Newly formed cell plate	[24]
		AtMAP65-5 promotes bundling of anti-parallel MTs		Confocal imaging of p35S driven MAP65-5-GFP in BY-2 cells	PPB, perinuclear MTs, aster-like MTs, spindle and phragmoplast midzone	[58]

		AtMAP65-8		Confocal imaging of p35S driven MAP65-8-GFP in BY-2 cells	Dots on CMT, PPB, spindle, phragmoplast MT (−) ends	[160]
γ-Tubulin	Arabidopsis wheat, soybean, BY-2 cells	Part of γ-TurC complexes, involved in MT nucleation 53 kDa	Gametophytic lethal in Arabidopsis. γ-tubulin depletion affects all MT arrays	Immunolocalization using anti-γ-tubulin antibodies	NE. All MT arrays along the entire MT length	[54, 77, 95, 96]
26S proteasome	BY-2 cells	20S core protease and Rpn10 (regulatory subunit)		Immunolocalization using anti-Rpn10 and anti-20S catalytic proteasome antibodies	NE, PPB, spindle and phragmoplast	[181]
AtMAP70	Arabidopsis	Stabilizes MT 69 kDa	Defects in anisotropic cell growth in RNAi plants	Confocal imaging of p35S driven GFP-AtMAP70 in BY-2 cells	All MT arrays	[87, 88]
Tobacco p90	Tobacco	Phospholipase D 90 kDa		Immunolocalization using a monoclonal anti-p90 antibody on BY-2 cells	All MT arrays	[61]

Techniques used to determine protein subcellular localization are indicated. When available, mutant phenotype and complementation analysis of the mutant phenotype performed to validate the localization of GFP or other fluorescent proteins fusions are mentioned. ND: localization not determined

In animal cells, the initial activation of cyclin B1-Cdk1 in early prophase takes place at the centrosome, before spreading into the cell for entry into mitosis [74]. *FASS*, *DCD1* and *ADD1* genes encode a putative PP2A regulatory subunit [25, 178]. PP2A phosphatases are key regulators of various mitotic processes in all eukaryotes [39]. RSA-1, the *C. elegans* FASS/DCD1/ADD1 homolog, localizes to centrosome, where it recruits PP2A complexes to regulate spindle assembly and MT outgrowth [139]. The fact that FASS encodes a PP2A regulatory subunit points to a role of protein phosphorylation/dephosphorylation cascades in PPB formation. Indeed, studies using PP2A inhibitors and kinase inhibitors have shown that inhibition of protein phosphatases and kinases disturbs PPB formation [10, 82].

In the same lines, several recent studies indicate that protein phosphorylation plays a crucial role in MT organization and dynamics [114, 138, 146, 167]. Two Mitogen-Activated protein kinases (MAPK) have been implicated in cortical MT functions. *mpk18* mutant analysis revealed that MPK18 plays a role in interphase CMT functions [167]. Mutation in the *MPK6* gene induces a shift in PPB, spindle and phragmoplast positioning, inducing abnormal division planes [114]. Moreover, MPK6 localizes at the PPB (Table 7.2). Possible candidates for downstream targets of these kinase activities are Microtubule Associated Proteins. Regulation by phosphorylation has been demonstrated for proteins of the MAP65 family in *Arabidopsis* and tobacco, during spindle and phragmoplast formation [99, 138, 146]. Nine AtMAP65 family members are present in the *Arabidopsis* genome [71]. Overall sequence conservation is poor, suggesting that AtMAP65 proteins accomplish a diversity of functions. Several proteins from the MAP65 family appear to localize to the PPB but none of them is specific of this structure, since most are also present on other MT arrays (Table 7.2). Several members have been shown to form cross-bridges between adjacent MT and to promote MT bundling [58, 100, 147]. This bundling activity is potentially important during G2/M transition, since PPB formation and narrowing involves cross-bridges (readily observed in early EM studies on PPBs; reviewed in [110]) and, most likely, bundling-dependent stabilization of adjacent MTs.

The MOR1 protein also possesses canonical MAPK phosphorylation sites. MOR1 and CLASP are two proteins containing TOG (tumor-overexpressed gene) domains, and both of them localize to the PPB in *Arabidopsis*. In mammals, MOR1 and CLASP homologues localize to MT plus ends during interphase, modulating MT growth and dynamics in a cell cycle-dependent manner [145]. The *mor1-1* mutation affects PPB formation: nearly one-half of *mor1-1* cells do not form a PPB prior to mitosis, and those that do form a PPB often display a disturbed organization of this array. *Arabidopsis* MOR1 affects cortical microtubule dynamics by promoting rapid growth and shrinkage, while reducing pause events [84], but its precise function at the PPB remains to be further characterized. Moreover, MOR1 seems to influence the association of the (+) end binding protein EB1 with MTs, either by modulating MT (+) structure or by interacting with EB1 [84]. Direct interactions have been documented in animal cells between EB1 and XMAP215, the MOR1 homolog [121]. The function of EB1 during plant mitosis is not clear. Despite EB1 localization on all mitotic MT arrays, surprisingly enough, mutant analysis in

Arabidopsis did not reveal a role for EB1 proteins during mitosis [15], perhaps because other MT binding proteins functionally overlap with EB1. CLASP, another TOG domain-containing protein, has been proposed to have a role in cortical MT organization and in PPB formation [8]. Indeed, in *clasp* mutant cells, broad PPBs are often observed, resulting from abnormal and delayed PPB narrowing [7]. CLASP has been proposed to mediate MT-cortex attachment, at least for interphase CMT. A similar role in CMT anchoring at the plasma membrane has been postulated for Phospholipase D [61]. Whether a modification of MOR1, CLASP or other MAPs activity in plant cells during G2/M transition can account for MT dynamics changes during PPB formation remains to be established.

Other proteins have been localized to the PPB but to date, there is no indication that they may play a role in its formation. This includes RUNKEL and AIR9, which have been proposed to be required respectively for cell plate expansion and cell plate attachment to the cell cortex, and whose function at the PPB has not been further investigated [22, 89].

7.4.3.3 Proteins Potentially Involved in CDS Establishment

Co-localization of a protein with the PPB may also reflect a role in the determination of the cortical division zone, especially when the protein persists at the CDS after PPB disassembly. The nature of the information deposited pre-mitotically at the cortical zone defined by the PPB, and remaining after PPB disassembly, has long remained a mystery to plant cell biologists since, apart from the controversial absence of actin in the ADZ, no marker was available for this region after PPB disassembly. This is beginning to be unraveled with the recent discovery of three proteins persisting at the CDS after PPB disassembly. This includes DCD1 and its homologue ADD1, TAN and RanGAP1 proteins [168, 178, 180]. As discussed earlier, DCD1 and ADD1 are undoubtedly involved in PPB formation. Whether DCD1-ADD1 localization at the CDS reveals an additional function for these proteins later during cytokinesis is still unclear. Conversely, TAN and RanGAP1 persistent localization at the CDS, together with the cytokinesis defects observed in seedlings of *Arabidopsis tan* and *ranGAP1* mutants, strongly argue for a role of these proteins in cell plate establishment, likely through phragmoplast guidance to the site previously occupied by the PPB. However, a function of these proteins in PPB assembly cannot be ruled out, since both of them are also present at the PPB. In this context, two other proteins appear to play an essential role in TAN and RanGAP1 pathways. These are the *Arabidopsis* kinesins POK1 and POK2 that are able to interact both with TAN1 [115] and RanGAP1 [180]. *pok1-pok2* double mutants display misoriented cell divisions, reflecting guidance defects of the phragmoplast towards the CDS. POK1 and POK2 appear to be required for RanGAP1 persistence at the CDS after PPB breakdown, but not for the initial recruitment of RanGAP1 to the PPB [180]. On the other hand, POK1 and POK2 are required for TAN localization at the PPB, suggesting that TAN and RanGAP1 could be cargos of these two kinesins [115].

The KCA1 kinesin provides an interesting example of a protein, which seems specifically excluded from the CDS, marking the division site in a negative manner [164]. KCA1 is a CDKA;1 interacting kinesin that locates at the expanding cell plate and the plasma membrane. Mutagenesis of serines of the two putative CDKA;1 phosphorylation sites mimicking constitutive phosphorylation prevents KCA1 association with the plasma membrane [164]. At the onset of mitosis, during PPB formation, the plasma membrane-associated KCA1 is excluded from the cortical zone spanning the PPB, thus defining a KCA1 depleted zone (KDZ). After PPB disassembly, the KDZ persists at the CDS independently from the cytoskeleton [164].

7.5 The PPB and the Division Plane of Plant Cells

7.5.1 Introduction

The strong spatial correlation between the PPB and the final division site observed in a variety of land plant species and cell types during 40 years of research, together with several experimental manipulations of PPB formation and function, have led to identify the PPB as a key player in premitotic establishment of the division site in land plant cells. In addition to these correlative approaches, the recent isolation of *Arabidopsis* and maize mutants and their cellular and molecular characterization has begun to shed some light on the proteins, functions and activities involved in the building and role(s) of this plant-specific array.

7.5.2 Mutations Affecting the PPB

Among mutations affecting PPB formation (Table 7.3), the *Arabidopsis ton1*, *fass/ton2* and the *Petunia trapu* mutants are the most spectacular ones since they are the only viable plant mutants unable to form a PPB [51, 154, 155]. This defect correlates with defective phragmoplast guidance and abnormal positioning of the cell plate between daughter cells. These mutants are characterized by the same strong developmental phenotype: mutant plants are extremely dwarf, the orientation of cell division is completely disorganized, but the general architecture of the plant, as well as cell differentiation, remain surprisingly correct [155]. The mutated locus of the petunia *tra1* mutant has not yet been identified. The *Arabidopsis ton1* mutation is caused by a double mutation affecting two homologous and redundant genes organized in tandem [11]. Interestingly, disruption of the *Physcomitrella patens* TON1 homologue leads to similar developmental and cellular phenotypes, as well as an absence of PPB formation in the moss gametophore. On the other hand, cell division in the protonema (which does not imply PPB) is not affected. In addition,

Table 7.3 Mutations affecting PPB formation

Mutant name (full name)	Species	Developmental and cellular phenotype	Cytoskeletal phenotype	References
trapu (*tra1*)	*Petunia hybrida*	Dwarf, short and thick organs; disorganized meristems, cell shape and size	Absence of PPB, random CMT	[51]
tonneau1 (*ton1*) (*double mutant*)	*Arabidopsis*	Dwarf and misshapen seedlings; abnormal positioning of division planes; abnormal cellular organization	Absence of PPB, random CMT, normal spindles; slight alterations in phragmoplast morphology, oblique phragmoplasts	[11, 155]
fass/tonneau2 (*fass/ton2*)	*Arabidopsis*	Dwarf and misshapen seedlings; abnormal positioning of division planes; abnormal cellular organization	Absence of PPB; random CMT; normal spindles; oblique, misshapen phragmoplasts	[25, 106, 107, 154, 155]
discordia1 (*dcd1*)	*Maize*	Affects asymmetric cell divisions in leaf epidermis (stomata formation)	In asymmetric division: frailed or incomplete PPBs; normal spindles and early phragmoplasts; abnormal positioning of late phragmoplasts	[60, 178]
alternative discordia1 (*add1*)	*Maize*	No noticeable phenotype	ND	[178]
dcd1 add1 (*RNAi*)	*Maize*	Defects in plant growth, cell growth and epidermal cell pattern; misorientation of symmetric and asymmetric cell divisions	In symmetric and asymmetric divisions: abnormal, frailed or absent PPBs; curved or tilted phragmoplasts	[178]
tangled1 (*tan1*)	*Maize*	Shorter plants; rough leaf texture; variable leaf epidermal cell shapes; Abnormal cell division orientations	Variable orientation of PPBs, spindles and phragmoplasts	[35, 148, 149]
tangled (*tan*)	*Arabidopsis*	Morphologically normal seedlings; abnormal cell division orientations in root tips	Normal PPBs; 11% of abnormally oriented phragmoplasts	[168]

(continued)

Table 7.3 (continued)

Mutant name (full name)	Species	Developmental and cellular phenotype	Cytoskeletal phenotype	References
microtubule organization 1/ gemini pollen 1 (*mor1/gem1*)	*Arabidopsis*	Stunted seedlings, isotropic cell expansion, and abnormal division planes for mor1-1 at restrictive temperature; cytokinesis defects at pollen mitosis for the gem1-1 homozygous lethal allele	CMT disruption, absent or frailed PPBs; shorter spindles and phragmoplasts; crooked or fragmented phragmoplasts	[83, 84,157, 173]
clasp	*Arabidopsis*	Dwarf plant; reduced cell number in root division zone; defects in directional cell expansion	Sparse interphase CMTs; broad PPBs, retarded PPB narrowing; shorter spindles and mature phragmoplasts	[7, 8,86]
atk1 (*Arabidopsis thaliana kinesin1*)	*Arabidopsis*	Normal vegetative development; reduced male fertility	Absence of PPB narrowing, disorganized prophase and metaphase spindles. Spindle abnormalities rectified by anaphase. Abnormal meiotic spindles	[31,103]
mpk6 (*mitogen-activated protein kinase6*)	*Arabidopsis*	"Short root" phenotype; root growth delay; ectopic and aberrant cell divisions	Shifted PPB, spindle and phragmoplast	[114]

cross-complementation experiments between *Arabidopsis* and moss show the conservation of TON1's function over land plant evolution [150].

Two genes similar to the *Arabidopsis FASS* gene are present in the maize genome. *DCD1* mutation induces cell division defects in asymmetrically dividing cells during stomata formation in maize leaves, whereas mutation of the nearly identical *ADD1* gene gives no phenotype [60, 178]. Depletion of DCD1 and ADD1 using RNAi leads to mutant phenotype strongly resembling *fass/ton2* mutant seedlings. Indeed, knocking down both genes prevents PPB formation and causes misorientation of both symmetric and asymmetric division planes [178]. However, unlike *fass Arabidopsis* mutants, *dcd1 add1* double mutants are not viable, suggesting that the loss of FASS-like function leads to early arrest of embryo development in maize. This difference may reflect differences in embryo development between monocots and dicots. It also remains possible that these proteins may not have the exact same function in the two species [178], or that some yet unidentified activity may partially compensate for FASS/TON1 loss of function in *Arabidopsis*, allowing development to proceed further than in maize. Interestingly, no transmission defects of the mutant alleles have been reported in both species, a further argument for a PPB-independent cytoskeletal pathway at work during generative and gametophytic divisions [21].

Based on mutant phenotype and protein localization, TON1, FASS, DCD1 and ADD1 appear to be the only genes identified so far whose mutation specifically disrupts PPB formation without affecting other mitotic MT arrays. Defects in cell plate positioning and phragmoplast guidance observed in these mutants do not indicate defects in spindle or phragmoplast functioning, but most probably result from the absence of PPB at the beginning of mitosis and absence of a properly defined CDS. Organization of the CMT is also strongly affected in the *ton1* and *fass* mutants, suggesting that the TON1/FASS pathway is specifically involved in the organization of cortical arrays, in interphase and at the premitotic stage.

The few other mutations described to significantly affect the PPB appear to have less specific functions, as they also affect spindle, and/or phragmoplast, and/or CMT function (Table 7.3). The maize *tangled* mutation not only affects PPB position, but also spindle and phragmoplast orientations in leaf epidermal cells, which results in abnormal division planes and, at the plant level, in rough leaf texture [35, 149]. Mutation in the *Arabidopsis* orthologue is even less severe, and the overall morphology of seedlings is unchanged, although slight abnormalities of cell division orientation can be noted in root tips. At the cellular level, the PPB and spindle seem unaffected, and only 10% of phragmoplasts are abnormally oriented in mutant cells [168]. The *Arabidopsis mpk6* mutant also displays aberrant positioning of PPBs, spindles and phragmoplasts in the root division zone, leading to misplaced divisions and to a "short root" phenotype [114].

Mutation in MOR1 affects all MT arrays [83, 173]. In the *mor*1-1 temperature sensitive mutant of *Arabidopsis*, one half of dividing cells fail to form PPBs prior to spindle formation, and when PPBs are formed, a proportion of PPBs are frailed and aberrant. Spindles are shorter and phragmoplasts appears crooked or fragmented. In addition, switching *mor1-1* seedling to restrictive temperature disrupts

interphase CMT arrays. Therefore, mor1-1 mutants grown at restrictive temperature are extremely squat and stunted, and aberrant cell shapes and incomplete cross walls are visible. In the *gem1-1* allele, MOR1 mutation is homozygous lethal and strong cytokinesis defects are observed during pollen mitosis [157]. Thus, as discussed above, MOR1 is likely to have a major and general function in the regulation of MT dynamics, including during the PPB stage [84].

Arabidopsis clasp mutant plants exhibit a dwarfed stature, with all organs being shorter than wild type [7, 86]. The *clasp* mutation reduces the cell number in the root division zone and causes defects in directional cell expansion. Although correctly organized, MT density in interphase CMT arrays is decreased, and partial detachment of MTs from the cortex is observed [8]. This correlates with hypersensitivity to an anti-MT drug. During mitosis, PPB narrowing is retarded, prophase spindles are irregular and spindle and phragmoplasts length is reduced [7].

The ATK1 kinesin was first described as necessary for spindle formation during male meiosis in *Arabidopsis* [31]. ATK1 also plays a role during mitosis, since *atk1-1* mutation affects MT organization during prophase and metaphase [103]. Mutant cells display PPBs that fail to narrow, lack MT accumulation at the predicted spindle poles during prophase and have reduced spindle bipolarity during prometaphase. However, spindle abnormalities are completely rectified by anaphase [103].

As a general conclusion from these genetic studies, and with the exception of the *clasp* and *atk1* mutants which present rather mild PPB defects, all mutations described so far which have a significant effect on PPB formation lead to aberrant cell division plane positioning, further reinforcing the idea that the PPB, as part of the cytokinetic apparatus of land plants, is a key determinant of division plane positioning.

7.5.3 The PPB and Cortical Division Site Establishment

In every cell types, the position of the PPB consistently and precisely predicts the insertion of the future division plane. This correlation between the PPB site and position of the CDS, based initially on cytological and pharmacological studies [68, 112] is further strengthened by recent genetic analyses. However, the exact timing and mechanism of CDS determination remain to be clarified. Several experiments suggest that whereas division plane orientation seems to be fixed during broad PPB formation, the precise CDS position is specified by PPB narrowing (reviewed in [110]). However, a recent study denies the role of PPB narrowing in CDS determination, and rather favours a function in spindle positioning [102]. In any case, the sequence of events, mechanisms and molecular network that initially determine the position of the PPB, and then of the CDS are still to be uncovered. Nuclear position, cell geometry, cell polarity and extracellular signals are all suspected to play a role in this process [110, 179].

How positional information is maintained during mitosis at the CDS after PPB disassembly is long standing question in plant cell biology, and is just beginning to be unveiled with the recent discovery of both positive (RanGAP1, TAN, DCD1) and negative markers (KCA1-depleted zone and the so-called ADZ) of the CDS after PPB disappearance [161] (see Sect. 7.4.3.3). These recent pieces of work all contribute to establish the existence of a narrow, ring-shaped cortex/plasma membrane domain that persists after PPB disassembly. It is currently defined by (1) a localization strictly coincident with the PPB plane and (2) a specific protein composition. Determining the structure and composition of this cortical zone during mitosis, including the plasma membrane and the cytoplasmic region associated to it, will be a challenge for the following years.

7.5.4 The PPB and the Prophase Spindle

Several studies have recently shed light on a putative role for the PPB in prophase spindle organization, that was proposed several years ago [175]. In addition to PPB formation, a characteristic of cells entering mitosis is the accumulation of MTs at the nuclear surface. These MTs first radiate in every directions, connecting the nucleus to the cell cortex. As the cell progresses into mitosis, they bridge the nucleus to the PPB (Fig. 7.1). During prophase, perinuclear MTs are progressively sorted into two poles opposite the PPB plane, which prefigure the future spindle axis. This structure, called the prophase spindle, contributes to formation of the metaphase spindle after NEB. The PPB appears to be involved in setting the prophase spindle bipolarity [5, 12], since in all reported cases where the PPB is experimentally perturbed – either genetically or by application of drugs – prophase spindle bipolarity is lost [11, 25, 27, 53, 101, 128]. This is for example the case of the *ton1* and *fass/ton2* mutants, which are unable to form a PPB, and instead display high accumulation of uniformly distributed perinuclear MTs [11, 25].

How PPB helps defining spindle bipolarity is still unclear. A role for MTs that connect nucleus to PPB has been recently proposed. These bridging MTs are proposed to transmit tensive forces facilitating organization and sorting of perinuclear MTs at the future spindle poles [4, 5]. The PPB has also been proposed to cause specific removal or dislocation of MTs or MAPs from the perinuclear equator facing the PPB, thereby contributing to the formation of an oriented bipolar spindle, but molecular mechanisms underlying this process remain unknown [63, 159]. The localization of RanGAP1 (GTP hydrolysis-activating protein of Ran) at the PPB is interesting in this respect. RanGAP1 is an effector of the RanGTP/GDP cycle, which plays an important role in MT biology during animal mitosis and cytokinesis [26, 33, 177], the general theme being that a high local concentration of RanGDP promotes MT depolymerization. RanGAP1 could thus establish a RanGDP gradient that could either reduce MT polymerization at the nuclear equator, thereby favoring the establishment of a bipolar prophase spindle [159] or participating in PPB disassembly after NEB [180].

The PPB, here viewed as a prophase spindle organizer and anchoring it to the cell cortex, is reminiscent of the mitotic centrosome of animal cells [5]. The centrosome, apart from its essential role in MT nucleation, also play a major role in spindle positioning and orientation in animal cells, and in anchoring the spindle to the cell cortex via astral MTs [5]. In this respect, it is striking that two proteins that have been shown to participate in PPB formation, TON1 and FASS/DCD1/ADD1 have similarity with proteins present at the centrosome of animal cells [11, 178].

7.6 Conclusions

Recent years have seen a number of advances which modify our view of division plane determination and PPB function in plants: several proteins are now identified to decorate the PPB and/or the CDS, and these can be used as starting points for further functional studies. The characterization of several mutations which affect the PPB and the cortical division site, together with determination of long-sought links with the cell-cycle machinery, have already contributed to clarify the sequence of events and to identify some key molecular partners involved. Despite these progresses, many aspects of PPB positioning and CDS establishment remain mysterious, our current view being obviously very partial and made from disconnected pieces of information. In order to complete the picture and to propose a coherent model of premitotic division plane determination in plant cells, a number of major questions should be tackled, among which:

- What are the initial intra/extracellular cues for PPB timing and spatial positioning?
- What connection with the cell cycle, which role(s) for cell-cycle proteins at the PPB?
- What are the mechanics and biochemistry of PPB assembly, how MT dynamics is controlled and what is the role of actin in the process?
- What are the relationships and cross-talks between the PPB, the nucleus, the phragmosome? What is the role of PPB-associated proteins?
- What is the sequence of events and molecular partners involved in CDS establishment?
- What is the specific structure and composition of the cortical division site, and how is it recognized during cytokinesis?

Recent work provides a wealth of genetic material and markers to address these points. Answering these questions will also rely in part on technical issues and progresses. Studies in the field, for example would tremendously benefit from being able to purify and characterize the PPB- and/or CDS-associated proteome, from high resolution and time-resolved microscopy, etc. In addition, new genetic screens using PPB-specific markers and mutants should be devised, in order to identify novel and unsuspected players in PPB formation and CDS establishment.

References

1. Abdel-Ghany SE, Day IS, Simmons MP, Kugrens P, Reddy AS (2005) Origin and evolution of kinesin-like calmodulin-binding protein. Plant Physiol 138:1711–1722
2. Allard JF, Wasteneys GO, Cytrynbaum EN (2010) Mechanisms of self-organization of cortical microtubules in plants revealed by computational simulations. Mol Biol Cell 21:278–286
3. Ambrose JC, Cyr R (2007) The kinesin ATK5 functions in early spindle assembly in Arabidopsis. Plant Cell 19:226–236
4. Ambrose JC, Cyr R (2008) Mitotic spindle organization by the preprophase band. Mol Plant 1:950–960
5. Ambrose JC, Cyr R (2008) Mitotic spindle assembly and function. In: Verma DPS, Hong Z (eds) Plant cell monographs, vol. 9 cell division control in plants. Springer, Berlin, pp 141–167
6. Ambrose JC, Li W, Marcus A, Ma H, Cyr R (2005) A minus-end-directed kinesin with plus-end tracking protein activity is involved in spindle morphogenesis. Mol Biol Cell 16:1584–1592
7. Ambrose JC, Shoji T, Kotzer AM, Pighin JA, Wasteneys GO (2007) The Arabidopsis CLASP gene encodes a microtubule-associated protein involved in cell expansion and division. Plant Cell 19:2763–2775
8. Ambrose JC, Wasteneys GO (2008) CLASP modulates microtubule-cortex interaction during self-organization of acentrosomal microtubules. Mol Biol Cell 19:4730–4737
9. Asada T, Kuriyama R, Shibaoka H (1997) TKRP125, a kinesin-related protein involved in the centrosome-independent organization of the cytokinetic apparatus in tobacco BY-2 cells. J Cell Sci 110:179–189
10. Ayaydin F, Vissi E, Meszaros T, Miskolczi P, Kovacs I, Feher A, Dombradi V, Erdodi F, Gergely P, Dudits D (2000) Inhibition of serine/threonine-specific protein phosphatases causes premature activation of cdc2MsF kinase at G2/M transition and early mitotic microtubule organisation in alfalfa. Plant J 23:85–96
11. Azimzadeh J, Nacry P, Christodoulidou A, Drevensek S, Camilleri C, Amiour N, Parcy F, Pastuglia M, Bouchez D (2008) Arabidopsis TONNEAU1 proteins are essential for preprophase band formation and interact with centrin. Plant Cell 20:2146–2159
12. Bannigan A, Lizotte-Waniewski M, Riley M, Baskin TI (2008) Emerging molecular mechanisms that power and regulate the anastral mitotic spindle of flowering plants. Cell Motil Cytoskeleton 65:1–11
13. Barroso C, Chan J, Allan V, Doonan J, Hussey P, Lloyd C (2000) Two kinesin-related proteins associated with the cold-stable cytoskeleton of carrot cells: characterization of a novel kinesin, DcKRP120-2. Plant J 24:859–868
14. Binarova P, Dolezel J, Draber P, Heberle-Bors E, Strnad M, Bogre L (1998) Treatment of Vicia faba root tip cells with specific inhibitors to cyclin-dependent kinases leads to abnormal spindle formation. Plant J 16:697–707
15. Bisgrove SR, Lee YR, Liu B, Peters NT, Kropf DL (2008) The microtubule plus-end binding protein EB1 functions in root responses to touch and gravity signals in Arabidopsis. Plant Cell 20:396–410
16. Boruc J, Mylle E, Duda M, De Clercq R, Rombauts S, Geelen D, Hilson P, Inze D, Van Damme D, Russinova E (2009) Systematic localization of the Arabidopsis core cell cycle proteins reveals novel cell division complexes. Plant Physiol 152:553–565
17. Boudolf V, Barroco R, Engler JD, Verkest A, Beeckman T, Naudts M, Inze D, De Veylder L (2004) B1-type cyclin-dependent kinases are essential for the formation of stomatal complexes in Arabidopsis thaliana. Plant Cell 16:945–955
18. Boudolf V, Inze D, De Veylder L (2006) What if higher plants lack a CDC25 phosphatase? Trends Plant Sci 11:474–479
19. Bowser J, Reddy AS (1997) Localization of a kinesin-like calmodulin-binding protein in dividing cells of Arabidopsis and tobacco. Plant J 12:1429–1437
20. Brown RC, Lemmon BE (1990) Monoplastidic cell-division in lower land plants. Am J Bot 77:559–571

21. Brown RC, Lemmon BE (2001) The cytoskeleton and spatial control of cytokinesis in the plant life cycle. Protoplasma 215:35–49
22. Buschmann H, Chan J, Sanchez-Pulido L, Andrade-Navarro MA, Doonan JH, Lloyd CW (2006) Microtubule-associated AIR9 recognizes the cortical division site at preprophase and cell-plate insertion. Curr Biol 16:1938–1943
23. Buschmann H, Fabri CO, Hauptmann M, Hutzler P, Laux T, Lloyd CW, Schaffner AR (2004) Helical growth of the Arabidopsis mutant tortifolia1 reveals a plant-specific microtubule-associated protein. Curr Biol 14:1515–1521
24. Caillaud MC, Lecomte P, Jammes F, Quentin M, Pagnotta S, Andrio E, de Almeida Engler J, Marfaing N, Gounon P, Abad P, Favery B (2008) MAP65-3 microtubule-associated protein is essential for nematode-induced giant cell ontogenesis in Arabidopsis. Plant Cell 20:423–437
25. Camilleri C, Azimzadeh J, Pastuglia M, Bellini C, Grandjean O, Bouchez D (2002) The Arabidopsis TONNEAU2 gene encodes a putative novel protein phosphatase 2A regulatory subunit essential for the control of the cortical cytoskeleton. Plant Cell 14:833–845
26. Carazo-Salas RE, Gruss OJ, Mattaj IW, Karsenti E (2001) Ran-GTP coordinates regulation of microtubule nucleation and dynamics during mitotic-spindle assembly. Nat Cell Biol 3:228–234
27. Chan J, Calder G, Fox S, Lloyd C (2005) Localization of the microtubule end binding protein EB1 reveals alternative pathways of spindle development in Arabidopsis suspension cells. Plant Cell 17:1737–1748
28. Chan J, Calder GM, Doonan JH, Lloyd CW (2003) EB1 reveals mobile microtubule nucleation sites in Arabidopsis. Nat Cell Biol 5:967–971
29. Chan J, Jensen CG, Jensen LC, Bush M, Lloyd CW (1999) The 65-kDa carrot microtubule-associated protein forms regularly arranged filamentous cross-bridges between microtubules. Proc Natl Acad Sci USA 96:14931–14936
30. Chang HY, Smertenko AP, Igarashi H, Dixon DP, Hussey PJ (2005) Dynamic interaction of NtMAP65-1a with microtubules in vivo. J Cell Sci 118:3195–3201
31. Chen C, Marcus A, Li W, Hu Y, Calzada JP, Grossniklaus U, Cyr RJ, Ma H (2002) The Arabidopsis ATK1 gene is required for spindle morphogenesis in male meiosis. Development 129:2401–2409
32. Chytilova E, Macas J, Sliwinska E, Rafelski SM, Lambert GM, Galbraith DW (2000) Nuclear dynamics in Arabidopsis thaliana. Mol Biol Cell 11:2733–2741
33. Ciciarello M, Mangiacasale R, Lavia P (2007) Spatial control of mitosis by the GTPase Ran. Cell Mol Life Sci 64:1891–1914
34. Cleary AL, Gunning BES, Wasteneys GO, Hepler PK (1992) Microtubule and F-actin dynamics at the division site in living Tradescantia stamen hair cells. J Cell Sci 103:977–988
35. Cleary AL, Smith LG (1998) The Tangled1 gene is required for spatial control of cytoskeletal arrays associated with cell division during maize leaf development. Plant Cell 10:1875–1888
36. Colasanti J, Cho S-H, Wick S, Sundaresan V (1993) Localization of the functional $p34^{cdc2}$ homolog of maize in root tip and stomatal complex cells: association with predicted division sites. Plant Cell 5:1101–1111
37. Crowell EF, Bischoff V, Desprez T, Rolland A, Stierhof YD, Schumacher K, Gonneau M, Hofte H, Vernhettes S (2009) Pausing of Golgi bodies on microtubules regulates secretion of cellulose synthase complexes in Arabidopsis. Plant Cell 21:1141–1154
38. De Schutter K, Joubes J, Cools T, Verkest A, Corellou F, Babiychuk E, Van Der Schueren E, Beeckman T, Kushnir S, Inze D, De Veylder L (2007) Arabidopsis WEE1 kinase controls cell cycle arrest in response to activation of the DNA integrity checkpoint. Plant Cell 19:211–225
39. De Wulf P, Montani F, Visintin R (2009) Protein phosphatases take the mitotic stage. Curr Opin Cell Biol 21:806–815
40. DeBolt S, Gutierrez R, Ehrhardt DW, Melo CV, Ross L, Cutler SR, Somerville C, Bonetta D (2007) Morlin, an inhibitor of cortical microtubule dynamics and cellulose synthase movement. Proc Natl Acad Sci USA 104:5854–5859
41. Dewitte W, Murray JA (2003) The plant cell cycle. Annu Rev Plant Biol 54:235–264

42. Dhonukshe P, Baluska F, Schlicht M, Hlavacka A, Samaj J, Friml J, Gadella TW Jr (2006) Endocytosis of cell surface material mediates cell plate formation during plant cytokinesis. Dev Cell 10:137–150
43. Dhonukshe P, Laxalt AM, Goedhart J, Gadella TWJ, Munnik T (2003) Phospholipase D activation correlates with microtubule reorganization in living plant cells. Plant Cell 15:2666–2679
44. Dhonukshe P, Mathur J, Hulskamp M, Gadella TW Jr (2005) Microtubule plus-ends reveal essential links between intracellular polarization and localized modulation of endocytosis during division-plane establishment in plant cells. BMC Biol 3:11
45. Dissmeyer N, Nowack MK, Pusch S, Stals H, Inze D, Grini PE, Schnittger A (2007) T-loop phosphorylation of Arabidopsis CDKA;1 is required for its function and can be partially substituted by an aspartate residue. Plant Cell 19:972–985
46. Dixit R, Chang E, Cyr R (2006) Establishment of polarity during organization of the acentrosomal plant cortical microtubule array. Mol Biol Cell 17:1298–1305
47. Dixit R, Cyr R (2002) Golgi secretion is not required for marking the preprophase band site in cultured tobacco cells. Plant J 29:99–108
48. Dixit R, Cyr R (2004) Encounters between dynamic cortical microtubules promote ordering of the cortical array through angle-dependent modifications of microtubule behavior. Plant Cell 16:3274–3284
49. Dixit R, Cyr RJ (2002) Spatio-temporal relationship between nuclear-envelope breakdown and preprophase band disappearance in cultured tobacco cells. Protoplasma 219:116–121
50. Doonan JH, Cove DJ, Corke FMK, Lloyd CW (1987) Pre-prophase band of microtubules, absent from tip-growing moss filaments, arises in leafy shoots during transition to intercalary growth. Cell Motil Cytoskeleton 7:138–153
51. Dubois F, Bui Dang Ha D, Sangwan RS, Durand J (1996) The Petunia tra1 gene controls cell elongation and plant development, and mediates responses to cytokinins. Plant J 10:47–59
52. Ehrhardt DW (2008) Straighten up and fly right: microtubule dynamics and organization of non-centrosomal arrays in higher plants. Curr Opin Cell Biol 20:107–116
53. Eleftheriou EP, Palevitz BA (1992) The effect of cytochalasin D on preprophase band organization in root tip cells of Allium. J Cell Sci 103:989–998
54. Erhardt M, Stoppin-Mellet V, Campagne S, Canaday J, Mutterer J, Fabian T, Sauter M, Muller T, Peter C, Lambert AM, Schmit AC (2002) The plant Spc98p homologue colocalizes with gamma-tubulin at microtubule nucleation sites and is required for microtubule nucleation. J Cell Sci 115:2423–2431
55. Ferreira PC, Hemerly AS, Villarroel R, Van Montagu M, Inze D (1991) The Arabidopsis functional homolog of the p34cdc2 protein kinase. Plant Cell 3:531–540
56. Flanders DJ, Rawlins DJ, Shaw PJ, Lloyd CW (1990) Nucleus-associated microtubules help determine the division plane of plant epidermal cells: avoidance of four-way junctions and the role of cell geometry. J Cell Biol 110:1111–1122
57. Fulop K, Tarayre S, Kelemen Z, Horvath G, Kevei Z, Nikovics K, Bako L, Brown S, Kondorosi A, Kondorosi E (2005) Arabidopsis anaphase-promoting complexes – multiple activators and wide range of substrates might keep APC perpetually busy. Cell Cycle 4:1084–1092
58. Gaillard J, Neumann E, Van Damme D, Stoppin-Mellet V, Ebel C, Barbier E, Geelen D, Vantard M (2008) Two microtubule-associated proteins of Arabidopsis MAP65s promote antiparallel microtubule bundling. Mol Biol Cell 19:4534–4544
59. Galatis B, Apostolakos P (2004) The role of the cytoskeleton in the morphogenesis and function of stomatal complexes. New Phytol 161:613–639
60. Gallagher K, Smith LG (1999) Discordia mutations specifically misorient asymmetric cell divisions during development of the maize leaf epidermis. Development 126:4623–4633
61. Gardiner JC, Harper JD, Weerakoon ND, Collings DA, Ritchie S, Gilroy S, Cyr RJ, Marc J (2001) A 90-kD phospholipase D from tobacco binds to microtubules and the plasma membrane. Plant Cell 13:2143–2158
62. Graham L (1996) Green algae to land plants: an evolutionary transition. J Plant Res 109:241–251
63. Granger C, Cyr R (2001) Use of abnormal preprophase bands to decipher division plane determination. J Cell Sci 114:599–607

64. Gupton SL, Collings DA, Allen NS (2006) Endoplasmic reticulum targeted GFP reveals ER organization in tobacco NT-1 cells during cell division. Plant Physiol Biochem 44:95–105
65. Gutierrez R, Lindeboom JJ, Paredez AR, Emons AM, Ehrhardt DW (2009) Arabidopsis cortical microtubules position cellulose synthase delivery to the plasma membrane and interact with cellulose synthase trafficking compartments. Nat Cell Biol 11:797–806
66. Hamada T, Igarashi H, Itoh TJ, Shimmen T, Sonobe S (2004) Characterization of a 200 kDa microtubule-associated protein of tobacco BY-2 cells, a member of the XMAP215/MOR1 family. Plant Cell Physiol 45:1233–1242
67. Hepler PK, Wolniak SM (1984) Membranes in the mitotic apparatus: their structure and function. Int Rev Cytol 90:169–238
68. Hoshino H, Yoneda A, Kumagai F, Hasezawa S (2003) Roles of actin-depleted zone and preprophase band in determining the division site of higher-plant cells, a tobacco BY-2 cell line expressing GFP-tubulin. Protoplasma 222:157–165
69. Hush J, Wu LP, John PCL, Hepler LH, Hepler PK (1996) Plant mitosis promoting factor disassembles the microtubule preprophase band and accelerates prophase progression in Tradescantia. Cell Biol Int 20:275–287
70. Hush JM, Wadsworth P, Callaham DA, Hepler PK (1994) Quantification of microtubule dynamics in living plant-cells using fluorescence redistribution after photobleaching. J Cell Sci 107:775–784
71. Hussey PJ, Hawkins TJ, Igarashi H, Kaloriti D, Smertenko A (2002) The plant cytoskeleton: recent advances in the study of the plant microtubule-associated proteins MAP-65, MAP-190 and the Xenopus MAP215-like protein, MOR1. Plant Mol Biol 50:915–924
72. Inze D, De Veylder L (2006) Cell cycle regulation in plant development. Annu Rev Genet 40:77–105
73. Iwakawa H, Shinmyo A, Sekine M (2006) Arabidopsis CDKA;1, a cdc2 homologue, controls proliferation of generative cells in male gametogenesis. Plant J 45:819–831
74. Jackman M, Lindon C, Nigg EA, Pines J (2003) Active cyclin B1-Cdk1 first appears on centrosomes in prophase. Nat Cell Biol 5:143–148
75. Jeganathan KB, Malureanu L, van Deursen JM (2005) The Rae1-Nup98 complex prevents aneuploidy by inhibiting securin degradation. Nature 438:1036–1039
76. Jeong SY, Rose A, Joseph J, Dasso M, Meier I (2005) Plant-specific mitotic targeting of RanGAP requires a functional WPP domain. Plant J 42:270–282
77. Joshi HC, Palevitz BA (1996) Gamma-tubulin and microtubule organization in plants. Trends Cell Biol 6:41–44
78. Kaplan D (1992) The relationship of cells to organisms in plants: problem and implications of an organismal perspective. Int J Plant Sci 153:S28–S37
79. Karahara I, Suda J, Staehelin LA, Mineyuki Y (2009) Technical note quantitative analysis of vesicles in the preprophase band by electron tomography. Cytologia 74:113–114
80. Katsuta J, Hashiguchi Y, Shibaoka H (1990) The role of the cytoskeleton in positioning of the nucleus in premitotic tobacco BY-2 Cells. J Cell Sci 95:413–422
81. Katsuta J, Shibaoka H (1988) The roles of the cytoskeleton and the cell wall in nuclear positioning in tabaccco BY-2 Cells. Plant Cell Physiol 29:403–413
82. Katsuta J, Shibaoka H (1992) Inhibition by kinase inhibitors of the development and the disappearance of the preprophase band of microtubules in tobacco BY-2 cells. J Cell Sci 103:397–405
83. Kawamura E, Himmelspach R, Rashbrooke MC, Whittington AT, Gale KR, Collings DA, Wasteneys GO (2006) MICROTUBULE ORGANIZATION 1 regulates structure and function of microtubule arrays during mitosis and cytokinesis in the Arabidopsis root. Plant Physiol 140:102–114
84. Kawamura E, Wasteneys GO (2008) MOR1, the Arabidopsis thaliana homologue of Xenopus MAP215, promotes rapid growth and shrinkage, and suppresses the pausing of microtubules in vivo. J Cell Sci 121:4114–4123
85. Ketelaar T, Faivre-Moskalenko C, Esseling JJ, de Ruijter NC, Grierson CS, Dogterom M, Emons AM (2002) Positioning of nuclei in Arabidopsis root hairs: an actin-regulated process of tip growth. Plant Cell 14:2941–2955

86. Kirik V, Herrmann U, Parupalli C, Sedbrook JC, Ehrhardt DW, Hulskamp M (2007) CLASP localizes in two discrete patterns on cortical microtubules and is required for cell morphogenesis and cell division in Arabidopsis. J Cell Sci 120:4416–4425
87. Korolev AV, Buschmann H, Doonan JH, Lloyd CW (2007) AtMAP70-5, a divergent member of the MAP70 family of microtubule-associated proteins, is required for anisotropic cell growth in Arabidopsis. J Cell Sci 120:2241–2247
88. Korolev AV, Chan J, Naldrett MJ, Doonan JH, Lloyd CW (2005) Identification of a novel family of 70 kDa microtubule-associated proteins in Arabidopsis cells. Plant J 42:547–555
89. Krupnova T, Sasabe M, Ghebreghiorghis L, Gruber CW, Hamada T, Dehmel V, Strompen G, Stierhof YD, Lukowitz W, Kemmerling B, Machida Y, Hashimoto T, Mayer U, Jurgens G (2009) Microtubule-associated kinase-like protein RUNKEL needed for cell plate expansion in Arabidopsis cytokinesis. Curr Biol 19:518–523
90. Kutsuna N, Hasezawa S (2002) Dynamic organization of vacuolar and microtubule structures during cell cycle progression in synchronized tobacco BY-2 cells. Plant Cell Physiol 43:965–973
91. Kutsuna N, Kumagai F, Sato MH, Hasezawa S (2003) Three-dimensional reconstruction of tubular structure of vacuolar membrane throughout mitosis in living tobacco cells. Plant Cell Physiol 44:1045–1054
92. Lee J, Das A, Yamaguchi M, Hashimoto J, Tsutsumi N, Uchimiya H, Umeda M (2003) Cell cycle function of a rice B2-type cyclin interacting with a B-type cyclin-dependent kinase. Plant J 34:417–425
93. Lee JY, Lee HS, Wi SJ, Park KY, Schmit AC, Pai HS (2009) Dual functions of Nicotiana benthamiana Rae1 in interphase and mitosis. Plant J 59:278–291
94. Li H, Zeng X, Liu ZQ, Meng QT, Yuan M, Mao TL (2009) Arabidopsis microtubule-associated protein AtMAP65-2 acts as a microtubule stabilizer. Plant Mol Biol 69:313–324
95. Liu B, Joshi HC, Wilson TJ, Silflow CD, Palevitz BA, Snustad DP (1994) Gamma-tubulin in Arabidopsis: gene sequence, immunoblot, and immunofluorescence studies. Plant Cell 6:303–314
96. Liu B, Marc J, Joshi HC, Palevitz BA (1993) A gamma-tubulin-related protein associated with the microtubule arrays of higher plants in a cell cycle-dependent manner. J Cell Sci 104:1217–1228
97. Lloyd C (1991) Cytoskeletal elements of the phragmosome establish the division plane in vacuolated higher plant cells. In: Lloyd C (ed) The cytoskeletal basis of plant growth and form. Academic, London, pp 245–257
98. Lunn JE (2007) Compartmentation in plant metabolism. J Exp Bot 58:35–47
99. Mao G, Chan J, Calder G, Doonan JH, Lloyd CW (2005) Modulated targeting of GFP-AtMAP65-1 to central spindle microtubules during division. Plant J 43:469–478
100. Mao T, Jin L, Li H, Liu B, Yuan M (2005) Two microtubule-associated proteins of the Arabidopsis MAP65 family function differently on microtubules. Plant Physiol 138:654–662
101. Marcus AI, Ambrose JC, Blickley L, Hancock WO, Cyr RJ (2002) Arabidopsis thaliana protein, ATK1, is a minus-end directed kinesin that exhibits non-processive movement. Cell Motil Cytoskeleton 52:144–150
102. Marcus AI, Dixit R, Cyr RJ (2005) Narrowing of the preprophase microtubule band is not required for cell division plane determination in cultured plant cells. Protoplasma 226:169–174
103. Marcus AI, Li W, Ma H, Cyr RJ (2003) A kinesin mutant with an atypical bipolar spindle undergoes normal mitosis. Mol Biol Cell 14:1717–1726
104. Marty F (1999) Plant vacuoles. Plant Cell 11:587–600
105. Mathur J, Mathur N, Kernebeck B, Srinivas BP, Hulskamp M (2003) A novel localization pattern for an EB1-like protein links microtubule dynamics to endomembrane organization. Curr Biol 13:1991–1997
106. Mayer Y, Torres Ruiz RA, Berleth T, Misera S, Jurgens G (1991) Mutations affecting body organization in the Arabidopsis embryo. Nature 353:402–407

107. McClinton RS, Sung ZR (1997) Organization of cortical microtubules at the plasma membrane in Arabidopsis. Planta 201:252–260
108. Meszaros T, Miskolczi P, Ayaydin F, Pettko-Szandtner A, Peres A, Magyar Z, Horvath GV, Bako L, Feher A, Dudits D (2000) Multiple cyclin-dependent kinase complexes and phosphatases control G2/M progression in alfalfa cells. Plant Mol Biol 43:595–605
109. Mews M, Sek FJ, Moore R, Volkmann D, Gunning BES, John PCL (1997) Mitotic cyclin distribution during maize cell division: implications for the sequence diversity and function of cyclins in plants. Protoplasma 200:128–145
110. Mineyuki Y (1999) The preprophase band of microtubules: its function as a cytokinetic apparatus in higher plants. Int Rev Cytol 187:1–49
111. Mineyuki Y, Aioi H, Yamashita M, Nagahama Y (1996) A comparative study on stainability of preprophase bands by the PSTAIR antibody. J Plant Res 109:185–192
112. Mineyuki Y, Palevitz BA (1990) Relationship between preprophase band organization, F-actin and the division site in Allium. J Cell Sci 97:283–295
113. Mineyuki Y, Yamashita M, Nagahama Y (1991) P34cdc2 kinase homolog in the preprophase band. Protoplasma 162:182–186
114. Muller J, Beck M, Mettbach U, Komis G, Hause G, Menzel D, Samaj J (2010) Arabidopsis MPK6 is involved in cell division plane control during early root development, and localizes to the pre-prophase band, phragmoplast, trans-Golgi network and plasma membrane. Plant J 61:234–248
115. Muller S, Han SC, Smith LG (2006) Two kinesins are involved in the spatial control of cytokinesis in Arabidopsis thaliana. Curr Biol 16:888–894
116. Muller S, Smertenko A, Wagner V, Heinrich M, Hussey PJ, Hauser MT (2004) The plant microtubule-associated protein AtMAP65-3/PLE is essential for cytokinetic phragmoplast function. Curr Biol 14:412–417
117. Murata T, Sonobe S, Baskin TI, Hyodo S, Hasezawa S, Nagata T, Horio T, Hasebe M (2005) Microtubule-dependent microtubule nucleation based on recruitment of gamma-tubulin in higher plants. Nat Cell Biol 7:961–968
118. Murata T, Wada M (1991) Effects of centrifugation on preprophase band formation in *Adiantum* protonemata. Planta 183:391–398
119. Nakajima K, Furutani I, Tachimoto H, Matsubara H, Hashimoto T (2004) SPIRAL1 encodes a plant-specific microtubule-localized protein required for directional control of rapidly expanding Arabidopsis cells. Plant Cell 16:1178–1190
120. Nebenfuhr A, Frohlick JA, Staehelin LA (2000) Redistribution of golgi stacks and other organelles during mitosis and cytokinesis in plant cells. Plant Physiol 124:135–151
121. Niethammer P, Kronja I, Kandels-Lewis S, Rybina S, Bastiaens P, Karsenti E (2007) Discrete states of a protein interaction network govern interphase and mitotic microtubule dynamics. PLoS Biol 5:e29
122. Nogami A, Mineyuki Y (1999) Loosening of a preprophase band of microtubules in onion (Allium cepa L.) root tip cells by kinase inhibitors. Cell Struct Funct 24:419–424
123. Nogami A, Suzaki T, Shigenaka Y, Nagahama Y, Mineyuki Y (1996) Effects of cycloheximide on preprophase bands and prophase spindles in onion (Allium cepa L) root tip cells. Protoplasma 192:109–121
124. Nowack MK, Grini PE, Jakoby MJ, Lafos M, Koncz C, Schnittger A (2006) A positive signal from the fertilization of the egg cell sets off endosperm proliferation in angiosperm embryogenesis. Nat Genet 38:63–67
125. O'Farrell PH (2001) Triggering the all-or-nothing switch into mitosis. Trends Cell Biol 11:512–519
126. Palevitz BA (1987) Actin in the preprophase band of Allium cepa. J Cell Biol 104:1515–1519
127. Panteris E (2008) Cortical actin filaments at the division site of mitotic plant cells: a reconsideration of the "actin-depleted zone". New Phytol 179:334–341
128. Panteris E, Apostolakos P, Galatis B (1995) The effect of taxol on triticum preprophase root cells: preprophase microtubule band organization seems to depend on new microtubule assembly. Protoplasma 186:72–78

129. Panteris E, Apostolakos P, Galatis B (2006) Cytoskeletal asymmetry in Zea mays subsidiary cell mother cells: a monopolar prophase microtubule half-spindle anchors the nucleus to its polar position. Cell Motil Cytoskeleton 63:696–709
130. Panteris E, Apostolakos P, Quader H, Galatis B (2004) A cortical cytoplasmic ring predicts the division plane in vacuolated cells of Coleus: the role of actomyosin and microtubules in the establishment and function of the division site. New Phytol 163:271–286
131. Papaseit C, Pochon N, Tabony J (2000) Microtubule self-organization is gravity-dependent. Proc Natl Acad Sci USA 97:8364–8368
132. Paradez A, Wright A, Ehrhardt DW (2006) Microtubule cortical array organization and plant cell morphogenesis. Curr Opin Plant Biol 9:571–578
133. Pastuglia M, Bouchez D (2007) Molecular encounters at microtubule ends in the plant cell cortex. Curr Opin Plant Biol 10:557–563
134. Pickett-Heaps JD, Gunning BES, Brown RC, Lemmon BE, Cleary AL (1999) The cytoplast concept in dividing plant cells: cytoplasmic domains and the evolution of spatially organized cell division. Am J Bot 86:153–172
135. Pickett-Heaps JD, Northcote DH (1966) Organization of microtubules and endoplasmic reticulum during mitosis and cytokinesis in wheat meristems. J Cell Sci 1:109–120
136. Preuss ML, Delmer DP, Liu B (2003) The cotton kinesin-like calmodulin-binding protein associates with cortical microtubules in cotton fibers. Plant Physiol 132:154–160
137. Sano T, Higaki T, Oda Y, Hayashi T, Hasezawa S (2005) Appearance of actin microfilament "twin peaks" in mitosis and their function in cell plate formation, as visualized in tobacco BY-2 cells expressing GFP-fimbrin. Plant J 44:595–605
138. Sasabe M, Soyano T, Takahashi Y, Sonobe S, Igarashi H, Itoh TJ, Hidaka M, Machida Y (2006) Phosphorylation of NtMAP65-1 by a MAP kinase down-regulates its activity of microtubule bundling and stimulates progression of cytokinesis of tobacco cells. Genes Dev 20:1004–1014
139. Schlaitz AL, Srayko M, Dammermann A, Quintin S, Wielsch N, MacLeod I, de Robillard Q, Zinke A, Yates JR 3rd, Muller-Reichert T, Shevchenko A, Oegema K, Hyman AA (2007) The C. elegans RSA complex localizes protein phosphatase 2A to centrosomes and regulates mitotic spindle assembly. Cell 128:115–127
140. Sedbrook JC, Ehrhardt DW, Fisher SE, Scheible WR, Somerville CR (2004) The Arabidopsis SKU6/SPIRAL1 gene encodes a plus end-localized microtubule-interacting protein involved in directional cell expansion. Plant Cell 16:1506–1520
141. Segui-Simarro JM, Staehelin LA (2006) Cell cycle-dependent changes in Golgi stacks, vacuoles, clathrin-coated vesicles and multivesicular bodies in meristematic cells of Arabidopsis thaliana: a quantitative and spatial analysis. Planta 223:223–236
142. Shaw SL, Kamyar R, Ehrhardt DW (2003) Sustained microtubule treadmilling in Arabidopsis cortical arrays. Science 300:1715–1718
143. Shoji T, Narita NN, Hayashi K, Asada J, Hamada T, Sonobe S, Nakajima K, Hashimoto T (2004) Plant-specific microtubule-associated protein SPIRAL2 is required for anisotropic growth in arabidopsis. Plant Physiol 136:3933–3944
144. Sinnott EW, Bloch R (1940) Cytoplasmic behavior during division of vacuolate plant cells. Proc Natl Acad Sci USA 26:223–227
145. Slep KC (2009) The role of TOG domains in microtubule plus end dynamics. Biochem Soc Trans 37:1002–1006
146. Smertenko AP, Chang HY, Sonobe S, Fenyk SI, Weingartner M, Bogre L, Hussey PJ (2006) Control of the AtMAP65-1 interaction with microtubules through the cell cycle. J Cell Sci 119:3227–3237
147. Smertenko AP, Chang HY, Wagner V, Kaloriti D, Fenyk S, Sonobe S, Lloyd C, Hauser MT, Hussey PJ (2004) The Arabidopsis microtubule-associated protein AtMAP65-1: molecular analysis of its microtubule bundling activity. Plant Cell 16:2035–2047
148. Smith LG, Gerttula SM, Han SC, Levy J (2001) TANGLED1: a microtubule binding protein required for the spatial control of cytokinesis in maize. J Cell Biol 152:231–236
149. Smith LG, Hake S, Sylvester AW (1996) The tangled-1 mutation alters cell division orientations throughout maize leaf development without altering leaf shape. Development 122:481–489

150. Spinner L, Pastuglia M, Belcram K, Pegoraro M, Goussot M, Bouchez D, Schaefer D (2010) The function of TONNEAU1 in moss reveals ancient mechanisms of division plane specification and cell elongation in land plants. Development 137:2733–2742
151. Stals H, Bauwens S, Traas J, Van Montagu M, Engler G, Inze D (1997) Plant CDC2 is not only targeted to the pre-prophase band, but also co-localizes with the spindle, phragmoplast, and chromosomes. FEBS Lett 418:229–234
152. Stoppin V, Vantard M, Schmit AC, Lambert AM (1994) Isolated plant nuclei nucleate microtubule assembly: the nuclear surface in higher plants has centrosome-like activity. Plant Cell 6:1099–1106
153. Takatsuka H, Ohno R, Umeda M (2009) The Arabidopsis cyclin-dependent kinase-activating kinase CDKF;1 is a major regulator of cell proliferation and cell expansion but is dispensable for CDKA activation. Plant J 59:475–487
154. Torres-Ruiz RA, Jürgens G (1994) Mutations in the Fass gene uncouple pattern formation and morphogenesis in *Arabidopsis* development. Development 120:2967–2978
155. Traas J, Bellini C, Nacry P, Kronenberger J, Bouchez D, Caboche M (1995) Normal differentiation patterns in plants lacking microtubular preprophase bands. Nature 375:676–677
156. Traas JA, Doonan JD, Rawlins DJ, Shaw PJ, Watts J, Lloyd CW (1987) An actin network is present in the cytoplasm throughout the cell cycle of carrot cells and associates with the dividing nucleus. J Cell Biol 105:387–395
157. Twell D, Park SK, Hawkins TJ, Schubert D, Schmidt R, Smertenko A, Hussey PJ (2002) MOR1/GEM1 has an essential role in the plant-specific cytokinetic phragmoplast. Nat Cell Biol 4:711–714
158. Umeda M, Shimotohno A, Yamaguchi M (2005) Control of cell division and transcription by cyclin-dependent kinase-activating kinases in plants. Plant Cell Physiol 46:1437–1442
159. Van Damme D (2009) Division plane determination during plant somatic cytokinesis. Curr Opin Plant Biol 12:745–751
160. Van Damme D, Bouget FY, Van Poucke K, Inze D, Geelen D (2004) Molecular dissection of plant cytokinesis and phragmoplast structure: a survey of GFP-tagged proteins. Plant J 40:386–398
161. Van Damme D, Vanstraelen M, Geelen D (2007) Cortical division zone establishment in plant cells. Trends Plant Sci 12:458–464
162. van der Vaart B, Akhmanova A, Straube A (2009) Regulation of microtubule dynamic instability. Biochem Soc Trans 37:1007–1013
163. Van Leene J, Stals H, Eeckhout D, Persiau G, Van Slijke E, Van Isterdael G, De Clercq A, Bonnet E, Laukens K, Remmerie N, Hendrickx K, De Vijlder T, Adbelkrim A, Pharazyn A, Van Onckelen H, Inze D, Witters E, De Jaeger G (2007) A tandem affinity purification-based technology platform to study the cell cycle interactome in Arabidopsis thaliana. Mol Cell Proteomics 6:1226–1238
164. Vanstraelen M, Van Damme D, De Rycke R, Mylle E, Inze D, Geelen D (2006) Cell cycle-dependent targeting of a kinesin at the plasma membrane demarcates the division site in plant cells. Curr Biol 16:308–314
165. Venverloo CJ, Libbenga KR (1987) Regulation of the plane of cell division in vacuolated cells. I. The function of nuclear positioning and phragmosome formation. J Plant Physiol 131:267–284
166. Vos JW, Dogterom M, Emons AM (2004) Microtubules become more dynamic but not shorter during preprophase band formation: a possible "search-and-capture" mechanism for microtubule translocation. Cell Motil Cytoskeleton 57:246–258
167. Walia A, Lee JS, Wasteneys G, Ellis B (2009) Arabidopsis mitogen-activated protein kinase MPK18 mediates cortical microtubule functions in plant cells. Plant J 59:565–575
168. Walker KL, Muller S, Moss D, Ehrhardt DW, Smith LG (2007) Arabidopsis TANGLED identifies the division plane throughout mitosis and cytokinesis. Curr Biol 17:1827–1836
169. Wang H, Cutler AJ, Fowke LC (1991) DNA-replication and the development of preprophase bands in soybean protoplast cultures. Physiol Plant 82:150–156
170. Wang H, Fowke LC, Crosby WL (1997) A plant cyclin-dependent kinase inhibitor gene. Nature 386:451–452

171. Wang H, Zhou Y, Bird DA, Fowke LC (2008) Functions, regulation and cellular localization of plant cyclin-dependent kinase inhibitors. J Microsc 231:234–246
172. Weingartner M, Binarova P, Drykova D, Schweighofer A, David JP, Heberle-Bors E, Doonan J, Bogre L (2001) Dynamic recruitment of Cdc2 to specific microtubule structures during mitosis. Plant Cell 13:1929–1943
173. Whittington AT, Vugrek O, Wei KJ, Hasenbein NG, Sugimoto K, Rashbrooke MC, Wasteneys GO (2001) MOR1 is essential for organizing cortical microtubules in plants. Nature 411:610–613
174. Wick SM (1991) Spatial aspects of cytokinesis in plant cells. Curr Opin Cell Biol 3:253–260
175. Wick SM, Duniec J (1983) Immunofluorescence microscopy of tubulin and microtubule arrays in plant cells. II. Transition between the pre-prophase band and the mitotic. Protoplasma 122:45–55
176. Wightman R, Turner SR (2007) Severing at sites of microtubule crossover contributes to microtubule alignment in cortical arrays. Plant J 52:742–751
177. Wilde A, Lizarraga SB, Zhang L, Wiese C, Gliksman NR, Walczak CE, Zheng Y (2001) Ran stimulates spindle assembly by altering microtubule dynamics and the balance of motor activities. Nat Cell Biol 3:221–227
178. Wright AJ, Gallagher K, Smith LG (2009) discordia1 and alternative discordia1 function redundantly at the cortical division site to promote preprophase band formation and orient division planes in maize. Plant Cell 21:234–247
179. Wright AJ, Smith LG (2008) Division plane orientation in plant cells. In: Verma DPS, Hong Z (eds) Plant cell monographs, vol. 9 cell division control in plants. Springer, Berlin, pp 33–57
180. Xu XFM, Zhao Q, Rodrigo-Peiris T, Brkljacic J, He CS, Muller S, Meier I (2008) RanGAP1 is a continuous marker of the Arabidopsis cell division plane. Proc Natl Acad Sci USA 105:18637–18642
181. Yanagawa Y, Hasezawa S, Kumagai F, Oka M, Fujimuro M, Naito T, Makino T, Yokosawa H, Tanaka K, Komamine A, Hashimoto J, Sato T, Nakagawa H (2002) Cell-cycle dependent dynamic change of 26S proteasome distribution in tobacco BY-2 cells. Plant Cell Physiol 43:604–613
182. Yao M, Wakamatsu Y, Itoh TJ, Shoji T, Hashimoto T (2008) Arabidopsis SPIRAL2 promotes uninterrupted microtubule growth by suppressing the pause state of microtubule dynamics. J Cell Sci 121:2372–2381
183. Zachariadis M, Quader H, Galatis B, Apostolakos P (2001) Endoplasmic reticulum preprophase band in dividing root-tip cells of Pinus brutia. Planta 213:824–827
184. Zhou Y, Fowke LC, Wang H (2002) Plant CDK inhibitors: studies of interactions with cell cycle regulators in the yeast two-hybrid system and functional comparisons in transgenic Arabidopsis plants. Plant Cell Reports 20:967–975

Chapter 8
Acentrosomal Spindle Formation Through the Heroic Age of Microscopy: Past Techniques, Present Thoughts, and Future Directions

Jennelle L. Malcos and Richard Cyr

Mazia [48] remarked, "In the heroic age of microscopy, the discoveries of the roles of chromosomes and of centrosomes went hand-in-hand." Cooperation between these two cellular components was found to be the driving force of cell division. The desire to further understand the fundamental process of cell division also lead to the discovery of a third, unifying component between the chromosomes and centrosomes; namely, the mitotic spindle. Through these discoveries, found primarily in animal cells, the dogma arose that successful cell division requires all three components: chromosomes, centrosomes, and the spindle. Few would argue that mitosis could be successful without chromosomes and the mitotic spindle, however, the essential function of the centrosome is, with increasing frequency, being questioned.

In higher plant cells, the formation of a bipolar mitotic spindle occurs in the absence of a discrete centrosome. The search for an animal-like centrosome in plant cells has lead to the hypothesis of a flexible centrosome. A flexible centrosome would change shape and location during cell division to produce and organize microtubule arrays. This hypothesis is based on the view that microtubules act as "pointers," defining the centrosome based on the final organized shape of the nucleated microtubules [48]. In animal cells, astral and spindle microtubules point to the centrioles, an easily identified marker of the animal centrosome. The same logic can be applied to plant microtubules (i.e., microtubules of a broad spindle point to a broad centrosome), leading to an arbitrary assignment of a centrosome.

Because there are no discrete centrosomal structures in plant cells, the question arises of how to distinguish a flexible centrosome. It can be argued that the plant centrosome is defined by the presence of γ-tubulin, yet nucleation must be coupled with organization capabilities to capture the full functionality of a centrosome. We question if it is possible that the idea of centrosomes in plant cells is an artificial constraint created by early discoveries in animal cells. Proper tissue morphogenesis

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in plants is dependent on highly organized divisions, rather than the cell migrations observed in animal tissue morphogenesis. Therefore, it is logical to hypothesize that plants may require unique organizing components, in addition to the spindle and chromosomes, for successful cell division.

In this review we strive not to address the existence of centrosomes in plants cells, but rather, ask the more fundamental question of what components are necessary for acentrosomal spindle organization. Evidence gleaned from the past and present "heroic age of microscopy" suggests that interactions between microtubules of the preprophase band (PPB), microtubules nucleated by the nuclear envelope forming the prophase spindle, and bridge microtubules between the two all play an important role in spindle formation. These three components, not observed in animal mitotic cells, may represent components of a system that functions to establish spindle bipolarity. Addressing this hypothesis involves a journey through a variety of past and present experimental techniques and approaches concerning the study of plant mitosis, as well as consideration for alternative mechanisms of spindle organization.

8.1 Early Fixation Techniques

Early studies of mitosis and spindle formation in plants were limited to tissues that were fixed, sectioned and stained. The dehydration step in fixation techniques resulted in the detection of fibrous structures around the chromosomes in dividing cells; therefore, the existence of the later-termed mitotic spindle in plant cells was documented at the turn of twentieth century [49]. Despite the static limitations of these studies, observations were surprisingly similar to the current fundamental details known of spindle formation.

In addition to the mitotic spindle, the fibrous nature of the later-termed prophase spindle was also described. In onion root tip cells the developing halves of the prophase spindle were described as, "flattened dome-shaped prominences" or "cone shape masses of fibers" on the opposite sides of the nuclei (Fig. 8.1) [49, 63]. Fibers from the prophase spindle remained outside the nucleus until the disappearance of the nuclear membrane, at which point they associated with chromosomes and formed the bipolar spindle. These observations highlighted the fact that the mitotic spindle is derived from the prophase spindle and that bipolarity is established prior to nuclear envelope breakdown, although the mechanism of this process was not yet understood.

The role and/or existence of centrosomes in plant spindle formation was debated at this time. Schaffner [63] included animal-like centrosomes in his drawings of onion root tips and specifically described granular substances at the poles of the spindle (Fig. 8.2). Conversely, McComb [49] emphasized the fact that she did not observe centrosome-like structures and specifically noted, "…it seems well-nigh incredible that any one should be able at the present day to read centrospheres or centrosomes into the process," attributing such observations to

Fig. 8.1 A drawing representing the prophase spindle present during mitosis in an *Allium cepa* cell, as visualized with early fixation techniques. Reproduced from McComb [49] with permission from Torrey Botanical Society

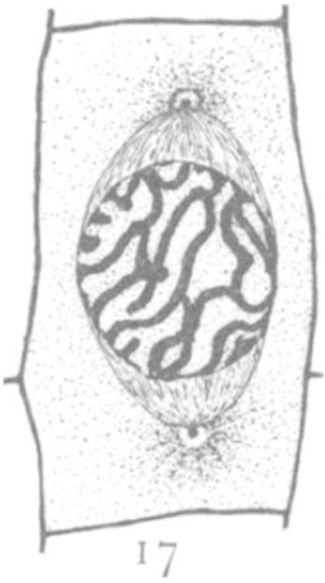

Fig. 8.2 A drawing representing the prophase spindle and centrosomes thought to be present during mitosis in an *A. cepa* cell, as visualized with early fixation techniques. Reproduced from Schaffner [63] with permission from the University of Chicago

poor fixation and staining techniques. Mitotic studies of plant cells were limited; however, extensive studies dedicated to bryophyte spermatogenesis concluded that centrosomes were at least present in plant species that still possessed motile sperm [37]. This view was adopted by many, even though some argued that the existence of animal-like centrosomes in higher plants should not be dismissed without further investigation utilizing more advanced techniques [22].

With the opinion that higher plants lack a centrosome, researchers sought to discover which mechanisms or structures contributed to acentrosomal spindle formation. Strasburger hypothesized that the spindle fibers were derived from a dynamic kinoplasm surrounding the plant cell nucleus during division, which had attributes similar to centrosomes in animal cells [27]. Another hypothesis was that the monoplastid in quillworts, which divided and migrated to polar regions prior to cell division, acted as centrosomes and established bipolarity in this specific lineage of plants [46]. The search for centrosomes continued as techniques improved to better visualize activities within cells.

Concurrent with searching for a centrosome-like structure is plants, research began to move in a direction away from fixed samples toward living tissues. The spindle fibers easily visualized in fixed samples were difficult, if not impossible, to observe in living cells via simple transmission light microscopy. Debates began concerning the reality of spindle fibers and whether they were artifacts of fixation or represented actual structures in living cells [10, 64].

Where visualization was limited, researchers used other techniques to address this question. Work completed by Belar (1929) and Schade (1930) addressed the reality of spindle fibers through chemical or mechanical distortions of the spindle during division, respectively [as cited in 64]. Treatments of *Tradescantia* stamen hairs in hypertonic solutions and centrifugation of onion root tips caused bends and splits in the spindle, parallel to the long axis of the cell. These observations provided indirect evidence that the spindle was an anisotropic structure because the resultant splits were always observed in a longitudinal manner. A directionally dependent structure suggested the spindle was "heterogenous," a term used to infer spindle fibers were distinct from the surrounding cytoplasm, not fixation artifacts of an otherwise homogenous environment [64]. Additionally, the fibers observed in fixed samples represented a distinct component in cells because "..if spindle fibers can be bent in the living cell, they cannot be interpreted simply as lines of force," a common argument of the time [64].

Confirmation of the fibrous nature of the plant mitotic spindle depended on the combined efforts of studies in light microscopy and transmission electron microscopy (TEM). Both techniques have inherent limitations; however, both have contributed greatly to the study of spindle formation and the search for centrosome-like structures in plant cells. For this reason, the contributions of these techniques will be discussed individually.

8.2 Light Microscopy Techniques

Early work involving fixation indicated the mitotic spindle was anisotropic and organized of parallel units (i.e., fibers). The ordered nature of the spindle units resulted in the optic phenomenon of birefringence; a characteristic observable through polarization microscopy. Application of this technique afforded the first glimpse of spindle structures in living animal cells, confirming the existence of

spindle fibers. However, the first polarizing microscopes lacked the resolution necessary to observe individual fibers [30]. Inoue [28] developed a polarizing microscope that was capable of such resolution. He was able to visual the spindle during meiosis in lilies; however, application of the technique to plant mitosis did not occur until several years later in *Haemanthus* endosperm cells (Fig. 8.3) [29]. Polarizing microscopy, therefore, helped confirm the existence of spindle fibers in plant cells.

Prior to work with polarizing microscopy, Bajer [6] used phase contrast microscopy to analyze plant cell division in *Haemanthus* endosperm tissue. Although spindle fibers could not be observed, careful analysis of the nucleus during prophase revealed several important features of spindle formation. During prophase, a region void of small particles forms around the nucleus, known at the clear zone (Fig. 8.4) [7]. As prophase progresses, the clear zone in *Haemanthus* expands equally until

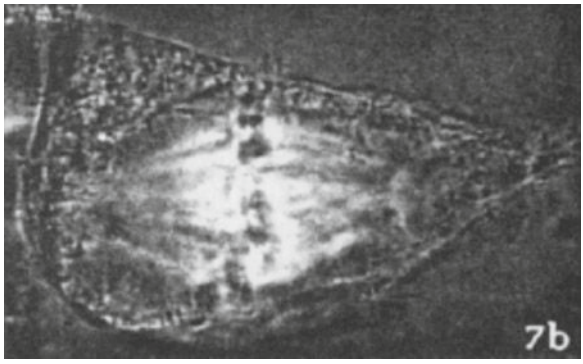

Fig. 8.3 The acentrosomal mitotic spindle of *Haemanthus* endosperm cells visualized with polarizing microscopy. Reproduced from Inoue and Bajer [29] with permission from Springer

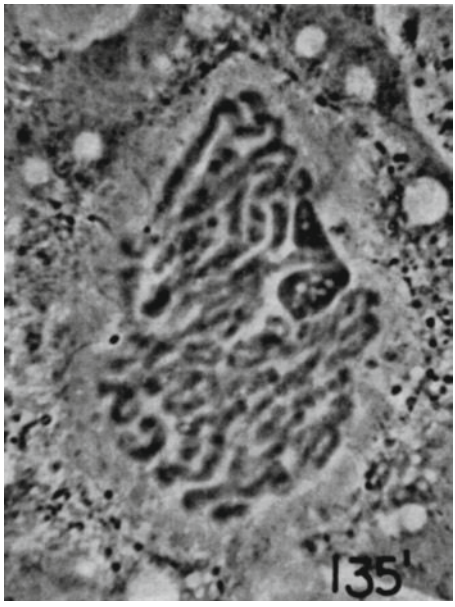

Fig. 8.4 A clear zone forms around the nucleus during prophase in *Haemanthus* endosperm cells when visualized with light microscopy. Reproduced from Bajer [7] with permission from Elsevier. Time in minutes is indicated on the micrograph from the start of prophase

nuclear envelope breakdown. At this time, the clear zone material mixes with the nuclear material, resulting in the formation of the spindle [7]. Polarizing microscopy revealed birefringent material in the clear zone, suggesting that the fibers of the prophase and mitotic spindles were of the same organized substance.

A notable difference was observed in elongated cells compared to endosperm cells that are isodiametric and free of cells walls. In the former cell types, the clear zone does not expand equally. Martens [47] observed that the clear zone accumulated at the site of the future spindle poles in *Tradescantia* stamen hair cells and called these structures "polar caps." The polar caps observed *in vivo* mirrored the fibrous structures of the prophase spindle observed with early fixation techniques. Bajer [7] later argued that the polar caps were specialized regions of the clear zone defining future poles in elongated cells, but not isodiametric cells.

8.3 Transmission Electron Microscopy (TEM) Techniques

Early TEM examinations of onion root tip cells revealed the fibrillar nature of the polar caps and mitotic spindle [61, 65]. They also revealed that the spindle fibers were collections of multiple fibers arranged longitudinally in the cell [65]. However, further examination of the ultrastructure of spindle fibers in these specimens was hindered due to poor preservation of cellular components in the fixed samples. Further successes with TEM depended heavily on advancements in fixation techniques, specifically the use of glutaraldehyde [62].

Ledbetter and Porter [36] utilized these new fixation methods and determined that the individual spindle fibers identified with early TEM work actually consisted of multiple filamentous units (i.e., protofilaments) joined together to form a tubular structure. "Micro-tubules," as they were named, appeared as parallel fibers in the mitotic spindle during division and as "hoops" or "rings" in the cortex of interphase cells [24, 26, 36, 53, 68]. These observations suggested that microtubule distribution was temporally regulated with cellular division, highlighting the flexible and/or plastic nature of microtubule organization. The authors also correlated the parallel organization of microtubules in the spindle with the birefringence observed by polarizing light microscopy. The identification of microtubules was a first breakthrough in understanding plant spindle organization based on TEM. This work initiated a series of studies concerned with microtubule organization in a variety of fixed plant tissues.

A second breakthrough in spindle formation using TEM was the discovery of the preprophase band (PPB). Through examination of meristematic wheat cells, Pickett-Heaps and Northcote [59] noted many microtubules "gracefully" encircling the nucleus, lying close to the cell wall. The PPB was subsequently identified in Timothy grass root tip cells [12], tobacco epidermal cells [15], aquatic ferns [11], onion root tip cells [55] and maize guard cells [19], demonstrating the ubiquitous nature of this microtubule array (Fig. 8.5). Interestingly, PPBs were not observed

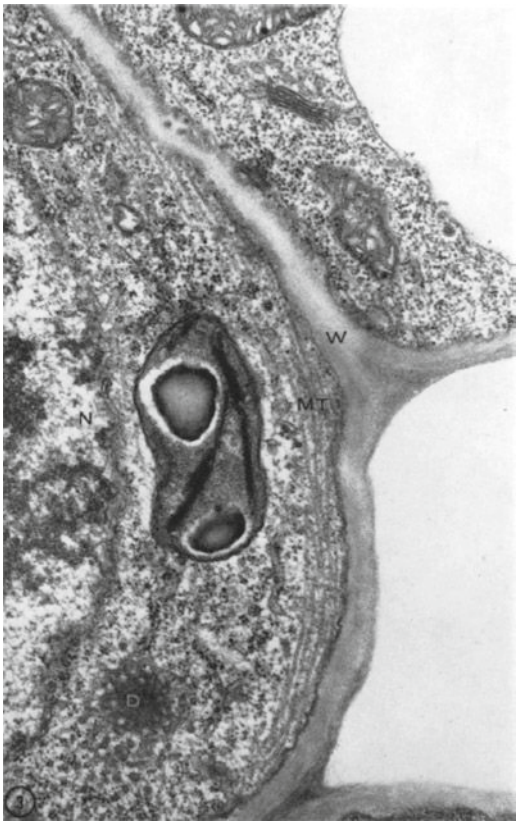

Fig. 8.5 Microtubules of the preprophase band curve around the periphery of a *Nicotiana tabacumi*, visualized with transmission electron microscopy. Reproduced from Cronshaw and Esau [15] with permission from Springer

with TEM analysis of the *Haemanthus* endosperm cells extensively used in light microscopy studies, an observation later reexamined with immunofluorescence microscopy [8, 25].

Microtubules associated with the nuclear envelope were also observed during prophase. The nuclear-associated microtubules organized as prophase progressed, aligning with the future spindle axis, perpendicular to the PPB and tangential to the nucleus [8, 11, 15, 42, 58]. This arrangement resulted in "a cone-shaped umbrella" of microtubules defining each of the future spindle poles, forming the prophase spindle [58]. As was observed with early fixation techniques, the microtubules did not penetrate the nucleus until nuclear envelope breakdown, at which point they invaded the nuclear area and intermingled with condensed chromosomes [42, 58].

TEM analysis of asymmetrical cell divisions in wheat and aquatic ferns brought to light a possible function for the PPB and nuclear-associated microtubules in cell patterning. Wheat stomatal complexes are formed through a series of asymmetrical cell divisions in which the nucleus becomes displaced during prophase and cytokinesis results in two different sizes of cells [59]. The PPB was predictably located around the nucleus, even when the nucleus was displaced from the center of the

cell, and consistently marked the location of the future cell wall. The same phenomenon was observed in the asymmetrical cell divisions in the epidermis of aquatic ferns, with the added detail that nuclear displacement was preceded by PPB formation, suggesting an interplay between the two structures [23]. Other researchers argued that the PPB did not play a role in positioning of the nucleus. Palevitz and Hepler [55] proposed that the PPB was an accumulation of microtubules forecasting an important cortical nucleating site involved in the later stages of mitosis, not in nuclear positioning.

Observations of the microtubule connections between the PPB and nucleus favored the former hypothesis; that is, the PPB plays a role in nuclear positioning. An increase in nuclear-associated microtubules and transverse microtubules (i.e., parallel to the PPB) were found in the cytoplasm directly between the nuclear envelope and the PPB [11, 15]. Additionally, changes in the shape of the prophase nucleus from spherical to elliptical were attributed to microtubule connections between the PPB and the nuclear envelope [11, 19]. How these connections worked and in what way they influenced spindle formation were not completely resolved using TEM; these important questions were addressed later.

Ultrastructural studies of plant cells using TEM had two major limitations: first, microtubule arrays could not be readily viewed in their entirety because fixed tissue had to be thinly sectioned; and second, these sections could only reveal the static organization of the microtubule arrays so their dynamic behavior could only be inferred. These two-dimensional limitations would eventually be eliminated with new techniques that preserved whole cellular organization and afforded a temporal component to address the transitions between arrays. Immunofluorescence microscopy techniques provided the technical advancements necessary to take this next step.

8.4 Fluorescence Microscopy: Immunofluorescence

Immunofluorescence microscopy exploits the specificity of antibodies to visualize structures in the cell. Franke et al. [18] were the first to demonstrate that antibodies produced using animal tubulin bind to plant microtubules in the prophase and mitotic spindles, suggesting a conservation of tubulin structure in eukaryotes. Work with *Haemanthus* endosperm cells demonstrated a complex radial array of cytoplasmic microtubules during interphase, which give way to nuclear-associated microtubules forming the clear zone and eventually a multi-polar prophase spindle [16]. There was, however, no observation of PPBs in endosperm cells [16, 18]. Because prepared endosperm cells lack cell walls, the absence of structures involved in division planes was not surprising.

Researchers soon sought to apply immunofluorescence techniques to plant cells bound by cell walls, both in suspension cultures and *in planta*. These cell types retained their morphological structure, compared to endosperm cells; however, the

cell wall prevented penetration of antibodies into the cytoplasm. Lloyd et al. [43] pioneered the application of cell wall degrading enzymes to fixed cells, allowing the profusion of antibodies into the cell. Complete degradation of the cell wall resulted in the formation of protoplasts and a loss of the hoop-like organization of microtubules along the long axis of the cell [41, 43, 60, 71]. Therefore, the use of these enzymes had limitations because the complete degradation of the wall resulted in the loss of cortical cytoskeleton organization [74]. Partial degradation of elongated carrot suspension cells revealed the hoop-like microtubule organization observed by TEM, therefore, validating the use of immunofluorescence in higher plant cells [43] (Fig. 8.6).

Cells retaining tissue-level morphological information (i.e., *in planta*) maintained subcellular structures believed important for appropriate cell division. The PPB was first observed in its entirety by Wick et al. [74] in the meristematic cells of onion root tips using immunofluorescence techniques. Their work highlighted the value of immunofluorescence microscopy to visualize the relationship between whole cytoskeletal arrays (compared with the sectioned material previously used in fixation and TEM) in *in planta* analyses. Additionally, cells at different stages of division could be visualized simultaneously, allowing for a temporal comparison of the transition of microtubules through cell division.

A combination of these improvements provided several insights into how the higher plant spindle formed, not just that specific components existed. Immunofluorescence microscopy revealed that early accumulation of microtubules to the PPB is accompanied by the presence of tubulin fluorescence at the nuclear envelope [72]. At this stage the PPB is wide and "barely" distinguishable from other cortical microtubules, demonstrating that tubulin association with the nuclear envelope occurs early in preprophase. Examination of cells in different stages of preprophase showed that the PPB narrows over time from a wide, loosely packed band to a tightly bundled band. As the PPB narrows, fluorescence at the nuclear envelope increases and eventually

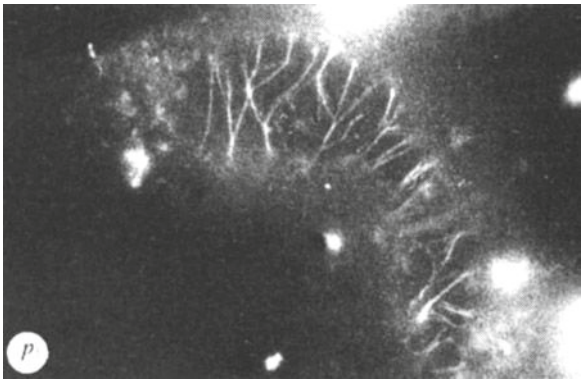

Fig. 8.6 Visualization of microtubules in elongated carrot cells from suspension cultures. Cells were treated for 30 min with cellulase, then extracted and stained with anti-tubulin. Reproduced from Lloyd et al. [43] with permission from Nature Publishing Group

overwhelms PPB fluorescence. The increase in nuclear fluorescence agrees with observations made in endosperm (using immunogold techniques) and meiotic cells, leading to the hypothesis that the nuclear envelope acts as a microtubule nucleating site in higher plant cells [16, 35, 72]. Additional work with antibodies specific for γ-tubulin, the isoform necessary for microtubule nucleation events in cells, showed an increase in fluorescence levels at the polar regions of the nuclear envelope [31, 39]. This observation further supported the hypothesis that the nuclear envelope acts as a microtubule nucleation site.

Distinct microtubules were rarely observed directly on the nuclear envelope during early preprophase, although microtubules approaching or emanating from the nucleus into the cytoplasm and/or the PPB region were noted [34, 51, 72]. As preprophase progresses into prophase, nuclear envelope-associated microtubules (i.e., perinuclear microtubules) increase in number and transition from random or parallel orientations to the plane of the PPB to tangential orientations approximating the future spindle [51, 73]. Work with centrifugation and squashing techniques further suggest there is a structural connection between the PPB and nucleus, likely through the microtubules transversing between the two structures [57, 73]. Therefore, a second characteristic observed through immunofluorescence is that microtubule connections between the PPB and nucleus occur throughout preprophase and prophase and that the organization of these microtubules transitions into a bipolar prophase spindle while the PPB is still intact. The fact that endosperm cells, lacking a PPB, form multi-polar prophase spindles that eventually establish bipolarity implies a role for the PPB in bipolar spindle formation [67].

In light of the existence of “bridge” microtubules (i.e., microtubules between the PPB and the prophase spindle), later immunofluorescence work demonstrated a relationship between PPB-narrowing and spindle bipolarity. Treatment of cells with cytochalasin D or cycloheximide prevents PPB-narrowing during prophase [17, 52, 54]. Cells subjected to these treatments had a lower percentage of cells with a bipolar prophase spindle in a population. Because of the static nature of fixed cells, the mechanism behind PPB-narrowing and spindle bipolarity was unknown. The relationship between the PPB and spindle bipolarity was further addressed with fluorescently tagged proteins, discussed in the next section.

A final characteristic revealed by immunofluorescence microscopy is that the poles of the prophase spindle do not completely converge to a point. Examination of cells through the axis of the developing prophase spindle demonstrate a non-fluorescent hole or annulus, which is invaded by microtubules after nuclear envelope breakdown [39, 73] (Fig. 8.7). This structure contrasts with the spindle pole in animal cells, which consists of a centriole as a focal point. Although these authors did not explore the function of this structure, current work suggests that the annulus is an integral structure involved in spindle bipolarity [2].

Immunofluorescence microscopy opened the door to three-dimensional analysis of arrays, with a temporal component available through comparison of many cells simultaneously; however, its major limitation (again) was the static nature due to fixation. The next hurdle in understanding acentrosomal spindle formation was the ability to observe transitional stages between prophase and metaphase. Work with

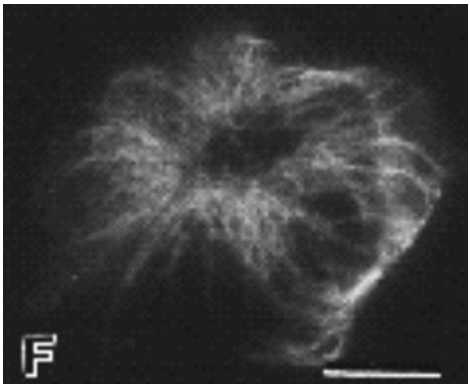

Fig. 8.7 The prophase spindle visualized by anti-tubulin staining and corresponding annulus located in the polar region. Reproduced from Liu et al. [39] with permission from the Journal of Cell Science

fluorescently labeled proteins allowed for real-time observations of microtubule arrays, thereby, overcoming this hurdle.

8.5 Fluorescent Microscopy Techniques: Intrinsic Fluorescent Proteins

Experiments utilizing fluorescently labeled proteins to visualize microtubule arrays consisted of two stages: first, after microinjection of cells with fluorescently labeled tubulin; and second, after green fluorescent protein (GFP) fusion proteins were stably expressed by the cell. Zhang et al. [75] were the first to inject *Tradescantia* stamen hair cells with fluorescein-labeled animal tubulin. After injection, all mitotic microtubule arrrays were visualized using a confocal laser scanning microscope. The patterns of fluorescence observed were very similar to techniques previously described. Later observations in *Tradescantia* cells focused on the division site and noted an accumulation of tubulin to the nuclear envelope and the presence of bridge microtubules [14].

These pioneering techniques resulted in several important technological advances. They validated the use of "reporter constructs" (i.e., fluorescently labeled proteins) to examine *in vivo* and real-time localization of proteins. The fact that the tubulin used this research was derived from animals paved the way for the application of heterologous systems when imaging spindle formation. Finally, this research also revealed the effectiveness of confocal laser scanning microscopy in time-lapse imaging, because cells were not damaged and fluorochromes did not bleach within the normal realms of usage. All of these advantages were important to the use of fluorescently labeled protein research [75].

Cortical arrays were first described in transgenic *Arabidopsis* plants expressing GFP:α-tubulin using epifluorescence microscopy, and the first examination of mitotic arrays was in tobacco BY-2 suspension culture cells expressing GFP fused to the microtubule binding domain (MBD) of the mammalian MAP4

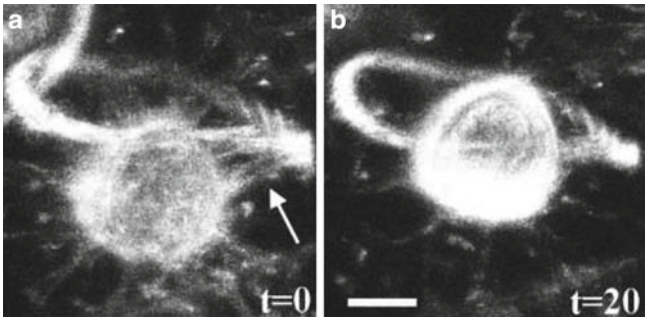

Fig. 8.8 Confocal microscopy images show microtubules interplay between the PPB and the nucleus ((**a**), *arrows*). As prophase progresses, the nucleus is centered and bridge microtubules are no longer visible (**b**). Time is indicated in minutes. Reproduced from Granger and Cyr [21] with permission from the Journal of Cell Science

protein [20, 44, 69]. In-depth studies of the relationship between the PPB and the future division plane concluded that cross talk exists between the PPB, nucleus and developing prophase spindle [21]. This interplay, mediated by bridge microtubules, allowed for "fine" adjustments in the placement of the nucleus and the subsequent spindle location and division plane independent of actin (Fig. 8.8). In situations where the nucleus is centered vertically, but not horizontally (i.e., the nucleus is in the plane of the PPB, but closer to one side of the cell), more bridge microtubules form between the nucleus nearer to the PPB and the developing spindle is displaced toward that side [2]. An important implication of these observations is that movement of a structure requires the generation of force, which may be created by microtubule motor proteins. A role for these proteins is further examined in mutational studies.

The studies described above also revealed that some *Arabidopsis* cells (expressing GFP:MBD) and some tobacco BY-2 culture cells (overexpressing GFP:End-binding protein 1) lack PPBs [2, 13]. In this situation, the perinuclear microtubules, which are still present, do not demonstrate a bipolar distribution before nuclear envelope breakdown. It is only after nuclear envelope breakdown that a bipolar metaphase spindle forms.

The success of fluorescence microscopy in conjunction with labeled proteins in plant cells lead to the development of effective reporter constructs for the microtubule cytoskeleton. These reporter constructs were utilized to better understand acentrosomal spindle formation in plants lacking specific functional proteins, either through T-DNA insertional mutagenesis or single point mutations. The function of two *Arabidopsis* kinesins in acentrosomal spindle formation were elucidated through the use of reporter constructs in mutant plants. The *atkin14a* homozygous mutant plants demonstrate broadened PPBs during prophase, visualized through the use of the GFP:MBD reporter construct, and a loss of bipolarity during prophase and prometaphase [45]. This loss of bipolar organization is ultimately corrected by anaphase; however, mutant cells take twice as long to proceed to anaphase,

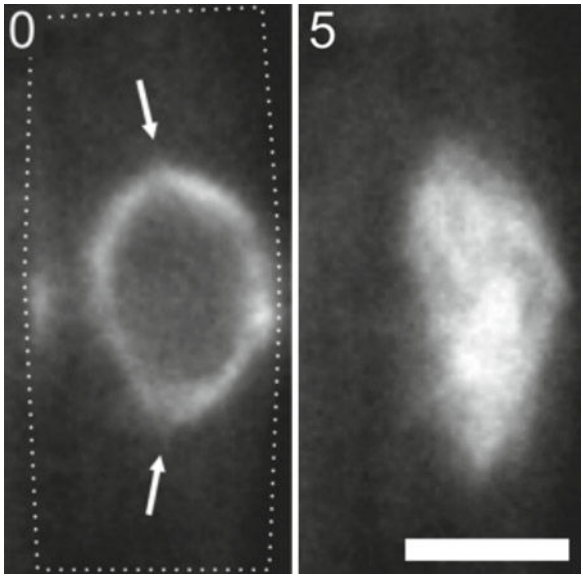

Fig. 8.9 An *Arabidopsis* root cell in an *atkin14b* mutant expressing GFP:TUB6. The disruption of forces at the midzone results in prophase spindles bent toward the PPB. Reproduced from Ambrose and Cyr [2] with permission from Oxford University Press

compared to wild-type cells, because microtubules are not organized into a bipolar spindle until after nuclear envelope breakdown.

The *atkin14b* homozygous mutants plants have normal PPBs, visualized through the use of the GFP:β-tubulin isoform 6 (TUB6) reporter construct, and develop bipolarity but lack spindle integrity at the midzone [1, 3]. This is exemplified by prophase and prometaphase spindles, which are bent toward the closest region of the PPB, and metaphase spindles that are broader, compared with wild-type spindles (Fig. 8.9). The bent spindle is attributed to unbalanced forces, in such a way that the force created between perinuclear and bridge microtubules of the PPB overcome the force needed to maintain the spindle midzone (which is weakened due to the lack of *AtKIN14b*). Both studies of *AtKIN14a* and *AtKIN14b* corroborate the interactions between the PPB and the perinuclear microtubules, while introducing the notion that microtubule motor proteins (i.e., kinesins) play an important role in acentrosomal spindle formation.

Immunofluorescence microscopy techniques have also been used to complement studies completed with real-time fluorescence microscopy. Two microtubule-associated proteins (MAPs), which may play a role in spindle formation, were characterized utilizing both techniques. Immunofluorescence microscopy of cells with a temperature-sensitive, mutant allele for *MOR1* revealed a population of cells either lacking PPBs or with split PPBs [32]. Real-time anaylsis of *mor1* cells expressing the GFP:TUB6 reporter construct showed an overall reduction of microtubule dynamics, suggesting a role for microtubule dynamicity in the formation of PPBs [33]. Cells with a mutant allele for *CLASP* lack PPB-narrowing during prophase, resulting in abnormal prophase spindle formation [4]. Real-time anaylsis of *clasp-1* cells expressing the GFP:TUB6 reporter construct showed a decrease in microtubule/

cortex interactions and microtubule stability [5]. The juxtaposition of dynamicity and stability mirrors the need for balanced forces within the structures involved with spindle formation.

8.6 Acentrosomal Bipolarity Organized by the PPB

Throughout the historic age of microscopy, we have gleaned much information on the components of acentrosomal spindle formation, however, an understanding of the mechanism by which these components organize is still in its infancy. It is clear that the PPB is critical for establishing early bipolarity through interactions with bridge and perinuclear microtubules. These interactions organize perinuclear microtubules into the prophase spindle such that microtubules run tangential to the nucleus and between the PPB and polar regions of the nucleus. Therefore, the PPB not only forecasts the future plane of cell division, but acts as an equatorial organizer for mitosis [2, 9]. As prophase progesses, the PPB narrows and the prophase spindle becomes more defined. Work by Ambrose and Cyr [2] has revealed that PPB-narrowing is accompanied by closing of the polar annulus. At nuclear envelope breakdown, the established bipolarity is quickly translated into the broadened acentrosomal spindle of higher plants.

A recent model suggests that the above events are organized through a delicate balance of forces generated by microtubule motor proteins [2]. Bridge microtubules provide two interrelated functions: first, to act as a template for organization; and second, to facilitate the transmission of force between the PPB and prophase spindle. Through the sliding action of kinesins, the initially unorganized perinuclear microtubules co-align with bridge microtubules to form a bipolar structure. The polar region is likely tapered through the action of minus-end-directed kineins, producing an annular structure. As a result of the action of molecular motor organization, force is generated between the PPB and the prophase spindle via the bridge microtubules. Support for this hypothesis can be seen in early immunofluorescence work with lysed *Arabidopsis* cells. Metaphase spindles in these cells were more focused, compared to walled cells. Additionally, a specific minus-end-directed kinesin (i.e., *AtKIN14a*) accumulated at the focused pole in the lysed cells, but not in walled cells [38]. The differences observed were attributed to the disruption of balanced forces normally working before lysis occurred and the subsequent loss of spatial signals associated with the cell wall.

In a three-dimensional system, equal production of force toward the annulus, as well as toward the PPB, centers the nucleus in the plane of the PPB. As the PPB begins to degrade (i.e., narrows) during progression from prophase to prometaphase, the concurrent loss of bridge microtubules causes annular forces to dominate, resulting in closing of the annulus. These events are typically concurrent with nuclear envelope breakdown and progression into prometaphase. In situations where the balance of force is disrupted, abnormal events occur (e.g., the bent spindles observed in *AtKIN14b* mutants).

Evidence also demonstrates that the non-uniform distribution of bridge microtubules results in nuclear migration toward the side of the cell with more connections. The lack of PPB-narrowing or complete absence of a PPB prevents bipolar organization. In these situations, bridge microtubules are either ineffective or not present to organize the perinuclear microtubules. Because bipolarity is eventually achieved after nuclear envelope breakdown, it is likely that alternative correcting mechanisms exist [2, 40].

The events contributing to acentrosomal spindle formation occur without the specifc structure of a centrosome. In fact, implying that a centrosome is necessary for spindle formation eliminates the apparent communication between a cortical spatial cue (the PPB) and the site of microtubule nucleation (the nuclear envelope). Because the PPB forecasts the future divison plane and positions the prophase nucleus for subsequent division, this mechanism for spindle formation reflects the importance of proper cell wall placement in plants. In contrast, spindle formation in animal cells begins with microtubule nucleation at the centrosomes to form astal arrays prior to or at nuclear envelope breakdown [50]. The astral arrays move to opposite ends of the nucleus by means of microtubule motor proteins, therefore, bipolarity is not dependent on cortical information. Reorientation and ultimate placement of the spindle occurs through interactions with the astral microtubules and the cell cortex after the metaphase spindle forms [56, 66]. The evolution of an acentrosomal mechanism in plants reflects the importance of organizing the spindle in a tissue-specific context, based on proper cell wall placement.

8.7 The Future Directions in Acentrosomal Spindle Formation

In acentrosomal spindle formation, microtubule nucleation and organization is mediated by the nuclear envelope and complex interactions between the PPB, bridge microtubules, and perinuclear microtubules. It is clear that many proteins, including microtubule motor proteins, play a role in these interactions [9]. Understanding how forces within the developing spindle are generated and maintained will require the use of the microscopy and mutagenesis techniques described herein.

Additional techniques may also play a critical role in this understanding acentrosomal spindle formation. Research is underway to develop an *in vitro* system that produces an artificial spindle [70]. Under specifc conditions, microtubules in an electric field align to form spindle-like structures (Fig. 8.10). Experimental designs, where proteins can be added to assembled spindles, will help elucidate the individual function of individual proteins from the complex interactions during spindle organization. The combination of *in vivo* and *in vitro* techniques will undoubtedly usher in the next age of understanding acentrosomal spindle formation in plants.

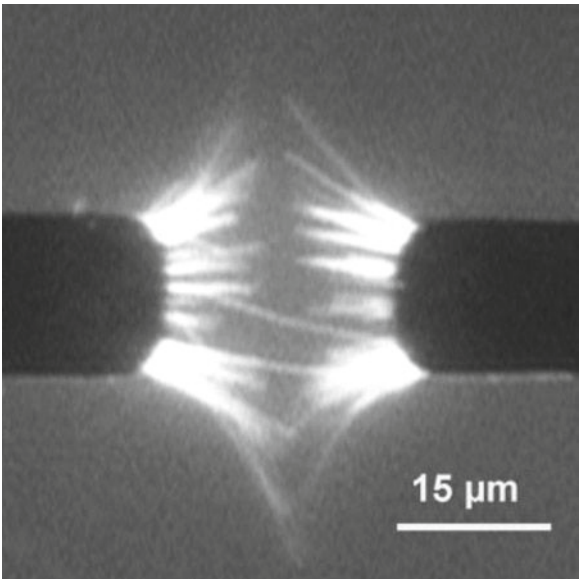

Fig. 8.10 The spindle-like accumulation of microtubules generated by dielectrophoretic forces. Reproduced from Uppalapati et al. [70] with permission from Wiley-VCH Verlag Gmbh & Company

References

1. Ambrose JC, Cyr R (2007) The kinesin ATK5 functions in early spindle assembly in *Arabidopsis*. Plant Cell 19(1):226–236
2. Ambrose JC, Cyr RJ (2008) Mitotic spindle organization by the preprophase band. Mol Plant 1:950–960
3. Ambrose JC, Li W et al (2005) A minus-end-directed kinesin with plus-end tracking protein activity is involved in spindle morphogenesis. Mol Biol Cell 16(4):1584–1592
4. Ambrose JC, Shoji T et al (2007) The *Arabidopsis* CLASP gene encodes a microtubule-associated protein involved in cell expansion and division. Plant Cell 19(9):2763–2775
5. Ambrose JC, Wasteneys GO (2008) CLASP modulates microtubule-cortex interaction during self-organization of acentrosomal microtubules. Mol Biol Cell 19(11):4730–4737
6. Bajer A (1955) Living smears from endosperm. Experientia 11:221–222
7. Bajer A (1957) Cine-micrographic studies on mitosis in endosperm. III. The origin of the mitotic spindle. Exp Cell Res 13(3):493–502
8. Bajer A, Mole-Bajer J (1969) Formation of spindle fibers, kinetochore orientation, and behavior of nuclear envelope during mitosis in endosperm – fine structural and in vitro studies. Chromosoma 27(4):448–484
9. Bannigan A, Lizotte-Waniewski M et al (2007) Emerging molecular mechanisms that power and regulate the anastral mitotic spindle of flowering plants. Cell Motil Cytoskeleton 65(1):1–11
10. Becker W (1938) Recent investigations in vivo on the division of plant cells. Bot Rev 4(8):446–472
11. Burgess J (1970) Interactions between microtubules and the nuclear envelope during mitosis in a fern. Protoplasma 71:77–89
12. Burgess J, Northcote DH (1967) A function of the preprophase band of microtubules in *Phleum pratense*. Planta 75:319–326
13. Chan J, Calder G et al (2005) Localization of the microtubule end binding protein EB1 reveals alternative pathways of spindle development in *Arabidopsis* suspension cells. Plant Cell 17(6):1737–1748

14. Cleary AL, Gunning BES et al (1992) Microtubule and F-Actin dynamics at the division site in living *Tradescantia* stamen hair cells. J Cell Sci 103:977–988
15. Cronshaw J, Esau K (1968) Cell division in leaves of *Nicotiana*. Protoplasma 65(1):1–24
16. De Mey J, Lambert AM et al (1982) Visualization of microtubules in interphase and mitotic plant cells of *Haemanthus* endosperm with the immuno-gold staining method. Proc Natl Acad Sci USA 79:1898–1902
17. Eleftheriou E, Palevitz B (1992) The effect of cytochalasin D on preprophase band organization in root tip cells of *Allium*. J Cell Sci 103:989–998
18. Franke WW, Seib E et al (1977) Tubulin-containing structures in anastral mitotic apparatus of endosperm cells of plant *Leucojum aestivum* as revealed by immunofluorescence microscopy. Cytobiologie 15(1):24–48
19. Galatis B (1980) Microtubules and guard-cell morphogenesis in *Zea mays* L. J Cell Sci 45:211–244
20. Granger CL (2000) Development and use of a GFP-labeled reporter protein for observing microtubule behavior in living cells. Doctoral Disseration, Pennsylvania State University, University Park, PA. 240pgs.
21. Granger CL, Cyr RJ (2000) Use of abnormal preprophase bands to decipher division plane determination. J Cell Sci 114:599–607
22. Guignard L (1989) Centrosomes in plants. Bot Gaz 25(3):158–164
23. Gunning BES, Harding AR et al (1978) Pre-prophase bands of microtubules in all categories of formative and proliferative cell division in *Azolla* roots. Planta 143:145–160
24. Hardham AR, Gunning BES (1978) Structure of cortical microtubule arrays in plant cells. J Cell Biol 77:14–34
25. Harris P, Bajer A (1965) Fine structure studies on mitosis in endosperm metaphase of *Haemanthus* Katherinae Bak. Chromosoma 16:624–636
26. Hepler P, Palevitz B (1974) Microtubules and microfilaments. Annu Rev Plant Physiol 25:309–362
27. Humphrey J (1895) Some recent cell literature. Bot Gaz 20(5):222–228
28. Inoue S (1953) Polarization optical studies of the mitotic spindle. 1. The demonstration of spindle fibers in living cells. Chromosoma 5:487–500
29. Inoue S, Bajer A (1961) Birefringence in endosperm mitosis. Chromosoma 12:48–63
30. Inoue S, Dan K (1951) Birefringence of the dividing cell. J Morphol 89(3):423–455
31. Joshi HC, Palacios MJ et al (1992) Gamma-tubulin is a centrosomal protein required for cell cycle-dependent microtubule nucleation. Nature 356:80–83
32. Kawamura E, Himmelspach R et al (2006) MICROTUBULE ORGANIZATION 1 regulates structure and function of microtubule arrays during mitosis and cytokinesis in the *Arabidopsis* root. Plant Physiol 140(1):102–114
33. Kawamura E, Wasteneys GO (2008) MOR1, the *Arabidopsis thaliana* homologue of Xenopus MAP215, promotes rapid growth and shrinkage, and suppresses the pausing of microtubules in vivo. J Cell Sci 121(pt 24):4114–4123
34. Kubiak J, Debrabander M et al (1986) Origin of the mitotic spindle in onion root-cells. Protoplasma 130(1):51–56
35. Lambert AM (1980) Role of chromosomes in anaphase trigger and nuclear-envelope activity in spindle formation. Chromosoma 76(3):295–308
36. Ledbetter MC, Porter KR (1963) A "microtubule" in plant cell fine structure. J Cell Biol 19:239–250
37. Lepper R (1956) The plant centrosome and the centrosome-blepharoplast homology. Bot Rev 22(6):375–417
38. Liu B, Cyr RJ et al (1996) A kinesin-like protein, KatAp, in the cells of *Arabidopsis* and other plants. Plant Cell 8:119–132
39. Liu B, Marc J et al (1993) A γ-tubulin-related protein associated with the microtubule arrays of higher plants in a cell cycle-dependent manner. J Cell Sci 104:1217–1228
40. Lloyd C, Chan J (2006) Not so divided: the common basis of plant and animal mitosis. Nat Rev Mol Cell Biol 7:147–152

41. Lloyd C, Slabas A et al (1980) Microtubules, protoplasts, and plant cell shape: an immunofluorescent study. Planta 147:500–506
42. Lloyd C, Slabas A et al (1982) Novel features of the plant cytoskeleton. Cell Biol Int Rep 6:171–175
43. Lloyd CW, Slabas AR et al (1979) Cytoplasmic microtubules of higher-plant cells visualized with anti-tubulin antibodies. Nature 279(5710):239–241
44. Marc J, Granger CL et al (1998) A *GFP-MAP4* reporter gene for visualizing cortical microtubule rearrangements in living epidermal cells. Plant Cell 10:1927–1939
45. Marcus AI, Li W et al (2003) A kinesin mutant with an atypical bipolar spindle undergoes normal mitosis. Mol Biol Cell 14(4):1717–1726
46. Marquette W (1907) Manifestations of polarity in plant cells which are apparently without centrosomes. Bot Zentralbl 21(1):281–303
47. Martens P (1929) New experimental studies on mitosis in the living cell. Cellule 39:167–216
48. Mazia D (1984) Centrosomes and mitotic poles. Exp Cell Res 153(1):1–15
49. McComb A (1900) The development of the karyokinetic spindle in vegetative cells of higher plants. Bull Torrey Bot Club 27(8):451–459
50. Merdes A, Cleveland DW (1997) Pathways of spindle pole formation: different mechanisms; conserved components. J Cell Biol 138(5):953–956
51. Mineyuki Y, Marc J et al (1991) Relationship between the preprophase band, nucleus and spindle in dividing *Allium cotyledon* cells. J Plant Physiol 138:640–649
52. Mineyuki Y, Palevitz BA (1990) Relationship between preprophase band organization, F-actin and the division site in *Allium.* Fluorescence and morphometric studies on cytochalasin treated cells. J Cell Sci 97:283–295
53. Newcomb EH (1969) Plant microtubules. Annu Rev Plant Physiol 20:253–288
54. Nogami A, Suzaki T et al (1996) Effects of cycloheximide on preprophase bands and prophase spindles in onion (*Allium cepa* L.) root tip cells. Protoplasma 192:109–121
55. Palevitz BA, Hepler PK (1974) The control of the plane of division during stomatal differentiation in *Allium*. I. Spindle reorientation. Chromosoma 46:297–326
56. Pearson CG, Bloom K (2004) Dynamic microtubules lead the way for spindle positioning. Nat Rev Mol Cell Biol 5(6):481–492
57. Pickett-Heaps JD (1969) Preprophase microtubules and stomatal differentiation; some effects of centrifugation on symmetrical and asymmetrical cell division. J Ultrastruct Res 27:24–44
58. Pickett-Heaps JD, Northcote DH (1966) Organization of microtubules and endoplasmic reticulum during mitosis and cytokinesis in wheat meristems. J Cell Sci 1:109–120
59. Pickett-Heaps JD, Northcote DH (1966) Cell division in the formation of the stomatal complex of the young leaves of wheat. J Cell Sci 1(1):121–128
60. Powell AJ, Lloyd CW et al (1980) Demonstration of the microtubular cytoskeleton of the moss, *Physcomitrella patens*, using antibodies against mammalian brain tubulin. Plant Sci Lett 18(4):401–404
61. Rosza G, Wyckoff R (1950) The electron microscopy of dividing cells. Biochim Biophys Acta 6:334–339
62. Sabatini DD, Miller F et al (1964) Aldehyde fixation for morphological and enzyme histochemical studies with the electron microscope. J Histochem Cytochem 12:57–71
63. Schaffner J (1898) Karyokinesis in the root tips of *Allium cepa*. Bot Gaz 26(4):225–238
64. Schrader F (1934) On the reality of spindle fibers. Biol Bull 67(3):519–533
65. Sedar AW, Wilson DF (1950) An electron microscope study of mitosis in the onion root tip. Anat Rec 108(3):531–532
66. Siller HK, Doe CQ (2009) Spindle orientation during asymmetric cell division. Nat Cell Biol 11:365–374
67. Smirnova EA, Bajer AS (1994) Microtubule converging centers and reorganization of the interphase cytoskeleton and the mitotic spindle in higher plant *Haemanthus*. Cell Motil Cytoskeleton 27(3):219–233
68. Srivasta LM, Singh AP (1972) Certain aspects of xylem differentiation in corn. Can J Bot 50(9):1795–1804

69. Ueda K, Matsuyama T et al (1999) Visualization of microtubules in living cells of transgenic *Arabidopsis thaliana*. Protoplasma 206:201–206
70. Uppalapati M, Huang YM et al (2008) Microtubule alignment and manipulation using AC electrokinetics. Small 4(9):1371–1381
71. Van der Valk P, Rennie PJ et al (1980) Distribution of cortical microtubules in tobacco protoplasts – an immunofluorescence microscopic and ultrastructural study. Protoplasma 105(1–2):27–43
72. Wick S, Duniec J (1983) Immunofluorescence microscopy of tubulin and microtubule arrays in plant cells. I. Pre-prophase band development and concomitant appearance of nuclear envelope-associated tubulin. J Cell Biol 97:235–243
73. Wick S, Duniec J (1984) Immunofluorescence microscopy of tubulin and microtubule arrays in plant cells. II. Transition between the pre-prophase band and the mitotic spindle. Protoplasma 122:45–55
74. Wick SM, Seagull RW et al (1981) Immunofluorescence microscopy of organized microtubule arrays in structurally stabilized meristematic plant cells. J Cell Biol 89:685–690
75. Zhang DH, Wadsworth P et al (1990) Microtubule dynamics in living dividing plant-cells – confocal imaging of microinjected fluorescent brain tubulin. Proc Natl Acad Sci USA 87(22):8820–8824

Chapter 9
Microtubule Organization in the Phragmoplast

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9.1 Introduction

Plant cytokinesis is manifested by the formation of a cross-wall called the cell plate which is brought about by the phragmoplast. The phragmoplast first appeared in advanced green algae like *Coleochaete* and *Chara* to replace the phycoplast as the cytokinetic apparatus. This remarkable apparatus marks the advancement toward land plants [27]. It consists of arrayed microtubules (MTs) and actin microfilaments (MFs) along which materials necessary for cell plate assembly are delivered in the form of vesicles. In the phycoplast, MTs are aligned in parallel to the division plane. In the phragmoplast, however, MTs are arranged perpendicularly to the division plane (Fig. 9.1). Such an arrangement of phragmoplast MTs was first described when the structure of MTs was reported [48]. The magnificent bipolar arrangement of phragmoplast MTs was further revealed in the *Haemanthus* endosperm cells, and these MTs were arranged in an anti-parallel pattern with their plus ends pointing at the division site [24, 32]. Coordinated activities of MTs and MFs allow the cell plate, formed by joining of membranous compartments resulted from Golgi vesicle fusion and trafficking, to be inserted into developmentally meaningful sites [37, 38, 102]. Ultimately, the cell plate acts as the physical barrier between two daughter cells.

The phragmoplast MT array is established *via* consecutive steps of MT polymerization and rearrangement starting from MTs with mixed polarities in the central spindle at late anaphase and telophase [123]. These midzone MTs slide against each other by a motor-driven mechanism to result in an anti-parallel configuration [5, 6]. Phragmoplast MTs are highly dynamic, and α- and β-tubulin heterodimers are continuously polymerized onto their plus ends [6, 35, 111]. Earlier examination of the *Haemanthus* endosperm cells indicates that phragmoplast MTs interdigitate near their plus ends by "cross-bridges" [32]. However, recent tomographic studies illustrate that plus ends of most MTs do not overlap in the middle of a mature phragmoplast in meristematic cells in *Arabidopsis thaliana* [7]. Nevertheless, the

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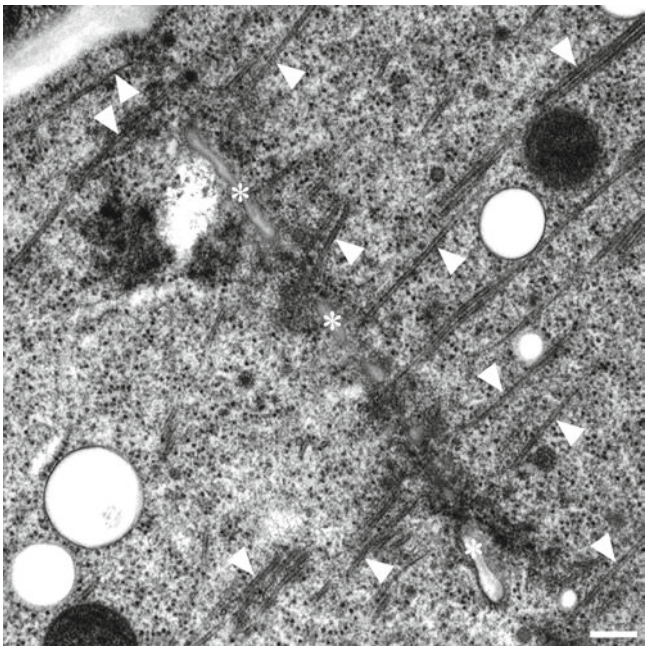

Fig. 9.1 A transmission EM image illustrating anti-parallel MTs (*arrowheads*) flanking the developing cell plate (*). *Scale bar*, 0.2 μm

bipolar feature of phragmoplast MTs is ubiquitously found in all plant cells, even when the phragmoplast MT array is established by MTs radiating from neighboring nuclear envelopes when building syncytial-type cell plates [7, 78, 79, 84]. Electron tomographic data show that a great number of MT plus ends terminate at ribosome-excluding cell plate assembly matrix which may function in stabilizing MT plus ends [79, 89]. However, the molecular constituents of this matrix are yet to be characterized [94].

Concomitant with the centrifugal buildup of the cell plate during cytokinesis, the phragmoplast MT array expands toward the cortex. In the central region where Golgi-derived vesicles have already been fused to form membranous sacks at the cell division site, MTs quickly depolymerize after fulfilling their duties of delivering the vesicles. In the meantime, new MTs are assembled at the advancing front of the phragmoplast toward its periphery. According to FRAP (Fluorescent Recovery After Photobleaching) data, tubulin subunits released from the depolymerization may be subsequently incorporated into polymerizing MTs [35]. This is particularly remarkable as polymerization and depolymerization take place simultaneously at very close sites, within ~5 μm in distance in the phragmoplast. Thus, plant cells have developed sophisticated mechanisms that regulate precisely positioned MT polymerization and depolymerization events. Unfortunately, very little if any has been learned about how such spatially coordinated events take place at the same time.

The assembly of the cell plate is intelligently coordinated with the activity of phragmoplast MTs. Active transport of Golgi-derived vesicles is accompanied by robust polymerization of phragmoplast MTs. Moreover, the bipolar MT array allows the vesicles to be transported uni-directionally toward MT plus ends at or near the division site [101, 112]. A battery of proteins involved in membrane/vesicle fusion and fission play essential roles in building up the cell plate [11, 36–38, 90]. While hemicelluloses like xyloglycans are transported by vesicles after being synthesized in the Golgi apparatus, other complex carbohydrates like callose are synthesized in situ after the biosynthesis machinery is delivered to the division site [34]. Some features of membrane trafficking during cytokinesis are conserved in plants and animals [59, 80]. While MTs govern the transport of vesicles, membrane trafficking during cell plate assembly also influences MT reorganization. For example, inhibition of cell plate formation by Brefeldin A can prevent the disassembly of phragmoplast MTs [121]. Thus, MT reorganization is coordinated with dynamic membrane remodeling events in the phragmoplast during cytokinesis.

In this review, we summarize current knowledge on MT reorganization in the phragmoplast by emphasizing on proteins regulating specific events during rapid progression of cytokinesis in flowering plants. A mini-phragmoplast hypothesis is presented at the end, aiming at unifying observations made on MT reorganization in the phragmoplast. Concomitant events of vesicle trafficking required for cell plate formation are not discussed here as they have been expertly summarized by colleagues in several recent review articles [11, 37, 38, 80, 90, 94].

9.2 Microtubule-Interacting Factors in the Phragmoplast

How many proteins are associated with the phragmoplast? How many proteins are required for the operation of the phragmoplast? In recent years, the list of proteins interacting with phragmoplast MTs has been growing significantly. Microtubule-associated proteins or MAPs and other MT-interacting factors were detected in the phragmoplast by two ways. A classical approach is to use specific antibodies against the MT-interacting factors to probe the endogenous proteins by immunolocalization. Since the successful application of the GFP (Green Fluorescent Protein)-tagging technique [63], other proteins have been shown to decorate the phragmoplast or are enriched in it when GFP fusion proteins are expressed in transformed or transfected cells. Not all detected proteins are uniformly associated with phragmoplast MTs. In other words, some proteins exhibit biased localizations in preference for certain portions of phragmoplast MTs.

MAPs uniformly decorating phragmoplast MTs. The following mentioned MAPs exhibit more or less even distribution patterns along phragmoplast MTs when their native or ectopically expressed forms were detected in cells undergoing cytokinesis. These MAPs are either essential for MT assembly, or exert specific roles in certain aspects of MT organization. Some are orthologs of MAPs identified in fungi and/or animals, while others turn out to be plant specific [29, 30]. As one of the

most critical factors promoting MT polymerization, MOR1/GEM1 of the XMAP215/TOG family decorates MTs in all arrays including the phragmoplast [31, 41]. Although the temperature sensitive *mor1* mutation affects MT activities at all stages under non-permissive conditions, the allelic *gem* mutation specifically blocked cytokinesis during male gametogenesis [106, 117]. However, it is unclear whether either mutation leads to a complete loss of the protein's function. At restrictive temperatures, the *mor1* mutant cells failed to complete cytokinesis by leaving behind stubbed or branched cell plates, but did not abolish the bipolarity of the phragmoplast MT array [23, 41]. Depletion of the tobacco ortholog of MOR1/GEM1 recapitulated the phenotypes observed in the *Arabidopsis* mutants, which included misoriented, fragmented, and branched phragmoplasts [75]. But microscopically, the bipolarity of the phragmoplast MT array was still established in the mutant cells. Interestingly, the HEAT (Huntington, Elongation factor-3, A subunit of PR65, Tor Kinase) repeat commonly present in the XMAP215 family proteins is also found in plant specific MAPs called TOR1/SPR2 and its homologs [19, 96]. Again, they decorate along phragmoplast MTs uniformly.

A number of plant MAPs are related to the TPX2 protein which acts as the targeting factor for the mitotic kinase Aurora A to the centrosome in animal cells [83]. Their MT-binding site bears the KLEEK motif. One of them, WVD2 (WaVe-Dampened 2) appears along phragmoplast MTs as well as MTs of other arrays [83]. Although the *wvd2* mutant only exhibited defects in anisotropic cell expansion, its role in phragmoplast MT organization may be redundant with those of homologous proteins. Intriguingly, the true homolog of the animal TPX2 exhibits specific association with spindle MTs, but not phragmoplast MTs [113]. Another group of plant specific MAPs, namely MAP70s, also bear the KLEEK motif. One of them exhibits localization along phragmoplast MTs when expressed in a GFP fusion [45]. The same can be said for its functionality in the phragmoplast as for WVD2.

It should be indicated that as MAPs the auxin-induced AIR9 protein as well as the EDE1 protein also decorated phragmoplast MTs when they were expressed in GFP fusions [18, 85]. Whether they play a role in phragmoplast MT organization, unfortunately, is currently unknown.

Proteins preferentially appearing toward MT minus ends. The most noticeable protein at the MT minus end is γ-tubulin [55]. In the phragmoplast, it has a gradient distribution pattern with the highest concentration toward the MT distal ends facing the daughter nuclei [54, 56]. When examined by immunofluorescence, the anti-γ-tubulin signal leaves a much wider dark gap in the phragmoplast midzone compared to that of anti-α/β-tubulin. Proteins in the γ-tubulin complex are expected to exhibit identical localizations as γ-tubulin since they form a tight complex [44, 68, 95]. Genetic studies show that the function of γ-tubulin is absolutely required for MT nucleation and organization during mitosis and cytokinesis [12, 82].

In plant cells, the γ-tubulin complex can initiate MT nucleation on the wall of extant MTs at angles of ~40° [67]. Whether the complex directly interacts with the MT wall or indirectly *via* other proteins is an open question. A WD40 repeat protein named NEDD1 (or GCP-WD) interacts with the γ-tubulin complex and plays a critical role in MT organization in animal cells [28, 60]. The examination of the

NEDD1 protein in *Arabidopsis* showed that it shares an identical localization pattern as γ-tubulin, which further supports its role in γ-tubulin-dependent activities [122]. A recent study even suggested that NEDD1 may directly interact with MTs [58], which would make it a potential linker mediating the interaction between the γ-tubulin complex and MTs. In *Arabidopsis*, the loss of NEDD1 leads to collapse of phragmoplast MT array, leaving behind randomly arranged MT bundles between two daughter nuclei [122]. It remains to be tested whether the plant NEDD1 mediates the interaction between the γ-tubulin complex and MT walls in order to establish MT branching at ~40°.

While it is being debated about whether NEDD1 directly interacts with MTs, it is also known to interact with the FAM29A protein in the augmin complex in mammalian cells [124]. The augmin complex interacts with MT walls *via* its MAP subunit called Hice1/hDgt4 in human cells [118]. Such an interaction would be ideal for establishing the link from γ-tubulin to MT walls *via* NEDD1 and the augmin complex. Although a few plant proteins show limited homology with several augmin subunits (unpublished data), they await experimental tests to show whether plant cells produce functional augmin and if so whether such a complex functions in MT nucleation and organization during mitosis and cytokinesis.

Proteins acting at MT plus ends in the phragmoplast. Proteins appearing specifically at polymerizing MT plus ends are collectively called plus end-tracking proteins or +TIPs [1]. Some of them are conserved in plants [13, 39]. Among them the most intensively studied one is the End-Binding 1 or EB1 protein. Plants have two classes of EB1 proteins [14, 29]. *Arabidopsis* EB1a and EB1b of the first class track plus ends of cortical MTs during interphase, EB1c resides in the nucleus prior to nuclear envelope breakdown during the cell division cycle [22, 43]. These three EB1 proteins decorate MT plus ends in spindles and the phragmoplast [14, 21]. Both native and ectopically expressed EB1-GFP fusion proteins show localizations across the phragmoplast with pronounced appearance in the midline [14, 21, 43].

Unlike animals, plants live nearly normally in the absence of all EB1 proteins [14, 43]. In *Arabidopsis*, it seems that only EB1c plays a critical role in cell growth as the *eb1c* mutant shows hypersensitivity to the microtubule-depolymerizing agent Oryzalin [14]. When challenged by 100 nM Oryzalin, the *eb1c* mutant cells undergoing cytokinesis formed fragmented phragmoplasts (Fig. 9.2), besides having serious defects in mitotic spindles [43]. However, fragments of phragmoplast had MTs arranged in regular bipolar fashion leaving behind a dark line in anti-tubulin immunofluorescence (arrowheads, Fig. 9.2).

In animal cells, the +TIP CLASP plays a critical role in MT-kinetochore interaction and consequently in spindle organization during mitosis [61]. Although a CLASP homolog has been identified in *Arabidopsis* as an important regulator of cortical MT organization, the protein was undetectable in spindles and the phragmoplast [3, 42]. The plant specific +TIP SPR1 and its relatives also function in the organization cortical MTs, but whether they play a role in the phragmoplast remains to be tested [69, 70, 93].

MT-bundling proteins of the MAP65/Ase1 family exhibit intriguing localization patterns in the phragmoplast with a preference toward MT plus ends [100, 109].

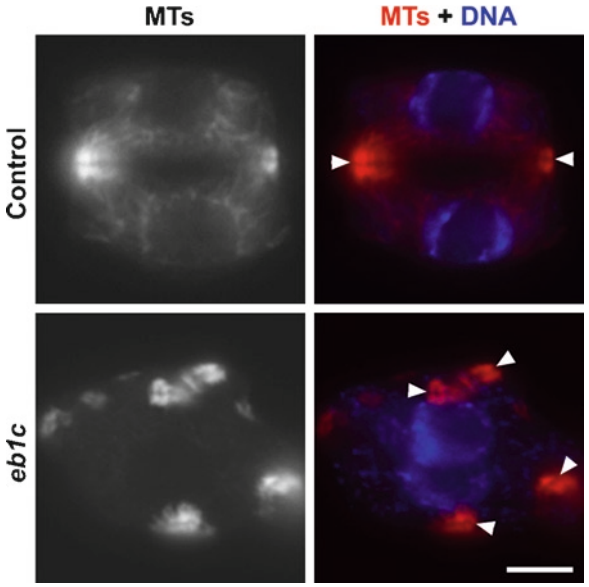

Fig. 9.2 Fragmented phragmoplasts in the *eb1c* mutant. Anti-tubulin immunofluorescence shows MTs in a control cell and an *eb1c* mutant cell after treatment with Oryzalin. In *merged images*, MTs are pseudocolored in *red* and DNA in *blue*. The *dark lines* in the *middle* of the phragmoplast are highlighted by *arrowheads*. *Scale bar*, 5 μm

Among nine MAP65 isoforms in *Arabidopsis*, MAP65-3 has the most exclusive localization at or near MT plus ends in the phragmoplast compared to several other MAP65s which also decorate phragmoplast MTs [20, 65, 100]. Activities of MAP65 proteins during cell division are likely regulated by protein phosphorylation [98]. For example, a phosphorylated form of MAP65-1 appears exclusively at MT plus ends in the phragmoplast while the unphosphorylated protein decorates phragmoplast MTs in a more uniform manner [92]. To date, only the loss of MAP65-3 has been shown to cause cytokinetic defects by producing cell wall stubs and multinucleate cells [20, 65]. The cytokinetic phenotype is coincident with a wider dark midline of anti-tubulin immunofluorescence in the phragmoplast in mutant cells when compared to the phragmoplast image of wild type cells [65]. How different MAP65 isoforms contribute to phragmoplast MT organization remains to be elucidated.

Microtubule-based motor kinesins in the phragmoplast. Plants surpass animals and fungi for the number of kinesin genes in their genomes [29, 87]. Among 61 kinesin genes in the *Arabidopsis* genome, more than 20 show preferential expression in mitotically active cells [110]. Both plus end- and minus end-directed motors are included. Several such mitotic kinesins have already been tested for having activities in the phragmoplast.

Early immunofluorescence attempts showed that evolutionarily conserved minus end-directed Kinesin-14 motors closely related to the NCD/Kar3 proteins of

animals and fungi exhibited a gradient localization pattern in the phragmoplast with the greatest accumulation toward MT plus end [53, 57]. But unlike MAP65-3, the Kinesin-14 signal does not occupy the phragmoplast midline indicating that it avoids the plus ends of the MTs there. Another member of the Kinesin-14 subfamily, the calmodulin-binding kinesin KCBP/ZWI also appears in the phragmoplast [15, 86]. When KCBP/ZWI was artificial activated by blocking its self-inhibitory domain by antibodies, phragmoplast formation was significantly delayed, indicating that it plays a precisely regulated role during cytokinesis [114]. However, in the absence of this kinesin, the *zwi* mutant plants grow healthily with defects in trichome morphogenesis in the leaf but not in cell division [77].

The plus end-directed Kinesin-5/BIMC motors have long been suspected to be the ones responsible for establishing the bipolar configuration of phragmoplast MTs [5, 6]. Such motors are expected to form bipolar homotetramers to interact with anti-parallel MTs in order to slide them apart [4]. Multiple isoforms of Kinesin-5 in plants have complicated the interpretation of their specific functions. Based on localization data, certain members of Kinesin-5 decorate phragmoplast MTs evenly, and others show preference toward MT plus ends [4, 9, 10]. The temperature sensitive *rsw7* mutation in the gene encoding AtKRP125c (one of the four Kinesin-5s in *Arabidopsis*) can cause collapses of bipolar spindles and mistakenly deployed fragmented phragmoplasts in the cytoplasm at restrictive temperatures [9]. However, the wrongly placed phragmoplasts still contained bipolar MT arrays with a clear dark midline by anti-tubulin immunofluorescence. Thus, if the role of Kinesin-5 is critical for establishing the bipolar MT array, it must be shared with other Kinesin-5 isoforms or motors of other kinesin subfamilies.

Two paralogous plus end-directed motors of the Kinesin-7 subfamily appear specifically in the phragmoplast midline in *Arabidopsis* and tobacco cells [73]. These NACK kinesins are responsible for the localization of cytokinesis important cascaded MAP kinases to the midline as well [73]. The loss of NACK1 led severe defects in cytokinesis in somatic cells, and the loss of NACK2 led to disorganized phragmoplast MT arrays during male meiosis [105, 120]. The aforementioned MAP65-1 is a substrate of this MAP kinase cascade, and its phosphorylation directly contributes to the turnover of phragmoplast MTs [91, 92]. It has been interpreted that the failure of robust MT turnovers in cells lacking NACK may be the ultimate cause of cytokinetic failure.

Compared to other kinesins in the phragmoplast, the plus end-directed Kinesin-12 members exhibit more exclusive association with MT plus ends in the phragmoplast [52, 81]. At least two homologous motors function redundantly at MT plus ends in the phragmoplast to ensure that the plus ends are maintained at the division site [51]. When such a role is lost, Golgi-derived vesicles would not be able to produce a cell plate. Instead, cell wall materials would form large aggregates. It is unknown whether Kinesin-12 directly interacts with MAP65-3 for its localization.

Other mitotic kinesins likely contribute to different aspects of cytokinesis in plants. For example the POK1 and POK2 kinesins may play a redundant role in regulating the orientation of the phragmoplast [64]. Unfortunately, it is unknown whether they directly contribute to MT organization as their intracellular localization is unknown.

9.3 Organization of Microtubule Minus Ends in the Phragmoplast

In animal cells, the midbody elongates and the diameter of its MT array narrows down concomitant with the constriction of actomyosin ring [103]. In comparison, the phragmoplast MT array exhibits opposite phenomena. The overall length of the array reduces while its diameter increases. In other words, the number of MTs increases while their lengths are shortened. Thus, it is not surprising that γ-tubulin-dependent MT generation is essential for the progression of cytokinesis. The function of γ-tubulin in MT generation and subsequent organization in the phragmoplast is dependent on other components of the γ-tubulin complex as well as NEDD1 [44, 71, 122].

In the absence of a structurally defined MT-organizing center or MTOC, MT nucleation may take place predominantly on existing MTs in plant cells [66]. Does MT nucleation take place on the wall of preexisting MTs in the phragmoplast? Based on the localization of γ-tubulin and its associated factors, MT minus ends are distributed very much across phragmoplast MTs, but avoiding a region of ~1 μm from the phragmoplast midline (Fig. 9.3). Therefore, we can conclude that MT minus ends do not appear in the vicinity of the phragmoplast midline. The localization of γ-tubulin in the phragmoplast also differs from that of MTs by emphasizing regions toward the center where "old" MTs are (Fig. 9.3). We interpret the abundance of the γ-tubulin signal as representing the richness of MT nucleation events. Therefore, in addition to new MTs generated at distal ends of the phragmoplast toward the daughter nuclei, MT nucleation events likely take place on the wall of pre-existing MTs in the phragmoplast as well.

The significance of MT-dependent MT generation in mitosis and cytokinesis has gradually been recognized in animal cells, especially after the discovery of the augmin complex [26, 107]. Is such an augmin-NEDD1-γ-tubulin complex continuum conserved in plant cells? If so, it may be accountable for the majority of MT nucleation events in the phragmoplast as well as in the spindle.

We wish to envisage MT-dependent MT nucleation during the development of the phragmoplast MT array. If an augmin complex interacts with MT walls, the

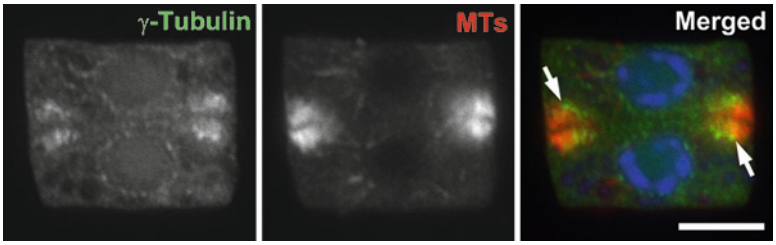

Fig. 9.3 Localization of γ-tubulin in the phragmoplast. The anti-γ-tubulin signal avoid the narrow region near the phragmoplast midline. It also preferentially appears toward the central/inner part of the phragmoplast (*arrows*). In *merged images*, γ-tubulin is pseudocolored in *green*, MTs in *red*, and DNA in *blue*. *Scale bar*, 5 μm

interaction would allow one or more γ-tubulin complexes to be anchored there, possibly through NEDD1 which acts as the linker. The establishment of such a continuum would serve as an anchoring site for MT minus ends so that a new MT could polymerize/branch off from the wall of an extant MT. Such events probably are initiated on midzone MTs upon the completion of anaphase. Consequently, rapid accumulation of newly born MTs can be seen as an MT-coalescing phenomenon in the central spindle.

We would also like to propose that a similar mechanism is responsible for the expansion of the phragmoplast MT array during the progression of cytokinesis. As discussed earlier, new MTs are nucleated toward the periphery of the phragmoplast while those in the central region are gradually depolymerized. If new MTs are generated from the wall of anti-parallel MTs, they grow toward the midline of the phragmoplast. Some of them may be captured by anti-parallel MT specific cross-linkers like MAP65 and stabilized. Others may be depolymerized once they encounter membranous structures of the developing cell plate. Gradual depolymerization of extant MTs in the centrifugal direction would lead to the loss of bases where the augmin-NEDD1-γ-tubulin complex continuum is anchored. Consequently, the activity of generating new MTs would be lost centrifugally until cytokinesis is completed.

We conclude that continuous MT nucleation is an indispensible process for the organization of phragmoplast MTs. Disorganized nucleation events may lead to collapse of phragmoplast MT array.

9.4 Organization of Microtubule Plus Ends in the Phragmoplast

In order for Golgi-derived vesicles and their fusion products to faithfully arrive at the cell division site, it is essential to have MT plus ends checked so that they would not pass through the midline. It is mysterious how plant cells achieve this seemingly simple but realistically challenging task as phragmoplast MTs are highly dynamic.

Many proteins specifically interact with MT plus ends and regulate MT dynamics and the interaction between the plus ends and other cellular entities. As a +TIP, EB1 is often considered to be essential for MT plus end activities. But the phragmoplast MT array develops normally in the absence of all three EB1 proteins in *Arabidopsis* [14, 43]. Thus, other MT-interacting factors must play critical roles in promoting MT polymerization and plus end organization.

MT plus ends in the phragmoplast are likely regulated by members of the MAP65 family based on their localization [100, 108]. The most studied member is MAP65-1 from both *Arabidopsis* and tobacco since their sequences were identified initially [97]. MAP65 proteins form homodimers *in vitro* and specifically bundles anti-parallel MTs [25, 99]. Such an anti-parallel MT specific bundling activity is well conserved among MAP65/Ase1 proteins in different kingdoms [40]. How they

distinguish anti-parallel MTs from parallel ones is unknown. Because MAP65 isoforms exhibit distinct localization patterns in the phragmoplast midzone, they probably have taken on different tasks in regulating MT organization there. In the absence of MAP65-3 in *Arabidopsis*, two sets of anti-parallel MTs do not seem to be connected [20, 65]. Thus, the engagement of anti-parallel MTs in the phragmoplast midline is primarily dependent on MAP65-3. Because most phragmoplast MTs do not overlap according to EM tomographic studies [7], we postulate that phragmoplast MTs consist of both interdigitating and non-interdigitating ones. Only the interdigitating MTs are cross-linked by MAP65-3 plus other proteins near their plus ends. Apparently such a bundling activity by MAP65-3 is critical for cytokinesis. The non-interdigitating MTs are essential for the bulk delivery of vesicles and their derivatives for the buildup of the cell plate.

In the moss *Physcomitrella patens*, two homologous kinesins play a critical role in MT interdigitation in the phragmoplast [33]. The motor domain of these moss kinesins are related to that of AtPAKRP2 in *A. thaliana*, which is associated with vesicles in the phragmoplast [50]. Recently, we have learned that in three null mutants of *AtPAKRP2* the bipolar MT organization pattern is not altered in the phragmoplast (Lee and Liu, unpublished). The moss and *Arabidopsis* kinesins likely have undertaken different tasks. This conclusion is also supported by the fact that their sequences outside their motor domains show no homology.

9.5 Stability and Turnovers of Microtubules in the Phragmoplast

As soon as the cell plate is being assembled, MTs exhibit a rapid turnover phenomenon. Toward the middle, MTs depolymerize from their minus ends upon the mission of vesicle delivery is accomplished. In the mean time, MT polymerization takes place at the advancing edge toward the periphery of the phragmoplast. As a result, the MT array of a mature phragmoplast with partially formed cell plate in the center exhibits the appearance of a concave lens when a longitudinal section is taken in the middle. How do the polymerization and depolymerization events take place simultaneously within several micrometers in the phragmoplast? A reasonable explanation would be having factors promoting polymerization and those stimulating depolymerization differentially localized.

Proteins like EB1, MOR1/TMAP200, and Kinesin-5 are likely among the critical factors that regulate MT dynamics in the phragmoplast. In *Arabidopsis*, the *eb1c* mutant cells show fragments of phragmoplast MT arrays after challenged by Oryzalin (Fig. 9.2). Similar defects were observed in the *mor1* mutant cells at restrictive temperatures [23, 41]. The *rsw7* mutation at a locus encoding the Kinesin-5 member AtKRP125c, which localizes along phragmoplast MTs, causes phragmoplast array to become fragmented as well [9]. Although these proteins are not required for maintaining the bipolar figure of MTs in these fragmented phragmoplasts, they likely contribute to the integrity of the phragmoplast MT array by promoting MT polymerization and/or stability.

Two highly homologous members of the Kinesin-14 subfamily, KATA/ATK1 and ATK5 likely act as MT-stabilizing factors in the phragmoplast as well. A fluorescent ATK5 fusion protein preferentially localizes to the advancing edge of the phragmoplast, toward the periphery [2]. Because ATK5 exhibits plus end-tracking activity, the motor probably plays a role in promoting MT polymerization at the advancing edge. Because it is a minus end-directed motor, it has been postulated that ATK5 could cross-link anti-parallel MTs and align the cross-linked MTs [2].

How are phragmoplast MTs depolymerized? The best known MT-depolymerizing or severing agents during mitosis are katanin and related proteins [88]. In *Arabidopsis*, the loss of katanin does not seem to affect MT organization in the phragmoplast [17]. So the protein responsible for stimulating MT depolymerization during cytokinesis is yet to be found. Whether katanin and its related proteins act on phragmoplast MTs awaits further investigations.

9.6 Regulation of Phragmoplast MT Dynamics by Post-Translational Modifications

Functions of proteins like MAP65 and kinesins may be regulated by phosphorylation during cell division. Both CDKA/Cdc2 and CDKB, the type A and B cyclin-dependent kinases, are associated with phragmoplast MTs [49, 115]. However, the mitotic cyclin CycB2 is rapidly degraded after metaphase [49]. It is known that multiple isoforms of MAP65 are phosphorylated by the CDK kinases. For example, MAP65-1 is hyperphosphorylated by CDK, and the phosphorylation events inhibit its association with MTs [98]. The artificial presence of non-phosphorylatable form would cause premature binding to the central spindle and consequently inhibit the progression of mitosis [62, 98]. This phenotype is consistent with the finding that the expression of a non-degradable form of cyclin B1 inhibits the formation of the phragmoplast [116]. Thus, the association of MAP65-1 with the central spindle and the phragmoplast MTs is likely triggered by a phosphatase-driven protein dephosphorylation event [62]. The impact of phosphorylation on the functions of other MAP65s, especially MAP65-3, is yet to be examined.

Besides CDKs, several novel kinases also act in the phragmoplast specifically. A MAP-kinase cascade forms a positive regulatory network during cytokinesis [47, 72]. This network is delivered to the phragmoplast by members of the Kinesin-7 subfamily, and acts at the phragmoplast midline [73, 104]. One of the substrates of the kinase cascade is MAP65-1 in tobacco cells [92]. Once MAP65-1 is phosphorylated near its C-terminus by the terminal kinase of the cascade, its MT-bundling activity is greatly compromised. Both the kinase and the phosphorylated form of MAP65-1 specifically localize to the phragmoplast midline [92]. A likely consequence is upon phosphorylation, MAP65-1's MT-bundling activity is down-regulated in regions near MT plus ends so that the turnover of phragmoplast MTs is stimulated.

Two other novel kinases localize to the phragmoplast and specifically regulate cytokinesis as well. The TIO kinase decorates the phragmoplast midline, and is

critical for phragmoplast operation [74]. The RUN kinase, which is a MAP, acts along phragmoplast MTs as well as MTs of other mitotic arrays [46]. Although both of them play critical roles in cytokinesis, their substrates are yet to be identified.

The appearance of the 26S proteasome subunits in the phragmoplast suggests that proteins associated with the apparatus like the Kinesin-5 member TKRP125 could be eliminated by the this protein degradation pathway upon completing their duties [76, 119]. Inhibition of the pathway would lead to the formation of extra phragmoplasts, probably due to the failure of down-regulation of factors promoting phragmoplast MT formation.

9.7 Conclusion: Assembly of the Phragmoplast by Mini-Phragmoplasts

It is concluded here that there are two populations of anti-parallel MTs in the phragmoplast: interdigitating MTs and non-interdigitating ones. The interdigitating MTs are cross-linked by factors like MAP65-3 and therefore become highly stable. They form the framework of the phragmoplast MT array, and are surrounded by non-interdigitating MTs which continuously polymerize toward the phragmoplast midline and depolymerize when encountering obstacles. Inspired by the mini-phragmoplast concept proposed a decade ago to describe the phragmoplast MT array during endosperm cellularization [79], we wish to view the phragmoplast MT array as a modular structure. Each module is a mini-phragmoplast containing an MT array in which a core of interdigitating MTs is surrounded by highly dynamic non-interdigitating MTs. Collectively, mini-phragmoplast modules are consolidated to establish a phragmoplast (Fig. 9.4). A new mini-phragmoplast MT

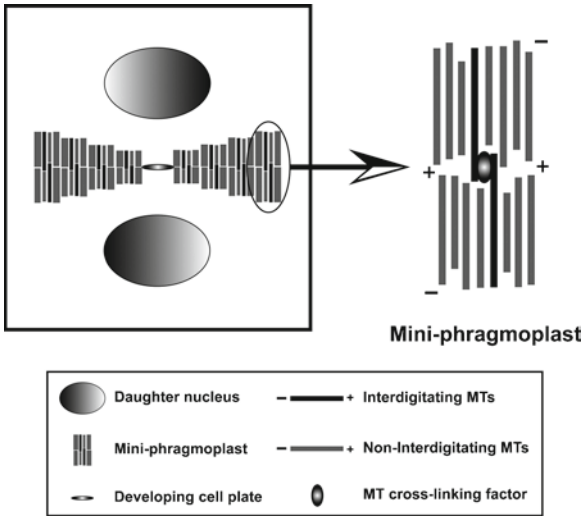

Fig. 9.4 Formation of the phragmoplast MT array by modules of mini-phragmoplasts. Keys of the diagram are shown on the *bottom*

module can be born when actively polymerizing anti-parallel MTs are stabilized by cross-linkers near their plus ends. The resulting interdigitating MTs will then be surrounded by more non-interdigitating MTs.

The formation of a mini-phragmoplast MT array may not require the preceding central spindle. When anti-parallel MTs encounter each other and are cross-linked near their plus ends, the core of a mini-phragmoplast is formed. Such events take place frequently between endosperm nuclei when the cellularization program is initiated for syncytial cytokinesis [16, 78, 79]. Similar events may occur elsewhere in the cytoplasm of a dividing cell. For example, fragments resembling mini-phragmoplasts were formed when MT polymerization were artificially induced [8]. Those so-called accessory phragmoplasts could be resulted from encountered anti-parallel MTs at sites where no such encountering would take place under normal circumstances.

Ultimately, the phragmoplast MT array serves as the powerhouse for the assembly of the cell plate using Golgi-derived vesicles as parts. Vesicles may be delivered along both populations of MTs. They may also surf at the plus ends of non-interdigitating MTs when the MTs are polymerized toward the cell division site. In the absence of interdigitating MTs, vesicles can still be delivered toward the phragmoplast midline. Only in the presence of interdigitating MTs, however, a dividing cell can harness the actions of individual mini-phragmoplasts to efficiently assemble the cell plate.

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References

1. Akhmanova A, Steinmetz MO (2008) Tracking the ends: a dynamic protein network controls the fate of microtubule tips. Nat Rev Mol Cell Biol 9:309–322
2. Ambrose JC, Li W, Marcus A, Ma H, Cyr R (2005) A minus-end-directed kinesin with plus-end tracking protein activity is involved in spindle morphogenesis. Mol Biol Cell 16:1584–1592
3. Ambrose JC, Shoji T, Kotzer AM, Pighin JA, Wasteneys GO (2007) The *Arabidopsis* CLASP gene encodes a microtubule-associated protein involved in cell expansion and division. Plant Cell 19:2763–2775
4. Asada T, Kuriyama R, Shibaoka H (1997) TKRP125, a kinesin-related protein involved in the centrosome-independent organization of the cytokinetic apparatus in tobacco BY-2 cells. J Cell Sci 110:179–189
5. Asada T, Shibaoka H (1994) Isolation of polypeptides with microtubule-translocating activity from phragmoplasts of tobacco BY-2 cells. J Cell Sci 107:2249–2257
6. Asada T, Sonobe S, Shibaoka H (1991) Microtubule translocation in the cytokinetic apparatus of cultured tobacco cells. Nature 350:238–241
7. Austin JR, Segui-Simarro JM, Staehelin LA (2005) Quantitative analysis of changes in spatial distribution and plus-end geometry of microtubules involved in plant-cell cytokinesis. J Cell Sci 118:3895–3903

8. Bajer AS, Smirnova EA, Mole-Bajer J (1993) Microtubule-converging centers-implications for microtubule dynamics in higher plants. In: Vig BK (ed) Chromosome segregation and aneuploidy. Springer, Berlin, pp 225–239
9. Bannigan A, Scheible W-R, Lukowitz W, Fagerstrom C, Wadsworth P, Somerville C, Baskin TI (2007) A conserved role for kinesin-5 in plant mitosis. J Cell Sci 120:2819–2827
10. Barroso C, Chan J, Allan V, Doonan J, Hussey P, Lloyd C (2000) Two kinesin-related proteins associated with the cold-stable cytoskeleton of carrot cells: characterization of a novel kinesin, DcKRP120-2. Plant J 24:859–868
11. Bednarek SY, Falbel TG (2002) Membrane trafficking during plant cytokinesis. Traffic 3:621–629
12. Binarova P, Cenklova V, Prochazkova J, Doskocilova A, Volc J, Vrlik M, Bogre L (2006) γ-Tubulin is essential for acentrosomal microtubule nucleation and coordination of late mitotic events in *Arabidopsis*. Plant Cell 18:1199–1212
13. Bisgrove S, Hable W, Kropf D (2004) +TIPs and microtubule regulation. The beginning of the plus end in plants. Plant Physiol 136:3855–3863
14. Bisgrove SR, Lee Y-RJ, Liu B, Peters N, Kropf DL (2008) The microtubule plus-end binding protein EB1 functions in root responses to touch and gravity signals in *Arabidopsis*. Plant Cell 20:396–410
15. Bowser J, Reddy ASN (1997) Localization of a kinesin-like calmodulin-binding protein in dividing cells of *Arabidopsis* and tobacco. Plant J 12:1429–1437
16. Brown RC, Lemmon BE (1992) Cytoplasamic domain: a model for spatial control of cytokinesis in reproductive cells of plants. EMSA Bull 22:48–53
17. Burk DH, Liu B, Zhong R, Morrison WH, Ye Z-H (2001) A katanin-like protein regulates normal cell wall biosynthesis and cell elongation. Plant Cell 13:807–827
18. Buschmann H, Chan J, Sanchez-Pulido L, Andrade-Navarro MA, Doonan JH, Lloyd CW (2006) Microtubule-associated AIR9 recognizes the cortical division site at preprophase and cell-plate insertion. Curr Biol 16:1938–1943
19. Buschmann H, Fabri CO, Hauptmann M, Hutzler P, Laux T, Lloyd CW, Schäffner AR (2004) Helical growth of the *Arabidopsis* mutant *tortifolia1* reveals a plant-specific microtubule-associated protein. Curr Biol 14:1515–1521
20. Caillaud M-C, Lecomte P, Jammes F, Quentin M, Pagnotta S, Andrio E, de Almeida Engler J, Marfaing N, Gounon P, Abad P, Favery B (2008) MAP65-3 microtubule-associated protein is essential for nematode-induced giant cell ontogenesis in *Arabidopsis*. Plant Cell 19:423–437
21. Chan J, Calder GM, Doonan JH, Lloyd CW (2003) EB1 reveals mobile microtubule nucleation sites in *Arabidopsis*. Nat Cell Biol 5:967–971
22. Dixit R, Chang E, Cyr R (2006) Establishment of polarity during organization of the acentrosomal plant cortical microtubule array. Mol Biol Cell 17:1298–1305
23. Eleftheriou EP, Baskin TI, Hepler PK (2005) Aberrant cell plate formation in the *Arabidopsis thaliana* microtubule organization 1 mutant. Plant Cell Physiol 46:671–675
24. Euteneuer U, McIntosh JR (1980) Polarity of midbody and phragmoplast microtubules. J Cell Biol 87:509–515
25. Gaillard J, Neumann E, Van Damme D, Stoppin-Mellet V, Ebel C, Barbier E, Geelen D, Vantard M (2008) Two microtubule-associated proteins of *Arabidopsis* MAP65s promote antiparallel microtubule bundling. Mol Biol Cell 19:4534–4544
26. Goshima G, Kimura A (2010) New look inside the spindle: microtubule-dependent microtubule generation within the spindle. Curr Opin Cell Biol 22:44–49
27. Graham LE, Cook ME, Busse JS (2000) The origin of plants: body plan changes contributing to a major evolutionary radiation. Proc Natl Acad Sci USA 97:4535–4540
28. Gunawardane RN, Martin OC, Zheng YX (2003) Characterization of a new γTuRC subunit with WD repeats. Mol Biol Cell 14:1017–1026
29. Guo L, Ho C-MK, Kong Z, Lee Y-RJ, Qian Q, Liu B (2009) Evaluating the microtubule cytoskeleton and its interacting proteins in monocots by mining the rice genome. Ann Bot 103:387–402
30. Hamada T (2007) Microtubule-associated proteins in higher plants. J Plant Res 120:79–98

31. Hamada T, Igarashi H, Itoh TJ, Shimmen T, Sonobe S (2004) Characterization of a 200 kDa microtubule-associated protein of tobacco BY-2 cells, a member of the XMAP215/MOR1 family. Plant Cell Physiol 45:1233–1242
32. Hepler PK, Jackson WT (1968) Microtubules and early stages of cell-plate formation in the endosperm of *Haemanthus katherinae* Baker. J Cell Biol 38:437–446
33. Hiwatashi Y, Obara M, Sato Y, Fujita T, Murata T, Hasebe M (2008) Kinesins are indispensable for interdigitation of phragmoplast microtubules in the moss *Physcomitrella patens*. Plant Cell 20:3094–3106
34. Hong Z, Verma DPS (2007) Molecular analysis of the cell plate forming machinery. In: Verma DPS, Hong Z (eds) Cell division control in plants. Springer, Berlin, pp 303–320
35. Hush JM, Wadsworth P, Callaham DA, Hepler PK (1994) Quantification of microtubule dynamics in living plant cells using fluorescence redistribution after photobleaching. J Cell Sci 107:775–784
36. Jürgens G (2004) Membrane trafficking in plants. Annu Rev Cell Dev Biol 20:481–504
37. Jürgens G (2005) Cytokinesis in higher plants. Annu Rev Plant Biol 56:281–299
38. Jürgens G (2005) Plant cytokinesis: fission by fusion. Trends Cell Biol 15:277–283
39. Kaloriti D, Galva C, Parupalli C, Khalifa N, Galvin M, Sedbrook JC (2007) Microtubule associated proteins in plants and the processes they manage. J Integr Plant Biol 49:1164–1173
40. Kapitein LC, Janson ME, van den Wildenberg S, Hoogenraad CC, Schmidt CF, Peterman EJG (2008) Microtubule-driven multimerization recruits ase1p onto overlapping microtubules. Curr Biol 18:1713–1717
41. Kawamura E, Himmelspach R, Rashbrooke MC, Whittington AT, Gale KR, Collings DA, Wasteneys GO (2006) MICROTUBULE ORGANIZATION 1 regulates structure and function of microtubule arrays during mitosis and cytokinesis in the *Arabidopsis* root. Plant Physiol 140:102–114
42. Kirik V, Herrmann U, Parupalli C, Sedbrook JC, Ehrhardt DW, Hülskamp M (2007) CLASP localizes in two discrete patterns on cortical microtubules and is required for cell morphogenesis and cell division in *Arabidopsis*. J Cell Sci 120:4416–4425
43. Komaki S, Abe T, Coutuer S, Inzé D, Russinova E, Hashimoto T (2010) Nuclear-localized subtype of end-binding 1 protein regulates spindle organization in *Arabidopsis*. J Cell Sci 123:451–459
44. Kong Z, Hotta T, Lee YR, Horio T, Liu B (2010) The γ-tubulin complex protein GCP4 is required for organizing functional microtubule arrays in *Arabidopsis thaliana*. Plant Cell 22:191–204
45. Korolev AV, Chan J, Naldrett MJ, Doonan JH, Lloyd CW (2005) Identification of a novel family of 70 kDa microtubule-associated proteins in *Arabidopsis* cells. Plant J 42:547–555
46. Krupnova T, Sasabe M, Ghebreghiorghis L, Gruber CW, Hamada T, Dehmel V, Strompen G, Stierhof YD, Lukowitz W, Kemmerling B, Machida Y, Hashimoto T, Mayer U, Jürgens G (2009) Microtubule-associated kinase-like protein RUNKEL needed for cell plate expansion in *Arabidopsis* cytokinesis. Curr Biol 19:518–523
47. Krysan P, Jester P, Gottwald J, Sussman M (2002) An *Arabidopsis* mitogen-activated protein kinase kinase kinase gene family encodes essential positive regulators of cytokinesis. Plant Cell 14:1109–1120
48. Ledbetter MC, Porter KR (1963) A "microtubule" in plant cell fine structure. J Cell Biol 19:239–250
49. Lee J, Das A, Yamaguchi M, Hashimoto J, Tsutsumi N, Uchimiya H, Umeda M (2003) Cell cycle function of a rice B2-type cyclin interacting with a B-type cyclin-dependent kinase. Plant J 34:417–425
50. Lee Y-RJ, Giang HM, Liu B (2001) A novel plant kinesin-related protein specifically associates with the phragmoplast organelles. Plant Cell 13:2427–2439
51. Lee YRJ, Li Y, Liu B (2007) Two *Arabidopsis* phragmoplast-associated kinesins play a critical role in cytokinesis during male gametogenesis. Plant Cell 19:2595–2605
52. Lee YRJ, Liu B (2000) Identification of a phragmoplast-associated kinesin-related protein in higher plants. Curr Biol 10:797–800

53. Liu B, Cyr RJ, Palevitz BA (1996) A kinesin-like protein, KatAp, in the cells of *Arabidopsis* and other plants. Plant Cell 8:119–132
54. Liu B, Joshi HC, Palevitz BA (1995) Experimental manipulation of γ-tubulin distribution in *Arabidopsis* using anti-microtubule drugs. Cell Motil Cytoskeleton 31:113–129
55. Liu B, Joshi HC, Wilson TJ, Silflow CD, Palevitz BA, Snustad DP (1994) γ-Tubulin in *Arabidopsis*-gene sequence, immunoblot, and immunofluorescence studies. Plant Cell 6:303–314
56. Liu B, Marc J, Joshi HC, Palevitz BA (1993) A γ-tubulin-related protein associated with the microtubule arrays of higher plants in a cell cycle-dependent manner. J Cell Sci 104:1217–1228
57. Liu B, Palevitz BA (1996) Localization of a kinesin-like protein in generative cells of tobacco. Protoplasma 195:78–89
58. Liu L, Wiese C (2008) Xenopus NEDD1 is required for microtubule organization in Xenopus egg extracts. J Cell Sci 121:578–589
59. Lloyd C, Chan J (2006) Not so divided: the common basis of plant and animal cell division. Nat Rev Mol Cell Biol 7:147–152
60. Lüders J, Patel UK, Stearns T (2006) GCP-WD is a γ-tubulin targeting factor required for centrosomal and chromatin-mediated microtubule nucleation. Nat Cell Biol 8:137–147
61. Maiato H, Fairley EA, Rieder CL, Swedlow JR, Sunkel CE, Earnshaw WC (2003) Human CLASP1 is an outer kinetochore component that regulates spindle microtubule dynamics. Cell 113:891–904
62. Mao GJ, Chan J, Calder G, Doonan JH, Lloyd CW (2005) Modulated targeting of GFP-AtMAP65-1 to central spindle microtubules during division. Plant J 43:469–478
63. Marc J, Granger CL, Brincat J, Fisher DD, Kao T-h, McCubbin AG, Cyr RJ (1998) A GFP-MAP4 reporter gene for visualizing cortical microtubule rearrangements in living epidermal cells. Plant Cell 10:1927–1940
64. Müller S, Han S, Smith LG (2006) Two kinesins are involved in the spatial control of cytokinesis in *Arabidopsis thaliana*. Curr Biol 16:888–894
65. Müller S, Smertenko A, Wagner V, Heinrich M, Hussey P, Hauser M (2004) The plant microtubule-associated protein AtMAP65-3/PLE is essential for cytokinetic phragmoplast function. Curr Biol 14:412–417
66. Murata T, Hasebe M (2007) Microtubule-dependent microtubule nucleation in plant cells. J Plant Res 120:73–78
67. Murata T, Sonobe S, Baskin TI, Hyodo S, Hasezawa S, Nagata T, Horio T, Hasebe M (2005) Microtubule-dependent microtubule nucleation based on recruitment of γ-tubulin in higher plants. Nat Cell Biol 7:961–968
68. Murata T, Tanahashi T, Nishiyama T, Yamaguchi K, Hasebe M (2007) How do plants organize microtubules without a centrosome? J Integr Plant Biol 49:1154–1163
69. Nakajima K, Furutani I, Tachimoto H, Matsubara H, Hashimoto T (2004) *SPIRAL1* encodes a plant-specific microtubule-localized protein required for directional control of rapidly expanding *Arabidopsis* cells. Plant Cell 16:1178–1190
70. Nakajima K, Kawamura T, Hashimoto T (2006) Role of the *SPIRAL1* gene family in anisotropic growth of *Arabidopsis* thaliana. Plant Cell Physiol 47:513–522
71. Nakamura M, Hashimoto T (2009) A mutation in the *Arabidopsis* γ-tubulin-containing complex causes helical growth and abnormal microtubule branching. J Cell Sci 122: 2208–2217
72. Nishihama R, Ishikawa M, Araki S, Soyano T, Asada T, Machida Y (2001) The NPK1 mitogen-activated protein kinase kinase kinase is a regulator of cell-plate formation in plant cytokinesis. Genes Dev 15:352–363
73. Nishihama R, Soyano T, Ishikawa M, Araki S, Tanaka H, Asada T, Irie K, Ito M, Terada M, Banno H, Yamazaki Y, Machida Y (2002) Expansion of the cell plate in plant cytokinesis requires a kinesin-like protein/MAPKKK complex. Cell 109:87–99
74. Oh SA, Johnson A, Smertenko A, Rahman D, Park SK, Hussey PJ, Twell D (2005) A divergent cellular role for the FUSED kinase family in the plant-specific cytokinetic phragmoplast. Curr Biol 15:2107–2111

75. Oh SA, Pal MD, Park SK, Johnson JA, Twell D (2010) The tobacco MAP215/Dis1-family protein TMBP200 is required for the functional organization of microtubule arrays during male germline establishment. J Exp Bot 61:969–981
76. Oka M, Yanagawa Y, Asada T, Yoneda A, Hasezawa S, Sato T, Nakagawa H (2004) Inhibition of proteasome by MG-132 treatment causes extra phragmoplast formation and cortical microtubule disorganization during M/G1 transition in synchronized tobacco cells. Plant Cell Physiol 45:1623–1632
77. Oppenheimer DG, Pollock MA, Vacik J, Szymanski DB, Ericson B, Feldmann K, Marks MD (1997) Essential role of a kinesin-like protein in *Arabidopsis* trichome morphogenesis. Proc Natl Acad Sci USA 94:6261–6266
78. Otegui M, Staehelin LA (2000) Cytokinesis in flowering plants: more than one way to divide a cell. Curr Opin Plant Biol 3:493–502
79. Otegui M, Staehelin LA (2000) Syncytial-type cell plates: a novel kind of cell plate involved in endosperm cellularization of *Arabidopsis*. Plant Cell 12:933–947
80. Otegui MS, Verbrugghe KJ, Skop AR (2005) Midbodies and phragmoplasts: analogous structures involved in cytokinesis. Trends Cell Biol 15:404–413
81. Pan R, Lee YRJ, Liu B (2004) Localization of two homologous *Arabidopsis* kinesin-related proteins in the phragmoplast. Planta 220:156–164
82. Pastuglia M, Azimzadeh J, Goussot M, Camilleri C, Belcram K, Evrard JL, Schmit AC, Guerche P, Bouchez D (2006) γ-Tubulin is essential for microtubule organization and development in *Arabidopsis*. Plant Cell 18:1412–1425
83. Perrin RM, Wang Y, Yuen CY, Will J, Masson PH (2007) WVD2 is a novel microtubule-associated protein in *Arabidopsis thaliana*. Plant J 49:961–971
84. Pickett-Heaps JD, Gunning BE, Brown RC, Lemmon BE, Cleary AL (1999) The cytoplast concept in dividing plant cells: cytoplasmic domains and the evolution of spatially organized cell division. Am J Bot 86:153–172
85. Pignocchi C, Minns GE, Nesi N, Koumproglou R, Kitsios G, Benning C, Lloyd CW, Doonan JH, Hills MJ (2009) ENDOSPERM DEFECTIVE1 is a novel microtubule-associated protein essential for seed development in *Arabidopsis*. Plant Cell 21:90–105
86. Preuss ML, Delmer DP, Liu B (2003) The cotton kinesin-like calmodulin-binding protein associates with cortical microtubules in cotton fibers. Plant Physiol 132:154–160
87. Richardson DN, Simmons MP, Reddy AS (2006) Comprehensive comparative analysis of kinesins in photosynthetic eukaryotes. BMC Genomics 7:18
88. Roll-Mecak A, McNally FJ (2010) Microtubule-severing enzymes. Curr Opin Cell Biol 22:96–103
89. Samuels AL, Giddings TH, Staehelin LA (1995) Cytokinesis in tobacco BY-2 and root tip cells – a new model of cell plate formation in higher plants. J Cell Biol 130:1345–1357
90. Sanderfoot AA (2007) Vesicle traffic at cytokinesis. In: Verma DPS, Hong Z (eds) Cell division control in plants. Springer, Berlin, pp 289–302
91. Sasabe M, Machida Y (2006) MAP65: a bridge linking a MAP kinase to microtubule turnover. Curr Opin Plant Biol 9:563–570
92. Sasabe M, Soyano T, Takahashi Y, Sonobe S, Igarashi H, Itoh TJ, Hidaka M, Machida Y (2006) Phosphorylation of NtMAP65-1 by a MAP kinase down-regulates its activity of microtubule bundling and stimulates progression of cytokinesis of tobacco cells. Genes Dev 20:1004–1014
93. Sedbrook JC, Ehrhardt DW, Fisher SE, Scheible WR, Somerville CR (2004) The *Arabidopsis* SKU6/SPIRAL1 gene encodes a plus end-localized microtubule-interacting protein involved in directional cell expansion. Plant Cell 16:1506–1520
94. Seguí-Simarro JM, Otegui MS, Austin JR, Staehelin LA (2007) Plant cytokinesis-insights gained from electron tomography studies. In: Verma DPS, Hong Z (eds) Cell division control in plants. Springer, Berlin, pp 251–287
95. Seltzer V, Janski N, Canaday J, Herzog E, Erhardt M, Evrard JL, Schmit AC (2007) *Arabidopsis* GCP2 and GCP3 are part of a soluble gamma-tubulin complex and have nuclear envelope targeting domains. Plant J 52:322–331

96. Shoji T, Narita NN, Hayashi K, Asada J, Hamada T, Sonobe S, Nakajima K, Hashimoto T (2004) Plant-specific microtubule-associated protein SPIRAL2 is required for anisotropic growth in *Arabidopsis*. Plant Physiol 136:3933–3944
97. Smertenko A, Saleh N, Igarashi H, Mori H, Hauser-Hahn I, Jiang C-J, Sonobe S, Lloyd CW, Hussey PJ (2000) A new class of microtubule-associated proteins in plants. Nat Cell Biol 2:750–753
98. Smertenko AP, Chang HY, Sonobe S, Fenyk SI, Weingartner M, Bogre L, Hussey PJ (2006) Control of the AtMAP65-1 interaction with microtubules through the cell cycle. J Cell Sci 119:3227–3237
99. Smertenko AP, Chang HY, Wagner V, Kaloriti D, Fenyk S, Sonobe S, Lloyd C, Hauser MT, Hussey PJ (2004) The *Arabidopsis* microtubule-associated protein AtMAP65-1: molecular analysis of its microtubule bundling activity. Plant Cell 16:2035–2047
100. Smertenko AP, Kaloriti D, Chang HY, Fiserova J, Opatrny Z, Hussey PJ (2008) The C-terminal variable region specifies the dynamic properties of *Arabidopsis* microtubule-associated protein MAP65 isotypes. Plant Cell 20:3346–3358
101. Smith LG (2002) Plant cytokinesis: motoring to the finish. Curr Biol 12:R206–R208
102. Staehelin LA, Hepler PK (1996) Cytokinesis in higher plants. Cell 84:821–824
103. Steigemann P, Gerlich DW (2009) Cytokinetic abscission: cellular dynamics at the midbody. Trends Cell Biol 19:606–616
104. Strompen G, El Kasmi F, Richter S, Lukowitz W, Assaad FF, Jurgens G, Mayer U (2002) The *Arabidopsis* HINKEL gene encodes a kinesin-related protein involved in cytokinesis and is expressed in a cell cycle-dependent manner. Curr Biol 12:153–158
105. Tanaka H, Ishikawa M, Kitamura S, Takahashi Y, Soyano T, Machida C, Machida Y (2004) The *AtNACK1/HINKEL* and *STUD/TETRASPORE/AtNACK2* genes, which encode functionally redundant kinesins, are essential for cytokinesis in *Arabidopsis*. Genes Cells 9:1199–1211
106. Twell D, Park SK, Hawkins TJ, Schubert D, Schmidt R, Smertenko A, Hussey PJ (2002) MOR1/GEM1 has an essential role in the plant-specific cytokinetic phragmoplast. Nat Cell Biol 4:711–714
107. Uehara R, Nozawa RS, Tomioka A, Petry S, Vale RD, Obuse C, Goshima G (2009) The augmin complex plays a critical role in spindle microtubule generation for mitotic progression and cytokinesis in human cells. Proc Natl Acad Sci USA 106:6998–7003
108. Van Damme D, Bouget FY, Van Poucke K, Inze D, Geelen D (2004) Molecular dissection of plant cytokinesis and phragmoplast structure: a survey of GFP-tagged proteins. Plant J 40:386–398
109. Van Damme D, Van Poucke K, Boutant E, Ritzenthaler C, Inze D, Geelen D (2004) In vivo dynamics and differential microtubule-binding activities of MAP65 proteins. Plant Physiol 136:3956–3967
110. Vanstraelen M, Inze D, Geelen D (2006) Mitosis-specific kinesins in *Arabidopsis*. Trends Plant Sci 11:167–175
111. Vantard M, Levilliers N, Hill AM, Adoutte A, Lambert AM (1990) Incorporation of paramecium axonemal tubulin into higher plant cells reveals functional sites of microtubule assembly. Proc Natl Acad Sci USA 87:8825–8829
112. Verma DPS (2001) Cytokinesis and building of the cell plate in plants. Annu Rev Plant Physiol Plant Mol Biol 52:751–784
113. Vos JW, Pieuchot L, Evrard JL, Janski N, Bergdoll M, de Ronde D, Perez LH, Sardon T, Vernos I, Schmit AC (2008) The plant TPX2 protein regulates prospindle assembly before nuclear envelope breakdown. Plant Cell 20:2783–2797
114. Vos JW, Safadi F, Reddy ASN, Hepler PK (2000) The kinesin-like calmodulin binding protein is differentially involved in cell division. Plant Cell 12:979–990
115. Weingartner M, Binarova P, Drykova D, Schweighofer A, David JP, Heberle-Bors E, Doonan J, Bögre L (2001) Dynamic recruitment of Cdc2 to specific microtubule structures during mitosis. Plant Cell 13:1929–1943
116. Weingartner M, Criqui MC, Mészáros T, Binarova P, Schmit AC, Helfer A, Derevier A, Erhardt M, Bögre L, Genschik P (2004) Expression of a nondegradable cyclin B1 affects

plant development and leads to endomitosis by inhibiting the formation of a phragmoplast. Plant Cell 16:643–657
117. Whittington AT, Vugrek O, Wei KJ, Hasenbein NG, Sugimoto K, Rashbrooke MC, Wasteneys GO (2001) MOR1 is essential for organizing cortical microtubules in plants. Nature 411:610–613
118. Wu G, Lin YT, Wei R, Chen Y, Shan Z, Lee WH (2008) Hice1, a novel microtubule-associated protein required for maintenance of spindle integrity and chromosomal stability in human cells. Mol Cell Biol 28:3652–3662
119. Yanagawa Y, Hasezawa S, Kumagai F, Oka M, Fujimuro M, Naito T, Makino T, Yokosawa H, Tanaka K, Komamine A, Hashimoto J, Sato T, Nakagawa H (2002) Cell-cycle dependent dynamic change of 26S proteasome distribution in tobacco BY-2 cells. Plant Cell Physiol 43:604–613
120. Yang C, Spielman M, Coles J, Li Y, Ghelani S, Bourdon V, Brown R, Lemmon B, Scott R, Dickinson H (2003) *TETRASPORE* encodes a kinesin required for male meiotic cytokinesis in *Arabidopsis*. Plant J 34:229–240
121. Yasuhara H, Shibaoka H (2000) Inhibition of cell-plate formation by brefeldin A inhibited the depolymerization of microtubules in the central region of the phragmoplast. Plant Cell Physiol 41:300–310
122. Zeng CJ, Lee YR, Liu B (2009) The WD40 repeat protein NEDD1 functions in microtubule organization during cell division in *Arabidopsis thaliana*. Plant Cell 21:1129–1140
123. Zhang DH, Wadsworth P, Hepler PK (1990) Microtubule dynamics in living dividing plant cells – confocal imaging of microinjected fluorescent brain tubulin. Proc Natl Acad Sci USA 87:8820–8824
124. Zhu H, Coppinger JA, Jang CY, Yates JR, Fang G (2008) FAM29A promotes microtubule amplification via recruitment of the NEDD1-gamma-tubulin complex to the mitotic spindle. J Cell Biol 183:835–848

Part III
The Cytoskeleton in Plant Growth and Development

Chapter 10
Signaling to the Cytoskeleton in Diffuse Cell Growth

Ying Fu and Zhenbiao Yang

Plant cells develop into various shapes to fulfill their specialized functions. Due to the presence of the cell wall, plant cells achieve their final morphology via polar cell expansion. Polar cell growth can be broadly grouped into two categories: tip growth and diffuse growth. Root hairs and pollen tubes are typical tip-growing cells, in which growth is locally focused at the leading edge of the cell (i.e., the tip) by F-actin dependent localized exocytosis. For most plant cells, membrane growth occurs over a large or entire area of the cell surface while cells expand directionally due to differential cell wall extensibility, a mode of cell expansion termed polar diffuse growth. This mode of cell expansion is believed to be responsible for cell elongation in roots, stems, and other organs. For certain cells such as the complex interlocking the pavement cells, a mixture of both tip growth and diffuse growth may be involved [52].

The plant cytoskeleton plays a critical role in cell morphogenesis and is composed of microtubules (MTs) and actin filaments (microfilaments, MFs). Evidence suggests that the cytoskeleton is capable of responding to various developmental cues and external environmental signals, and modulates many aspects of plant cell development such as cell division, organelle movement, cell polarity formation, polar cell growth, cell shape changes, and cellular responses to biotic and abiotic stresses [37, 48, 52, 61, 77, 83, 94, 96, 106, 110]; #106. The roles of the cytoskeleton have been inferred from genetic mutants as well as pharmacological treatments using the cytoskeleton-interacting drugs [37, 52, 83, 98].

The deformation of plant cells due to defects in the organization and dynamics of the cytoskeleton is well-documented [37, 52, 83, 86, 97, 98, 106]. Cortical MTs are thought to play a primary role in the regulation of polar diffusion growth, but increasing evidence supports a role for F-actin in this process as well [106]. As discussed elsewhere in this volume, each cell may contain multiple structurally and functionally distinct populations of F-actin or MTs. Mutations in G-actin or tubulin or pharmacological treatments usually do not distinguish these subpopulations and

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have limited values in pinpointing the precise function of the cytoskeleton. Furthermore, genetic mutations result in terminal phenotypes and thus are inherently limited in resolving cause and effect between cell shape and the cytoskeleton. Understanding specific signals and their signaling pathways leading to specific changes in the reorganization and dynamics of MTs and MFs can produce direct insights into cytoskeletal functions. In principle the targets of these signaling pathways are actin-binding proteins (ABPs) or microtubule associated proteins (MAPs), proteins that directly regulate the cytoskeletal structure [28, 31, 39, 83, 106]. Thus signals and pathways that specifically regulate a subpopulation of the cytoskeleton that influences polar diffuse growth will be the focus of this chapter.

10.1 Signals Modulating Diffuse Cell Growth

Diffuse growth-based polar cell expansion in plants is regulated by numerous signals, including phytohormones, mechanical forces, developmental signals and various environmental cues such as light, gravity, pathogens, and abiotic stresses. Environmental signals may regulate cell expansion via their effects on plant hormones [1, 10, 38, 78, 85]. The mechanisms by which various signals regulate polar cell expansion are unclear, but accumulating evidence supports the participation of the cytoskeleton [24, 38, 106].

10.1.1 Phytohormones

Treatments with various phytohormones, such as auxin, brassinosteroids (BR), gibberellic acid (GA) and ethylene, induce re-organization of the cytoskeleton and alter cell elongation/expansion, which imply that these phytohormones act as signals to regulate polar cell growth through modulating the plant cytoskeleton. Leaf epidermal cells and hypocotyl and root cells have been favorite systems for the investigation of how phytohormones regulate cell and organ morphogenesis, and how they coordinate these physiological processes through their complex interactions [17, 18, 23, 92, 102, 106]. In these cell systems, well-ordered cortical MTs are associated with cell elongation and appear to be essential for anisotropic growth to form elongated cells. The organization of such cortical MTs seems to be regulated by multiple hormones. For example, brassinolide (BL) alone can promote transversely orientated MTs in epidermal cells of azuki bean epicotyls [55]. A brassinosteriod (BR) biosynthesis mutant (*bul1-1/dwf7-3/ste1-4*) is defective in α-tubulin expression and cortical MT organization [13, 14]. Treatment of this mutant with brassinosteroid causes reorganization of MT into correct orientation, which is a process that independent of the activation of α-tubulin expression or the increase in tubulin content [13, 14]. GA is another hormone that promotes cell elongation and expansion. GA-insensitive mutants *ga-1* and *gai* display dwarf phenotype whereas

a constitutive-GA-response mutant *spy* exhibits elongated phenotype. GA was reported be able to align cortical MT transversely to the elongating direction in growing cells [34, 57, 58, 76, 99]. Similarly, auxin is able to stimulate the formation of transverse MTs in shoot cells of different plants [63, 71, 89, 107]. Auxin can also induce the cortical MT randomization in root hair-forming cells, and this process can be promoted by another phytohormone, ethylene [88]. In addition, ethylene treatment-induced MT alignment as well as cell elongation has also been observed in leaf epidermal cells at 23°C, but not at 31°C [74]. There is genetic evidence as well that ethylene has a role in MT regulation during cell growth: a loss-of-function mutant of a ethylene receptor ETR2 (*etr2-3*) exhibits random and disorganized MT network in a subset of trichomes with fewer branches in contrast to longitudinally orientated MTs observed in WT trichomes. Furthermore, stabilization of MTs by application of paclitaxol or overexpression of MAP4 partially rescued the *etr2-3* defects in the MT organization and trichome branching [65]. These observations indicate that ethylene regulates polar cell growth at least partially via modulating MT cytoskeleton.

Phytohormones also modulate the organization of actin microfilaments. Auxin-mediated actin dynamics and reorganization may participate in cell expansion, division and differentiation. Auxin was reported to induce differential expression of an actin gene, *ACT7* [42]. Addition of exogenous auxin induced the formation of fine MF strands in the epidermal cells of rice coleoptile segments, which suggests a role for auxin in stimulating the dynamics of MFs [38, 93, 95]. Interestingly, Rahman et al. reported that different forms of auxin caused opposite effects on MFs organization. IAA (indole acetic acid) and NAA (1-naphthalene acetic acid) subtly increase MFs bundling, as does the auxin transport inhibitor TIBA (tri-iodobenzoic acid), whereas 2,4-D (2,4-dichlorophenoxyacetic acid) tended to remove MF, which mimics the effect of the actin-depolymerization drug, latrunculin B [68]. One possible explanation for these observations is that different forms of auxin activate distinct auxin signaling pathways that impact different types of actin dynamics/reorganization. Work from Peter Nick's group suggests that the form of auxin that promotes rapid cell expansion is different from that regulates cell division [12]. Auxin promotion of cell expansion may involve changes in cortical fine MFs that is known to associate with polar cell expansion such as in pavement cells [30, 32].

ABA is another hormone that may play a role in actin regulation. Exogenous application of ABA induces swollen root hairs and cortical cells in rice roots, as well as the expression of several proteins including ADF (actin depolymerization factor). ADF is an ABP that has been suggested to promote MF disassembly. It is proposed that ABA has a role in controlling the phosphorylation status of ADF, and thereby affects MF rearrangement [15]. However, it is still unclear whether ABA-ADF mediated MF rearrangement accounts for the abnormal polar growth of root hairs and cortex cells. Dong et al. reported that overexpression of AtADF1 in *Arabidopsis* resulted in the disappearance of thick actin cables and reduced the elongation of cells and organs, but the radial expansion of certain cells and organs was increased. In contrast, reduction of AtADF1 promoted formation of actin cables

and stimulated the elongation of cells as well as organs [22]. These results suggest that ABA may have role in regulating MF organization via ADF to control polar cell growth.

On the other hand, strong evidence suggests that the plant cytoskeleton may play an important role in feedback regulation of phytohormone signaling. For example, GA treatment increased mRNA level of an MT-severing protein AtKSS1 [6]. MT-severing activity modulates MT bundling and alignment [84, 100, 101]. It is interesting that AtKSS activity or MT seems to be involved in feedback modulation of GA biosynthesis via the expression of a biosynthetic GA20-oxidase, AtGA20ox1 [6, 56]. The AtKSS protein mutant *leu1/fra2/bot1* displayed high levels of *AtGA20ox1* mRNA.

The actin cytoskeleton has been implicated in the regulation of polar auxin transport by modulating polar targeting of PIN proteins [5, 43, 60]. In rice plants, expression of an ABP talin, which caused bundling of actin filaments, reduced auxin polar transport [64]. Interestingly normal organization of MFs was restored by addition of transportable auxin in the transgenic rice plants, and this treatment also rescued auxin transport. A self-regulatory model (i.e., auxin promotes its own transport by modulating actin filaments) was proposed to explain these observations [64], but the molecular mechanism by which auxin regulates its own transport remains to be determined.

MTs also have a role in polar targeting of PIN proteins. Disruption of MTs with de-polymerizing drug oryzalin interfered basal PIN2 targeting in cortex cells and PIN1 targeting in stele cells [43]. Prolonged oryzalin treatment induced ectopic mislocalization of PIN2 and PIN1 in cortex and stele cells [43]. These findings provide evidence that MTs are required for polar targeting of PIN proteins and polar trafficking of auxin [43].

10.1.2 Mechanical Signals

Mechanical stimuli are believed to act as signals to impact plant responses at cellular and organismal levels. The most noticeable behaviors are those touch responses of carnivorous plants (such as the Venus' Flytrap (*Dionaea muscipula*)) and sensitive plants (*Mimosa pudica*). However the cellular responses to mechanical signal which leads to changes in cell expansion and morphogenesis are not clearly documented, and the mechanism of perception and inter- or intra-cellular transduction of endogenous mechanical signals are not well understood [7], although a number of studies has implicated MTs in the transduction of mechanical signals in plants. Wymer et al. demonstrated that application of a unidirectional centrifugal force can reset a preferential expansion direction in tobacco protoplasts [104]. They also found that this response was mediated by MT reorganization, since application of MT-disrupting drug amiprophos-methyl before and during centrifugation caused protoplasts elongated without a preferential growth axis. A recent study by Hamant and colleagues provided further support for mechanical

stress being a signal that controls MT dynamics during shoot apical meristem morphogenesis [24, 36]. It is predicated that the meristem surface is under considerable tension, which comes from cell walls of individual cells and the turgor pressure they exerted. Hamant et al. found that the MT-alignment is paralleled to the direction of the predicated principal tensional stress. They modified mechanical stress by either compressing the entire meristem laterally or by ablating a single or a few epidermal cells. In these experiments, realignment of the cortical MTs in response to the stress tension has been detected [24, 36]. These results strongly suggest that mechanical force can act as a signal for directional cell expansion through its effect on microtubule organization.

10.2 Signal Transduction Pathways Regulating MFs

A complex signaling network has been demonstrated to regulate MFs dynamics in tip-growing cells, especially in pollen tubes. This network is composed of multiple cross-linked signaling pathways mediated by ROP (*R*h*o*-related GTPases from *p*lants) GTPases-, Ca^{2+}, phosphoinositide-, reactive oxygen species (ROS), etc. [16, 29, 45, 50, 83, 106]. However much less is known about signaling mechanisms regulating MFs in diffusely-growing cells. Recent studies suggest that ROP signaling pathway plays a general role in regulation of MFs in both tip growing cells and diffusely-growing cells [106] (Fig. 10.1).

ROP GTPases belong to the conserved family of Rho GTPases. In eukaryotic kingdoms, Rho-family GTPases have conserved functions in the signaling pathways regulating MFs during cell morphogenesis and cell movement. In these pathways, a conserved actin-nucleation complex, ARP2/3 complex, is involved. In animal cells, CDC42 and RAC subfamilies of Rho GTPases activate the ARP2/3 complex through the regulatory WASPs (Wiskott–Aldrich syndrome proteins) and WAVE (Wiskott–Aldrich syndrome protein family verprolin homologous) complex, and subsequently promote formation of MFs in filopodia as well as in lamellipodia, respectively [20, 25, 47, 49, 66, 69]. In plants, the subunits of plant ARP2/3 and WAVE complex are conserved [8, 19]. Genetic screens have identified a large collection of mutants for subunits of the ARP2/3 complex and the WAVE complex in *Arabidopsis*. These mutants display highly similar defects in the morphogenesis of diffusely-growing cells, such as trichomes, leaf pavement cells as well as hypocotyl cells (reviewed in [39, 83, 86]). The most striking phenotypes are found in trichomes, including swollen stalk and defects in branch elongation, which are associated with abnormal actin organization and mimic cell shape changes caused by actin depolymerizing drugs [53, 87]. It is interesting that defects in cell shapes of pavement cells partially resemble those caused by loss of ROP2 and ROP4 function [3, 9, 21, 26, 30, 32, 54, 109]. These observations hint a possible connection between ROPs and ARP2/3-dependent MFs formation as in Rho GTPase (RAC/CDC42) regulation of this complex in animals. Furthermore, the WAVE complex might be involved in this connection (Fig. 10.1).

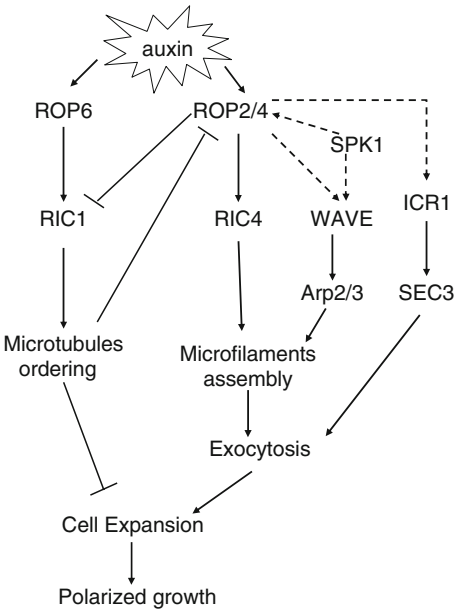

Fig. 10.1 ROPs signaling to cytoskeleton elements to control diffusely polar cell growth in *Arabidopsis*. ROP proteins coordinately regulate MFs and MTs organization and dynamics, eventually control polarized cell growth. ROP2 and ROP4 may function redundantly in stimulating MFs assembly and subsequently the exocytosis and cell expansion, yet they inhibit RIC1's function in promoting MT ordering. However ROP6 works antagonistically with ROP2 and ROP4 to promote MT ordering through RIC1. Well-ordered MTs may also have a role in feedback regulation of ROP2/4 activity

Genetic studies imply that ROP2 and SRA1 (a WAVE complex protein) might function in the same pathway to regulate actin-dependent cell growth. In in vitro pull-down assays, SRA1 was found to bind to GTP-ROP2 and GTP-ROP4 [4]. This pathway may be activated by SPK1, the only DOCK family guanine nucleotide exchange factor (GEF) found in plants. SPK1 was reported to associate with the nucleotide-depleted or the GDP-bound ROP2 [4, 67, 86]. It is interesting that NAP1, another component of WAVE complex, was detected in the immunoprecipitates of solubilized endogenous SPK1, which suggests an association of SPK1 with the WAVE complex. It has been proposed that the direct Rho effector-binding of a Rho GEF may contribute the regulation specificity by providing a general scaffolding strategy [4, 75]. Direct interaction between ROPs and subunits of ARP2/3 complex and WAVE complex were also investigated using yeast two-hybrid (Y2H) assay and bimolecular fluorescence complementation (BiFC) method. Basu and his colleagues detected a direct interaction between ROP2 and WAVE complex subunit PIR121/SRA1/KLK. However Uhrig and colleagues reported that PIR121/SRA1/KLK did not interact with neither ROP2 nor ROP5, ROP7, ROP8, ROP11. This controversy may be caused by different vectors, yeast strains

or assay conditions that used in two separated studies [3, 90]. Another WAVE complex component, SCAR, was detected to interact with ROP5, ROP7, ROP8, ROP11 by Uhrig et al, which suggests other ROPs may participate in the WAVE-ARP2/3 signaling pathway in regulation of MFs dynamic during cell morphogenesis. Nonetheless in vivo studies are clearly needed to investigate the possible connection of ROPs with the WAVE complex and ARP2/3 complex.

In addition to the common Rho GTPases-WAVE-ARP2/3 pathway in the regulation of MFs, plants have developed its specific pathways, in which the plant specific players are involved. RIC (*R*OP *i*nteractive *C*RIB motif containing proteins) is a family of plant-specific ROP effectors [103]. One member of this family, RIC4, was found directly interact with active form of ROP1 and ROP2, and promote fine MFs formation when overexpressed in pollen tubes and leaf pavement cells, respectively. These fine MFs is required for tip growth in pollen tube and the lobe outgrowth in pavement cells. Pavement cells from both *ric4* and *rop2* loss of function mutants contained fewer fine MFs and shallower lobes [32, 35]. To date, the exact mechanism by which RIC4 regulates MFs is still unknown, but is expected to act through an ABP. An inspiring report from Yang's lab demonstrates that the RIC4-dependent MFs assembly was responsible for vesicle accumulation in pollen tube and involved in the regulation of exocytosis [41, 46]. This may be a general mechanism used by ROP-RIC4-actin signaling pathway to regulate polar cell growth. In pavement cells, a novel ROP effector ICR1 (*i*nteractor of *c*onstitutive active *R*OPs 1) has been characterized [44]. ICR1 binds to the active form of ROPs and interacts with a subunit of exocyst vesicle tethering complex, SEC3. Knocking down or silencing ICR1 leads to cell deformation, and ectopic expression of ICR1 alters cell morphology of pavement cells and root cells (including root hairs) as well [44]. These results support the hypothesis that ROP-RIC4-actin pathway may act coordinately with the ROP-ICR1-SEC3 pathway to regulate exocytosis for polar cell growth in both pavement cells and root hairs. A recent most exciting finding in the field of cytokeleton signaling in plants is the demonstration that auxin activates the ROP-RIC4 signaling pathway in the regulation of MFs dynamics [105] (Fig. 10.1).

10.3 Signal Transduction Pathways that Transmit Signals to MTs

It has been observed in numerous cases that ROP signaling, protein phosphorylation and dephosphorylation and Ca^{2+} signaling play an important role in the transduction of internal developmental and external environmental signals to MAPs, subsequently regulating MT organization and dynamics during the cell division and cell expansion (reviewed in [39]). Here we highlight known pathways involved in the regulation of diffuse cell growth.

10.3.1 ROP GTPase Signaling

Given a critical role for well-ordered cortical MTs in determining the direction of cell expansion based on diffuse growth, the mechanism of signaling to the organization and re-organization of these MT is highly sought but poorly understood. An important breakthrough in this area is a recent study uncovering the first known signaling pathway that promotes the formation of highly-ordered cortical MTs in plants (Fig. 10.1). RIC1 is a novel MAP belongs to the RIC family effectors [32, 103]. In leaf pavement cells (PCs), RIC1 is responsible for well-organized transverse MTs formation [32]. A ROP-family GTPase, ROP6 was demonstrated the RIC1-activating ROP to promote the MT ordering. Deletion of ROP6 led to more randomly-orientated cortical MT arrays as well as wider necks in pavement cells, which are similar to that resulted from lacking of RIC1. Whereas overexpression of ROP6 induced well-ordered MTs and the inhibition of lobe elongation, just like overexpression of RIC1 did. Importantly, ROP6 is required for RIC1 association with MTs [33]. These observations strongly support the hypothesis that the ROP6-RIC1 pathway leads to the rearrangement of MTs into transversely ordered parallel arrays in pavement cells. Since transversely oriented MT is believed to restrict anisotropic cell expansion in the direction perpendicular to the MTs, which is an essential process for plant development and morphogenesis. An important question is whether the ROP-RIC1-MT pathway provides a general spatial-control mechanism in elongating cells. Fu et al. performed further investigation and found that hypocotyl epidermal cells in ROP6 and RIC1 loss-of-function mutants are support the notion that the ROP6-RIC1-MT ordering pathway play a broad role in the spatial regulation of cell expansion [33] (Fig. 10.1). A recent breakthrough in our understanding of signaling mechanisms that regulate the organization of cortical MTs is the finding that the ROP6-RIC1 pathway is activated by auxin [105].

It is interesting that another ROP-family GTPase, ROP2, which belongs to the same clade of the ROP family as ROP6, plays an opposing role that inhibit RIC1 to associate with MTs to fulfill its function [32] (Fig. 10.1). It is proposed that distinct subcellular localization patterns, which are guided by the HVC (hypervariable C-terminal) region of ROPs, are account for the different functions of ROP2 and ROP6 in regulation of MT ordering [32, 33, 108]. Since the ROP2 pathway is also activated by auxin, auxin regulates the morphogenesis of diffusely-growing cells by coordinating two antagonistic pathways: the ROP2-RIC4 and the ROP6-RIC1 pathways.

ROP2 inhibition of RIC1's binding to MTs indicates a central role of ROP signaling in crosstalk between MFs and MTs arrays in pavement cells, which is also played by other Rho family members in yeast and animal cells [27, 70]. On the other hand, ROP6-RIC1 promoted well-ordered MTs can inhibit the activity of ROP2 and subsequently reduced the cortical fine MFs [32], indicates a feedback regulation of ROP activity carried by cytoskeleton element (Fig. 10.1).

10.3.2 Protein Kinases and Phosphatases

Protein phosphorylation by protein kinases and de-phosphorylation by phosphatases play major roles in signal transduction during plant development. Evidence that suggests the involvement of protein kinases and phosphatases in the regulation of MTs during diffuse cell growth. For example it was reported that treatment with a kinase inhibitor or with a phosphatase inhibitor induced the disruption of MT arrays and caused swollen root cells [2]. A mutation in *TON2/FASS* gene, which encodes a protein similar to a subunit of PP2A, induced defects in cell elongation and cortical MT orientation [11]. Another MAPK phosphatase mutant *phs1-1* also displayed less ordered and more fragmented MT arrays [62]. It is interesting that GA_3-induced MT re-orientation in epicotyl segments of azuki bean was blocked by kinases inhibitors such as 6-dimethylaminopurine [59], hinting a potential role of protein kinases and/or phophatases in hormone signaling to MTs. However, the signaling pathways that modulate protein phosphorylation-mediated MT organization and dynamics during diffuse growth remain to be understood.

Much more information is available on the phosphorylation of MAPs (such as MT bundling proteins MAP-65 and kinesin-like proteins) by protein kinases (especially mitogen-activated protein kinases (MAPKs) and cyclin-dependent kinases (CDKs)) during cell division (reviewed by [39, 73]). There are 9 MAP65 proteins found in *Arabidopsis* and three isoforms of MAP65-1 in tobacco and 11 MAP65 in rice, which exhibit different MT bundling activities and subcellular localization [40, 51, 80, 91]. It is proposed that MAP65 proteins are key players regulating the maintenance and dynamics of central spindles and phragmoplasts [39, 72, 73]. AtMAP65-1 is phosphorylated by MAPK and CDK, which reduce its interaction with MTs and the MT bundling activity. Overexpression of a non-phosphorylatable mutant of AtMAP65-1 resulted in excessive accumulation of MTs in the metaphase spindle midzone and a delay in cell division progression [81]. Similar scenario was observed in tobacco cells, a tobacco MAPK, NRK1/NTF6 can phosphorylate NtMAP65-1 proteins and subsequently reduce the lateral's MT bundling activity in vitro. Phosphorylated NtMAP65-1 was found more accumulated at the midzone of the phragmoplast compared to non-phosphorylated NtMAP65-1 using specific antibodies.

The fact that both tobacco and *Arabidopsis* MAP65-1 proteins were found to localize to interphase cortical MTs as well [79, 82], it is highly possible that phosphorylation of MAP65 proteins by CDK or MAP kinase signaling cascade may play a important role in regulation of cortical MTs organization in diffuse polar cell growth.

10.3.2.1 Perspective

Important breakthroughs have been made in studies of developmental signal and cellular signaling in polarized cell growth. A framework of a complex network has been

established including signals and their perception, transduction and regulation of the cell growth via cytoskeleton and vesicular trafficking and so on. However, there are many missing links in this network. For example, for diffuse growth which involved so many signals, how does the cell distinguish each signal to react and eventually integrate all cues to form the proper shape? What are those cellular receptors of hormone or other signals that mediate the downstream signaling to the cytoskeleton? Are they the same as known hormone receptors that trigger the transcriptional responses or novel ones that act at the cellular level? What is the mechanism for a given receptor relays the developmental cue? It has also been implicated that the cytoskeleton not only plays critical roles as a downstream target to manipulate the cell growth, but also have a role in feedback regulation of the signaling pathways. But little is known about the molecular detail of the feedback loop. In addition there must be other players including ABPs, MAPs, protein kinases and phosphotases, etc. remains to be identified. It is no doubt that further investigation with combined tools of genetic, cytological, molecular, biochemical as well as computational modeling approaches will shed a light on our understanding of these questions.

References

1. Achard P, Cheng H, De Grauwe L, Decat J, Schoutteten H, Moritz T, Van Der Straeten D, Peng J, Harberd NP (2006) Integration of plant responses to environmentally activated phytohormonal signals. Science 311:91–94
2. Baskin TI, Wilson JE (1997) Inhibitors of protein kinases and phosphatases alter root morphology and disorganize cortical microtubules. Plant Physiol 113:493–502
3. Basu D, El-Assal Sel D, Le J, Mallery EL, Szymanski DB (2004) Interchangeable functions of *Arabidopsis* PIROGI and the human WAVE complex subunit SRA1 during leaf epidermal development. Development 131:4345–4355
4. Basu D, Le J, Zakharova T, Mallery EL, Szymanski DB (2008) A SPIKE1 signaling complex controls actin-dependent cell morphogenesis through the heteromeric WAVE and ARP2/3 complexes. Proc Natl Acad Sci USA 105:4044–4049
5. Blakeslee JJ, Peer WA, Murphy AS (2005) Auxin transport. Curr Opin Plant Biol 8:494–500
6. Bouquin T, Mattsson O, Naested H, Foster R, Mundy J (2003) The *Arabidopsis* lue1 mutant defines a katanin p60 ortholog involved in hormonal control of microtubule orientation during cell growth. J Cell Sci 116:791–801
7. Braam J (2005) In touch: plant responses to mechanical stimuli. New Phytol 165:373–389
8. Brembu T, Winge P, Bones AM (2005) Catching the WAVEs of plant actin regulation. J Plant Growth Regul 24:55–66
9. Brembu T, Winge P, Seem M, Bones AM (2004) NAPP and PIRP encode subunits of a putative wave regulatory protein complex involved in plant cell morphogenesis. Plant Cell 16:2335–2349
10. Buer CS, Sukumar P, Muday GK (2006) Ethylene modulates flavonoid accumulation and gravitropic responses in roots of *Arabidopsis*. Plant Physiol 140:1384–1396
11. Camilleri C, Azimzadeh J, Pastuglia M, Bellini C, Grandjean O, Bouchez D (2002) The *Arabidopsis* TONNEAU2 gene encodes a putative novel protein phosphatase 2A regulatory subunit essential for the control of the cortical cytoskeleton. Plant Cell 14:833–845
12. Campanoni P, Nick P (2005) Auxin-dependent cell division and cell elongation. 1-Naphthaleneacetic acid and 2, 4-dichlorophenoxyacetic acid activate different pathways. Plant Physiol 137:939–948

13. Catterou M, Dubois F, Schaller H, Aubanelle L, Vilcot B, Sangwan-Norreel BS, Sangwan RS (2001) Brassinosteroids, microtubules and cell elongation in *Arabidopsis thaliana*. II. Effects of brassinosteroids on microtubules and cell elongation in the bul1 mutant. Planta 212:673–683
14. Catterou M, Dubois F, Schaller H, Aubanelle L, Vilcot B, Sangwan-Norreel BS, Sangwan RS (2001) Brassinosteroids, microtubules and cell elongation in *Arabidopsis thaliana*. I. Molecular, cellular and physiological characterization of the *Arabidopsis* bull mutant, defective in the delta 7-sterol-C5-desaturation step leading to brassinosteroid biosynthesis. Planta 212:659–672
15. Chen CW, Yang YW, Lur HS, Tsai YG, Chang MC (2006) A novel function of abscisic acid in the regulation of rice (*Oryza sativa* L.) root growth and development. Plant Cell Physiol 47:1–13
16. Cheung AY, Wu HM (2008) Structural and signaling networks for the polar cell growth machinery in pollen tubes. Annu Rev Plant Biol 59:547–572
17. Chilley PM, Casson SA, Tarkowski P, Hawkins N, Wang KL, Hussey PJ, Beale M, Ecker JR, Sandberg GK, Lindsey K (2006) The POLARIS peptide of *Arabidopsis* regulates auxin transport and root growth via effects on ethylene signaling. Plant Cell 18:3058–3072
18. Collett CE, Harberd NP, Leyser O (2000) Hormonal interactions in the control of *Arabidopsis* hypocotyl elongation. Plant Physiol 124:553–562
19. Deeks MJ, Hussey PJ (2005) Arp2/3 and SCAR: plants move to the fore. Nat Rev Mol Cell Biol 6:954–964
20. Disanza A, Steffen A, Hertzog M, Frittoli E, Rottner K, Scita G (2005) Actin polymerization machinery: the finish line of signaling networks, the starting point of cellular movement. Cell Mol Life Sci 62:955–970
21. Djakovic S, Dyachok J, Burke M, Frank MJ, Smith LG (2006) BRICK1/HSPC300 functions with SCAR and the ARP2/3 complex to regulate epidermal cell shape in *Arabidopsis*. Development 133:1091–1100
22. Dong CH, Xia GX, Hong Y, Ramachandran S, Kost B, Chua NH (2001) ADF proteins are involved in the control of flowering and regulate F-actin organization, cell expansion, and organ growth in *Arabidopsis*. Plant Cell 13:1333–1346
23. Dugardeyn J, Vandenbussche F, Van Der Straeten D (2008) To grow or not to grow: what can we learn on ethylene-gibberellin cross-talk by in silico gene expression analysis? J Exp Bot 59:1–16
24. Dumais J (2009) Plant morphogenesis: a role for mechanical signals. Curr Biol 19:R207–R208
25. Eden S, Rohatgi R, Podtelejnikov AV, Mann M, Kirschner MW (2002) Mechanism of regulation of WAVE1-induced actin nucleation by Rac1 and Nck. Nature 418:790–793
26. El-Din El-Assal S, Le J, Basu D, Mallery EL, Szymanski DB (2004) DISTORTED2 encodes an ARPC2 subunit of the putative *Arabidopsis* ARP2/3 complex. Plant J 38:526–538
27. Etienne-Manneville S (2004) Actin and microtubules in cell motility: which one is in control? Traffic 5:470–477
28. Fu Y (2010) ROP GTPases and the cytoskeleton. In: Yalovsky S (ed) Integrated G proteins signaling in plants. Springer, Berlin
29. Fu Y (2010) The actin cytoskeleton and signaling network during pollen tube tip growth. J Integr Plant Biol 52(2):131–137
30. Fu Y, Li H, Yang Z (2002) The ROP2 GTPase controls the formation of cortical fine F-actin and the early phase of directional cell expansion during *Arabidopsis* organogenesis. Plant Cell 14:777–794
31. Fu Y, Kawasaki T, Shimamoto K, Yang Z (2008) ROP/RAC GTPases. In: Yang Z (ed) Intracellular signaling in plants. Wiley-Blackwell, Hoboken, pp 64–99
32. Fu Y, Gu Y, Zheng Z, Wasteneys G, Yang Z (2005) *Arabidopsis* interdigitating cell growth requires two antagonistic pathways with opposing action on cell morphogenesis. Cell 120:687–700
33. Fu Y, Xu T, Zhu L, Wen M, Yang Z (2009) A ROP GTPase signaling pathway controls cortical microtubule ordering and cell expansion in *Arabidopsis*. Curr Biol 19:1827–1832

34. Fujino K, Koda Y, Kikuta Y (1995) Reorientation of cortical microtubules in the sub-apical region during tuberization in single-node stem segments of potato in culture. Plant Cell Physiol 36:891–895
35. Gu Y, Fu Y, Dowd P, Li S, Vernoud V, Gilroy S, Yang Z (2005) A Rho family GTPase controls actin dynamics and tip growth via two counteracting downstream pathways in pollen tubes. J Cell Biol 169:127–138
36. Hamant O, Heisler MG, Jonsson H, Krupinski P, Uyttewaal M, Bokov P, Corson F, Sahlin P, Boudaoud A, Meyerowitz EM, Couder Y, Traas J (2008) Developmental patterning by mechanical signals in *Arabidopsis*. Science 322:1650–1655
37. Hashimoto T, Kato T (2006) Cortical control of plant microtubules. Curr Opin Plant Biol 9:5–11
38. Holweg C, Susslin C, Nick P (2004) Capturing in vivo dynamics of the actin cytoskeleton stimulated by auxin or light. Plant Cell Physiol 45:855–863
39. Hussey P, Hashimoto T (2008) The cytoskeleton and signal transduction: role and regulation of plant actin- and microtubule-binding proteins. In: Yang Z (ed) Intracellular signaling in plants. Wiley-Blackwell, Hoboken, pp 244–272
40. Hussey PJ, Hawkins TJ, Igarashi H, Kaloriti D, Smertenko A (2002) The plant cytoskeleton: recent advances in the study of the plant microtubule-associated proteins MAP-65, MAP-190 and the *Xenopus* MAP215-like protein, MOR1. Plant Mol Biol 50:915–924
41. Hwang JU, Gu Y, Lee YJ, Yang Z (2005) Oscillatory ROP GTPase activation leads the oscillatory polarized growth of pollen tubes. Mol Biol Cell 16:5385–5399
42. Kandasamy MK, Gilliland LU, McKinney EC, Meagher RB (2001) One plant actin isovariant, ACT7, is induced by auxin and required for normal callus formation. Plant Cell 13:1541–1554
43. Kleine-Vehn J, Langowski L, Wisniewska J, Dhonukshe P, Brewer PB, Friml J (2008) Cellular and molecular requirements for polar PIN targeting and transcytosis in plants. Mol Plant 1:1056–1066
44. Lavy M, Bloch D, Hazak O, Gutman I, Poraty L, Sorek N, Sternberg H, Yalovsky S (2007) A Novel ROP/RAC effector links cell polarity, root-meristem maintenance, and vesicle trafficking. Curr Biol 17:947–952
45. Lee YJ, Yang Z (2008) Tip growth: signaling in the apical dome. Curr Opin Plant Biol 11:662–671
46. Lee YJ, Szumlanski A, Nielsen E, Yang Z (2008) Rho-GTPase-dependent filamentous actin dynamics coordinate vesicle targeting and exocytosis during tip growth. J Cell Biol 181:1155–1168
47. Li H, Yang Z (2000) Rho GTPases and the Actin Cytoskeleton. In: Staiger CJ, Baluska F, Volkmann D, Barlow PW (eds) Actin: a dynamic framework for multiple plant cell functions. Dordrecht, Kluwer, pp 301–321
48. Lipka V, Panstruga R (2005) Dynamic cellular responses in plant-microbe interactions. Curr Opin Plant Biol 8:625–631
49. Lodish H, Berk A, Kaiser C, Krieger M, Scott M, Bretscher A, Ploegh H, Matsudaira P (2007) Molecular cell biology. W.H. Freeman, New York
50. Malho R, Liu Q, Monteiro D, Rato C, Camacho L, Dinis A (2006) Signalling pathways in pollen germination and tube growth. Protoplasma 228:21–30
51. Mao G, Chan J, Calder G, Doonan JH, Lloyd CW (2005) Modulated targeting of GFP-AtMAP65-1 to central spindle microtubules during division. Plant J 43:469–478
52. Mathur J (2004) Cell shape development in plants. Trends Plant Sci 9:583–590
53. Mathur J, Spielhofer P, Kost B, Chua N (1999) The actin cytoskeleton is required to elaborate and maintain spatial patterning during trichome cell morphogenesis in *Arabidopsis thaliana*. Development 126:5559–5568
54. Mathur J, Mathur N, Kernebeck B, Hulskamp M (2003) Mutations in actin-related proteins 2 and 3 affect cell shape development in *Arabidopsis*. Plant Cell 15:1632–1645
55. Mayumi K, Shibaoka H (1995) A possible double role for Brassinolide in the reorientation of cortical microtubules in the epidermal cells of azuki bean epicotyls. Plant Cell Physiol 36:173–181

56. Meier C, Bouquin T, Nielsen ME, Raventos D, Mattsson O, Rocher A, Schomburg F, Amasino RM, Mundy J (2001) Gibberellin response mutants identified by luciferase imaging. Plant J 25:509–519
57. Mita T, Shibaoka H (1984) Gibberellin stabilizes microtubules in onion leaf sheath cells. Protoplasma 119:100–109
58. Mita T, Katsumi M (1986) Gibberellin control of microtubule arrangement in the mesocotyl epidermal cells of the d_5 mutant of *Zea mays* L. Plant Cell Physiol 24:109–117
59. Mizuno K (1994) Inhibition of gibberellin-induced elongation, reorientation of cortical microtubules and change of isoform of tubulin in epicotyl segments of azuki bean by protein kinase inhibitors. Plant Cell Physiol 35:1149–1157
60. Muday GK, Murphy AS (2002) An emerging model of auxin transport regulation. Plant Cell 14:293–299
61. Muller S, Wright AJ, Smith LG (2009) Division plane control in plants: new players in the band. Trends Cell Biol 19:180–188
62. Naoi K, Hashimoto T (2004) A semidominant mutation in an *Arabidopsis* mitogen-activated protein kinase phosphatase-like gene compromises cortical microtubule organization. Plant Cell 16:1841–1853
63. Nick P, Schafer E, Furuya M (1992) Auxin redistribution during first positive phototropism in corn coleoptiles: microtubule reorientation and the Cholodny–Went theory. Plant Physiol 99:1302–1308
64. Nick P, Han MJ, An G (2009) Auxin stimulates its own transport by shaping actin filaments. Plant Physiol 151:155–167
65. Plett JM, Mathur J, Regan S (2009) Ethylene receptor ETR2 controls trichome branching by regulating microtubule assembly in *Arabidopsis thaliana*. J Exp Bot 60:3923–3933
66. Pollard TD, Borisy GG (2003) Cellular motility driven by assembly and disassembly of actin filaments. Cell 112:453–465
67. Qiu JL, Jilk R, Marks MD, Szymanski DB (2002) The *Arabidopsis* SPIKE1 gene is required for normal cell shape control and tissue development. Plant Cell 14:101–118
68. Rahman A, Bannigan A, Sulaman W, Pechter P, Blancaflor EB, Baskin TI (2007) Auxin, actin and growth of the *Arabidopsis thaliana* primary root. Plant J 50:514–528
69. Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, Parsons JT, Horwitz AR (2003) Cell migration: integrating signals from front to back. Science 302:1704–1709
70. Rosales-Nieves AE, Johndrow JE, Keller LC, Magie CR, Pinto-Santini DM, Parkhurst SM (2006) Coordination of microtubule and microfilament dynamics by *Drosophila* Rho1, Spire and Cappuccino. Nat Cell Biol 8:367–376
71. Sakoda M, Hasegawa K, Ishizuka K (1992) Mode of action of natural growth inhibitors in radish hypocotyl elongation-influence of raphanusanin on auxin-induced microtubule orientation. Physiol Plant 84:509–513
72. Sasabe M, Machida Y (2006) MAP65: a bridge linking a MAP kinase to microtubule turnover. Curr Opin Plant Biol 9:563–570
73. Sasabe M, Machida Y (2008) Signaling by protein phosphorylation in cell division. In: Yang Z (ed) Intracellular signaling in plants. Wiley-Blackwell, Hoboken, pp 64–99
74. Sato M, Tsutsumi M, Ohtsubo A, Nishii K, Kuwabara A, Nagata T (2008) Temperature-dependent changes of cell shape during heterophyllous leaf formation in *Ludwigia arcuata* (Onagraceae). Planta 228:27–36
75. Schmidt A, Hall A (2002) Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. Genes Dev 16:1587–1609
76. Shibaoka H (1993) Regulation by gibberellins of the orientation of cortical microtubules in plant cells. Aust J Plant Physiol 20:461–470
77. Shoji T, Suzuki K, Abe T, Kaneko Y, Shi H, Zhu JK, Rus A, Hasegawa PM, Hashimoto T (2006) Salt stress affects cortical microtubule organization and helical growth in *Arabidopsis*. Plant Cell Physiol 47:1158–1168
78. Sibout R, Sukumar P, Hettiarachchi C, Holm M, Muday GK, Hardtke CS (2006) Opposite root growth phenotypes of hy5 versus hy5 hyh mutants correlate with increased constitutive auxin signaling. PLoS Genet 2:e202

79. Smertenko A, Saleh N, Igarashi H, Mori H, Hauser-Hahn I, Jiang CJ, Sonobe S, Lloyd CW, Hussey PJ (2000) A new class of microtubule-associated proteins in plants. Nat Cell Biol 2:750–753
80. Smertenko AP, Kaloriti D, Chang HY, Fiserova J, Opatrny Z, Hussey PJ (2008) The C-terminal variable region specifies the dynamic properties of *Arabidopsis* microtubule-associated protein MAP65 isotypes. Plant Cell 20:3346–3358
81. Smertenko AP, Chang HY, Sonobe S, Fenyk SI, Weingartner M, Bogre L, Hussey PJ (2006) Control of the AtMAP65-1 interaction with microtubules through the cell cycle. J Cell Sci 119:3227–3237
82. Smertenko AP, Chang HY, Wagner V, Kaloriti D, Fenyk S, Sonobe S, Lloyd C, Hauser MT, Hussey PJ (2004) The *Arabidopsis* microtubule-associated protein AtMAP65-1: molecular analysis of its microtubule bundling activity. Plant Cell 16:2035–2047
83. Smith LG, Oppenheimer DG (2005) Spatial control of cell expansion by the plant cytoskeleton. Annu Rev Cell Dev Biol 21:271–295
84. Stoppin-Mellet V, Gaillard J, Vantard M (2006) Katanin's severing activity favors bundling of cortical microtubules in plants. Plant J 46:1009–1017
85. Sukumar P, Edwards KS, Rahman A, Delong A, Muday GK (2009) PINOID kinase regulates root gravitropism through modulation of PIN2-dependent basipetal auxin transport in *Arabidopsis*. Plant Physiol 150:722–735
86. Szymanski DB (2005) Breaking the WAVE complex: the point of *Arabidopsis* trichomes. Curr Opin Plant Biol 8:103–112
87. Szymanski DB, Marks MD, Wick SM (1999) Organized F-actin is essential for normal trichome morphogenesis in *Arabidopsis*. Plant Cell 11:2331–2347
88. Takahashi H, Kawahara A, Inoue Y (2003) Ethylene promotes the induction by auxin of the cortical microtubule randomization required for low-pH-induced root hair initiation in lettuce (*Lactuca sativa* L.) seedlings. Plant Cell Physiol 44:932–940
89. Takesue K, Shibaoka H (1999) Auxin-induced longitudinal-to-transverse reorientation of cortical microtubules in nonelongating epidermal cells of azuki bean epidotyls. Protoplasma 206:27–30
90. Uhrig JF, Mutondo M, Zimmermann I, Deeks MJ, Machesky LM, Thomas P, Uhrig S, Rambke C, Hussey PJ, Hulskamp M (2007) The role of *Arabidopsis* SCAR genes in ARP2-ARP3-dependent cell morphogenesis. Development 134:967–977
91. Van Damme D, Van Poucke K, Boutant E, Ritzenthaler C, Inze D, Geelen D (2004) In vivo dynamics and differential microtubule-binding activities of MAP65 proteins. Plant Physiol 136:3956–3967
92. Vandenbussche F, Verbelen JP, Van Der Straeten D (2005) Of light and length: regulation of hypocotyl growth in *Arabidopsis*. Bioessays 27:275–284
93. Waller F, Riemann M, Nick P (2002) A role for actin-driven secretion in auxin-induced growth. Protoplasma 219:72–81
94. Wang C, Li J, Yuan M (2007) Salt tolerance requires cortical microtubule reorganization in *Arabidopsis*. Plant Cell Physiol 48:1534–1547
95. Wang QY, Nick P (1998) The auxin response of actin is altered in the rice mutant Yin-Yang. Protoplasma 204:22–33
96. Wang X (2005) Regulatory functions of phospholipase D and phosphatidic acid in plant growth, development, and stress responses. Plant Physiol 139:566–573
97. Wasteneys GO (2000) The cytoskeleton and growth polarity. Curr Opin Plant Biol 3:503–511
98. Wasteneys GO, Galway ME (2003) Remodeling the cytoskeleton for growth and form: an overview with some new views. Annu Rev Plant Biol 54:691–722
99. Wenzel CL, Williamson RE, Wasteneys GO (2000) Gibberellin-induced changes in growth anisotropy precede gibberellin-dependent changes in cortical microtubule orientation in developing epidermal cells of barley leaves. Kinematic and cytological studies on a gibberellin-responsive dwarf mutant, M489. Plant Physiol 124:813–822

100. Wightman R, Turner SR (2007) Severing at sites of microtubule crossover contributes to microtubule alignment in cortical arrays. Plant J 52:742–751
101. Wightman R, Turner SR (2008) A novel mechanism important for the alignment of microtubules. Plant Signal Behav 3:238–239
102. Wolters H, Jurgens G (2009) Survival of the flexible: hormonal growth control and adaptation in plant development. Nat Rev Genet 10:305–317
103. Wu G, Gu Y, Li S, Yang Z (2001) A genome-wide analysis of *Arabidopsis* Rop-interactive CRIB motif-containing proteins that act as Rop GTPase targets. Plant Cell 13:2841–2856
104. Wymer CL, Wymer SA, Cosgrove DJ, Cyr RJ (1996) Plant cell growth responds to external forces and the response requires intact microtubules. Plant Physiol 110:425–430
105. Xu T, Wen M, Nagawa S, Fu Y, Chen JG, Wu MJ, Perrot-Rechenmann C, Friml J, Jones AM, Yang Z (2010) Cell surface- and Rho GTPase-based auxin signaling controls cellular interdigitation in *Arabidopsis*. Cell doi:10.1016/j.cell.2010.09.003
106. Yang Z (2008) Cell polarity signaling in *Arabidopsis*. Annu Rev Cell Dev Biol 24:551–575
107. Zandomeni K, Schopfer P (1993) Reorientation of microtubules at the outer epidermal wall of maize coleoptiles by phytochrome, blue-light photoreceptor and auxin. Protoplasma 173:103–112
108. Zheng ZL, Yang Z (2000) The Rop GTPase: an emerging signaling switch in plants. Plant Mol Biol 44:1–9
109. Zimmermann I, Saedler R, Mutondo M, Hulskamp M (2004) The *Arabidopsis* GNARLED gene encodes the NAP125 homolog and controls several actin-based cell shape changes. Mol Genet Genomics 272:290–296
110. Wasteneys GO, Yang Z (2004) The cytoskeleton becomes multidisciplinary. Plant Physiol 136:3853–3854

Chapter 11
Microtubule and Cell Shape Determination

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11.1 Microtubules Determine Cell Shape

The final shape of an expanding plant cell is determined by the biochemical responses of the cell wall to tensile stress produced by turgor pressure. Different cell shapes can be generated by restricting the expanding area to large or small regions of a cell [18]. Over the years, the microtubule (MT) cytoskeleton has been shown to be critical for the anisotropic growth and thus for determining cell shape.

Essential roles of cortical MTs in growth anisotropy have been well demonstrated when most MTs in interphase cells are depolymerized. Structurally diverse herbicides, including dinitroanilines (e.g., oryzalin), benzamides (e.g., propyzamide, also known as pronamide), and phosphoric amides (e.g., amiprophos-methyl), bind the plant tubulin heterodimers and thus inhibit the formation of MT polymers in vitro [23, 38, 56]. These MT-depolymerizing herbicides produce gross morphological effects in whole plants, but the most characteristic abnormality is the swollen root tips where cortical and epidermal cells become more isodiametric [4, 37, 51]. These drugs also effectively depolymerized cortical MTs and inhibited anisotropic growth in axially elongating cultured tobacco BY-2 cells (e.g., [2]). Among dozens of *Arabidopsis* tubulin mutants with an impaired MT organization, several severe mutants show profound abnormalities in cell division and cell morphogenesis [25]. In these dominant-negative tubulin mutants, axially elongating epidermal cells, leaf epidermal pavement cells, and trichomes do not attain distinct cell shapes, and become highly isotropic. A weak mutant allele of *Arabidopsis* tubulin-folding cofactor A produces pleiotropic MT-related phenotypes, including reduced growth anisotropy in trichomes and rapidly elongating hypocotyl cells [28]. In addition, mutations in a variety of MT-associated proteins (MAPs) cause abnormal cell shapes which manifest as stunted or twisted organ growth [48]. These pharmacological and

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genetic results strongly support that intact MTs are essential for the morphogenesis of the distinct cell types found in plants.

How can interphase MTs control cell morphogenesis and determine the growth axis? The most prevalent explanation is that cortical MTs regulate the deposition of load-bearing cellulose microfibrils in specified directions as cells expand. MTs may control cellulose synthesis in several ways, including restraining movement of the cellulose synthase machinery, and directing delivery of the synthase and other relevant proteins to the plasma membrane. Detailed information and discussion are found in the accompanying review article.

11.2 Distinct MT Array Organization Correlates with Cell Shape: Wild-Type Cells

Cortical MTs in different plant cell types form distinct array patterns. Rapidly elongating epidermal cells of roots and etiolated hypocotyls show transverse arrays, whereas more slowly elongating cells of cylindrical shapes typically have oblique or longitudinal arrays in addition to substantial populations of cortical MTs with discordant orientations. Other arrangements of cortical MTs are found in leaf epidermal pavement cells, trichomes, and root hairs. In this review, I will focus on the parallel arrays found in axially growing cells, and discuss how such an organization is important for anisotropic growth and the determination of cell shape.

When Ledbetter and Porter [31] first observed MTs in the cortical regions of root tip cells of two angiosperms and one gymnosperm, they found individual MTs to line up in a parallel array that mirrored the orientation of the cellulose microfibrils of the adjacent cell walls. Since then, numerous papers have described bundles of overlapping cortical MTs encircling elongating plant cells, roughly in an orientation perpendicular to the growth axis of the cylindrical cell. The degree of the array alignment, however, varies among cell types and under growth conditions. For example in *Arabidopsis* seedlings, highly ordered, uniformly oriented transverse arrays are found in the epidermal cells at the elongation zone of the root (e.g., [49]), while cortical MTs are distributed much more broadly in the more slowly elongating epidermal cells of the light-grown hypocotyl and petiole (e.g., [55]). MT–MT interactions favor a parallel arrangement of the MT bundles, but how the parallel MT arrays tend to position in an orientation perpendicular to the growth axis is not well understood. If bundled MTs form loops winding round the entire body of the cylindrical cell, they might self-align transversely by generating bundling that maximizes MT overlap, in a mechanism called “self-cinching” [54]. However, cortical MTs in cylindrical cells form a helical alignment, instead of a stack of hoops; thus, such a self-cinching mechanism appears implausible [35, 54].

In wild-type *Arabidopsis* roots, cortical MTs in epidermal cells are distributed in various orientations, although the mean angle is close to the transverse

orientation, before the onset of cell elongation, and become consistently transversely aligned in parallel arrays when cells begin to expand [49]. In the distal elongation region where growth starts to decline, MTs become much less frequent and often obliquely oriented. Such an apparent correlation between MT orientation and longitudinal growth indicates the importance of the transverse cortical array in maintaining a high degree of growth anisotropy. The finding of helical MT arrays in twisting mutants and in drug-treated wild-type roots further supports that the orientation of MT arrays determines the direction of cell expansion.

11.3 Helical Growth as an Unusual Case of Growth Anisotropy

The cell shape in axially elongating organs can deviate from a long cylindrical stick to a oval barrel when the organization of cortical MTs is increasingly disrupted. Between these two extremes, mild disruption of MT functions often, but not always, results in twisted cell shapes. Examples of non-twisting MT-related mutants include mutations in Katanin, CLASP, and MAP65s. The epidermal cells at the elongation zone of *katanin* and *clasp* mutants lose anisotropic growth and are radially swollen, but grow parallel to the growth axis of the roots [3, 7]. Many other MT-related mutants do twist or cause organ twisting when overexpressed, with the prominent feature that their direction of twisting is fixed, to either the right or the left. Roots and etiolated hypocotyls of right-handed mutants twist so that the epidermal cell files form right-handed steep helices, whereas those of left-handed mutants have skewed epidermal cell files that follow left-handed helices (Fig. 11.1). When these mutant seedlings are grown on a hard agar plate that is placed vertically or at a slightly inclined angle, right-handed mutant roots grow toward the right side of the plate, while left-handed mutant roots grow toward the left side. The roots which accidentally grow into the agar still show helical cell files but grow to the direction of gravity through the agar [17], indicating that rotating root tips skid on the agar surface to the direction of overall root growth.

It should be noted that organ twisting is a complex process in which the growth of inner and outer cell files must be coordinated. Based on the observation that, in a twisting axial organ, anisotropic longitudinal growth was much more severely impaired in the endodermis and cortex (inner) cells than the outermost epidermal cells, a helical growth model has been proposed [17, 22]; the total length of inner cell files is shorter than that of the outer cell files, so that the inner cell files must tilt to either the right or the left to accommodate the differences in the longitudinal length of cell files (Fig. 11.2). It has been postulated that the organization of cortical MT arrays (or its effect on cell shape) is more sensitive to MT defects in the inner cell types than in the epidermal cells, but the molecular basis of this presumed cell-type-dependent difference is totally unknown. Importantly, this model itself

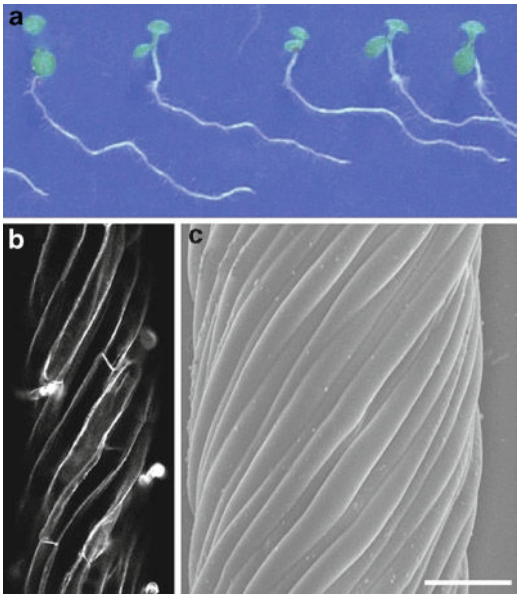

Fig. 11.1 Helical growth in a right-handed twisting mutant, *spiral 3*. This mutant is caused by an amino-acid missense mutation in the GCP2 subunit of the γ-tubulin nucleating complex, and results in wider and more divergent nucleating angles of newly formed MTs with respect to the wall of previously existing MTs. (**a**) Seedlings grown on a hard agar surface. Primary roots grow toward the right side of the plate when viewed from above. (**b**) Epidermal cell files of the mutant roots (stained with propidium iodide) twist to form right-handed helices at the differentiated region. Root hairs are stained intensely. (**c**) Epidermal cell files of the etiolated mutant hypocotyls (viewed with scanning electron microscopy) also form right-handed helices. Bar: 100 μm

does not specify the direction of skewing. A particular organization of cortical MT arrays (see below) is believed to confer the directional cue to this basic helical growth model. Indeed, there exist several non-directional twisting mutants in which direction is not fixed or apparently random [9, 12, 27, 44]. Individual roots even change the direction of twisting, either from the right to the left, or vice versa, during growth. In these mutants, responses and/or the transport of auxin are supposed to be altered, presumably leading to developmental defects in the vascular tissues (while maintaining the wild-type growth in the outer cell files), thus causing organ twisting. When MTs were examined in such a non-directional twisting mutant, *tornado1*/*lopped*, normal cortical MT organization was observed in twisting epidermal cells [26].

Although typically recognized as an intriguing growth pattern at the organ level in higher plants, helical growth can be seen at the level of individual cells. The long thin cylindrical cells of multicellular or filamentous algae, such as the internodal cells of Nitella, twist around their longitudinal axis as the cells elongate. The helical cell wall texture of these freely elongating cells has been proposed to be responsible

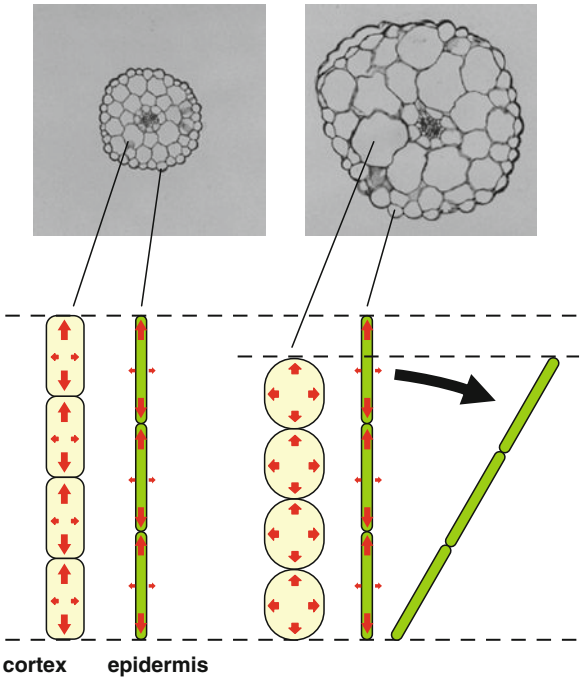

Fig. 11.2 Twisting model. Cross sections of upper hypocotyl regions of 5-day-old etiolated seedlings are shown in the upper panel. Wild-type cortex and epidermal cells (together with other cell types) elongate longitudinally and constitute a hypocotyl tissue. In twisting mutants, such as in *spr1-1*, cortex cells are more severely affected than epidermal cells, with regards to anisotropic cell expansion, and expand more radially. Shorter cortex cells are predicted to cause epidermal cells to twist, thus adjusting the height of both cell layers to the same length. This model itself does not specify the direction of twisting. Adapted from Ishida et al. [26]

for the helical growth of the rod-like structures [16, 45]. In an *Arabidopsis* twisting mutant with a dominant-negative tubulin missense mutation, twisting was observed in freely growing cells of trichomes and single suspension-cultured cells [8]. Thus, the helical expansion of individual cells may be the basis of the organ twisting with fixed orientations.

A variation of the helical growth model views the epidermis as an interconnected sheath that surrounds the axial organ, and primarily determines organ growth [36]. By manipulating brassinosteroid biosynthesis and signaling, it has been demonstrated that the epidermis both promotes and restricts shoot growth by providing a non-autonomous signal to the ground tissues [46]. Twisting of the epidermal cell layer may be a coordinated behavior of individual twisting epidermal cells, and the underlying inner layers could passively follow the expansion of the epidermis by means of mechanical stimuli. In this model, the radial expansion of the cortex cells in twisting mutants is a consequence, not a cause, of the organ twisting.

11.4 Helical MT Organization Underlies Skewed Cell Growth

Various defects in nucleation, dynamics, and signaling of cortical MTs cause the helical organization of MT arrays, in contrast to mostly transverse wild-type arrays. Most prevalent causes for the helical arrays are dominant-negative mutations in tubulins, probably because there exist a few dozen tubulin genes in a given plant species that can be potential targets for dominant mutations (e.g., six α-tubulin genes and nine β-tubulin genes in *Arabidopsis thaliana*). Thirty five *Arabidopsis* twisting mutants have been isolated that have either missense or amino acid deletion mutations in α- or β-tubulins [8, 24, 25, 50]. These twisting mutants are genetically semi-dominant; heterozygous mutants skew but less severely than homozygotes. Although weakly twisting mutants behave recessively in standard growth conditions, they show clear semi-dominant responses to MT-disrupting drugs. Mechanistically, the semi-dominant mutant phenotypes are caused by the incorporation of mutated tubulin proteins, along with wild-type tubulins, into MT polymers, thus compromising (or poisoning) MT functions. When mutant tubulins are tagged at either their N- or C-terminus with a Myc epitope and expressed in wild-type *Arabidopsis* plants, the helical MT arrays of the transgenic plants are found to contain the Myc-tagged mutant tubulins and to reproduce the organ twisting phenotypes of the original mutants. Null mutant alleles of single tubulin genes did not cause obvious growth defects [50], supporting the dominant-negative mechanisms of mutant tubulins in twisting mutants.

Mutated amino acid residues are generally found on the exposed surface of the tubulin 3D structures [43], and are concentrated at the longitudinal interface of the α- and β-tubulins within and between the αβ-heterodimer, and at the lateral interface between two adjacent MT protofilaments [25, 50]. It is often difficult to predict how MT dynamics and functions are altered by these tubulin mutations, but there are a few notable exceptions. The 251st aspartate residue in α-tubulins is located at the T7 loop, and is modeled to interact with phosphates of the GTP nucleotide in β-tubulin of the next αβ-heterodimer in the MT [43]. Thus, upon the arrival of a new dimer at the plus end of the MT, Asp-251 in α-tubulin of the incoming dimmer likely interacts with the GTP of the receiving β-subunit. This GTP is consequently hydrolyzed, and the resulting GDP becomes buried in the interface. The Asp^{251}-to-Asn mutation in α-tubulin 5 is predicted to interfere with the GTPase-activating function of α-tubulin, and may impair or delay GTP hydrolysis at the plus end [25]. Indeed, cortical MTs in the *tua5*^{D251N} mutant were decorated along the entire MT lattices with the end binding 1-green fluorescent protein (EB1-GFP) protein, which normally accumulates only at the plus end. Recently, GTP-bound tubulin was occasionally found in the MT lattice, suggesting that GTP hydrolysis is sometimes incomplete during MT polymerization [14]. A similar predictable delay in GTP hydrolysis can be found in the Arg-2-Lys mutation in α-tubulin 4 [8, 25]. In this case, the Arg-2 is modeled to form hydrogen bonds with the GTPase region of β-tubulin, and the EB1-GFP label persists to older parts of mutant MTs [8].

Mutations in or over-expression of MAPs also give rise to organ twisting with generally fixed chirality [26]. It should be noted that some of these MAPs, such as SPIRAL1 [40, 47], SPIRAL2 [55] and EB1 [5, 29], are partly or strongly concentrated at the plus end of growing MTs, implying that they affect the dynamics or fine structure of MTs at the plus end.

When cortical MT arrays in rapidly elongating epidermal cells were analyzed, right-handed twisting mutants consistently showed skewed arrays of shallow left-handed helices, while left-handed mutants always possessed shallow right-handed helical arrays ([8, 17, 24, 25, 30, 50, 55]; Fig. 11.3). Such a remarkable correlation between skewed direction of cortical helical arrays and growth direction of elongating organs is consistent with the currently popular model in which cortical MT arrays guide the movement of the cellulose synthase complex in the plasma membrane and deposition of cellulose, thereby dictating the direction of cell expansion.

MT dynamics in twisting mutants is typically monitored by the use of GFP-tubulin and EB1-GFP markers, and provides useful insights into the properties of helical MT arrays. The right-handed helical arrays of a *tua4* mutant in which Ser-178

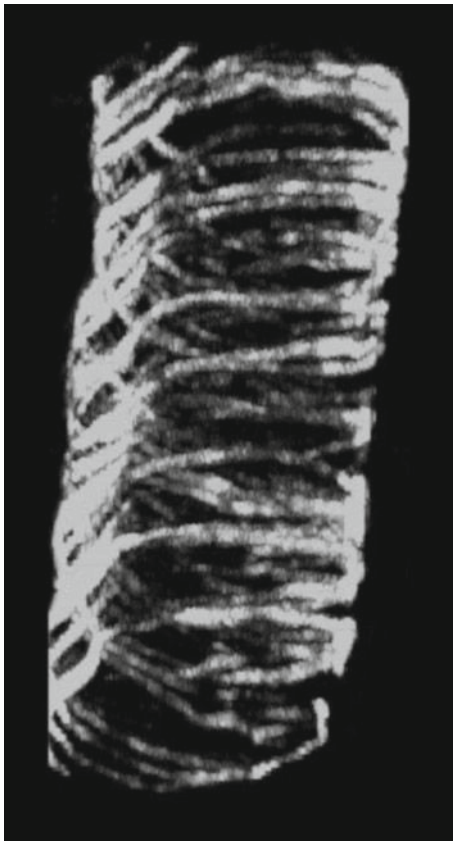

Fig. 11.3 Cortical MT arrays in an epidermal cell at the elongation zone of a left-handed twisting tubulin mutant. MTs are immunostained with a tubulin antibody, and serial confocal images are stacked to reveal the shallow right-handed helical arrays in a cell

is deleted showed more dynamic individual MTs (compared to wild-type MTs), whereas the left-handed arrays of *tua5*^{D251N} and transgenic *Arabidopsis* plants expressing the modified α-tubulin with an N-terminal appendage had less dynamic MTs [1, 25]. Such a correlation between MT dynamics and the helical organization is interesting but other MT properties should be considered before making a simple generalization. Low concentrations of MT-depolymerizing drugs cause less dynamic but more destabilized MTs and right-handed helical arrays [42]. Recombinant SPIRAL2 protein and its homologue suppress the pause state, in which MTs undergo neither growth nor shrinkage, at MT ends, thereby leading to enhanced MT growth in vitro [55]. Consequently, in the *spiral2* mutant background, cortical MTs are less dynamic, and the pause state increases at the plus end.

MT nucleation is yet another factor that contributes to the generation of helical MT arrays (see [39] for general review of MT nucleation events). A missense mutation in the GCP2 subunit of the *Arabidopsis* γ-tubulin-containing complex impairs its interaction with GCP3, another subunit of the core complex, and causes a left-handed helical organization of cortical MT arrays, and severe right-handed helical growth [41]. Interestingly, in this right-handed mutant called *spiral3*, MT dynamics and nucleation efficiency are not markedly affected, but nucleating angles of newly formed MTs in relation to the wall of preexisting MTs are wider and more divergently distributed, compared to nucleating angles of wild-type MTs (with an average of around 40°). Thus, in *spiral3*, defects in some aspects of MT nucleation, rather than in MT dynamics, appear to be the primary cause for the organization of helical MT arrays. MT nucleation angles and dynamic properties (including stability) at the plus end would affect the outcome of MT–MT interactions during the formation or maintenance of cortical arrays, but there is no satisfactory explanation of the mechanistic principles by which MT arrays with fixed chirality (in the form of either right- or left-handed helices) are generated.

11.5 Toward Elucidation of Symmetry-Breaking Factors

To transform transverse MT arrays into helical arrays with fixed handedness, there should exist chiral information within a cell. An elongating epidermal cell in a cylindrical organ is polarized along the basal-apical axis and along the inner-outer axis. It is not known (and is quite questionable) whether the left and right sides of a cell accumulate quantitatively or qualitatively different molecules that generate the left–right asymmetry. Even if such a left–right asymmetry exists and is used to guide the alignment of cortical MTs, this information, together with the other two established asymmetry, would align MTs in a stack of skewed hoops, instead of helices. Plant cortical arrays (in wild-type and mutant cells) can be arranged in helices but not in a stack of tilted hoops (Fig. 11.3; [15, 26, 33, 34]).

The origin of fixed asymmetry in helical arrays might be looked for in a MT polymer itself. If we postulate that a MT has a slight tendency to twist in the direction of a right-handed helical path, such an asymmetric MT would grow on the

plasma membrane with a projectory slightly deviated to the right. A macroscopic analogy would be a primary root of *Arabidopsis* right-handed twisting mutants that skids on the hard agar surface toward the right-side of the plate. When a skewed MT forms a parallel or anti-parallel bundle with another skewed MT of the same chirality, the asymmetric information of a single MT is preserved in the bundle; two right-handed single MTs would form a right-handed bundle (Fig. 11.4). Skewed MT bundles would crawl on the plasma membrane with a defined bias toward the direction originated in the individual MTs, and would eventually encircle the cell in the form of helices.

Is the MT polymer strictly symmetrical? Most MTs assembled in vivo, including cortical MTs in *Arabidopsis* root epidermal cells [26], appear to be composed of 13 protofilaments (pfs), although many exceptional MTs with pf numbers ranging from 8 to 19 have been noted in different organisms and cell types (for references, see [11]). Observations of MTs assembled in vitro by cryo-electron microscopy detected various MTs characterized by pf numbers ranging from 10 to 16, with 14-pf MTs as the dominant type [10, 53]. Importantly, only 13-pf MTs have unskewed pfs that align parallel with respect to the MT axis, while MTs with other pf numbers are slightly skewed along the MT axis, giving what is sometimes called a supertwist (Fig. 11.4a). For example, the pf in a 12-pf MT describes right-handed helices, and the pf in a 14-pf MT forms left-handed helices. The strong preference for the 13-pf MTs in vivo suggests that cellular factors highly promote generation of the straight 13-pf MTs and restrict formation of skewed MTs. In the currently favored "template" model, 13 γ-tubulin molecules, together with other accessory protein, form a ring or lock-washer-shaped complex, and define the number of pfs in the MT (see [39] for review). Recently, EB1 and its yeast homolog Mal3 were

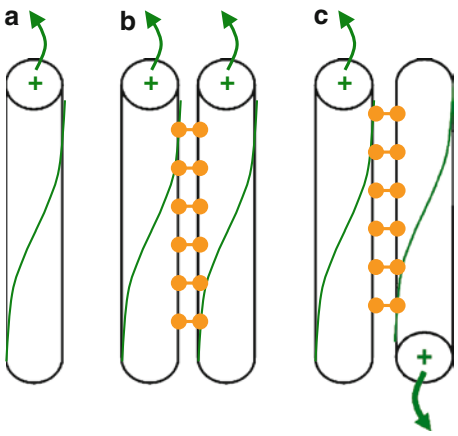

Fig. 11.4 Microtubule with right-handed pfs. (**a**) The plus end of MT (+) rotates to follow a right-handed helical path. One pf is shown in *green*. (**b**) Two MTs bundled in a parallel orientation by bundle-forming MAPs (*yellow*). The helical form of single MTs is maintained in the bundled MTs. (**c**) Two MTs bundled in an anti-parallel orientation also retain the helical form. Microtubule and cell shape determination

found to promote in vitro the assembly of pure tubulin into exclusively 13-pf MTs, possibly by eliminating stressed lattices during closure of the growing MT sheets at the plus tip to a tube configuration [19, 52]. Consequently, compromised functions of these regulated MT assembly and proof-reading mechanisms may increase in vivo frequencies of skewed MTs with a pf number other than 13. It may not be coincidental that some twisting mutants are caused by defects in a subunit of the γ-tubulin-containing nucleation complex and MAPs that accumulate, at least partly, at the plus tip.

How can we experimentally test whether the pf-number variation underlies the helical MT organization? To examine the pf numbers in MTs in vivo, a rather classical technique employs electron microscopy after fixation with glutaraldehyde and tannic acid, and then analyzes whether the MT cross-sectional image is enhanced by multiple rotary exposures with 13 equal arcs of a complete circle [32]. A critical disadvantage of this technique, however, is that the number of MTs analyzed is very limited. When cortical MTs in root epidermal cells of wild type and twisting tubulin mutants were analyzed by this method, we obtained, during the experimental period of 2–3 months, only a few dozen high-quality MT cross-sectional images, of which a handful were unambiguously assigned to be 13-pf MTs [25]. Although this analysis shows that the majority of cortical MTs have 13 pfs in both wild-type and mutant cells, it is not sensitive enough to detect minor MT populations with unusual pf numbers, if present. As cryo-electron microscopy and cryo-electron tomography have been recently used to investigate the ultrastructure of MTs in mammalian cells [6], the new technique may be applied to plant samples in the near future.

Because MTs occasionally switch from one type of lattice to another during elongation in vitro [10, 52], longitudinal images of the MT lattice (instead of cross sections) are preferable to detect possibly sporadic occurrences of skewed MT regions. To this end, cryo-electron microscopy of in vitro assembled MTs may be a practical analytical system. In vitro reconstitution of MT assembly and dynamics, and subsequent transformation of the local order into distinct array patterns, is a future challenge, and requires functional nucleation complexes and critical MT regulators that may be prepared as recombinant proteins or purified biochemically from plant cells. Once the dynamic MT organization process is reconstituted with wild-type proteins, the critical involvement of each component and effects of mutant proteins may be assessed, as well as the fine structure of MTs.

11.6 Tissue Morphology Affects Microtubule Alignment

When protoplasts prepared from cultured tobacco BY-2 cells were induced to expand under appropriate hormonal conditions, wall-regenerating cells began to align cortical MT arrays in a parallel organization and elongated in a direction perpendicular to the transverse arrays [21]. Since positional information is lost in the spherical protoplasts, the elongation axis appears to be selected randomly. In the context of a cylindrical organ, it is not well understood how the positional information

of a post-cytokinetic cell at the margin of an apical meristem is perceived and then interpreted to align initially dispersed cortical MTs so that the transverse arrays are eventually organized strictly perpendicular to the long axis of a root or stem. Previous transverse cell division events might provide a polarity cue, although experimental evidence is absent.

Mechanical modeling and experimental manipulation of the *Arabidopsis* shoot meristem now suggest that mechanical stress imposed by the tissue morphology regulates MT alignment [13, 20]. Cortical MTs were found to align parallel to the predicted directions of maximal stress; the principal stress direction and the MT orientations were radial or isotropic at the apex, and became anisotropic to an orthoradial (circumferential) pattern at the flanks of the meristem. The transverse MTs then promoted the growth of the stem into an elongated structure, thereby creating a feed-back loop. Although these experiments were done at the shoot apex, the same principal mechanisms should apply to root morphogenesis. The positional information of the epidermal cells at the flanks of the apex thus orients cortical MTs by way of physical stress. The next challenge will be to elucidate the molecular mechanisms by which a stress pattern in a cell orients MTs.

References

1. Abe T, Hashimoto T (2005) Altered microtubule dynamics by expression of modified α-tubulin protein causes right-handed helical growth in transgenic *Arabidopsis* plants. Plant J 43:191–204
2. Akashi T, Izumi K, Nagano E, Enomoto M, Mizuno K, Shibaoka H (1988) Effects of propyzamide on tobacco cell microtubules in vivo and in vitro. Plant Cell Physiol 29:1053–1062
3. Ambrose JC, Wasteneys GO (2008) CLASP modulates microtubule-cortex interaction during self-organization of acentrosomal microtubules. Mol Biol Cell 19:4730–4737
4. Baskin TI, Wilson JE, Cork A, Williamson RE (1994) Morphology and microtubule organization in *Arabidopsis* roots exposed to oryzalin or taxol. Plant Cell Physiol 35:935–942
5. Bisgrove SR, Lee Y-RJ, Liu B, Peters NT, Kropf DL (2008) The microtubule plus-end binding protein EB1 functions in root responses to touch and gravity signals in *Arabidopsis*. Plant Cell 20:396–410
6. Bouchet-Marquis C, Zuber B, Glynn A-M, Eltsov M, Grabenbauer M, Goldie KN, Thomas D, Frangakis AS, Dubochet J, Chrétien D (2007) Visualization of cell microtubules in their native state. Biol Cell 99:45–53
7. Burk DH, Liu B, Zhong R, Morrison WH, Ye Z (2001) A katanin-like protein regulates normal cell wall biosynthesis and cell elongation. Plant Cell 13:807–827
8. Buschmann H, Hauptmann M, Niessing D, Lloyd CW, Schäffner AR (2009) Helical growth of the *Arabidopsis* mutant *tortifolia2* does not depend on cell division patterns but involves handed twisting of isolated cells. Plant Cell 21:2090–2106
9. Carland FM, McHale NA (1996) *LOP1*: a gene involved in auxin transport and vascular patterning in *Arabidopsis*. Development 122:1811–1819
10. Chrétien D, Fuller SD (2000) Microtubules switch occasionally into unfavorable configurations during elongation. J Mol Biol 298:663–676
11. Chrétien D, Wade RH (1991) New data on the microtubule surface lattice. Biol Cell 71:161–174
12. Cnops G, Neyt P, Raes J, Petrarulo M, Nelissen H, Malenica N, Luschnig C, Tietz O, Ditengou F, Palme K, Azmi A, Prinsen E, van Lijsebettens M (2006) The *TORNADO1* and

TORNADO2 genes function in several patterning processes during early leaf development in *Arabidopsis thaliana*. Plant Cell 18:852–866
13. Corson F, Hamant O, Bohn S, Traas J, Boudaoud A, Couder Y (2009) Turning a plant tissue into a living cell froth through isotropic growth. Proc Natl Acad Sci USA 106:8453–8458
14. Dimitrov A, Quesnoit M, Moutel S, Cantaloube I, Poüs C, Perez F (2008) Detection of GTP-tubulin conformation in vivo reveals a role for GTP remnants in microtubule rescues. Science 322:1353–1356
15. Flanders DJ, Rawlins DJ, Shaw PJ, Lloyd CW (1989) Computer-aided 3-D reconstruction of interphase microtubules in epidermal cells of *Datura stramonium* reveals principles of array assembly. Development 106:531–541
16. Frei E, Preston RD (1961) Cell wall organization and wall growth in the filamentous green algae *Cladophora* and *Chaetomorpha* II. Proc R Soc Lond B 155:55–77
17. Furutani I, Watanabe Y, Prieto R, Masukawa M, Suzuki K, Naoi K, Thitamadee S, Shikanai T, Hashimoto T (2000) The *SPIRAL* genes are required for directional control of cell elongation in *Arabidopsis thaliana*. Development 127:4443–4453
18. Geitmann A, Ortega JKE (2009) Mechanisms and modeling of plant cell growth. Trends Plant Sci 14:467–478
19. des Georges A, Katsuki M, Drummond DR, Osei M, Cross RA, Amos LA (2008) Mal3, the *Schizosaccharomyces pombe* homolog of EB1, changes the microtubule lattice. Nature Struct Mol Biol 15:1102–1108
20. Hamant O, Heisler MG, Jönsson H, Krupinski P, Uyttewaal M, Bokov P, Corson F, Sahlin P, Boudaoud A, Meyerwitz EM, Couder Y, Traas J (2008) Developmental patterning by mechanical signals in *Arabidopsis*. Science 322:1650–1655
21. Hasezawa S, Hogetsu T, Syono K (1988) Rearrangement of cortical microtubules in elongating cells derived from tobacco protoplasts. J Plant Physiol 133:46–51
22. Hashimoto T (2002) Molecular genetic analysis of left-right handedness in plants. Philos Trans R Soc Lond B Biol Sci 357:799–808
23. Hugdahl JD, Morejohn LC (1993) Rapid and reversible high-affinity binding of the dinitroaniline herbicide oryzalin to tubulin from *Zea mays* L. Plant Physiol 102:725–740
24. Ishida T, Hashimoto T (2007) An *Arabidopsis thaliana* tubulin mutant with conditional root-skewing phenotype. J Plant Res 120:635–640
25. Ishida T, Kaneko Y, Iwano M, Hashimoto T (2007) Helical microtubule arrays in a collection of twisting tubulin mutants of *Arabidopsis thaliana*. Proc Natl Acad Sci USA 104:8544–8549
26. Ishida T, Thitamadee S, Hashimoto T (2007) Twisted growth and organization of cortical microtubules. J Plant Res 120:61–70
27. Kamphausen T, Fanghänel J, Neumann D, Schulz B, Rahfeld J (2002) Characterization of *Arabidopsis thaliana* AtFKBP42 that is membrane-bound and interacts with Hsp90. Plant J 32:263–276
28. Kirik V, Grini PE, Mathur J, Klinkhammer I, Adler K, Bechtold N, Herzog M, Bonneville J-M, Hülskamp M (2002) The *Arabidopsis TUBULIN-FOLDING COFACTOR A* gene is involved in the control of the α/β-tubulin monomer balance. Plant Cell 14:2265–2276
29. Komaki S, Abe T, Coutuer S, Inzé D, Russinova E, Hashimoto T (2010) Nuclear-localized subtype of end-binding 1 protein regulates spindle organization in *Arabidopsis*. J Cell Sci 123:451–459
30. Korolev AV, Buschmann H, Doonan JH, Lloyd CW (2007) AtMAP70-5, a divergent member of the MAP70 family of microtubule-associated proteins, is required for anisotropic cell growth in *Arabidopsis*. J Cell Sci 120:2241–2247
31. Ledbetter MC, Porter KR (1963) A "microtubule" in plant cell fine structure. J Cell Biol 19:239–250
32. Ledbetter MC, Porter KR (1964) Morphology of microtubules of plant cells. Science 144:872–874
33. Liang BM, Dennings AM, Sharp RE, Baskin TI (1996) Consistent handedness of microtubule helical arrays in maize and *Arabidopsis* primary roots. Protoplasma 190:8–15

34. Lloyd CW (1983) Helical microtubular arrays in onion root hairs. Nature 305:311–313
35. Lloyd CW (1984) Toward a dynamic helical model for the influence of microtubules on wall patterns in plants. Int Rev Cytol 86:1–51
36. Lloyd CW, Chan J (2002) Helical microtubule arrays and spiral growth. Plant Cell 14:2319–2324
37. Morejohn LC (1991) The molecular pharmacology of plant tubulin and microtubules. In: Lloyd CW (ed) The cytoskeletal basis of plant growth and form. Academic, London, pp 29–43
38. Morejohn LC, Fosket DE (1984) Inhibition of plant microtubule polymerization in vitro by the phosphoric amide herbicide amiprophos-methyl. Science 224:874–876
39. Murata T, Hasebe M (2010) Microtubule nucleation and organization in plant cells. In: Liu B (ed) The Plant Cytoskeleton, pp. 81–94. Springer, New York
40. Nakajima K, Furutani I, Tachimoto H, Matsubara H, Hashimoto T (2004) *SPIRAL1* encodes a plant-specific microtubule-localized protein required for directional control of rapidly expanding *Arabidopsis* cells. Plant Cell 16:1178–1190
41. Nakamura M, Hashimoto T (2009) A mutation in the *Arabidopsis* γ-tubulin-containing complex causes helical growth and abnormal microtubule branching. J Cell Sci 122:2208–2217
42. Nakamura M, Naoi K, Shoji T, Hashimoto T (2004) Low concentrations of propyzamide and oryzalin alter microtubule dynamics in *Arabidopsis* epidermal cells. Plant Cell Physiol 45:1330–1334
43. Nogales E, Whittaker M, Milligan RA, Downing KH (1999) High-resolution model of the microtubule. Cell 96:79–88
44. Pérez-Pérez JM, Ponce MR, Micol JL (2004) The *ULTRACURVATA2* gene of *Arabidopsis* encodes an FK506-binding protein involved in auxin and brassinosteroid signaling. Plant Physiol 134:101–107
45. Probine MC (1963) Cell growth and the structure and mechanical properties of the wall in internodal cells of *Nitella opaca.* III. Spiral growth and cell wall structure. J Exp Bot 14:101–113
46. Savaldi-Goldstein S, Peto C, Chory J (2007) The epidermis both drives and restricts plant shoot growth. Nature 446:199–202
47. Sedbrook JC, Ehrhardt DW, Fisher SE, Scheible W, Somerville CR (2004) The *Arabidopsis SKU6/SPIRAL1* gene encodes a plus end-localized microtubule-interacting protein involved in directional cell expansion. Plant Cell 16:1506–1520
48. Sedbrook JC, Kaloriti D (2008) Microtubules, MAPs and plant directional cell expansion. Trends Plant Sci 13:303–310
49. Sugimoto K, Williamson RE, Wasteneys GO (2000) New techniques enable comparative analysis of microtubule orientation, wall texture, and growth rate in intact roots of *Arabidopsis*. Plant Physiol 124:1493–1506
50. Thitamadee S, Tuchihara K, Hashimoto T (2002) Microtubule basis for left-handed helical growth in *Arabidopsis*. Nature 417:193–196
51. Vaughn KC (2000) Anticytoskeletal herbicides. In: Nick P (ed) Plant microtubules. Springer, Berlin, pp 193–205
52. Vitre B, Coquelle FM, Heichette C, Garnier C, Chrétien D, Arnal I (2008) EB1 regulates microtubule dynamics and tubulin sheet closure in vitro. Nat Cell Biol 10:415–421
53. Wade RH, Chrétien D (1990) Characterization of microtubule protofilament numbers. J Mol Biol 212:775–786
54. Williamson RE (1991) Orientation of cortical microtubules in interphase plant cells. Int Rev Cytol 129:135–206
55. Yao M, Wakamatsu Y, Itoh TJ, Shoji T, Hashimoto T (2008) *Arabidopsis* SPIRAL2 promotes uninterrupted microtubule growth by suppressing the pause state of microtubule dynamics. J Cell Sci 121:2372–2381
56. Young DH, Lewandowski VT (2000) Covalent binding of the benzamide RH-4032 to tubulin in suspension-cultured tobacco cells and its application in a cell-based competitive-binding assay. Plant Physiol 124:115–124

Chapter 12
Cytoskeleton and Root Hair Growth

Eunsook Park and Andreas Nebenführ

12.1 Introduction

Root hairs are highly polarized outgrowths of a subset of root epidermis cells, the so-called trichoblasts. The biological function of root hairs is to increase the surface area of roots in order to facilitate the absorption of water and nutrients from soil. Root hairs are also the site of initial interaction with microorganisms [22]. The patterning of root trichoblasts varies between plant species and can also be regulated by environmental factors [16]. These genetic and environmental regulatory mechanisms have been studied intensively and were reviewed elsewhere [31]. Trichoblasts are unique plant cells that first elongate by diffuse growth over their entire surface with the other root cells and subsequently form an outgrowth, the root hair, which elongates only at its very tip. This kind of tip growth also occurs in pollen tubes, which are discussed elsewhere in this volume.

The mechanism of root hair growth can be conceptually divided into two distinct events: root hair initiation, which breaks the symmetry of the root epidermis and results in the formation of a bulge, and unidirectional root hair elongation by tip growth in which all secretion of new cell wall material occurs in a small area at the tip of the hair. Root hairs are not critical for plant growth, so that plants can grow normally on growth media in the lab even with defective root hairs. This permitted studies about the molecular mechanisms underlying root hair development based on mutant screens for defects in root hair growth [55]. These mutants showed diverse phenotypes from abnormal length or shape of root hairs to additional root hair initiation. In independent studies, pharmacological analyses using chemicals to disrupt cytoskeletal organization revealed the importance of the cytoskeleton, especially actin filaments, for tip growth [2, 7]. More recently, studies with GFP-fused proteins related to root hair growth provided further support for the emerging regulatory network and opened up the additional dimension of temporal dynamics [11, 13, 29].

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Collectively, these studies have established that three factors are very important for this special type of cell morphogenesis. First, signaling from the trichoblast determination pathway leads to a rearrangement of the cytoskeleton in the root epidermis for bulge formation and ultimately for support of tip growth in the emerging hair [2, 7]. Second, polar membrane trafficking is required to provide new plasma membrane and cell wall components to the growing tip [44]. Finally, there is a unique distribution of regulatory factors, primarily calcium [18], reactive oxygen species [41], and pH [8] which regulate each other to modulate tip growth (reviewed in [13]). Pollen tubes, the other classical model for tip growth in plants, share a similar growth mechanism that involves similar key regulators [13]. However, there are also some distinct differences between pollen tubes and root hairs, such as vacuole position and growth rate. It remains to be seen whether a common model can simultaneously explain the specific behavior of root hairs and pollen tubes. However, in this chapter, we will forgo this comparison and focus on the mechanisms of root hair growth.

12.2 Root Hair Initiation

The first visible step during root hair initiation is that a part of the outer epidermis cell starts swelling in response to local signaling. The position of the initial swelling depends on the plant species. For example, *Arabidopsis* root hairs are initiated on the basal part of the trichoblast, i.e. closest to the root tip [11], while maize roots form the root hair approximately in the middle of an epidermal cell [2, 19]. The positioning of root hairs on trichoblasts depends at least partially on hormonal signaling as revealed by mutant analysis. As the first step of root hair initiation, a membrane trafficking effector, ROP-GTPase, and a cell wall loosening enzyme, expansin, accumulate at the initiation site [40]. Both actin and microtubule cytoskeleton then rearrange [69] and local alkalization of the cytosol occurs accompanied by acidification of the cell wall [8]. In addition, the cytosolic calcium concentration increases locally due to a massive uptake of calcium from the environment [70]. Finally, actin filaments accumulate and the cell bulges outward and eventually begins tip growth [31].

12.2.1 Selection of the Bulge Site

Auxin and ethylene signaling appears to be a key regulator in determining the position of root hairs, since many root hair defective mutants are directly or indirectly related to auxin or ethylene responses [26]. In particular, ethylene signaling mutants showed altered position of root hairs on a trichoblast, suggesting that bulge site selection occurs downstream of a hormone signaling pathway [38]. The auxin transport mutant, *aux1*, also displayed root hairs in an apically shifted position and often

carried two root hairs on a cell [25]. The apical and basal membrane localization of AUX1 on root epidermis cells appeared to be required for maintaining planar root hair polarity by facilitating the uptake of auxin from more distal cells to maintain a local proximal auxin maximum in the trichoblast [60]. Moreover, pharmacological interruption of auxin transport also disrupted proper root hair positioning, supporting the critical role of auxin in determining the root hair position [24].

How auxin and ethylene signaling affect cytoskeletal reorganization is still not clear. However, ROP-GTPase (Rho-like GTPase of plants) may be part of the signaling cascade. In *Arabidopsis*, immunolocalization of ROP proteins revealed their accumulation under the plasma membrane of the root hair initiation site even before swelling started, and this accumulation was maintained during root hair growth [21]. This localization, together with evidence that overexpression of ROP-GTPase resulted in multiple root hair formation on a single trichoblast [77], further supports the notion that ROP-GTPases are key regulators for root hair initiation. ROP accumulation was not sensitive to cytoskeleton-disrupting drugs suggesting that ROP accumulation is an upstream event of actin and microtubule rearrangement in trichoblasts [2, 40]. How localization and activity of ROP-GTPase is functionally regulated by polar auxin transport still remains an open question to be investigated. Recently, a mathematical simulation model hypothesized that the auxin gradient might function to balance the activity of ROPs, so that the root hair can be formed at the proper position of root epidermis cells [45]. Although there is as of yet little experimental support for this hypothesis, this model is a good starting point to identify the mechanisms that ultimately lead to selection of the bulge site.

12.2.2 The Cytoskeleton in Bulge Formation

Root epidermis cells have highly organized transverse cortical microtubule arrays. Once a trichoblast starts root hair formation, this microtubule array becomes irregular and randomized at the site of the future root hair bulge [2]. This is correlated with local wall acidification which leads to a change in cell wall formation and ultimately results in wall thinning so that the cell can swell in this region [8]. Remarkably, the apex of the bulge is devoid of microtubules when the root hair swelling is ready to initiate tip growth [2]. At the same time, actin filaments at the future bulge site become arranged parallel to the long axis of the cell [2]. G-actin and profilin also accumulate at the tip of the root hair bulge, as observed by immunofluorescence in maize [10], and a fine actin meshwork later forms within the bulge [2].

Actin mutants, *act2* and *act8*, showed root hair bulges but no proper tip growth suggesting that the actin cytoskeleton does not have a critical role in the early steps of root hair initiation [32, 51]. However, actin mutants often formed multiple sites of root hair bulges implying that actin might be involved in the process of determining the root hair initiation site on the trichoblast [32]. Lettuce seedlings that were germinated on media containing 10 μM cytochalasin B, an inhibitor of actin

polymerization, did not produce any root hairs [61]. This result suggests that longitudinal redistribution of actin filaments on the site of root hair emergence is necessary for root hair initiation. However, cytochalasin D treatment of vetch roots did not show any effects on root hair bulge formation [39]. Note, however, that cytochalasin variants have different side effects beside actin disruption. Thus, it is conceivable that long-term exposure of lettuce roots to cytochalasin B may have induced additional secondary effects that inhibited root hair development. The actin cytoskeleton is clearly more important in later steps of root hair initiation, namely, during the transition from bulge formation to tip growth since *actin* mutants as well as pharmacological interventions showed that a defect of actin organization prevented the initiation of root hair tip growth [2, 32, 39, 51].

12.3 Root Hair Tip Growth: General Considerations and Organelle Distribution

Once a bulge is established, root hairs continue to increase their surface area only at their tip, away from the root epidermis. *Arabidopsis* root hairs have been reported to be able to grow to a length of about 700 μm with the growth rate of 1–2 μm/min. However, these parameters can differ significantly depending on growth conditions [42, 43]. Conventionally, root hairs are divided into three zones from the tip toward the root epidermis, apex, subapex, and shank. This subcellular organization of growing root hairs shares some similarity with pollen tubes with some notable differences (compare Chap. 3 for a discussion of pollen tube growth). In growing root hairs, the dome-shaped apex, where active growth occurs, is predominantly filled with secretory vesicles. Large organelles are prevented from entering this region, presumably by mesh-like short, randomly distributed actin fragments in the subapex [4]. Unlike pollen tubes, apex and subapex of root hairs are often not clearly distinguished; the vesicle-filled apical zone is narrower than in pollen tubes and the actin mesh spreads more broadly. A major difference between root hairs and pollen tubes is the position of the large central vacuole. While the vacuole in pollen tubes is located far behind the tip in the shank, the vacuole in growing root hairs can reach into subapex [13].

When root hairs stop growing, the vesicle accumulation in the apex disappears and the vacuole can fill the entire root hair. These characteristic changes in organelle distribution and cell morphology depend strongly on the cytoskeleton which in turn is regulated by a feedback loop involving membrane transport and several signaling molecules.

12.3.1 Actin Cytoskeleton in Root Hair Tip Growth

Actin filaments are fundamental for root hair growth, similar to what was found in other cell types (reviewed in [30, 58]. Visualization of the actin cytoskeleton has

been performed with several different probes in fixed or living root hairs. While longitudinal actin bundles along the shank of growing root hairs are relatively consistent in images with different probes, the existence of actin at the subapex and apex is somewhat controversial. Confocal microscopy images of actin visualized with a freeze-shattering technique using actin antibodies showed high actin accumulation at the maize root hair apex [2]. Labeling of globular actin (G-actin) by fluorescein isothiocyanate (FITC)-DNase I also showed that G-actin accumulated extensively in the apex in growing root hairs of wheat. This accumulation disappeared and was replaced by thick filamentous actin (F-actin) extending into the tip in fully grown root hairs [27]. Co-visualization of G-actin and F-actin by labeling with FITC-DNase I and Tetramethyl Rhodamine Isothiocyanate (TRITC)-Phalloidin, respectively, showed distinct localization of G-actin and F-actin in growing root hairs. While G-actin accumulated at the apex, F-actin was presented in the shank of the growing root hairs and could not penetrate into the apex [27].

GFP-conjugated actin binding proteins also have been used to visualize actin filaments. This technique offers the advantage of revealing F-actin dynamics during root hair growth. However, this has to be balanced with potential artifacts resulting from over-expression of the labels. Initial experiments with GFP-talin transformed *Arabidopsis* displayed the high accumulation of actin at the apex of growing root hairs [2]. In subsequent years, it was revealed that over-expression of GFP-talin could cause severe developmental defects in transgenic plants [34]. Many studies now employ a fimbrin-based marker to visualize actin dynamics in all cells of several species (Fig. 12.1a). The second actin binding domain of fimbrin fused to the C-terminus of GFP (GFP-FABD2) displayed more fine actin filaments than

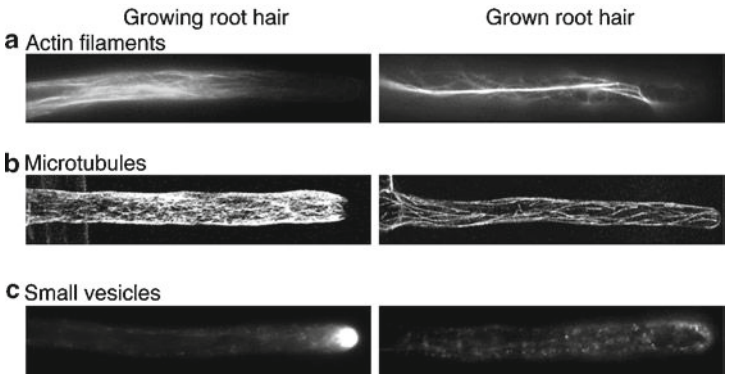

Fig. 12.1 Distinct distribution of cytoskeletal elements and vesicles during root hair tip growth. (**a**) Actin cytoskeleton, visualized with FABD2-YFP. In growing root hairs, cortical F-actin filaments array mostly longitudinally along the shank and are absent from subapex and apex of root hairs. In contrast, thick bundles of actin cables reach into the apex in fully-grown root hairs. (**b**) Microtubules, visualized by GFP-MBD. Growing root hairs display longitudinal or helical microtubules along the root hair shank. Note that microtubules do not reach the extreme apex of the growing root hair while they do so in growing root hairs (modified after [68]). (**c**) RabA4b-YFP-labeled vesicles accumulate at the tip of growing root hairs. This accumulation is tightly correlated with root hair growth; consequently, fully-grown root hairs lack the accumulation of RabA4b-YFP vesicles at the tip

GFP-talin and did not show any obvious developmental defects [71]. In contrast to GFP-talin, GFP-FABD2 (or GFP-FABD2-GFP for brighter signals) did not accumulate in the apex of growing root hairs of transgenic *Arabidopsis* seedlings [56, 72]. However, FABD2-GFP transformed *Medicago truncatula* displayed accumulation of actin at the apex [39, 65, 71], suggesting that actin organization might differ between the species. Recently, a new actin marker, Lifeact has been introduced which is based on the actin-binding domain of yeast ABP1 [17]. Lifeact-Venus revealed a similar actin cytoskeleton as GFP-FABD2, however, it had a better resolution at the root hair tip, so that an irregular actin mesh in the subapex and highly dynamic fine filaments reaching into the tip of the apex could be observed [17].

Based on results from a variety of cell types, it is generally assumed that a central function of actin filaments is to deliver membrane compartments to the apex of root hairs in order to provide cell wall components and membrane lipids necessary for growth, as well as several regulatory factors [3]. The contribution of actin dynamics to root hair tip growth has been exposed by treatment with actin disrupting drugs, Latrunculin B (LatB) [2, 9] and cytochalasin D [39]. The effect of LatB is dosage-dependent. A 1 h treatment of as little as 50 nM LatB reduced root hair growth by 25% while concentrations higher than 500 nM led to complete cessation of root hair growth in *Arabidopsis* [7]. Concentrations of more than 1 μM cytochalasin D also caused cytoplasmic streaming to stop within 30 min and 10 μM cytochalasin D could kill root hairs within 15 min in *Medicago* [39]. Overall, disruption of actin filaments causes root hairs to stop growing, suggesting that actin organization is critical for tip growth. Genetic studies have corroborated this conclusion. Over decades, numerous root hair mutants of *Arabidopsis* have been isolated in several studies (reviewed in Guimill and Dunand, 2007) and some of them are mutants of either components of actin filaments or regulators of actin dynamics. For example, *der1*, a mutant of *ACTIN2* (*ACT2*), displayed a cessation of root hair growth after bulging [51] with additional pleiotropic phenotypes in different tissues [23]. ACT8, the isoform most similar to ACT2, also contributes to root hair tip growth. *act8* mutants showed around 50% shorter root hairs than wild type and overexpression of *ACT8* could complement the *act2* mutant phenotype [32]. Mutation of several regulators of actin dynamics also displayed an arrest of tip growth in root hairs; induced overexpression of AIP1, a F-actin capping protein, resulted in short root hairs [33], and overexpression of *PFN1*, an *Arabidopsis* profilin isoform, stimulated root hair tip growth and resulted in root hairs that were twice as long as wild type [49]. Finally, mutation of *AtFH8*, an *Arabidopsis* group Ie formin known to regulate actin dynamics, caused an arrest of root hair tip growth after bulge formation [15].

12.3.2 Myosin in Root Hair Tip Growth

While the actin cytoskeleton has been recognized as an important factor for tip growth, the involvement of myosins, motor proteins that utilize F-actin as a track, was not clear until recently. Based on evidence from pollen tubes and circumstantial

evidence from the importance of the actin cytoskeleton [63, 78], a contribution of myosins on root hair tip growth has been proposed [67]. Recent studies with truncated class XI myosins fused to GFP variants at their N-terminus showed their localization on various membrane compartments suggesting a function in intracellular vesicle trafficking [1, 36]. Direct evidence supporting the involvement of myosin in root hair tip growth was provided by the identification of a mutant of *Arabidopsis thaliana*, *xi-k*. *xi-k* plants showed short root hairs and abnormal trichome branching patterns [43, 46]. *MYA2*, another one of the 13 class XI myosin genes in *Arabidopsis*, also seems to be involved in root hair tip growth based on the fact that *mya2* mutants also displayed short root hairs [46]. Interestingly, in these mutants, movements of three organelles, Golgi, peroxisomes, and mitochondria, were dramatically slower than in wild type [46]. It is still unknown how a single myosin can contribute to the movement of three distinct organelles, particularly, since none of the tested YFP-myosin tail fusions localized to these organelles [36, 50, 59]. A possible hypothesis is that one of the cargoes of XI-K might be a regulator of cytoskeleton dynamics, thus resulting in defects of movements of many organelles. In support of this contention, recent observation using variable-angle evanescent wave microscopy and spinning disc confocal microscopy showed that actin turnover might be required for myosin-based mitochondrial trafficking [80]. Thus, while it is now firmly established that myosins are involved in root hair tip growth, more research is needed to decipher the mechanism by which myosins operate in this process.

12.3.3 Microtubules and Root Hair Tip Growth

The organization of microtubules has been initially visualized in root hairs using electron microscopy and immunofluorescence with anti-tubulin antibodies [37, 68]. Later, transgenic plants expressing fluorescent proteins fused to α-tubulin 6 (GFP-TUA6) or to the microtubule binding domain of microtubule associated protein 4 (GFP-MBD), were used to reveal dynamic changes of microtubule organization during root hair growth [5, 65]. After the reorganization event during root hair bulging, dense cortical microtubules (CMTs) are arranged longitudinally along the shank of the root hairs. Similar to the organization of F-actin, CMTs have not been detected in the apex of growing root hairs (Fig. 12.1b). Once root hairs are fully grown and the central vacuole approaches the root hair tip, longer and less dense CMTs are arranged longitudinally or spirally along the root hairs and can reach to the very tip of the root hairs [65, 69]. Endoplasmic microtubules (EMTs) initially have been observed in CLSM images in the interior of root hairs expressing GFP-MBD in *Medicago truncatula* [57]. EMTs displayed a more irregular arrangement than CMTs and predominantly accumulated around the nucleus as well as in the subapex. This distribution later has also been shown to exist in *Arabidopsis* root hairs [69]. EMTs in the subapex of growing root hairs are highly dynamic. While the majority of CMTs array parallel to the shank of root hairs, EMTs at the subapex continuously change their directions and lengths [69].

In contrast to actin filaments, which play a major role in tip growth, microtubules are generally thought of being primarily important for maintaining the direction of root hair growth. Treatment with the microtubule depolymerizing drug, oryzalin, and the microtubule stabilizing drug, taxol, showed a loss of directionality of *Arabidopsis* root hair growth [7]. Both taxol and oryzalin-treated root hairs displayed wavy root hairs as well as branched root hairs. Both drugs showed a similar effect on the angular deviation from straight root hairs, however, taxol was more effective in triggering branching than oryzalin [7], suggesting that the two drugs can distinguish between two distinct roles of microtubules during root hair growth. Analysis of mutants in α-tubulin (*tua6*) [5] and in a microtubule-associated protein (*mor1*) [73] also revealed branched or wavy root hair growth, consistent with the pharmacological analysis. Thus, microtubules appear to be required for maintaining a stable polarity for straight growth.

The growth rates of *Arabidopsis* root hairs did not change during treatment with the microtubule disrupting drug treatments [7] although *Medicago truncatula* root hairs had only 60% of root hair growth rate of untreated root hairs [57]. Thus, it is likely that microtubules do not play a direct the role in enlarging the root hair cell surface. Recent studies of mutants in armadillo repeat-containing kinesins (ARKs or MRH2) of *Arabidopsis* showed abnormal root hair growth but no reduced root hair growth rate, further supporting a microtubule function in restricting the elongation zone of root hair tip growth [53, 77, 79].

Given their co-alignment patterns [22] and evidence from other cell types [14], an interaction between microtubules and the actin cytoskeleton has been proposed. Using propyzamide to depolymerize microtubules and cytochalasin B to destabilize actin filaments, the interaction between microtubules and actin cytoskeleton has been studied in detail in the root hairs of *Hydrocharis dubia* [66]. Simultaneous treatment with both inhibitors was sufficient to stop cytoplasmic streaming and growth. Washout of Cytochalasin B in the presence of propyzamide did not allow full recovery of the longitudinal actin cytoskeleton indicating that longitudinal microtubules are required to establish the actin cables normally found in the root hair shank [66]. At the same time, actin filaments appeared to be required for normal MT dynamics since longer treatment of root hairs with LatB permitted the formation of MT bundles in the apical or subapical regions [65]. A possible candidate for this interaction between actin filaments and microtubules is ARK/MRH2 kinesin, since it could be shown that ARK1/MRH2 can bind to actin filaments in vitro [77]. Further work will be necessary to confirm this hypothesis.

12.3.4 Membrane Trafficking and Tip Growth

To increase the cell size, massive secretion at the tip is required to provide membrane and cell wall components. Additionally, active endocytosis occurs at the apex of root hairs to recycle regulators and remove excess membrane [44]. These membrane trafficking activities are reflected in the accumulation of secretory and endocytic vesicles in the apex of growing root hairs. The small GTPase, RabA4b, has been

successfully used as a vesicle marker in root hairs (Fig. 12.1c). In growing root hairs, RabA4b-YFP labeled vesicles accumulated at the apex of root hairs and LatB treatment released this accumulation coincident with a cessation of growth [48].

It is not surprising that many root hair mutants are defective in genes encoding proteins involved in membrane trafficking [23]. Members of the Ras-like small GTPase superfamily have roles on various endomembrane compartments and several mutants of these proteins showed impaired tip growth. A major example is ROP, a Rho-like GTPase that coordinates actin organization and membrane trafficking by stimulating multiple signaling pathways [76]. Beside its contribution to root hair initiation (see Sect. 12.2.1), ROP-GTPase functions as a critical player in root hair tip growth [40]. ROP-GTPase is normally localized to almost the entire plasma membrane of plant cells but is restricted to the apical plasma membrane of growing root hairs. ROP-GTPase activates phosphatidyl-inositol (PtdIns)-monophosphate kinase (PIPK), a key regulator to maintain tip focused membrane trafficking and actin organization [76]. It also stimulates NADPH oxidase activity, which leads to the production of reactive oxygen species [56], a critical factor involved in regulating calcium gradients at the apex of root hairs [6]. Many regulators of ROP-GTPase activity were also found to be involved in root hair tip growth. An *Arabidopsis* root hair defective mutant, *supercentipede 1* (*scn1*), has a similar phenotype to ROP-GTPase mutants, such that mutants have multiple root hair initiation sites in a cell and root hair tip growth is aborted [12]. *SCN1* encodes a Rho-GTPase GDP dissociation inhibitor (RhoGDI) that restricts ROP activity to the apex in order to maintain a polarity for root hair growth. Similarly, ROP localization on the plasma membrane is a prerequisite for its function and S-acylation of ROP-GTPase is necessary for its membrane localization [76]. *TIP1* encodes a S-acyl transferase [28] and *Arabidopsis tip1* mutants were initially isolated based on their extremely short, sometimes branched root hair phenotype [54]. Since S-acyl transferases tend to modify only specific target proteins, it will be interesting to test whether ROPs are targets for TIP1.

Other regulators of root hair tip growth are specific phosphoinositides that can serve as a recognition landmark for certain cytosolic proteins, thus recruiting them to membrane patches where they then perform their functions. For example, PI(4,5)P_2 accumulates in the apical plasma membrane of root hairs and many modifying proteins of this lipids are important for the root hair tip growth mechanism [13, 75]. RabA4b interacts with PtdIns-4OH Kinase, PI-4Kβ1 that synthesizes a precursor of PI(4,5)P_2 and both proteins colocalize to small vesicles at the apex of growing root hairs [47]. A *pi4kβ1/2* double mutant displayed shorter root hairs than wild type, which were often branched or jagged. PI-4Kβ1 also binds to CBL1, a calcium sensor, implicating that activity of PI-4Kβ1 is depending on the calcium gradient present at the root hair apex. Another root hair defective mutant, *rhd4*, showed short and wavy root hairs and RabA4b-YFP accumulation at the apex was altered in the mutants [64]. *RHD4* encodes PI4P-phosphatase and might function to balance PI4P levels to maintain polar tip growth at the root hair apex. RHD4 might also have a function in actin organization since *rhd4* mutants showed more patchy actin organization and thinner filaments in root epidermis cells compared with wild type, however, the detailed mechanism of this interaction is not yet clear.

12.3.5 Signaling Factors in Tip Growth

It has long been known that intracellular calcium gradients in the root hair cytoplasm have an important role in root hair growth [29]. Besides a role of calcium as a second messenger in signaling cascades, it has been suggested that calcium might restrict the site of membrane trafficking and also modify the cytoskeleton at the root hair tip so that the root hair can grow straight [74]. For example, calcium can affect F-actin polymerization by controlling actin-binding protein activity, such as profilin, actin-depolymerizing factor (ADF), or villin (reviewed in Hussey et al., 2006). In support of this, profilin also accumulates in the root hair tip [2] and can lead to sequestration of G-actin in a calcium dependent manner [35]. Calcium may also control tip growth by several other mechanisms in addition to a modulation of F-actin dynamics. For example, G-actin accumulation at the apex of wheat root hairs as visualized with FITC-DNase I could be disrupted by treatment with dibromo-l,2-bis(o-aminophenoxy)ethane-*N*,*N*,*N*',*N*'-tetraacetic acid (BAPTA) [27]. BAPTA is well known to rapidly dissipate calcium gradients at the growing root hair tip and consequently stops root hair tip growth [18]. It was also shown that membrane trafficking is regulated by the calcium gradient since treatment with the calcium ionophore A23187 caused the release of the accumulation of RabA4b-YFP from the apex and a stop of root hair growth [47]. Despite these insights, the precise mechanism by which calcium regulates tip growth is still unknown. Given the role of calcium as a key regulatory factor for many cellular events, it is likely that the calcium gradient coordinates tip growth by acting through multiple regulatory factors at the same time.

Similar to pollen tubes, the tip-focused calcium gradient in root hairs has been shown to oscillate within a range from 0.2 μM to more than 1.5 μM [9, 74]. These calcium oscillations are tightly correlated with the cell growth rate [42]. Recent studies have investigated this connection in more detail by using the ratiometric calcium marker, yellow Cameleon 3.6 [42]. This study demonstrated that root hair growth rate oscillations are typically followed by cytosolic calcium oscillation at the root hair apex with about 5 s lag time. This observation suggests that a high concentration of cytosolic calcium at the root hair apex restricts root hair growth. This hypothesis was supported by observations that treatment with 200 μM La^{3+}, a blocker of Ca^{2+} channels, led to an acceleration of root hair growth. Similarly, treatment with 10 μM A23187, a calcium ionophore, blocked root hair growth [42]. Cytosolic calcium gradients are accompanied by pH changes and a ROS gradient in the cytosol as well as in the cell wall space. ROS are also considered a critical factor to sustain polar growth, since *rhd2*, a mutant of *Arabidopsis* NADPH oxidase, displayed short root hairs and the typical calcium gradient in root hair tip was absent [20]. Given that addition of external ROS can recover the root hair elongation and ROS can activate calcium channels, it is likely that accumulation of ROS at the tip is required to open calcium channels at the plasma membrane of the root hair apex in order to increase the cytosolic calcium concentration at the tip [20]. Interestingly, unlike animal NADPH oxidase, plant NADPH oxidases have two EF hand motifs at their N-terminus suggesting their regulation by calcium [52]. Indeed,

RHD2 can be activated by calcium in the growing root hair, resulting in a positive feedback loop [52, 62]. This inter-dependence of calcium and ROS signaling leads to alternating oscillations of ROS and Ca^{2+}gradients in the growing root hair tip, which appears to be necessary for maintaining polar root hair growth [41].

12.4 Conclusions

Root hairs are very dynamic cells which can grow relatively fast compared to other plant cells. They are also unique in their asymmetric outgrowth from a part of root epidermis cells. The growth of root hairs is regulated by a highly elaborate mechanism that involves many components, such as the cytoskeleton, membrane trafficking, and signaling factors. Two distinct steps of root hair development, root hair initiation and tip growth, are regulated in different ways by the regulatory factors (Fig. 12.2). However, there are common rules for this regulatory system. First, signals from genetic and environmental cues are translated into a change of the

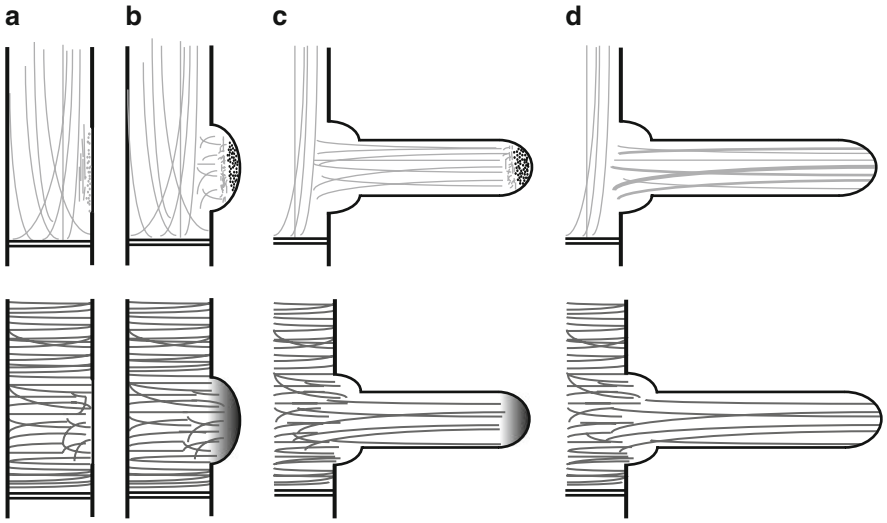

Fig. 12.2 Cellular architecture during root hair development. Schematic representation of root hairs during four stages of root hair development. Upper panels show actin filaments (*grey lines*) and secretory vesicles (*black dots*). Lower panels show microtubules (*darker grey lines*) and the tip-focused calcium gradient (*darker color* represents higher calcium concentration). (**a**) Early events after bulge site selection. Note the fragmentation of actin filaments and the local loss of microtubule organization. (**b**) Initial outgrowth and bulge formation. Actin filaments begin to form a dense mesh behind the tip region where vesicles accumulate and the calcium gradient forms. (**c**) Tip growth. Cytoskeletal elements are mostly longitudinal along the shank but do not reach into the apex. Vesicle accumulation and calcium accumulation are maximal at the apex. (**d**) Fully grown hair. Vesicle accumulation and calcium gradient at the tip disappear and longitudinal actin filaments and microtubules reach into the apex

cytoskeleton. Second, the dynamic cytoskeleton rearrangement results in polar trafficking of membrane compartments to facilitate surface increase at the tip. These processes are coordinated by signaling factors that help to maintain cell polarity and allow growth only at the root hair tip. Importantly, these functional steps are all interdependent, so that they reinforce each other in a series of interlocking feedback loops (Fig. 12.3).

ROPs are critical for both maintaining cell polarity and growth of root hairs since they determine the site of exocytosis and maintain calcium oscillation by activating ROS production which in turn activates calcium channels. The feedback loop of calcium and ROS oscillation is translated into a distinct organization of the actin cytoskeleton in different region of root hairs. High calcium concentration prevents F-actin formation at the apex but allows formation of a dynamic actin mesh in the subapex. Further back in the root hair, F-actin cables parallel to the shank of root hairs deliver organelles to the subapical region. Among these organelles, Golgi stacks provide membrane compartments that contain key regulators to the tip of root hairs. As a result, vesicles accumulate in the apex and exocytosis occurs preferentially at the tip. Exocytosis of calcium channels reinforces the calcium oscillations thereby stabilizing the feedback system. At the same time, PtdIns 4,5-P_2 (PIP_2), a derived membrane lipid that is deposited at the apex by exocytotic vesicles, activates ROP proteins to stimulate ROS production, while ROP activity is spatially limited by interaction with RhoGDI in the subapex. Remarkably, ROPs activate PIP-kinase to produce PIP_2 in the membrane, resulting in a positive feedback loop that stabilizes ROP activity at the apex. The net result

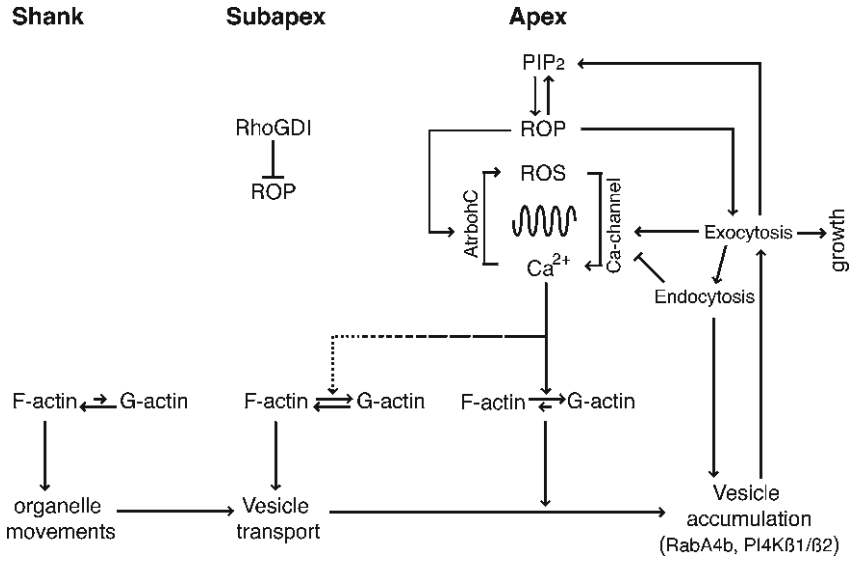

Fig. 12.3 Self-reinforcing feedback regulation of tip growth. Simplified model of regulatory mechanisms that affect the actin cytoskeleton in different areas of the root hair during tip growth. For details see text

of these interdependent feedback loops is a series of stable oscillations that ensures polar growth over long periods.

Every factor in this regulatory network plays a pivotal role, so that slight imbalances of any of these factors affect the entire system, resulting in failure of normal root hair growth. Recent studies have identified many of the genes responsible for root hair development and their functions, and made big strides in revealing the underlying molecular mechanisms of root hair development. However, it should be emphasized that many details still need to be clarified. For example, the precise role of myosins on root hair development and the contribution of endocytosis to tip growth still remain in question. Further genetic, cell biological, and biochemical studies to investigate the dynamic interplay of the various factors involved in root hair growth combined with the collection of quantitative data and computational modeling will be necessary to elucidate these and other questions.

References

1. Avisar D, Abu-Abied M, Belausov E, Sadot E, Hawes C, Sparkes IA (2009) A comparative study of the involvement of 17 *Arabidopsis myosin* family members on the motility of Golgi and other organelles. Plant Physiol 150:700–709
2. Baluska F, Salaj J, Mathur J, Braun M, Jasper F, Samaj J, Chua NH, Barlow PW, Volkmann D (2000) Root hair formation: F-actin-dependent tip growth is initiated by local assembly of profilin-supported F-actin meshworks accumulated within expansin-enriched bulges. Dev Biol 227:618–632
3. Baluska F, Volkmann D (2002) Pictures in cell biology. Actin-driven polar growth of plant cells. Trends Cell Biol 12:14
4. Baluska F, Wojtaszek P, Volkmann D, Barlow P (2003) The architecture of polarized cell growth: the unique status of elongating plant cells. Bioessays 25:569–576
5. Bao Y, Kost B, Chua NH (2001) Reduced expression of alpha-tubulin genes in *Arabidopsis thaliana* specifically affects root growth and morphology, root hair development and root gravitropism. Plant J 28:145–157
6. Baxter-Burrell A, Yang Z, Springer PS, Bailey-Serres J (2002) RopGAP4-dependent Rop GTPase rheostat control of *Arabidopsis* oxygen deprivation tolerance. Science 296:2026–2028
7. Bibikova TN, Blancaflor EB, Gilroy S (1999) Microtubules regulate tip growth and orientation in root hairs of *Arabidopsis thaliana*. Plant J 17:657–665
8. Bibikova TN, Jacob T, Dahse I, Gilroy S (1998) Localized changes in apoplastic and cytoplasmic pH are associated with root hair development in Arabidopsis thaliana. Development 125:2925–2934
9. Bibikova TN, Zhigilei A, Gilroy S (1997) Root hair growth in *Arabidopsis thaliana* is directed by calcium and an endogenous polarity. Planta 203:495–505
10. Braun M, Baluska F, von Witsch M, Menzel D (1999) Redistribution of actin, profilin and phosphatidylinositol-4, 5-bisphosphate in growing and maturing root hairs. Planta 209:435–443
11. Carol RJ, Dolan L (2002) Building a hair: tip growth in *Arabidopsis thaliana* root hairs. Philos Trans R Soc Lond B Biol Sci 357:815–821
12. Carol RJ, Takeda S, Linstead P, Durrant MC, Kakesova H, Derbyshire P, Drea S, Zarsky V, Dolan L (2005) A RhoGDP dissociation inhibitor spatially regulates growth in root hair cells. Nature 438:1013–1016

13. Cole RA, Fowler JE (2006) Polarized growth: maintaining focus on the tip. Curr Opin Plant Biol 9:579–588
14. Collings DA, Lill AW, Himmelspach R, Wasteneys GO (2006) Hypersensitivity to cytoskeletal antagonists demonstrates microtubule-microfilament cross-talk in the control of root elongation in *Arabidopsis thaliana*. New Phytol 170:275–290
15. Deeks MJ, Cvrckova F, Machesky LM, Mikitova V, Ketelaar T, Zarsky V, Davies B, Hussey PJ (2005) *Arabidopsis* group Ie formins localize to specific cell membrane domains, interact with actin-binding proteins and cause defects in cell expansion upon aberrant expression. New Phytol 168:529–540
16. Dolan L, Costa S (2001) Evolution and genetics of root hair stripes in the root epidermis. J Exp Bot 52:413–417
17. Era A, Tominaga M, Ebine K, Awai C, Saito C, Ishizaki K, Yamato KT, Kohchi T, Nakano A, Ueda T (2009) Application of Lifeact reveals F-actin dynamics in *Arabidopsis thaliana* and the liverwort, Marchantia polymorpha. Plant Cell Physiol 50:1041–1048
18. Felle HH, Hepler PK (1997) The Cytosolic Ca2+ Concentration Gradient of Sinapis alba Root Hairs as Revealed by Ca2+-Selective Microelectrode Tests and Fura-Dextran Ratio Imaging. Plant Physiol 114:39–45
19. Fischer U, Ikeda Y, Grebe M (2007) Planar polarity of root hair positioning in *Arabidopsis*. Biochem Soc Trans 35:149–151
20. Foreman J, Demidchik V, Bothwell JH, Mylona P, Miedema H, Torres MA, Linstead P, Costa S, Brownlee C, Jones JD, Davies JM, Dolan L (2003) Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature 422:442–446
21. Fu Y, Li H, Yang Z (2002) The ROP2 GTPase controls the formation of cortical fine F-actin and the early phase of directional cell expansion during *Arabidopsis* organogenesis. Plant Cell 14:777–794
22. Geitmann A, Emons AM (2000) The cytoskeleton in plant and fungal cell tip growth. J Microsc 198:218–245
23. Gilliland LU, Kandasamy MK, Pawloski LC, Meagher RB (2002) Both vegetative and reproductive actin isovariants complement the stunted root hair phenotype of the *Arabidopsis* act2-1 mutation. Plant Physiol 130:2199–2209
24. Grebe M (2004) Ups and downs of tissue and planar polarity in plants. Bioessays 26:719–729
25. Grebe M, Friml J, Swarup R, Ljung K, Sandberg G, Terlou M, Palme K, Bennett MJ, Scheres B (2002) Cell polarity signaling in *Arabidopsis* involves a BFA-sensitive auxin influx pathway. Curr Biol 12:329–334
26. Guimil S, Dunand C (2007) Cell growth and differentiation in *Arabidopsis* epidermal cells. J Exp Bot 58:3829–3840
27. He X, Liu YM, Wang W, Li Y (2006) Distribution of G-actin is related to root hair growth of wheat. Ann Bot 98:49–55
28. Hemsley PA, Kemp AC, Grierson CS (2005) The TIP GROWTH DEFECTIVE1 S-acyl transferase regulates plant cell growth in *Arabidopsis*. Plant Cell 17:2554–2563
29. Hepler PK, Vidali L, Cheung AY (2001) Polarized cell growth in higher plants. Annu Rev Cell Dev Biol 17:159–187
30. Hussey, P.J., T. Ketelaar, and M.J. Deeks (2006) Control of the actin cytoskeleton in plant cell growth. Annu Rev Plant Biol. 57:109–125
31. Ishida T, Kurata T, Okada K, Wada T (2008) A genetic regulatory network in the development of trichomes and root hairs. Annu Rev Plant Biol 59:365–386
32. Kandasamy MK, McKinney EC, Meagher RB (2009) A single vegetative actin isovariant overexpressed under the control of multiple regulatory sequences is sufficient for normal *Arabidopsis* development. Plant Cell 21:701–718
33. Ketelaar T, Allwood EG, Hussey PJ (2007) Actin organization and root hair development are disrupted by ethanol-induced overexpression of *Arabidopsis* actin interacting protein 1 (AIP1). New Phytol 174:57–62

34. Ketelaar T, Anthony RG, Hussey PJ (2004) Green fluorescent protein-mTalin causes defects in actin organization and cell expansion in *Arabidopsis* and inhibits actin depolymerizing factor's actin depolymerizing activity in vitro. Plant Physiol 136:3990–3998
35. Kovar DR, Drobak BK, Staiger CJ (2000) Maize profilin isoforms are functionally distinct. Plant Cell 12:583–598
36. Li JF, Nebenführ A (2007) Organelle targeting of myosin XI is mediated by two globular tail subdomains with separate cargo binding sites. J Biol Chem 282:20593–20602
37. Lloyd CW, Wells B (1985) Microtubules are at the tips of root hairs and form helical patterns corresponding to inner wall fibrils. J Cell Sci 75:225–238
38. Masucci JD, Schiefelbein JW (1994) The rhd6 Mutation of *Arabidopsis thaliana* Alters Root-Hair Initiation through an Auxin- and Ethylene-Associated Process. Plant Physiol 106:1335–1346
39. Miller D, de Ruijter NC, Bisseling T, Emons AM (1999) The role of actin in root hair morphogenesis: studies with lipochito-oligosaccharide as a growth stimulator and cytochalasin as an actin perturbing drug. Plant J 17:141–154
40. Molendijk AJ, Bischoff F, Rajendrakumar CS, Friml J, Braun M, Gilroy S, Palme K (2001) *Arabidopsis thaliana* Rop GTPases are localized to tips of root hairs and control polar growth. EMBO J 20:2779–2788
41. Monshausen GB, Bibikova TN, Messerli MA, Shi C, Gilroy S (2007) Oscillations in extracellular pH and reactive oxygen species modulate tip growth of *Arabidopsis* root hairs. Proc Natl Acad Sci USA 104:20996–21001
42. Monshausen GB, Messerli MA, Gilroy S (2008) Imaging of the Yellow Cameleon 3.6 indicator reveals that elevations in cytosolic Ca2+ follow oscillating increases in growth in root hairs of *Arabidopsis*. Plant Physiol 147:1690–1698
43. Ojangu EL, Jarve K, Paves H, Truve E (2007) *Arabidopsis thaliana* myosin XIK is involved in root hair as well as trichome morphogenesis on stems and leaves. Protoplasma 230:193–202
44. Ovecka M, Lang I, Baluska F, Ismail A, Illes P, Lichtscheidl IK (2005) Endocytosis and vesicle trafficking during tip growth of root hairs. Protoplasma 226:39–54
45. Payne RJ, Grierson CS (2009) A theoretical model for ROP localisation by auxin in *Arabidopsis* root hair cells. PLoS ONE 4:e8337
46. Peremyslov VV, Prokhnevsky AI, Avisar D, Dolja VV (2008) Two class XI myosins function in organelle trafficking and root hair development in *Arabidopsis*. Plant Physiol 146:1109–1116
47. Preuss ML, Schmitz AJ, Thole JM, Bonner HK, Otegui MS, Nielsen E (2006) A role for the RabA4b effector protein PI-4Kbeta1 in polarized expansion of root hair cells in *Arabidopsis thaliana*. J Cell Biol 172:991–998
48. Preuss ML, Serna J, Falbel TG, Bednarek SY, Nielsen E (2004) The *Arabidopsis* Rab GTPase RabA4b localizes to the tips of growing root hair cells. Plant Cell 16:1589–1603
49. Ramachandran S, Christensen HE, Ishimaru Y, Dong CH, Chao-Ming W, Cleary AL, Chua NH (2000) Profilin plays a role in cell elongation, cell shape maintenance, and flowering in *Arabidopsis*. Plant Physiol 124:1637–1647
50. Reisen D, Hanson MR (2007) Association of six YFP-myosin XI-tail fusions with mobile plant cell organelles. BMC Plant Biol 7:6
51. Ringli C, Baumberger N, Diet A, Frey B, Keller B (2002) ACTIN2 is essential for bulge site selection and tip growth during root hair development of *Arabidopsis*. Plant Physiol 129:1464–1472
52. Sagi M, Fluhr R (2001) Superoxide production by plant homologues of the gp91(phox) NADPH oxidase. Modulation of activity by calcium and by tobacco mosaic virus infection. Plant Physiol 126:1281–1290
53. Sakai T, Honing H, Nishioka M, Uehara Y, Takahashi M, Fujisawa N, Saji K, Seki M, Shinozaki K, Jones MA, Smirnoff N, Okada K, Wasteneys GO (2008) Armadillo repeat-containing kinesins and a NIMA-related kinase are required for epidermal-cell morphogenesis in *Arabidopsis*. Plant J 53:157–171

54. Schiefelbein J, Galway M, Masucci J, Ford S (1993) Pollen tube and root-hair tip growth is disrupted in a mutant of *Arabidopsis thaliana*. Plant Physiol 103:979–985
55. Schiefelbein JW, Somerville C (1990) Genetic control of root hair development in *Arabidopsis thaliana*. Plant Cell 2:235–243
56. Sheahan MB, Staiger CJ, Rose RJ, McCurdy DW (2004) A green fluorescent protein fusion to actin-binding domain 2 of *Arabidopsis* fimbrin highlights new features of a dynamic actin cytoskeleton in live plant cells. Plant Physiol 136:3968–3978
57. Sieberer BJ, Timmers AC, Lhuissier FG, Emons AM (2002) Endoplasmic microtubules configure the subapical cytoplasm and are required for fast growth of Medicago truncatula root hairs. Plant Physiol 130:977–988
58. Smith, L.G., and D.G. Oppenheimer (2005) Spatial control of cell expansion by the plant cytoskeleton. Annu Rev Cell Dev Biol. 21:271–295
59. Sparkes IA, Teanby NA, Hawes C (2008) Truncated myosin XI tail fusions inhibit peroxisome, Golgi, and mitochondrial movement in tobacco leaf epidermal cells: a genetic tool for the next generation. J Exp Bot 59:2499–2512
60. Swarup R, Friml J, Marchant A, Ljung K, Sandberg G, Palme K, Bennett M (2001) Localization of the auxin permease AUX1 suggests two functionally distinct hormone transport pathways operate in the *Arabidopsis* root apex. Genes Dev 15:2648–2653
61. Takahashi H, Hirota K, Kawahara A, Hayakawa E, Inoue Y (2003) Randomization of cortical microtubules in root epidermal cells induces root hair initiation in lettuce (Lactuca sativa L.) seedlings. Plant Cell Physiol 44:350–359
62. Takeda S, Gapper C, Kaya H, Bell E, Kuchitsu K, Dolan L (2008) Local positive feedback regulation determines cell shape in root hair cells. Science 319:1241–1244
63. Tang XJ, Hepler PK, Scordilis SP (1989) Immunochemical and immunocytochemical identification of a myosin heavy chain polypeptide in Nicotiana pollen tubes. J Cell Sci 92 (Pt 4):569–574
64. Thole JM, Vermeer JE, Zhang Y, Gadella TW Jr, Nielsen E (2008) Root hair defective4 encodes a phosphatidylinositol-4-phosphate phosphatase required for proper root hair development in *Arabidopsis thaliana*. Plant Cell 20:381–395
65. Timmers AC, Vallotton P, Heym C, Menzel D (2007) Microtubule dynamics in root hairs of Medicago truncatula. Eur J Cell Biol 86:69–83
66. Tominaga M, Morita K, Sonobe S, Yokota E, Shimmen T (1997) Microtubules regulate the organization of actin filaments at the cortical region in root hair cells of hydrocharis. Protoplasma 199:83–92
67. Tominaga M, Yokota E, Sonobe S, Shimmen T (2000) Mechanism of inhibition of cytoplasmic streaming by a myosin inhibitor, 2, 3-butanedione monoxime. Protoplasma 213:46–54
68. Traas JA, Braat P, Emons AM, Meekes H, Derksen J (1985) Microtubules in root hairs. J Cell Sci 76:303–320
69. Van Bruaene N, Joss G, Van Oostveldt P (2004) Reorganization and in vivo dynamics of microtubules during *Arabidopsis* root hair development. Plant Physiol 136:3905–3919
70. Very AA, Davies JM (2000) Hyperpolarization-activated calcium channels at the tip of *Arabidopsis* root hairs. Proc Natl Acad Sci USA 97:9801–9806
71. Voigt B, Timmers AC, Samaj J, Muller J, Baluska F, Menzel D (2005) GFP-FABD2 fusion construct allows in vivo visualization of the dynamic actin cytoskeleton in all cells of *Arabidopsis* seedlings. Eur J Cell Biol 84:595–608
72. Wang YS, Yoo CM, Blancaflor EB (2008) Improved imaging of actin filaments in transgenic *Arabidopsis* plants expressing a green fluorescent protein fusion to the C- and N-termini of the fimbrin actin-binding domain 2. New Phytol 177:525–536
73. Whittington AT, Vugrek O, Wei KJ, Hasenbein NG, Sugimoto K, Rashbrooke MC, Wasteneys GO (2001) MOR1 is essential for organizing cortical microtubules in plants. Nature 411:610–613
74. Wymer CL, Bibikova TN, Gilroy S (1997) Cytoplasmic free calcium distributions during the development of root hairs of *Arabidopsis thaliana*. Plant J 12:427–439

75. Xue HW, Chen X, Mei Y (2009) Function and regulation of phospholipid signalling in plants. Biochem J 421:145–156
76. Yalovsky S, Bloch D, Sorek N, Kost B (2008) Regulation of membrane trafficking, cytoskeleton dynamics, and cell polarity by ROP/RAC GTPases. Plant Physiol 147:1527–1543
77. Yang G, Gao P, Zhang H, Huang S, Zheng ZL (2007) A mutation in MRH2 kinesin enhances the root hair tip growth defect caused by constitutively activated ROP2 small GTPase in *Arabidopsis*. PLoS ONE 2:e1074
78. Yokota E, Muto S, Shimmen T (2000) Calcium-calmodulin suppresses the filamentous actin-binding activity of a 135-kilodalton actin-bundling protein isolated from lily pollen tubes. Plant Physiol 123:645–654
79. Yoo CM, Wen J, Motes CM, Sparks JA, Blancaflor EB (2008) A class I ADP-ribosylation factor GTPase-activating protein is critical for maintaining directional root hair growth in *Arabidopsis*. Plant Physiol 147:1659–1674
80. Zheng M, Beck M, Muller J, Chen T, Wang X, Wang F, Wang Q, Wang Y, Baluska F, Logan DC, Samaj J, Lin J (2009) Actin turnover is required for myosin-dependent mitochondrial movements in *Arabidopsis* root hairs. PLoS ONE 4:e5961

Chapter 13
Microtubules, MAPs and Xylem Formation

Edouard Pesquet and Clive Lloyd

13.1 Introduction

The evolution of plants from water onto land involved the development of systems for projecting their photosynthetic and reproductive organs up into the atmosphere. Xylem (from the ancient Greek "xylon" for wood) tissue is just such a system for it transports the water up to the leaves and its strength provides essential physical support. This vascular tissue is organized into vascular bundles (Fig. 13.1a) whose number and organization in the plant organ are species specific [30]. Similar to other plant tissues, xylem is comprised of several cell types with distinct morphological features including: (i) conducting cells named tracheary elements (TEs) (Fig. 13.1b, c), (ii) support cells named fibers (Fig. 13.1d) and (iii) associated parenchyma. The common morphological feature of all xylem cells is the development of a secondary cell wall which corresponds to supplementary deposition of cell wall polymers – cellulose, hemicellulose and lignin – between the primary cell wall and the plasma membrane [100]. The deposition of this secondary cell wall is either made on all sides of the cell (such cell wall organization is referred to as sclerenchyma) in the case of TEs and fibers, or restricted to one side of the cell (such cell wall organization is also referred to as collenchyma) for xylem parenchyma, which develop a transfer-cell like structure orientated around the xylem vessels. Cellulose – a linear β-1-4 glucan chain – is organized into cellulose microfibrils that will be deposited by plasma membrane-localized cellulose synthase complexes (CesA) – protein complexes that have been observed in electron microscopy to have a rosette-like structure ([105, 106] for review) [51]. Hemicellulose – a hexose and pentose polymer –synthesized in the Golgi then secreted will bind and link the cellulose microfibrils. Finally, lignin – an amorphous phenolic polymer – will encrust the entire secondary cell wall and confer supplementary resistance and impermeability [7]. The secondary cellulosic depositions

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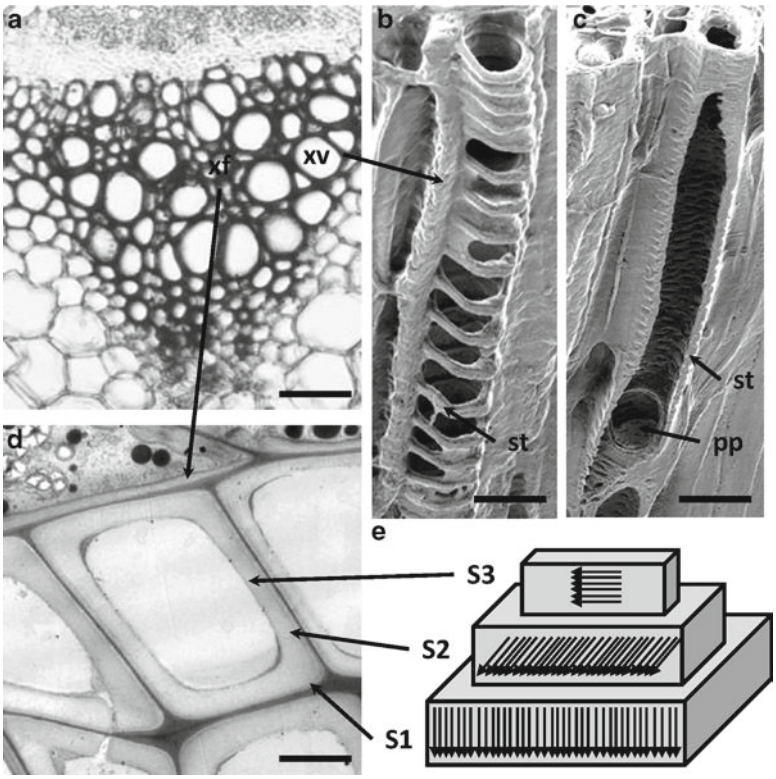

Fig. 13.1 Xylem cells in plants. (**a**) *Arabidopsis* vascular bundle characteristic of angiosperm primary vascular organization comprising both xylem vessels (xv) and xylem fibers (xf), bar = 40 μm. (**b**) Scanning electron microscopic (SEM) image of xylem vessel with annular secondary cell wall pattern; secondary thickenings are indicated by (st), bar = 6 μm. (**c**) SEM image of xylem vessel with reticulate secondary cell wall pattern; secondary thickenings are indicated by (st) and terminal perforation plate by (pp), bar = 6 μm. (**d**) xylem fiber and (**e**) the corresponding diagram of cellulose microfibril orientation in the three concentric layers forming the secondary cell wall of fibers, bar = 4 μm

in xylem fibers are arranged into three concentric layers, S1, S2 and S3, each exhibiting a different microfibril angle that confers supplementary mechanical strength to the fiber's wall (Fig. 13.1e).

Tracheary elements – so called because early microscopists thought the hollow, thickened tubes resembled our own trachea – are a type of xylem cell and they fulfilled two key functions in the evolutionary transition of plants from water onto land. These specialized cells represent a plastic modular cell unit that, once assembled end-to-end, will form throughout the plant a continuous vasculature in the form of xylem vessels [38, 107]. Thus, one, TEs conduct water and minerals from the soil to the aerial parts; two, they support the plant as it grows up into the atmosphere. These two roles are directly related to the particular pattern described by secondary wall thickenings (Fig. 13.1c). To allow them their conductive role,

these dead cells are hollowed by programmed cell death and perforated at each end to form a vascular cylinder with an accessible lumen. To keep this lumen open, TEs are reinforced by transverse deposition of secondary cell wall organized in a limited number of specific patterns (i.e., annular, spiral, reticulate and pitted; Fig. 13.2a–d). Thus, the secondary cell wall of TEs not only describes an overall pattern but at the subcellular level exhibits a regular symmetric organization with smooth secondary depositions. These depositions are evenly spaced and of similar thickness – about ten times thicker than the primary cell wall (Fig. 13.2e–g). Annular and spiral thickenings (Fig. 13.2b) are found in the protoxylem of young forming tissue associated with growth in height but are later crushed by growth of surrounding tissue. The reticulated and pitted patterns of secondary wall thickening (Fig. 13.2c, d) occur in both metaxylem and secondary xylem, which will form the final vascular conduit in both perennial and annual plant species [30]. At the ends of these cells, perforation plates – which can be simple or ornamented perforations depending on the plant species [107] – form to allow conduction of sap from one TE to the next. Modular addition of TEs therefore enables the long distance conduction of dissolved solutes while the mechanical strength of the thickened cell walls supports the

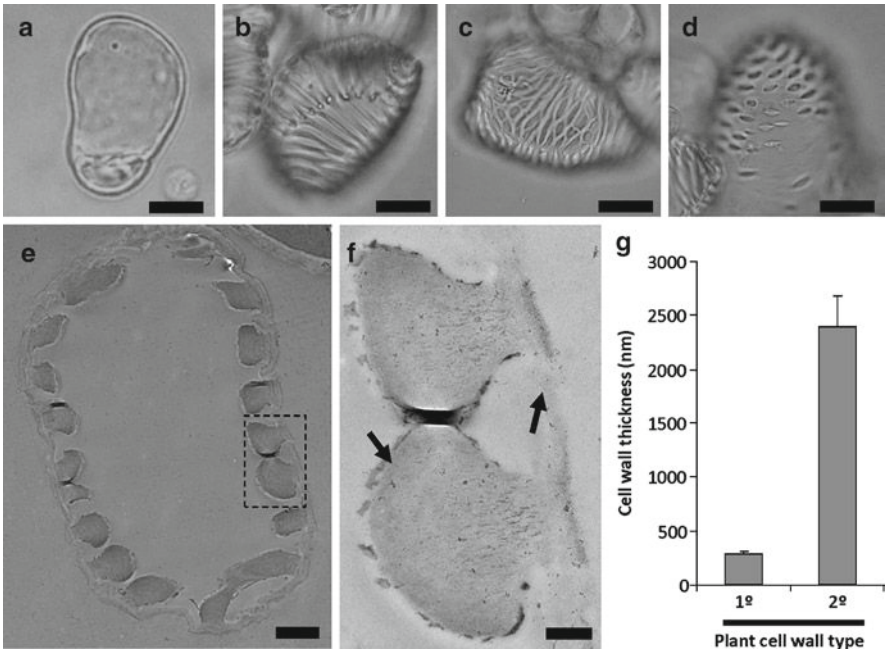

Fig. 13.2 Tracheary elements differentiating in vitro. (**a–d**) Cell with primary cell wall (**a**); xylem vessel element with spiral (**b**), reticulate (**c**) and pitted (**d**) secondary cell walls, bars = 8 μm. (**e–g**) TEM images of the cross-section of a xylem vessel element (**e**); illustrating the thickness of the secondary wall in-growth relative to the primary wall in a xylem vessel element (**f**) indicated by *arrows* corresponding to the *boxed area* in (**e**), bars = 250 nm. Histogram comparing primary and secondary cell wall thickness of TEs (**g**)

ever-increasing weight of tissue as the plant extends into space. Vascular continuity is ensured by the differentiation of new xylem cells immediately behind the advancing apical meristem (procambium), thereby continuously maintaining irrigation during the plant's increase in height.

Xylem is also formed from secondary meristem (cambium) along the flank and adds to the width of the plant and this represents the most active developing tissue of woody plants. Unfortunately, the fine study of xylem formation has been experimentally difficult due to several factors: (i) xylem occurs deep within the plant organs, (ii) the functional state of xylem cells is as a dead "cell corpse" (Roberts and McCann 2000) and (iii) few cells are actually forming at any one time and may not be at the same stage of differentiation. Two main experimental strategies have been developed to circumvent these experimental difficulties including: (1) the development of simplified in vitro systems allowing the controlled differentiation of TEs (i.e., the *Coleus* explants system or the *Zinnia* cell cultures) and (2) the use of wood-forming tissues that, compared to primary xylem, have the advantage of showing the entire differentiation gradient from the meristematic cell to the functional TEs and fibers in one longitudinal section. Thus the study of microtubules during xylem formation has been dependant on the development on new biological system.

In this review, we will examine the way that different functional classes of microtubules contribute to the cell wall sculpturings of differentiating wood cells. We will discuss what is known about the role of microtubule-associated proteins (MAPs), focusing on our recent work in which we developed a simplified system for the controlled induction of xylem cell differentiation.

13.2 Microtubule Organization in Xylem Cells

The description of cortical microtubule organization in xylem cells derived from the technical improvements associated first with transmission electron microcopy (TEM), (which showed the close correspondence between microtubules and the overlying accumulations of secondary wall [53]); then immunofluorescence (which showed the overall pattern of microtubules beneath thickenings in cells; [31, 32]); and more recently the development of fluorescent protein fusion reporters that show the disposition of microtubules in whole plants. The first TEM observations made on *Coleus* explants triggered to form TEs showed cortical microtubules organized in xylem cells as a dense network of parallel microtubule. These underlay the active sites of secondary cell wall deposition but were absent from areas exhibiting no secondary cell wall [53, 74]. Additional observations using TEM in TEs of *Triticum* and *Allium* seedlings [8] as well as in in vitro *Zinnia* TEs [39, 93] confirmed this specific organization. Our transmission electron microscopical (TEM) studies on *Arabidopsis* TEs differentiating in vitro illustrate these parallel microtubules restricted beneath the secondary cell wall thickenings (Fig. 13.3a, b, shown by arrows in b). In xylem fibers, observations using TEM showed a similarly dense network of parallel microtubules organized underneath the secondary cell wall

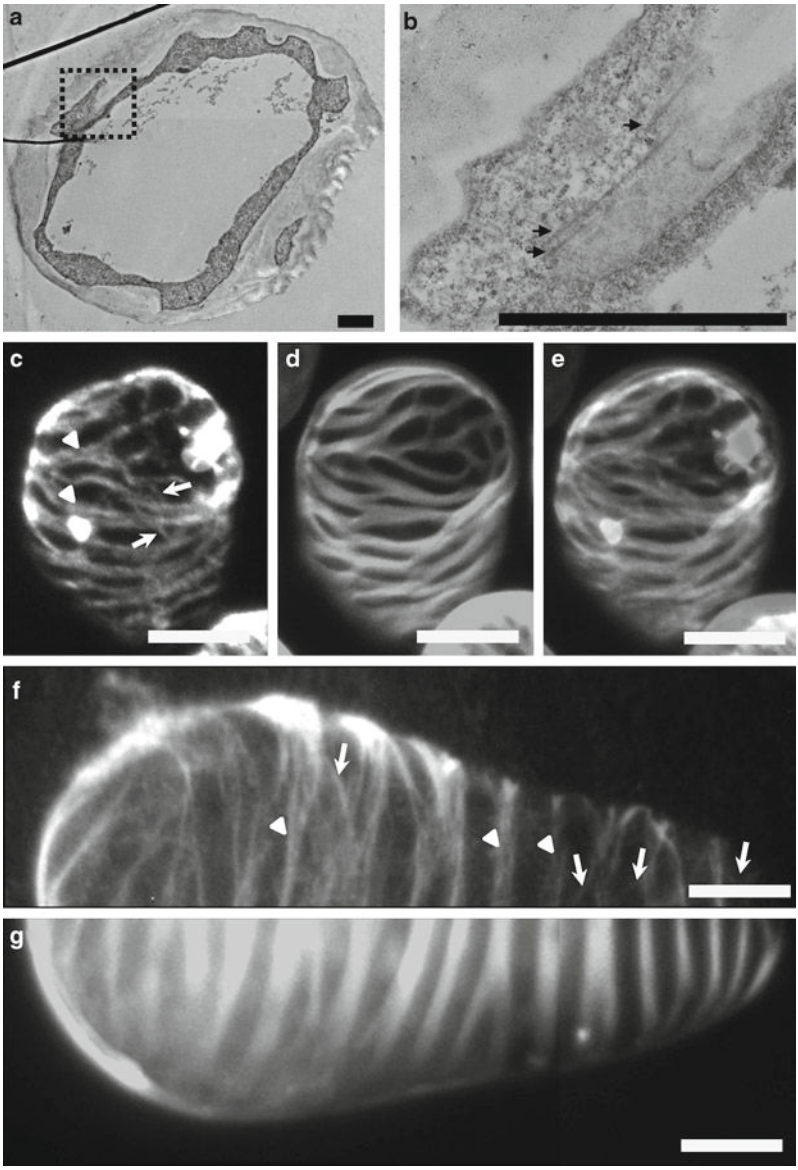

Fig. 13.3 Microtubules in plant tracheary elements. (**a**, **b**) TEM images of microtubules relative to the secondary cell wall in in vitro differentiating *Arabidopsis* TE. Overview of cell morphology (**a**) and focusing within the boxed area (**b**) where microtubules are indicated by arrows, bars = 700 nm. (**c–e**) confocal images of immunolocalisation of α-tubulin using YOL 1/34 antibody (**c**) on *Arabidopsis* TEs differentiating in vitro compared to the TE secondary cell wall stained with Calcofluor (**d**); the corresponding merged image of both channels is shown in (**e**), bars = 9 μm. (**f**, **g**) Confocal images of *Arabidopsis* TEs differentiating in vitro constitutively expressing α-tubulin-GFP. (**f**) Shows half of the cell's secondary cell wall stained with GFP and (**g**) shows the other half stained with Calcofluor. Note the *arrows* that indicate the interconnecting microtubules and the *arrowhead* that define the underlying microtubules bundle beneath the secondary thickenings, bars = 6 μm

([5, 17, 59]). Fluorescence immunolocalisation studies in gymnosperm trees [1, 40, 101], angiosperm trees [18, 55] and in vitro differentiation of *Zinnia* TEs ([31, 32]) not only provided confirmation of the specific presence of microtubules beneath the secondary cell wall thickenings but also defined the overall cell organization. In *Arabidopsis* TEs differentiating in vitro, we found that thick bundles of microtubules resided beneath the secondary cell wall thickenings (Fig. 13.3c, d, see arrowhead in c). In this case, gaps in the secondary cell wall, which were characteristic of the pitted TE pattern and referred to as border pits, were defined early on by a delimiting circle of cortical microtubules before any secondary cell wall was visible (Fig. 13.4) ([1, 16, 40, 101]). Similarly, simple or scalariform terminal perforation plates of TEs were also pre-determined by a set of microtubules defining the boundaries of the future perforation (Fig. 13.4) [16, 41]. More recently, GFP fusion reporters have provided more detailed information of how microtubules are organized in xylem cells as illustrated in *Arabidopsis* TEs differentiating in vitro and stably expressing α-tubulin-GFP (Figs. 13.3f, g and 13.4). Three recent studies have been made on the organization of cortical microtubules in *Arabidopsis* TEs labeled with a GFP-tubulin reporter [75, 84, 104]. As previously observed by both TEM and fluorescent immunolocalisation, all three studies confirm the presence of narrow transverse bundles of microtubules that form beneath the future sites of secondary cell wall formation prior to any secondary wall deposition (Fig. 13.5b). It is only with respect to the relationship between microtubules and the growing secondary cell wall thickenings that the observations differ in detail. Pesquet et al. [84] report that the initial narrow transverse microtubule bundles are gradually thickened by additional microtubules as the secondary cell wall is deposited, thus completely surrounding – in transverse section – the growth of the thickening in a U-shape cup within which the secondary cell wall is laid down (Fig. 13.5c, e). However, in their study [75] report that the initial narrow transverse microtubule bundle thickens then splits into two flanking bundles (known as "twin peaks") that gradually move apart (Fig. 13.5d, g) [104] reports an intermediate between these two views. In this case, the initial narrow transverse microtubule bundle is first thickened by additional microtubules at the beginning of secondary cell wall deposition and then split into two large bands of microtubules flanking the sides of the secondary deposition (Fig. 13.5c, f).

Recent observations reconcile these variations (Fig. 13.6) [75] had shown that bundles of microtubules, which were labeled with GFP-tubulin, split either side of the secondary wall thickening. In our studies on *Arabidopsis* TEs differentiating in vitro, we found that GFP fusions of the microtubule plus-tip marker EB1 or the binding partners AtMAP70-1 and AtAMP70-5 reproduced this split pattern (Fig. 13.6h, i) [84]. Careful through-focusing showed that the labeling with these probes was indeed confined the superficial lines of fluorescence flanking each rib of wall. However, when we used a different microtubule marker, GFP-α-tubulin6, these "twin peaks" either side of the thickenings were found in serial z-sections to join up to form a U-shaped mould that cupped the wall thickening as it ingressed into the cell (Fig. 13.6g). From our observations it seems that the "twin peaks" bands of microtubules seen by [75] are likely to represent the tips of

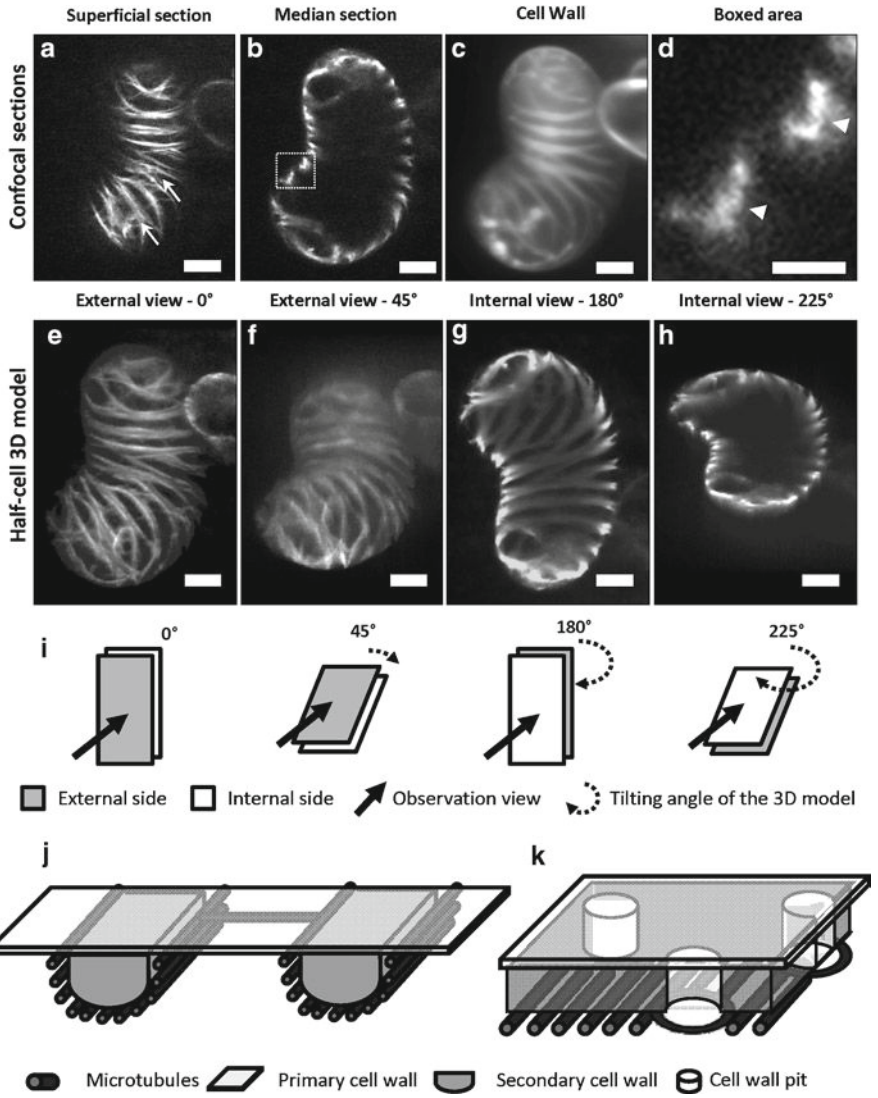

Fig. 13.4 Cortical microtubule organization in xylem tracheary elements. (**a–d**) confocal images of *Arabidopsis* TEs differentiating in vitro constitutively expressing α-tubulin-GFP. Superficial view (**a**), median view (**b**), corresponding secondary cell wall stained with Calcofluor (**c**) and magnification of the *boxed area* in (**b**) showing the U-shaped cups of microtubules beneath secondary cell wall thickenings (**d**). The *arrows* indicate the interconnecting microtubules joining the bundles that underlie the secondary thickenings. (**e–h**) Half-cell 3D reconstruction of the previous xyz confocal stack presented from the external side of the cell at 0° (**e**), at 45° (**f**), and from the internal side of the cell at 180° (**g**) and 225° (**h**). Bars = 6 μm. (**i**) simplified representation of the view and tilting angle of the above presented half-cell 3D reconstruction. (**j**) Schematic representation of the relationship between microtubules and secondary cell wall in annular/spiral type TEs. (**k**) Schematic representation of the relationship between microtubules and secondary cell wall in reticulate/pitted patterned type TEs

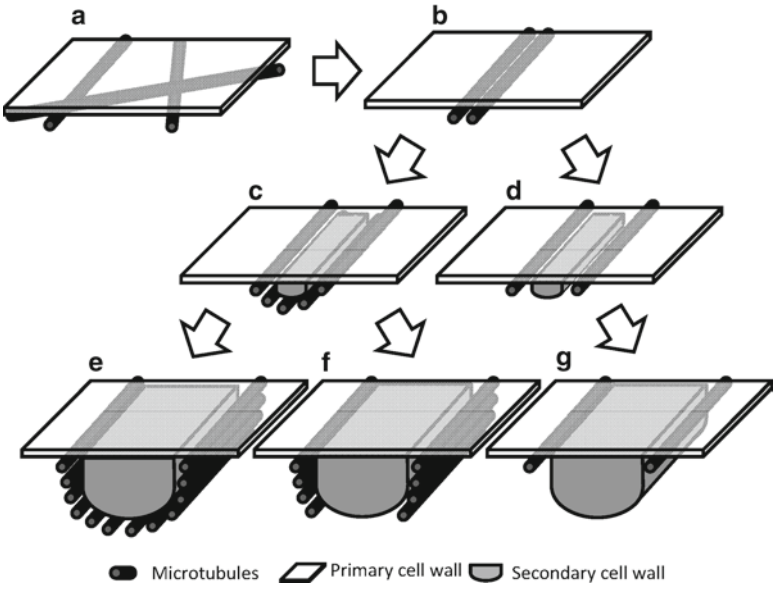

Fig. 13.5 Proposed model for the cortical microtubule organization in xylem tracheary elements. (**a**) Interphase cell displaying a randomly organized microtubule network. (**b**) Narrow transverse microtubule bundle defining the future site of secondary cell wall formation. (**c**) Initial secondary cell wall thickenings surrounded by microtubules compared to (**d**): where the initial bundle splits into two flanking microtubules. (**e**) Classical model where the secondary thickenings are within a U-shaped cup defined by microtubules compared to (**f**) [103] where microtubules are organized into two flanking bands and (**g**) Oda et al's model [75] where only two flanking microtubules are present

the U of a continuous cytoskeletal cradle. In that cradle, there may be two kinds of microtubules: more stable microtubules forming the cup that holds the entire cytoplasmic face of the ingrowing secondary thickening and a more dynamic class of microtubules restricted to the superficial layer at the tips of the U (Fig. 13.6j). The appearance of the microtubule bundle in living cells may therefore depend on the type of microtubule marker to which the fluorescent tag is attached. It is thought that splitting of the band focuses cellulose biosynthesis to the cortical edges of the band [55]. However, our interpretation is that while MAPs define the borders within which secondary cell wall deposition is restricted, microtubules are not absent from the area between the tramlines but continue to direct cellulose biosynthesis to this area as it continues to thicken and to invade the cytoplasm.

In addition to these microtubules concentrated at the wall thickening, another sub-population of microtubules has been described in several studies – microtubules forming a semi-circular connection between adjacent secondary thickenings (shown by fluorescent immunolocalisation in Falconer and Seagull [34, 88] and indicated by arrows on Fig. 13.3c; shown by GFP-microtubule reporters in [75, 76, 84, 104] and indicated by arrows on Figs. 13.3f and 13.4a). These microtubules are not associated with any deposition of secondary thickening as they

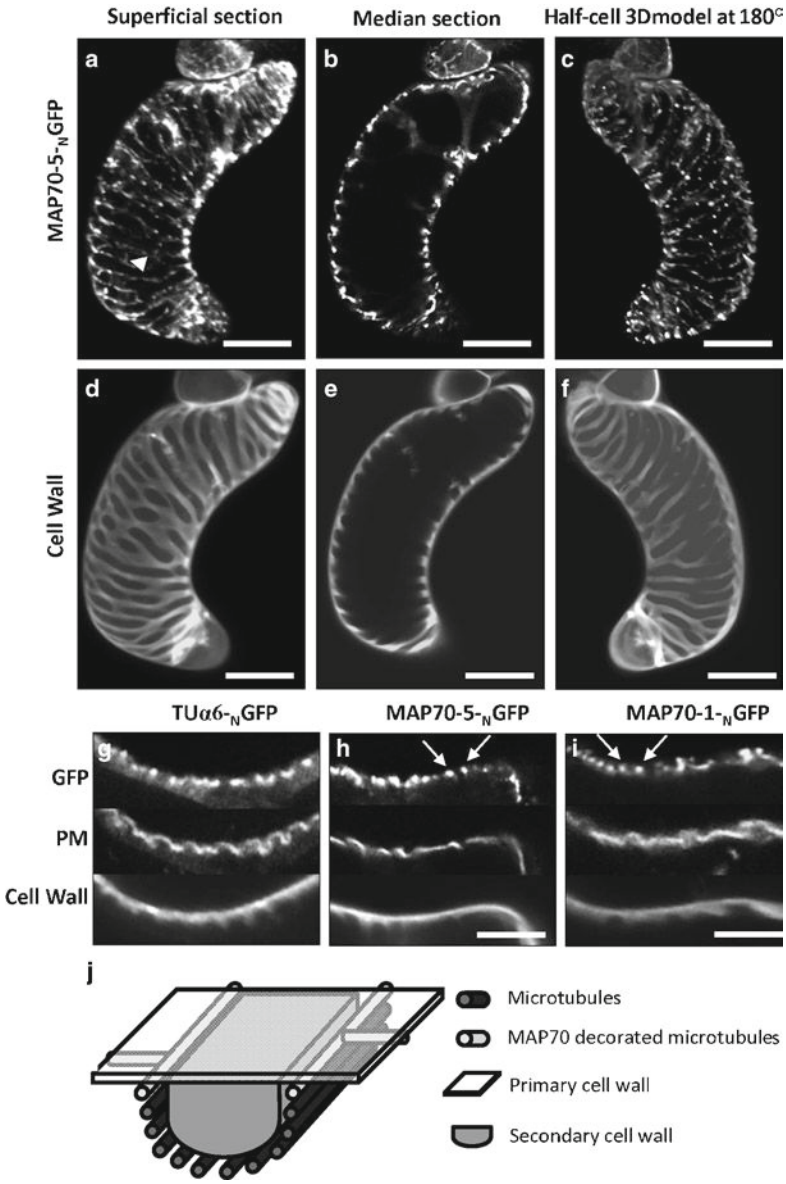

Fig. 13.6 Different sub-populations of microtubules in TEs. (**a–f**) Confocal images of in vitro differentiating *Arabidopsis* TE constitutively expressing MAP70-5-GFP in superficial view (**a**), median view (**b**), half-cell 3D reconstruction at 180° and corresponding secondary cell wall stained with Calcofluor (**d–f**). (**g–i**) Higher magnification of the relationship between microtubules (GFP), plasma membrane stained with FM4-64 (PM) and cell wall stained with calcofluor (cell wall) of in vitro differentiation of *Arabidopsis* TE constitutively expressing α-tubulin-GFP (**g**), MAP70-5-GFP (**h**) and MAP70-1-GFP (**i**), bars = 9 μm. Note that both MAP70-5 and MAP70-1 label the inter-connecting microtubule (*arrowhead* in **a**) and the flanking microtubules (*arrows* in **h**, **i**). (**j**) Schematic representation of the different sub-populations of microtubules defined both by their position relative to the secondary thickenings and by their MAP70 binding

underlie the intervening areas of primary cell wall only. In this way, at least three different sub-populations of microtubule can be defined in differentiating TE cells based on their position relative to the growing secondary thickenings: (i) "underlying" microtubules that completely follow the shape of the forming thickening in a U-shaped cup, (ii) more dynamic "flanking" microtubules, either side of the wall thickening, that appear to define the boundaries between primary and secondary cell wall and (iii) microtubules that form curvilinear interconnections that lie at right angles between the microtubules associated with the transverse ribs of thickening. Hence, not only do microtubules appear to act as a scaffold for each rib of secondary thickening but they define the boundary between primary and secondary wall and also seem to maintain the regular spacing between these primary ribs.

13.3 Impact of Pharmacological Treatments on Microtubules in Xylem Cells

The connection between microtubules and secondary thickening during TE differentiation first emerged from early studies on the effects of anti-microtubule drugs. Such treatments either stabilize microtubules (i.e., taxol) or destabilize microtubules (i.e., colchicine, amiprophos-methyl [APM], isopropyl-*N*-phenylcarbamate [IPC], isopropyl-*N*-(3-chlorophenyl)-carbamate [CIPC], propyzamide, oryzalin). However, the very first observations on the role of microtubules on xylem cells, obtained using drug treatments, were on whole developing seedlings [53]. Although colchicine is now recognized to be less effective on plant than on animal microtubules [71] pioneering studies with this drug on developing *Triticum* seedlings showed that the absence or reduction of cortical microtubules in xylem vessel cells led to a loss of the regularity with which cell wall was deposited without apparently affecting the fibrillar structure of the wall [8, 83]. Using the in vitro TE differentiation system derived from *Coleus* explants [35], colchicine was found to induce abnormal and asymmetrical secondary cell walls in TEs [52, 90]. Similarly, working with TEs induced by wounding in vitro [50] showed that colchicine affected secondary cell wall morphology in newly-formed xylem vessels by increasing the cell wall reticulation pattern. Similarly, in a simpler system obtained by inducing TEs in *Arabidopsis* cell cultures [75] likewise observed a complete loss of secondary cell wall patterning. Interestingly, when colchicine treatment was performed prior to secondary cell wall deposition the resulting TE secondary thickenings appeared irregular and asymmetrical with rough edges. But when treatment was performed while the secondary cell wall was being deposited the resulting TE exhibited new reticulation of the secondary cell wall as well as the loss of regular patterning. Complementary experiments using other microtubule-depolymerising drugs, such as APM and propyzamide, on differentiating TE cell cultures confirmed the general effects of colchicine as, for example, in *Pisum* and *Commelina* root TEs treated with APM [55]. Indeed, in differentiating *Zinnia* TEs, APM treatment

caused complete microtubule disappearance resulting in highly reticulated and irregular secondary cell wall deposition [33, 88]. Similarly [75] reported that differentiating *Arabidopsis* TE cell cultures treated with propyzamide presented abnormal secondary cell walls. Treatment of *Allium* and *Triticum* explants with IPC induced a high reduction of cortical microtubules leading to a complete disorganization of the secondary cell walls in xylem vessels [8]. However, the precise effect of phenyl carbamates on microtubule polymerization in particular, or on microtubule arrays in general, is unclear in plants since these drugs affect the number of spindle poles and possibly therefore microtubule nucleation sites [24].

These mostly early studies amply supported the idea that microtubule bundles are necessary for laying down corresponding bands of secondary thickening on a 1:1 basis but, over and above this, they also suggest that the global pattern of wall thickenings depends on microtubule organization. The current use of anti-microtubule drugs on living cells labeled with fluorescent fusion proteins is particularly insightful since it adds important detail to this general picture. When *Arabidopsis* seedlings were treated with oryzalin, Wightman and Turner [104] saw that the bands of YFP-IRX3 (A secondary cell wall specific CesA – AtCesA7/At5g17420) were lost concurrently with the loss of the CFP signal for microtubules. This demonstrates that microtubules are continuously required to maintain the linear banding of CESA complexes, for the CESA complexes appear unable to sustain their organization when microtubules depolymerize. In our studies on *Arabidopsis* TEs differentiating in vitro, we found that oryzalin treatment resulted in a complete loss of overall patterning and a disturbance of the symmetry, the thickness and the spacing of each thickenings at the sub-cellular level (Fig. 13.7b, e). Similarly [76] reported that oryzalin caused cell wall disorganization. Roberts et al. [88] nevertheless observed that in APM-treated differentiating *Zinnia* TEs, microtubules appeared not to be necessary for maintaining secondary cell wall deposition. Interestingly, they also reported that compounds interfering with cellulosic cell wall synthesis, such as Congo Red and Evans Blue, disturbed the symmetry and pattern of secondary cell walls and in turn affected the underlying organization of cortical microtubules. This suggests that cellulose microfibrils themselves could play a role in both maintaining the microtubule network and ensuring the integrity of the secondary cell wall bands as they are being deposited [88].

Microtubule stabilization with taxol has a different impact on the secondary cell wall. Differentiating *Zinnia* TEs treated with taxol exhibited an altered orientation of the secondary cell wall, with thickened bands appearing in the longitudinal axis of the cell rather than in the transverse [31, 32]. In differentiating *Arabidopsis* TEs, taxol treatment caused tilting of the secondary cell wall relative to the transverse axis and has also been described as simplifying the pattern of secondary thickenings in vessels [75]. In our studies on *Arabidopsis* TEs differentiating in vitro, and similarly to the *Zinnia* system, taxol treatment caused a mis-orientation of secondary cell wall to the longitudinal axis (Fig. 13.7c, f). Dynamic observation of secondary cell wall deposition in taxol-treated *Arabidopsis* TEs revealed that secondary cell wall deposition was slowed down [75] suggesting a requirement for microtubules to remain in a dynamic state during secondary cell wall deposition.

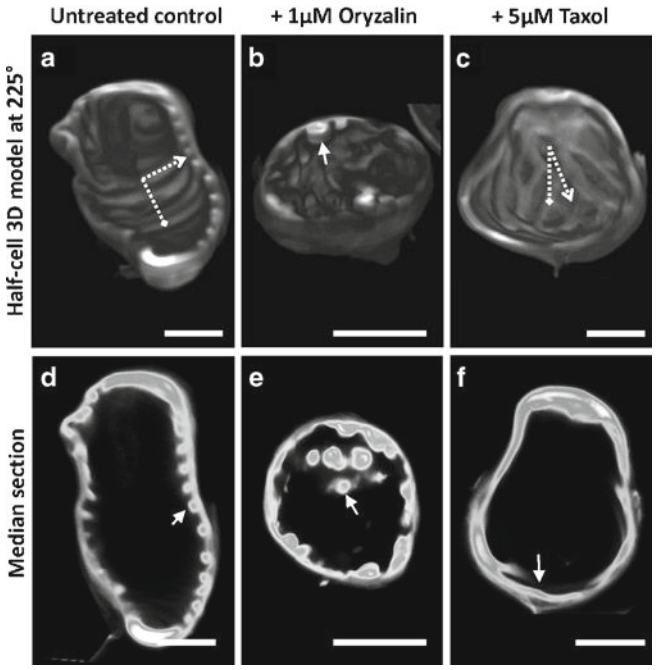

Fig. 13.7 Impact of microtubule-related pharmacological drug treatment on secondary cell wall formation in xylem tracheary elements. (**a–c**) Half-cell 3D confocal reconstruction at 225° of untreated *Arabidopsis* TE (**a**); treated with 1 μM of microtubule-destabilizing oryzalin (**b**); and treated 5 μM microtubule-stabilizing taxol (**c**). (**d–f**) Corresponding median confocal section of *Arabidopsis* TEs. Note the complete loss of pattern and symmetry when treated by oryzalin (*arrow*) in (**e**) compared to (**d**, **f**). Note the mis-orientation of the secondary cell wall orientation to the longitudinal axis of the cell between (**a**, **c**) (highlighted with *dotted arrows*). Bars = 8 μm

Contrary to the microtubule destabilizing drug treatments, which were shown to sometimes cause a more reticulated pattern, taxol was shown to cause less reticulation. This drug sometimes induced a more highly banded pattern [75] but in other cases did not affect pitted TE cell wall formation [76].

Altogether, these pharmacological treatments indicated that microtubules are essential for controlling the sites and patterns described by the secondary walls of xylem cells. The microtubule network of xylem cells therefore defines the symmetrical organization of the secondary cell wall, the pattern described by these secondary cell walls and the width, depth, and regularity of the wall depositions. However, questions still remain about the overall patterning and type of thickenings. Since annular and spiral patterns predominate in taxol-treated TEs and reticulate and pitted patterns form in APM, IPC and colchicine-treated TEs [34, 75] it might seem that the degree of microtubule stabilization describes the particular pattern of thickening. This raises the question of what are the mechanisms by which cortical microtubules ensure the even distribution of secondary cell wall thickenings.

13.4 Reorientation of the Microtubule Network Axis During Xylem Cell Formation

Microtubules are generally (but not invariably) transverse in rapidly elongating *Arabidopsis* epidermal cells and reorient towards longitudinal as growth ceases [65]. If this applies to xylem precuesor cells then, once cell elongation has ceased, it is essential for cortical microtubules to reorient back to transverse to form the various circumferential patterns of secondary thickening. Adding microtubule-stabilizing taxol [31, 32] stopped the reorientation of microtubules from longitudinal (possibly caused by the trauma of extracting mesophyll cells from *Zinnia* leaves) to transverse. This caused the formation of abnormal longitudinal secondary thickenings that would have been highly unlikely to have resisted lateral compression if this had occurred *in planta*. In differentiating *Zinnia* TEs, a gradual transition from a longitudinally oriented array of microtubules to a transverse array was reported by both [31, 32, 60] to occur prior to secondary cell wall formation. Observations of GFP-tagged tubulin in differentiating *Arabidopsis* TEs, which are less traumatized than *Zinnia* cells extracted by tissue homogenization, show that the microtubule network reoriented from random to transverse [75]. Nevertheless, taxol treatment in *Arabidopsis* TEs was shown to alter the overall orientation of the wall causing TE cell wall pits to be mis-orientated relative to the longitudinal axis of the cell [76]. Microtubule dynamics are therefore necessary to allow the tubules to organize and reorganize particular patterns. Similarly, reorientation of the microtubule network is seen along the wood differentiation gradient. In *Abies sachalinensis* and *Taxus cuspidata* TEs, rearrangement of the microtubule array went from an unordered network in the cambial cells (which were mostly orientated on the longitudinal axis), to an unordered network mostly orientated on the transverse axis during cell radial expansion. During the formation of the secondary cell wall this then evolved into a dense microtubule network gradually tilting from the transverse axis to a flat S-helix to a steep Z-helix and then back to a flat S-helix in a clockwise manner [1, 2, 42, 85]. These gradual changes of microtubule orientation from an S-helix to a Z-helix and back again to an S-helix coincided with the cellulose microfibril angles of the three concentric secondary cell wall layers S1, S2 and S3 [1, 2]. In reaction wood fibers (wood formed in response to gravitational constraints like wind or soil inclination), the microtubules of the S1 layer's S-helix reorients clockwise through a Z-helix to become parallel to the longitudinal axis of the fiber cell – these angles guiding in turn the well-defined angles of the cell wall layers [85]. Microtubules are now known to move along tracks that, over time, rotate [19] as newly-nucleated microtubules either move along the "mother" microtubule or branch to one side [21]. Such mechanisms are likely to underlie the way that microtubules adopt a new bundled pattern in forming xylem cells (although it is not known why microtubules adopt an essentially transverse alignment in TEs than a longitudinal one).

13.5 Expression of Microtubule and MAP Genes During Xylem Formation

Drug studies strongly suggested that changes in the dynamics or associative properties of microtubules occur as part of xylem cell differentiation. The stabilization, dynamicity, bundling and severing of microtubules have all subsequently been shown to be dependent on particular microtubules associated proteins or MAPs [49]. The dramatic rearrangement of cortical microtubules in xylem cells can be seen to depend on two main types of control: (i) the quantitative regulation of structural microtubule components, i.e., the relative quantity of α- and β-tubulins during sequential stages of wood formation; and (ii) the specific spatio-temporal expression of specific MAPs that alter the properties and/or organization of microtubules at a given stage and in a way that influences the differentiation of specific xylem cell types. Figures 13.9 and 13.10 presents a summarizing scheme of factors, which are detailed below, that directly influence microtubules during the formation of the secondary cell thickenings of wood cells.

Post-translational modifications potentially represent an additional level of control modifying the properties and behavior of both microtubules and their interacting MAPs. Microtubules can be severed by katanin [9]. Post-translational modifications can also directly target unsevered microtubules through tubulin acetylation [56], phosphorylation [6], tyrosination [28, 57], glutamylation and glycylation [94]. Little is known about post-translational modifications in xylem-forming tissues. In one study [18] reported that both tyrosinated and the detyrosinated form of tubulin could be immunolocalised in wood-forming cells of hybrid aspen but acetylated-tubulin was undetectable. However, it is not clear whether post-translational modification represents a passive marker of changes that have already occurred to microtubules or whether such modifications actively change their properties.

13.5.1 Tubulin During Xylem Formation

Both α- and β-tubulins (respectively referred to TUA and TUB) form small multigenic families of unequal size in plant species (i.e., counting 6 α and 9 β isoforms in *Arabidopsis thaliana* [22, 61, 96], 8 α and 20 β isoforms in *Populus* [77]). Tubulin proteins are highly conserved among plant species, sharing between 74 and 97% nucleotidic sequence identity and 88–98% protein sequence identity between TUAs and TUBs in *Populus* [77]; however, the amount and type of both α- and β-tubulin isoforms can be modified by cell- and tissue- specific patterns of differential expression. Xylem-specific isoforms of TUAs and TUBs have been characterized in the *Zinnia* TE differentiation system, in the vasculature of annual plants and during both normal and reaction wood formation in perennial woody species [27, 77, 108]. Genomic analysis, which allows the most abundant transcripts to be detected during normal and reaction wood formation, has shown specific isoforms of both TUA and TUB are increased in *Populus* ([3, 26, 54]), *Eucalyptus* [81, 82, 86, 98], *Pinus radiata* [67, 68] and *Pinus maritimus* ([79, 80]).

Proteomic analysis confirmed the accumulation of both TUAs and TUBs in xylem forming tissue in *Populus* [58], *Pinus maritimus* [46] and *Eucalyptus grandis* [15]. Tubulin promoter analysis fused to a GUS reporter has shown vascular-specific expression of TUB8 in *Arabidopsis thaliana* [23] and EgTUB1 in *Eucalyptus* [98]. The *Zinnia* in vitro xylogenesis system also showed a specific increase of both specific TUAs and TUBs isoforms at the transcriptional [108] and at the translational level ([36, 37]) prior to secondary cell wall formation in TEs.

A functional role for the induction of tubulin genes and protein in xylem-forming tissues has been suggested by only a few studies. Constitutive over-expression of EgTUB1 in whole *Eucalyptus* trees, which potentially causes homology-dependant gene silencing, has been found to directly affect the woody cell wall by lowering the cellulose microfibril angle [98]. Indeed, in *Eucalyptus* down-regulation of EgTUB1 was shown to be associated with an increased cellulose microfibril angle while up-regulation caused a decrease [102]. Similarly, in *Pinus taeda,* genetic analysis points to α-tubulin as genetic determinant associated with the angle of cellulose microfibrils in conifers [47]. The link between expression of a particular tubulin isoform and the angle with which cellulose microfibrils are deposited is not immediately obvious. One possibility is that specific tubulins, which are associated with a particular developmental program, coincidentally mark the change in growth rate and it is that which affects the change in cellulose alignment. In *Arabidopsis* roots, for example [69] have shown that microtubules reorient from net transverse in the zone of most rapid elongation but become more longitudinally aligned as the growth rate declines. The change in growth rate that accompanies the switch to secondary wall formation might therefore be linked with a differentiation-specific module of gene expression of which upregulation of a specific tubulin isoform(s) is a component. It is also conceivable that tubulins may not be functionally redundant so that differentiation-specific isoforms fulfill a particular function – their microtubules perhaps binding more readily to MAPs or being post-translationally modified. That subtle changes to tubulin molecules can have a dramatic effect on microtubules is illustrated by the *spiral* mutants of *Arabidopsis* [99]. A single amino acid substitution in α-tubulin genes *TUA6* and *TUA4*, for *lefty1* and *lefty2* respectively, is responsible for changes in microtubule stability, the helical angle of cortical microtubules and the handedness with which the entire growth axis twists.

Along the same lines, over-expression of single tubulin isoforms has been reported to be lethal in certain species (*Eucalyptus*-[98]; maize-[4]). Interestingly, constitutive over-expression of α-tubulin-GFP can in some cases lead to defects in cell wall formation causing reduction in the thickness of the xylem cell wall [11].

13.5.2 MAP70 Microtubule Associated Protein During Xylem Vessel Formation

Although expression analysis revealed xylem-specific expression and induction for both TUA and TUB during normal and reaction wood formation [54], genomic analyses of all known plant MAPs during TE and xylem formation did not show

significant xylem-specific expression [84]. Among the few specifically induced MAPs, MAP70-5 (At4g17220) – a member of the 70 kDa MAP70 family [62, 63] – was shown to be specifically upregulated in xylem-forming tissues in whole *Arabidopsis* plants [109]. This MAP isoform was co-regulated with all secondary cell wall cellulose synthases in two different *Arabidopsis* in vitro systems that allowed TE differentiation [64, 84]. In *Arabidopsis,* the multigenic family of MAP70s is composed of five isoforms, of which AtMAP70-1 has been shown to stabilize microtubules [63] (Fig. 13.8a). MAP70s are plant-specific proteins present only in land plants (in both bryophytes and tracheophytes); they are composed of four coil–coil regions covering a plant-specific myosin heavy chain domain (pfam PF07058) (Fig. 13.8a). Interestingly, MAP70-5 has the capacity to form protein complexes with itself but also with other MAP70 isoforms such as the constitutively expressed MAP70-1 [62, 63, 84]. As described above, in differentiating *Arabidopsis* TEs, MAP70-5 and MAP70-1 do not label the U-shaped cup underlying the secondary cell wall but are restricted to the flanking microtubules and the microtubules that interconnect adjacent microtubule bundles (Fig. 13.6) [84]. These MAP70s could therefore represent a class of protein that acts as a boundary marker between thickened and unthickened areas of wall. Consistent with this, silencing of MAP70 in differentiating *Arabidopsis* cell lines causes the regularly-spaced secondary cell wall pattern of TEs to be lost and, most strikingly, causes the ribs of secondary cell wall to invaginate, detach from the existing wall and traverse through the cytoplasm (Fig. 13.8b–f) [84]. Interestingly, MAP70 silencing causes the cortical microtubules and the plasma membrane to follow the intruding secondary cell wall strands [84]. MAP70-1 and MAP70-5 appear to act synergistically in controlling the pattern of TE secondary cell walls (Fig. 13.9).

13.5.3 MIDD1 Microtubule Plus-End Associated Protein During Xylem Vessel Formation

Microtubule depletion domain 1 MIDD1 – a plant specific protein of 394aa composed of two coil–coil domains (At3g53350) – has recently been shown to be upregulated during *Arabidopsis* TE secondary cell wall formation [64, 76]. Interestingly, MIDD1 was found to preferentially label the interconnecting microtubules in *Arabidopsis* TEs (Fig. 13.9) [76]. This labeling specificity appears to be due to the coil–coil domains in which the first binds microtubules and the second specifically binds the plasma membrane of the TE pits [76]. By contrast to the MAP70 coil–coil proteins, which have been shown to stabilize microtubules, MIDD1 has been shown to decrease cortical microtubules density when overexpressed and to be preferentially accumulated at the microtubule plus-end during microtubule shrinking [76]. Genetic modulation of MIDD1 by RNA silencing caused the secondary cell wall of TEs in vitro to be without pits [76]. This suggests that MIDD1 could regulate cell wall pit formation by locally destabilizing microtubules. Together, these results on the modification of cell wall patterning by both MAP70-5/MAP70-1 and MIDD1 suggest a

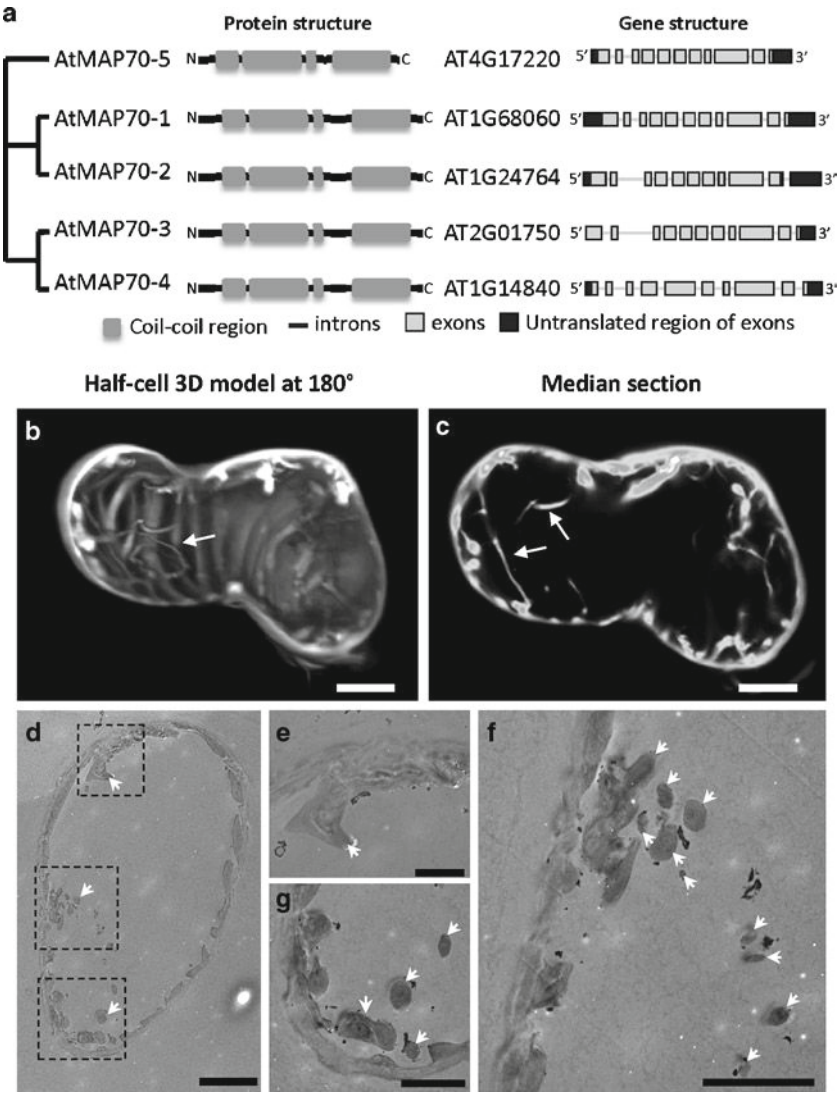

Fig. 13.8 Role of TE-specific microtubule associated protein MAP70-5. (**a**) Phylogenetic tree of the small MAP70 gene family in *Arabidopsis thaliana* including corresponding isoform protein structure with the position of coil–coil domains, the corresponding gene locus number and the intron/exon structure of each MAP70 gene. Half-cell 3D confocal reconstruction at 180° of *Arabidopsis* TE in which MAP70-5 has been constitutively silenced (**b**); the corresponding median confocal section (**c**), bars = 5 μm. *Arrows* indicate the presence of strands of secondary cell wall passing through the cytoplasm. (**d–f**) TEM images of *Arabidopsis* TE in which MAP70-5 has been constitutively silenced, showing internalized secondary cell wall strands indicated by *arrows*, bars = 5 μm

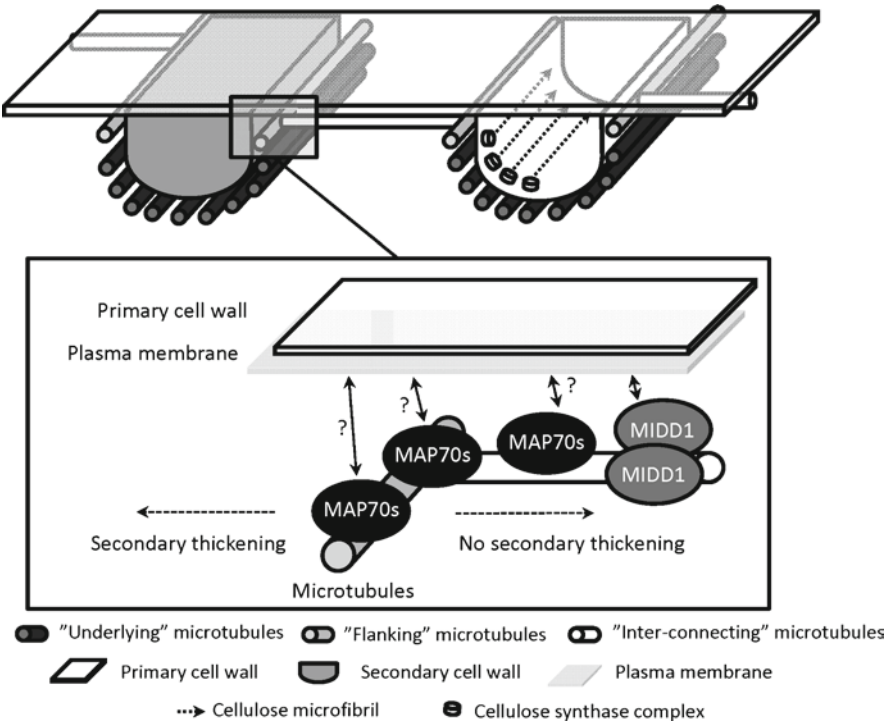

Fig. 13.9 Schematic representation of the role of "flanking" and "inter-connecting" microtubule types in TE secondary cell wall formation. There are three types of microtubules present during TE secondary cell wall formation: the underlying class in *dark gray*, the flanking microtubules in *light gray* and inter-connecting microtubules in *white*. Flanking microtubules labeled by MAP70 define the boundaries of secondary cell wall deposition while inter-connecting microtubules are labeled with MAP70s and MIDD1

tight relationship between microtubules, their anchoring to the plasma membrane and the overall pattern of TEs cell walls (Fig. 13.9).

13.5.4 The Cross-Bridging MAP65 During Xylem Vessel Formation

It is self-evident that just as future sites of secondary cell wall formation are determined by the bundling of underlying cortical microtubules so, in a complementary manner, does the absence of microtubule bundles signify an area devoid of overlying secondary deposition. In principle this demarcation between thickened and unthickened areas of wall could be effected by proteins that act as boundary markers between the two zones, keeping microtubules corralled in lanes, or by proteins that draw microtubules together into bundles. One obvious candidate for microtubule

bundling is MAP65. In carrot, isolated MAP65 has been shown to bundle microtubules made from pure brain tubulin by forming 25 nm cross-bridges that are regularly-spaced along the microtubule's axis [20] and AtMAP65-1 performs the same function in *Arabidopsis* [95]. In *Arabidopsis* this MAP family is composed of nine isoforms, all sharing the characteristic MAP65_ASE1 domain (pfam PF03999) that has the capacity to bundle microtubules. In vitro, AtMAP65-1 and AtMAP65-5 have both been shown to form homodimers that cross-link anti-parallel microtubules [43]. An association of MAP65 with secondary cell wall formation in TEs was provided by two studies. First, MAP65 was shown to be specifically up-regulated prior to secondary cell wall formation in the *Zinnia* TE differentiation system [70]. In forming TEs, immunolocalisation of MAP65, using a peptide antibody that recognized some but not all MAP65 isoforms, showed that MAP65 decorated both the "inter-connecting" microtubules and the "flanking" microtubules but it is difficult, retrospectively, to assess whether it labeled the microtubules underlying the U-shaped cup. Since it was not possible to transform *Zinnia* mesophyll cells in vitro, an *Arabidopsis* suspension was transiently overexpressed with ZeMAP65-1; this caused the microtubules to form bundles, phenocopying the pattern seen in TEs [70].

In another study, the *Arabidopsis* TE differentiation system was combined with a micro-array analysis and this showed that *Arabidopsis* MAP65-8 (At1g27920) was specifically co-regulated with secondary cellulose synthase [64]. However, our study [84] reported that none of the MAP65 isoforms was specific to TE formation during *Arabidopsis* in vitro TE differentiation as eight of the nine isoforms are also expressed in undifferentiated cell cultures. Clearly, MAP65s are capable of bundling microtubules but the evidence is equivocal that bundling in TEs is associated with upregulation of MAP65 isoforms (Fig. 13.10). If MAP65 is involved in TE formation might its activity be regulated at the post-translational level?

13.5.5 FRA1 Kinesin During Xylem Cell Wall Formation

The fragile fiber gene *FRA1*, was identified as a mutant in a forward genetic screen for inter-fascicular fiber defects [110]. *fra1*, which has a point mutation causing intron splicing defects, specifically affects a MAP with a kinesin motor domain (At5g47820) [110]. Kinesins represent the largest of the known MAP families with a total of 65 isoforms [66] all sharing the characteristic kinesin motor domain (pfam PF00225). Interestingly, the FRA1 mutation caused no changes in cell wall composition nor in secondary cell wall thickness [110]. The mutation did cause cellulose microfibrils to be disorganized and less tightly packed; the protein did decorate microtubules but its mutation caused no changes in microtubule organization and behavior [110]. FRA1 expression is ubiquitous and therefore not restricted to the vascular system. It is expressed in young expanding organs but in non-expanding organs is restricted to inter-fascicular fibers and xylem vascular bundles thereby implicating FRA1 in both cell expansion and secondary cell wall formation [111]. Understanding how FRA1 works was further pursued by constitutive over-expression

studies in whole *Arabidopsis* plants. In contrast to the effect of mutation, overexpression caused a reduction of secondary cell wall thickness in both xylem vessels and inter-fascicular fibers [111]. Interestingly, *Arabidopsis* plants over-expressing FRA1 exhibited extra secondary cell wall layers compared to wild-type [111]. Normally, secondary fibers are composed of three concentric layers of secondary cell wall each having a differently tilted cellulose microfibril angle, S1, S2 and S3, while FRA1 over-expression caused a strong reduction of the thickness of each layer and the formation of up to eight additional cell wall layers with concomitantly reduced cellulose content [111]. In FRA1 over-expressors the innermost cellulose layer showed a different angle to wild-type plants, consistent with the hypothesis that FRA1 might be a kinesin controlling the microfibril orientation of the different secondary cell wall layers of xylem cells (Fig. 13.10).

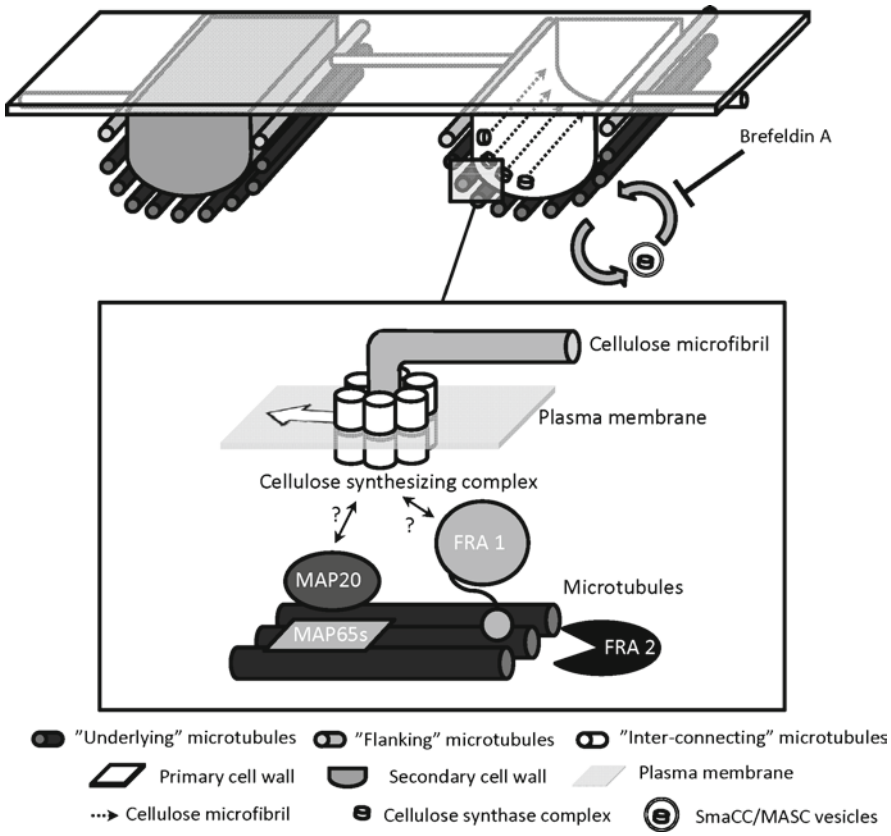

Fig. 13.10 Schematic representation of the role of the "underlying" class of microtubules in secondary cell wall formation of TEs. Cellulose synthase complexes are cycled from the trans-Golgi network through SmaCC/MASC vesicles. They are inserted into the plasma membrane alongside the "underlying" microtubules, then track along the membrane as they extrude microfibrils parallel to the microtubule bundles. The flanking microtubules define the borders within which microtubule-templated deposition can occur

13.5.6 FRA2 Katanin During Xylem Cell Wall Formation

The second mutant identified from a forward genetic screen for inter-fascicular fiber defects was *fragile fiber* 2 (*fra2*) [9]. The defect was a point mutation giving rise to a truncated protein lacking the last 78 C-terminal amino acids of AtKTN1 (At1g80350) – a protein homologous to the p60 subunit of the microtubule-severing protein, katanin of animals [92]. A promoter-trapping screen confirmed that AtKTN1 is specifically expressed in vascular tissue [73]. The FRA2 mutation causes a reduction of both the length of fibers and the thickness of their cell walls [9, 10]. Notably, the FRA2 mutation also completely disorganized the orientation of cellulose microfibrils and caused an "unsmoothed" deposition of secondary cell wall material [10]. Moreover, FRA2 mutation was shown to decrease both the cellulose and hemicellulose content [9]. Contrary to the FRA1 mutation, FRA2 mutation greatly affected microtubule organization and behavior such that microtubules appeared shorter and more randomly organized in the cell [9, 10]. Together these results provide genetic confirmation of the tight relationship between microtubules and the control and organization of secondary cell wall deposition (Fig. 13.10).

13.5.7 MAP20s During Xylem Cell Wall Formation

MAP20 – a small protein with a TPX2 domain (pfam PF06886) present in all plant species (*Arabidopsis* homolog At5g37478) – was identified by co-expression studies with secondary cell wall cellulose synthases in wood-forming tissues of poplar [87]. Immunological detection showed that MAP20 was present in all xylem cell types [87]. In transient expression studies, MAP20 produced uniform microtubule labeling but no evidence has yet been provided for its impact on microtubule stability. MAP20 overexpression [87] causes root twisting, classically observed in many other microtubule-associated mutants ([12]). Interestingly, MAP20 has been found to specifically bind the cellulose biosynthesis inhibitor 2,6-dichlorobenzonitrile (DCB), further underlining its potential role in cellulose synthesis [87]. Together, these results on the direct interaction of MAP20 with a cellulose synthase inhibitor, combined with the direct influence of FRA1 overexpression and FRA2 mutation on cellulose synthesis, suggests a tight relationship between microtubules and the synthesis and organization of cellulose microfibrils in TEs (Fig. 13.10).

13.6 Microtubules and Cellulose Synthesis in Xylem Cells

In interphase cells, microtubules attached to the inner face of the plasma membrane form a cortical array in which the microtubules are more or less evenly distributed over the membrane but during TE formation the microtubules bunch up into various

configurations, matching the overlying wall thickening. Because the secondary wall thickenings are so evident, this tissue is exemplary for showing the very direct relationship between microtubules and the cellulose-rich cell wall. This functional role of the microtubules directly beneath secondary cell wall was first provided by co-localization of these microtubules with all three secondary cell wall CesAs using fluorescent immunolocalisation [45]. Sandwiched between microtubule and cellulose microfibrils is a large cellulose-synthesizing complex embedded in the plasma membrane. In *Arabidopsis* there are ten *CESA* genes but a special subset of non-redundant *CESA* members *(CESA4, CESA7, CESA8*) contributes to the structure of the cellulose-synthesizing complexes utilized for extruding microfibrils into the secondary cell wall (see [105, 106], for a review). During primary growth, microtubules form tracks for the movement of cellulose synthases along the surface of the plasma membrane [78]. In transgenic *Arabidopsis* seedlings expressing both YFP-CesA7/IRX3 and CFP-tubulin fusions, CesA particles have been shown move within the zone confined by microtubules [104]. Due to their high density of CesA in secondary cell wall forming areas, individual CesA particles cannot be monitored and followed individually but overall these particles move at a tremendous speed (7 $\mu m\,s^{-1}$; [103]). Interestingly, both primary and secondary cell wall CesA are also associated in specialized Golgi vesicles named small CESA compartments (SmaCC; [48]) or microtubule-associated cellulose synthase compartments (MASC; [25]) which move unidirectionally to deliver new CesA complexes to the active site of cellulose synthesis (Fig. 13.10) [104]. SmaCC/MASC movement is estimated at 9 $\mu m\,min^{-1}$ which is similar to the rate of microtubule depolymerization [25, 48]. The exact nature of the SmaCC/MASC is still in question as a subpopulation of SmaCC/MASC colocalises with the trans-Golgi network [25, 48]. Brefeldin A treatment, which inhibits endomembrane traffic, has been shown to increase SmaCC/MASC accumulation [48]. In the *Zinnia* TE system, brefeldin A completely inhibits secondary cellulosic deposition but does not alter the cortical microtubule network, which still resides at the sites where secondary cell wall would supposedly be synthesized [91]. In poplar xylem fibers, endomembrane vesicles are associated with the microtubules beneath the secondary cell wall [59]. By defining the future secondary cell wall, microtubules in xylem cells represent acceptor sites for endomembrane vesicles that will deliver the all of the enzymatic apparatus for cellulose synthesis; then it will guide the movement of the CesA complex along the plasma membrane and hence the direction of the corresponding cellulose microfibril during thickening growth (Fig. 13.10).

13.7 Microtubules and the Global Patterning of the Xylem Cell Wall

As we have discussed, numerous observations have shown that microtubules define and delimit the sites and overall pattern of the future secondary cell wall, long before any thickenings can be detected [53]. This led [55] to suggest the existence

of two sub-populations of microtubules in TEs: underlying microtubules that surrounded and shape the growing secondary cell wall and flanking microtubules that form a boundary between the thickening and the microtubule-free domains in between the thickenings. This was confirmed in our recent study [84] in which we also further supported the presence of the "third" type of TE-specific microtubules, which was not overlain by secondary wall and formed curving loops that appeared to act as spacers between the transverse thickenings. Nevertheless, the exact role of each type of microtubules remains to be elucidated.

In MAP70-silenced lines induced to form TEs, 60–70% of differentiating cells exhibited strands of secondary cell wall detached from their normal cortical position [84]. The underlying microtubules were also disconnected from the cortex, instead coating the plasma membrane surrounding the invaginated wall strands (Fig. 13.8b–f). This suggests that MAP70-decorated microtubules that define the flanking limit of cell wall deposition might somehow interact with the plasma membrane and/or indirectly with the primary cell wall, forming a picket fence of tight transmembrane association (Fig. 13.9). Consistent with this, in early studies, tight interaction between plasma membrane, cytoplasmic vesicles and microtubules was observed at the future sites of secondary cell wall deposition in xylem vessels from *Allium* and *Triticum* seedlings [8]. In *Pinus strobus*, plasma membrane/cell wall interacting particles were described as being associated with microtubules in TEs developing their secondary cell wall [72]. Recently [59] reported that plasma membrane tubules underlying sites of active cell wall synthesis in developing poplar xylem fibers were co-aligned with cellulose microfibrils and microtubules. This suggested that microtubules might have increased attachment to the plasma membrane and the wall during the onset of secondary cell wall formation. Nevertheless MAP70s do not present any transmembrane domain (Fig. 13.8a). An indication of some association of microtubules with the plasma membrane comes from studies showing that tubulin seems to be a component of isolated plasma membranes [97]. MIDD1 has been shown to interact with the plasma membrane and to preferentially label the future pit sites of TEs [76]. In addition, the 90-kD phospholipase D is suggested to be a MAP that links microtubules to the plasma membrane [44] as well as a 161-kD tobacco pollen tube MAPs [13, 14]. This anchoring of the delimiting microtubules is essential for defining the boundaries of secondary cell wall deposition and, hence, the overall patterning of thickened and unthickened walls. Inspection of the four major patterns of TE secondary wall thickening shows that some occupy a greater proportion of the cell surface than others [84]. Spiral thickenings tend to be well-focussed and relatively narrow with the consequence that there are large clear spaces between. On the other hand, pitted thickenings – as the name implies – have small, pit-like clear areas set amongst broad thickenings that occupy a much larger proportion of the surface. Overexpression of AtMAP70-1 or AtMAP70-5 enhanced the proportion of spiral thickenings of induced *Arabidopsis* TEs and decreased pitted thickenings whereas silencing increased the number of pitted thickenings. Similarly, RNA silencing of MIDD1 completely abolished pit formation in *Arabidopsis* TEs [76]. Double overexpression of the MAP70 isoforms produced more spiral thickenings than was the case

for either protein alone. Interestingly, two proteins of unknown function with a transmembrane domain, TED6 and TED7, which are also co-regulated with secondary cell wall cellulose synthases in *Zinnia* differentiating TEs, have been shown to predict the future sites of secondary cell wall formation in *Arabidopsis* [29]. Genetic modulation of either TED6 or TED7 expression by RNA silencing leads to modification of cell wall patterns, reduction of cell wall thickness, smoothness and symmetry [29]. These proteins could therefore represent direct or indirect partners of the MIDD1/MAP70s in dictating the cell wall pattern. The width of the thickening can be imagined to be controlled by the number of underlying microtubules and the extent to which they are cross-bridged. However, the finding that MAP70s flank each thickening and that MIDD1 destabilizes microtubules in the future pit sites, together with the fact that their altered expression changes the width and type of individual thickenings, suggests that flanking and interconnecting MAPs play a role in specifying the overall pattern.

Spiral, scalariform, reticulate and pitted secondary thickenings appear to represent different parts of a spectrum based on the extent that areas of the plasma membrane are colonized by cortical microtubules. In this way, MAPs regulating the boundaries between thickenings must play a key developmental role. Not only do members of the MAP70 family appear to regulate the pattern of TE thickening but also the pattern of vascular strands that the TEs comprise *in planta*. RNA interference of *AtMAP70-5* reduced the number of vascular bundles in *Arabidopsis* plants by about 50% and retarded the formation of interfascicular fibers that interconnect the bundles into a ring [84]. From their phylogenetic analysis, [84] suggested that the MAP70 family arose with the first land plants, reinforcing the idea that these proteins are involved in the development of the water-conducting vasculature.

13.8 Conclusion

Xylem cell differentiation – especially during secondary cell wall deposition – has a strict requirement for microtubules to reorganize, define, position and guide the machinery for cell wall synthesis. Microtubules perform this function at the local level for each thickening but because microtubules interact to form global arrays they also influence the overall organization of the secondary wall. Ectopically expressed MAP65 causes regular interphase cortical microtubules to form TE-like super-bundles; this suggests a role for this cross-bridging MAP in TE bundle formation but the absence of changes in the level of expression suggests that any developmental influence is subtle [84]. Instead, other MAPs like AtMAP70-5 may be important for defining the boundaries between primary and secondary wall. The surprise is that although a wealth of genomic information is available from xylem development, only very few xylem-specific MAPs have been identified. If no other xylem-specific MAPs are discovered then regulation of the cortical microtubule network of xylem cells might – in addition to the induction of known xylem-specific MAPs – depend upon post-translational modification.

References

1. Abe H, Funada R, Imaizumi H, Ohtani J, Fukazawa K (1995) Dynamic changes in the arrangement of cortical microtubules in conifer tracheids during differentiation. Planta 197:418–421
2. Abe H, Funada R, Ohtani J, Fukazawa K (1995) Changes in the arrangement of microtubules and microfibrils in differentiating conifer tracheids during the expansion of cells. Ann Bot 75:305–310
3. Andersson-Gunneråas S, Mellerowicz EJ, Love J, Segerman B, Ohmiya Y, Coutinho PM, Nilsson P, Henrissat B, Moritz T, Sundberg B (2006) Biosynthesis of cellulose-enriched tension wood in Populus: global analysis of transcripts and metabolites identifies biochemical and developmental regulators in secondary wall biosynthesis. Plant J 45:144–165
4. Anthony RG, Hussey PJ (1998) Suppression of endogenous alpha and beta tubulin synthesis in transgenic maize calli overexpressing alpha and beta tubulins. Plant J 16:297–304
5. Arend M, Fromm J (2003) Ultrastructural changes in cambial cell derivatives during xylem differentiation in poplar. Plant Biol 5:255–264
6. Blume Y, Yemets A, Sulimenko V, Sulimenko T, Chan J, Lloyd C, Dräber P (2008) Tyrosine phosphorylation of plant tubulin. Planta 229:143–150
7. Boerjan W, Ralph J, Baucher M (2003) Lignin biosynthesis. Annu Rev Plant Biol 54:519–546
8. Brower DL, Hepler PK (1976) Microtubules and secondary wall deposition in xylem: the effects of isopropyl N-phenylcarbamate. Protoplasma 87:91–111
9. Burk DH, Liu B, Zhong R, Morrison WH, Ye ZH (2001) A katanin-like protein regulates normal cell wall biosynthesis and cell elongation. Plant Cell 13:807–827
10. Burk DH, Ye ZH (2002) Alteration of oriented deposition of cellulose microfibrils by mutation of a katanin-like microtubule-severing protein. Plant Cell 14:2145–2160
11. Burk DH, Zhong R, Morrison WH, Ye ZH (2006) Disruption of cortical microtubules by overexpression of green fluorescent protein-tagged a-tubulin 6 causes a marked reduction in cell wall synthesis. J Integr Plant Biol 48:85–98
12. Buschmann H, Lloyd CW (2008) Arabidopsis mutants and the network of microtubule-associated functions. Mol Plant 1:888–898
13. Cai G (2010) Assembly and disassembly of plant microtubules: tubulin modifications and binding to MAPs. J Exp Bot 61:623–626
14. Cai G, Ovidi E, Romagnoli S, Vantard M, Cresti M, Tiezzi A (2005) Identification and characterization of plasma membrane proteins that bind to microtubules in pollen tubes and generative cells of tobacco. Plant Cell Physiol 46:563–578
15. Celedon PAF, Andrade A, Meireles KGX et al (2007) Proteomic analysis of the cambial region in juvenile Eucalyptus grandis at three ages. Proteomics 7:2258–2274
16. Chaffey NJ, Barnett JR, Barlow PW (1997) Cortical microtubule involvement in bordered pit formation in secondary xylem vessel elements of *Aesculus hippocastanum* L. (Hippocastanaceae): a correlative study using electron microscopy and indirect immunofluorescence microscopy. Protoplasma 197:64–75
17. Chaffey NJ, Barnett JR, Barlow PW (1999) A cytoskeletal basis for wood formation in angiosperm trees: the involvement of cortical microtubules. Planta 208:19–30
18. Chaffey N, Barlow P, Sundberg B (2002) Understanding the role of the cytoskeleton in wood formation in angiosperm trees: hybrid aspen (*Populus tremula* x *P. tremuloides*) as the model species. Tree Physiol 22:239–249
19. Chan J, Calder G, Fox S, Lloyd C (2007) Cortical microtubule arrays undergo rotary movements in Arabidopsis hypocotyl epidermal cells. Nat Cell Biol 9:171–175
20. Chan J, Jensen CG, Jensen LC, Bush M, Lloyd CW (1999) The 65-kDa carrot microtubule-associated protein forms regularly arranged filamentous cross-bridges between microtubules. Proc Natl Acad Sci U S A 96:14931–14936
21. Chan J, Sambade A, Calder G, Lloyd C (2009) Arabidopsis cortical microtubules are initiated along, as well as branching from, existing microtubules. Plant Cell 21:2298–2306

22. Cheng Z, Snustad DP, Carter JV (2001) Temporal and spatial expression patterns of TUB9, a beta-tubulin gene of *Arabidopsis thaliana*. Plant Mol Biol 47:389–398
23. Chu B, Wilson TJ, McCune-Zierath C, Snustad DP, Carter JV (1998) Two beta-tubulin genes, TUB1 and TUB8, of Arabidopsis exhibit largely nonoverlapping patterns of expression. Plant Mol Biol 37:785–790
24. Clayton L, Lloyd CW (1984) The relationship between the division plane and spindle geometry in Allium cells treated with CIPC and griseofulvin: an anti-tubulin study. Eur J Cell Biol 34:248–253
25. Crowell EF, Bischoff V, Desprez T, Rolland A, Stierhof YD, Schumacher K, Gonneau M, Höfte H, Vernhettes S (2009) Pausing of Golgi bodies on microtubules regulates secretion of cellulose synthase complexes in Arabidopsis. Plant Cell 21:1141–1154
26. Déjardin A, Leplé JC, Lesage-Descauses MC, Costa G, Pilate G (2004) Expressed sequence tags from poplar wood tissues – a comparative analysis from multiple libraries. Plant Biol 6:55–64
27. Demura T, Tashiro G, Horiguchi G, Kishimoto N, Kubo M, Matsuoka N, Minami A, Nagata-Hiwatashi M, Nakamura K, Okamura Y, Sassa N, Suzuki S, Yazaki J, Kikuchi S, Fukuda H (2002) Visualization by comprehensive microarray analysis of gene expression programs during transdifferentiation of mesophyll cells into xylem cells. Proc Natl Acad Sci U S A 99:15794–15799
28. Duckett CM, Lloyd CW (1994) Gibberellic acid-induced microtubule reorientation in dwarf peas is accompanied by rapid modification of an a-tubulin isotype. Plant J 5:363–372
29. Endo S, Pesquet E, Yamaguchi M, Tashiro G, Sato M, Toyooka K, Nishikubo N, Udagawa-Motose M, Kubo M, Fukuda H, Demura T (2009) Identifying new components participating in the secondary cell wall formation of vessel elements in zinnia and Arabidopsis. Plant Cell 21:1155–1165
30. Esau K (1977) Anatomy of seed plants, 2nd edn. Wiley, New York
31. Falconer MM, Seagull RW (1985) Immunofluorescent and calcofluor white staining of developing tracheary elements in *Zinnia elegans* L. suspension cultures. Protoplasma 125:190–198
32. Falconer MM, Seagull RW (1985) Xylogenesis in tissue culture: taxol effects on microtubule reorientation and lateral association in differentiating cells. Protoplasma 128:157–166
33. Falconer MM, Seagull RW (1986) Xylogenesis in tissue culture II: microtubules, cell shape and secondary wall patterns. Protoplasma 133:140–148
34. Falconer MM, Seagull RW (1988) Xylogenesis in tissue culture III: continuing wall deposition during tracheary element differentiation. Protoplasma 144:10–16
35. Fosket DE, Roberts LW (1964) Induction of wound-vessel differentiation in isolated coleus stem segments in vitro. Am J Bot 51:19–25
36. Fukuda H (1987) A change in tubulin synthesis in the process of tracheary element differentiation and cell division of isolated zinnia mesophyll cells. Plant Cell Physiol 28:517–528
37. Fukuda H (1989) Regulation of tubulin degradation in isolated zinnia mesophyll cells in culture. Plant Cell Physiol 30:243–252
38. Fukuda H (2004) Signals that control plant vascular cell differentiation. Nat Rev Mol Cell Biol 5:379–391
39. Fukuda H, Kobayashi H (1989) Dynamic organization of the cytoskeleton during tracheary-element differentiation from single cells isolated from the mesophyll of *Zinnia elegans*. Dev Growth Differ 31:9–16
40. Funada R, Abe H, Furusawa O, Imaizumi H, Fukazawa K, Ohtani J (1997) The orientation and localization of cortical microtubules in differentiating conifer tracheids during cell expansion. Plant Cell Physiol 38:210–212
41. Funada R, Miura H, Shibagaki M, Furusawa O, Miura T, Fukatsu E, Kitin P (2001) Involvement of localized cortical microtubules in the formation of a modified structure of wood. J Plant Res 144:491–497
42. Furusawa O, Funada R, Murakami Y, Ohtani J (1997) Arrangement of cortical microtubules in compression wood tracheids of Taxus cuspidata visualized by confocal laser microscopy. J Wood Sci 44:230–233

43. Gaillard J, Neumann E, Van Damme D, Stoppin-Mellet V, Ebel C, Barbier E, Geelen D, Vantard M (2008) Two microtubule-associated proteins of Arabidopsis MAP65s promote antiparallel microtubule bundling. Mol Biol Cell 19:4534–4544
44. Gardiner JC, Harper JD, Weerakoon ND, Collings DA, Ritchie S, Gilroy S, Cyr RJ, Marc J (2001) A 90-kD phospholipase D from tobacco binds to microtubules and the plasma membrane. Plant Cell 13:2143–2158
45. Gardiner JC, Taylor NG, Turner SR (2003) Control of cellulose synthase complex localization in developing xylem. Plant Cell 15:1740–1748
46. Gion JM, Lalanne C, Le Provost G, Ferry-Dumazet H, Paiva J, Chaumeil P, Frigerio JM, Brach J, Barré A, de Daruvar A, Claverol S, Bonneu M, Sommerer N, Negroni L, Plomion C (2005) The proteome of maritime pine wood forming tissue. Proteomics 5:3731–3751
47. González-Martínez SC, Wheeler NC, Ersoz E, Nelson CD, Neale DB (2007) Association genetics in Pinus taeda L. I. Wood property traits. Genetics 175:399–409
48. Gutierrez R, Lindeboom JJ, Paredez AR, Emons AM, Ehrhardt DW (2009) Arabidopsis cortical microtubules position cellulose synthase delivery to the plasma membrane and interact with cellulose synthase trafficking compartments. Nat Cell Biol 11:797–806
49. Hamada T (2007) Microtubule-associated proteins in higher plants. J Plant Res 120:79–98
50. Hammersley DRH, McCully ME (1980) Differentiation of wound xylem in pea roots in the presence of colchicines. Plant Sci Lett 19:151–156
51. Heath W (1985) Plasma-membrane rosettes involved in localized wall thickening during xylem vessel formation of *Lepidium sativum* L. Planta 164:12–21
52. Hepler PK, Fosket DE (1971) The role of microtubules in vessel member differentiation in Coleus. Protoplasma 72:213–236
53. Hepler PK, Newcomb EH (1964) Microtubules and fibrils in the cytoplasm of coleus cells undergoing secondary wall deposition. J Cell Biol 20:529–532
54. Hertzberg M, Aspeborg H, Schrader J, Andersson A, Erlandsson R, Blomqvist K, Bhalerao R, Uhlén M, Teeri TT, Lundeberg J, Sundberg B, Nilsson P, Sandberg G (2001) A transcriptional roadmap to wood formation. Proc Natl Acad Sci U S A 98:14732–14737
55. Hogetsu T (1991) Mechanism for formation of the secondary wall thickening in tracheary elements: Microtubules and microfibrils of tracheary elements of *Pisum sativum* L. and *Commelina communis* L. and the effects of amiprophosmethyl. Planta 185:190–200
56. Huang RF, Lloyd CW (1999) Gibberellic acid stabilizes microtubules in maize suspension cells to cold and stimulates acetylation of a-tubulin. FEBS Lett 443:317–320
57. Jovanovic AM, Durst S, Nick P (2010) Plant cell division is specifically affected by nitrotyrosine. J Exp Bot 61:901–909
58. Juan D, Hong-Li X, De-Qiang Z, Xin-Qiang H, Min-Jie W, Ying-Zhang L, Ke-Ming C, Lu Meng-Zhu L (2006) Regeneration of the secondary vascular system in poplar as a novel system to investigate gene expression by a proteomic approach. Proteomics 6:881–895
59. Kaneda M, Rensing K, Samuels L (2010) Secondary cell wall deposition in developing secondary xylem of poplar. J Integr Plant Biol 52:234–243
60. Kobayashi H, Fukuda H, Shibaoka H (1988) Interrelation between the spatial disposition of actin filaments and microtubules during the differentiation of tracheary elements in cultured Zinnia cells. Protoplasma 143:29–37
61. Kopczak SD, Haas NA, Hussey PJ, Silflow CD, Snustad DP (1992) The small genome of Arabidopsis contains at least six expressed alpha-tubulin genes. Plant Cell 4:539–547
62. Korolev AV, Buschmann H, Doonan JH, Lloyd CW (2007) AtMAP70-5, a divergent member of the MAP70 family of microtubule-associated proteins, is required for anisotropic cell growth in Arabidopsis. J Cell Sci 120:2241–2247
63. Korolev AV, Chan J, Naldrett MJ, Doonan JH, Lloyd CW (2005) Identification of a novel family of 70 kDa microtubule-associated proteins in Arabidopsis cells. Plant J 42:547–555
64. Kubo M, Udagawa M, Nishikubo N, Horiguchi G, Yamaguchi M, Ito J, Mimura T, Fukuda H, Demura T (2005) Transcription switches for protoxylem and metaxylem vessel formation. Genes Dev 19:1855–1860

65. Le J, Vandenbussche F, De Cnodder T, Van der Straeten D, Verbelen J-P (2005) Cell elongation and microtubule behavior in Arabidopsis hypocotyl: responses to ethylene and auxin. J Plant Growth Reg 24:166–178
66. Lee YR, Liu B (2004) Cytoskeletal motors in Arabidopsis. Sixty-one kinesins and seventeen myosins. Plant Physiol 136:3877–3883
67. Li X, Wu HX, Dillon SK, Southerton SG (2009) Generation and analysis of expressed sequence tags from six developing xylem libraries in Pinus radiata D. Don. BMC Genomics 10:41–59
68. Li X, Wu HX, Southerton SG (2010) Seasonal reorganization of the xylem transcriptome at different tree ages reveals novel insights into wood formation in *Pinus radiata*. New Phytol 187:764–776
69. Liang BM, Dennings AM, Sharp RE, Baskin TI (1996) Consistent handedness of microtubule helical arrays in maize and Arabidopsis primary roots. Protoplasma 190:8–15
70. Mao G, Buschmann H, Doonan JH, Lloyd CW (2006) The role of MAP65-1 in microtubule bundling during Zinnia tracheary element formation. J Cell Sci 119:753–758
71. Morejohn LC, Bureau TE, Tocchi LP, Fosket DE (1984) Tubulins from different higher plant species are immunologically nonidentical and bind colchicine differentially. Proc Natl Acad Sci U S A 81:1440–1444
72. Murmanis L (1971) Particles and microtubules in vascular cells of *Pinus strobes* L. during cell wall formation. New Phytol 70:1089–1093
73. Nagawa S, Sawa S, Sato S, Kato T, Tabata S, Fukuda H (2006) Gene trapping in Arabidopsis reveals genes involved in vascular development. Plant Cell Physiol 47:1394–1405
74. Nelmes BJ, Preston RD, Ashworth D (1973) A possible function of microtubules suggested by their abnormal distribution in rubbery wood. J Cell Sci 13:741–751
75. Oda Y, Mimura T, Hasezawa S (2005) Regulation of secondary cell wall development by cortical microtubules during tracheary element differentiation in Arabidopsis cell suspensions. Plant Physiol 137:1027–1036
76. Oda Y, Lida Y, Kondo Y, Fukuda H (2010) Wood cell-wall structure requires local 2D-microtubule disassembly by a novel plasma membrane-anchored protein. Curr Biol 20:1197–1202
77. Oakley RV, Wang YS, Ramakrishna W, Harding SA, Tsai CJ (2007) Differential expansion and expression of alpha- and beta-tubulin gene families in Populus. Plant Physiol 145:961–973
78. Paredez AR, Somerville CR, Ehrhardt DW (2006) Visualization of cellulose synthase demonstrates functional association with microtubules. Science 312:1491–1495
79. Paiva JA, Garcés M, Alves A, Garnier-Géré P, Rodrigues JC, Lalanne C, Porcon S, Le Provost G, Perez Dda S, Brach J, Frigerio JM, Claverol S, Barré A, Fevereiro P, Plomion C (2008) Molecular and phenotypic profiling from the base to the crown in maritime pine wood-forming tissue. New Phytol 178:283–301
80. Paiva JA, Garnier-Géré PH, Rodrigues JC, Alves A, Santos S, Graça J, Le Provost G, Chaumeil G, Da Silva-Perez D, Bosc A, Fevereiro P, Plomion C (2008) Plasticity of maritime pine (*Pinus pinaster*) wood-forming tissues during a growing season. New Phytol 179:1080–1094
81. Paux E, Carocha V, Marques C, Mendes de Sousa A, Borralho N, Sivadon P, Grima-Pettenati J (2005) Transcript profiling of Eucalyptus xylem genes during tension wood formation. New Phytol 167:89–100
82. Paux E, Tamasloukht M, Ladouce N, Sivadon P, Grima-Pettenati J (2004) Identification of genes preferentially expressed during wood formation in Eucalyptus. Plant Mol Biol 55:263–280
83. Pickett-Heaps JD (1967) The effects of colchicine on the ultrastructure of dividing plant cell, xylem wall differentiation and distribution of cytoplasmic microtubules. Dev Biol 15:206–236
84. Pesquet E, Korolev AV, Calder G, Lloyd CW (2010) The microtubule-associated protein AtMAP70-5 regulates secondary wall patterning in Arabidopsis wood cells. Curr Biol 20:744–749
85. Prodham AKMA, Funada R, Ohtani J, Abe H, Fukazawa K (1995) Orientation of microfibrils and microtubules in developing tension-wood fibres of Japanese ash (*Fraxinus mandshurica* var.japonica). Planta 196:577–585

86. Qiu D, Wilson IW, Gan S, Washusen R, Moran GF, Southerton SG (2008) Gene expression in Eucalyptus branch wood with marked variation in cellulose microfibril orientation and lacking G-layers. New Phytol 179:94–103
87. Rajangam AS, Kumar M, Aspeborg H, Guerriero G, Arvestad L, Pansri P, Brown CJ, Hober S, Blomqvist K, Divne C, Ezcurra I, Mellerowicz E, Sundberg B, Bulone V, Teeri TT (2008) MAP20, a microtubule-associated protein in the secondary cell walls of hybrid aspen, is a target of the cellulose synthesis inhibitor 2, 6-dichlorobenzonitrile. Plant Physiol 148:1283–1294
88. Roberts AW, Frost AO, Roberts EM, Haigler CH (2004) Roles of microtubules and cellulose microfibril assembly in the localization of secondary-cell-wall deposition in developing tracheary elements. Protoplasma 224:217–229
89. Roberts K, McCann MC (2000) Xylogenesis: the birth of a corpse. Curr Opin Plant Biol 3:517–522
90. Roberts LW, Baba S (1968) IAA-induced xylem differentiation in the presence of colchicines. Plant Cell Physiol 9:315–321
91. Rojas M, Owen TP Jr, Lindahl KN (1999) Brefeldin A inhibits secondary cell wall synthesis in developing tracheary elements of *Zinnia elegans*. Int J Plant Sci 160:683–690
92. Roll-Mecak A, McNally FJ (2010) Microtubule-severing enzymes. Curr Opin Cell Biol 22:96–103
93. Salnikov VV, Grimson MJ, Delmer DP, Haigler CH (2001) Sucrose synthase localizes to cellulose synthesis sites in tracheary elements. Phytochemistry 57:823–833
94. Sharma N, Bryant J, Wloga D, Donaldson R, Davis RC, Jerka-Dziadosz M, Gaertig J (2007) Katanin regulates dynamics of microtubules and biogenesis of motile cilia. J Cell Biol 178:1065–1079
95. Smertenko AP, Chang HY, Wagner V, Kaloriti D, Fenyk S, Sonobe S, Lloyd C, Hauser MT, Hussey PJ (2004) The Arabidopsis microtubule-associated protein AtMAP65-1: molecular analysis of its microtubule bundling activity. Plant Cell 16:2035–2047
96. Snustad DP, Haas NA, Kopczak SD, Silflow CD (1992) The small genome of Arabidopsis contains at least nine expressed beta-tubulin genes. Plant Cell 4:549–556
97. Sonesson, A., Berglund, M., Staxén, I., Widell, S. 1997. The characterization of plasma membrane-bound tubulin of cauliflower using Triton X-114 fractionation. Plant Physiol. 115:1001–7.
98. Spokevicius AV, Southerton SG, MacMillan CP, Qiu D, Gan S, Tibbits JF, Moran GF, Bossinger G (2007) beta-tubulin affects cellulose microfibril orientation in plant secondary fibre cell walls. Plant J 51:717–726
99. Thitamadee S, Tuchihara K, Hashimoto T (2002) Microtubule basis for left-handed helical growth in Arabidopsis. Nature 417:193–196
100. Turner S, Gallois P, Brown D (2007) Tracheary element differentiation. Annu Rev Plant Biol 58:407–433
101. Uehara K, Hogetsu T (1993) Arrangement of cortical microtubules during formation of bordered pit in the tracheids of Taxus. Protoplasma 172:145–153
102. Washusen R, Evans R, Southerton S (2005) A study of *Eucalyptus grandis* and *Eucalyptus globulus* branch wood microstructure. IAWA J 26:203–210
103. Wightman R, Marshall R, Turner SR (2009) A cellulose synthase-containing compartment moves rapidly beneath sites of secondary wall synthesis. Plant Cell Physiol 50:584–594
104. Wightman R, Turner SR (2008) The roles of the cytoskeleton during cellulose deposition at the secondary cell wall. Plant J 54:794–805
105. Wightman R, Turner SR (2010) Trafficking of the plant cellulose synthase complex. Plant Physiol 153:427–432
106. Wightman R, Turner SR (2010) Trafficking of the cellulose synthase complex in developing xylem vessels. Biochem Soc Trans 38:755–760
107. Ye ZH, Freshour G, Hahn MG, Burk DH, Zhong R (2002) Vascular development in Arabidopsis. Int Rev Cytol 220:225–256

108. Yoshimura T, Demura T, Igarashi M, Fukuda H (1996) Differential expression of three genes for different β-tubulin isotypes during the initial culture of Zinnia mesophyll cells that divide and differentiate into tracheary elements. Plant Cell Physiol 37:1167–1176
109. Zhao C, Craig JC, Petzold HE, Dickerman AW, Beers EP (2005) The xylem and phloem transcriptomes from secondary tissues of the Arabidopsis root-hypocotyl. Plant Physiol 138:803–818
110. Zhong R, Burk DH, Morrison WH, Ye ZH (2002) A kinesin-like protein is essential for oriented deposition of cellulose microfibrils and cell wall strength. Plant Cell 14:3101–3117
111. Zhou J, Qiu J, Ye ZH (2007) Alteration in secondary wall deposition by overexpression of the FRA1 kinesin-like protein in Arabidopsis. J Integr Plant Biol 49:1235–1243

Chapter 14
The Cytoskeleton and Root Growth Behavior

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14.1 Introduction

As sessile organisms, plants have to face environmental challenges as they occur, without being able to run away from them and escape toward more favorable conditions. Consequently, evolution equipped plant organs with an extensive plasticity that allows them to cope with environmental changes. At the same time, however, plant organs are also capable of directed growth, a process that allows them to explore their immediate environment for optimal conditions where they can fulfill their primary functions.

Roots are proficient at directing their growth away from deleterious conditions, toward more favorable parameters that permit the uptake of water, mineral ions, and oxygen along with providing mechanical support for the plant. Growing in the heterogeneous environment of soil, roots have to avoid obstacles, extreme temperatures, and excessive or depleted levels of water, certain ions, and oxygen.

To grow downward into the soil immediately after germination and reach new sources of water and nutrients before seed reserves are exhausted, young primary roots use the direction of gravity as a growth guide. Most plant organs are capable of orienting their growth with respect to gravity, even though the gravity set point angle (angle between the growth and gravity vectors) differs significantly between organs, environmental conditions, and developmental stages of tissues. For instance, while primary roots tend to orient themselves downward, primary shoots tend to growth upward, toward light, where photosynthesis and gas exchange can occur more effectively. Lateral roots, on the other hand, will tend to grow along a vector that forms a defined angle from the primary root, at least for some period following its emergence from the primary root (reviewed in [32]).

While it is important for a root to use gravity as a growth guide, it is equally important for it to modulate its growth behavior depending on other directional cues.

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The soil is typically highly heterogeneous, and vertical downward growth may have to be replaced by another pattern of guided growth under defined conditions. For instance, a root growing along the gravity vector is likely to hit a rock at some time during its life. Such an obstacle will hinder the root's ability to grow and reach out for water and ions. This root should be able to redirect its growth to bypass the obstacle. A root facing a rock will sense the obstacle in its path, a process that will lead to a transient inhibition of gravitropism. Consequently, the root will now be free to bend away from the obstacle, against the gravity vector. As soon as the obstacle is bypassed, the root will be able to resume its gravitropically-driven downward growth [48, 51].

Of course, other directional parameters within a root's environment are critical to guiding its growth. Considering that its primary function is to take up the water and mineral ions needed for plant growth, development and reproduction, it is not surprising that gradients in water and nutrients can be sensed by a root and used as directional information to guide growth. Interestingly, here again, roots have been shown to respond to lower humidity environments by inhibiting their gravitropic response, a process that allows them to use water gradients as growth guides [17, 78]. Hence, the transduction pathways that lead to tropic responses in roots are multifaceted and interconnected, leading to dynamic behaviors that are believed to allow optimal navigation within a heterogeneous and changing environment.

Roots that are simultaneously exposed to multiple directional cues display strikingly complex growth patterns, as illustrated by *Arabidopsis thaliana* seedlings forced to grow on the surface of vertical hard agar-containing media. Under these conditions, the seedlings' primary roots are exposed to gravity and touch stimulation, along with multiple other surface-derived stimuli. Such roots still tend to grow downward, due mainly to gravitropism. However, they also tend to deviate their growth away from the gravity vector, always in the same direction (left when viewed from the front of the plate, right when viewed through the medium from the back of the plate). A more extreme root-growth behavior is seen when wild-type Arabidopsis seedlings are grown on horizontal hard surfaces. Under these conditions, the roots tend to form clockwise coils that are associated with strong epidermal cell file rotation [39].

When Arabidopsis seedlings are forced to grow on backward tilted hard agar surfaces, their roots also tend to meander left and right on the surface, along a wavy path, in addition to displaying the right-skewing behavior discussed above (illustrated in Fig. 14.1). Under most growth conditions, the curvatures that contribute to these movements are accompanied by a rotation of the tip about its axis whose handedness typically correlates with the direction of curvature: left-handed for curvatures to the right and right-handed for curvatures to the left (Fig. 14.1). It has been postulated that such complex behaviors allow roots to effectively forge their way around obstacles in soil [13, 49, 56, 68, 75].

Interestingly, Charles Darwin and his son reported a very similar pattern of root growth in 1880. These investigators germinated seeds from a number of diverse species on top of a backward-tilted smoked glass plate. As soon as they hit the plate, the roots continued to grow downward. However, hindrance from the glass

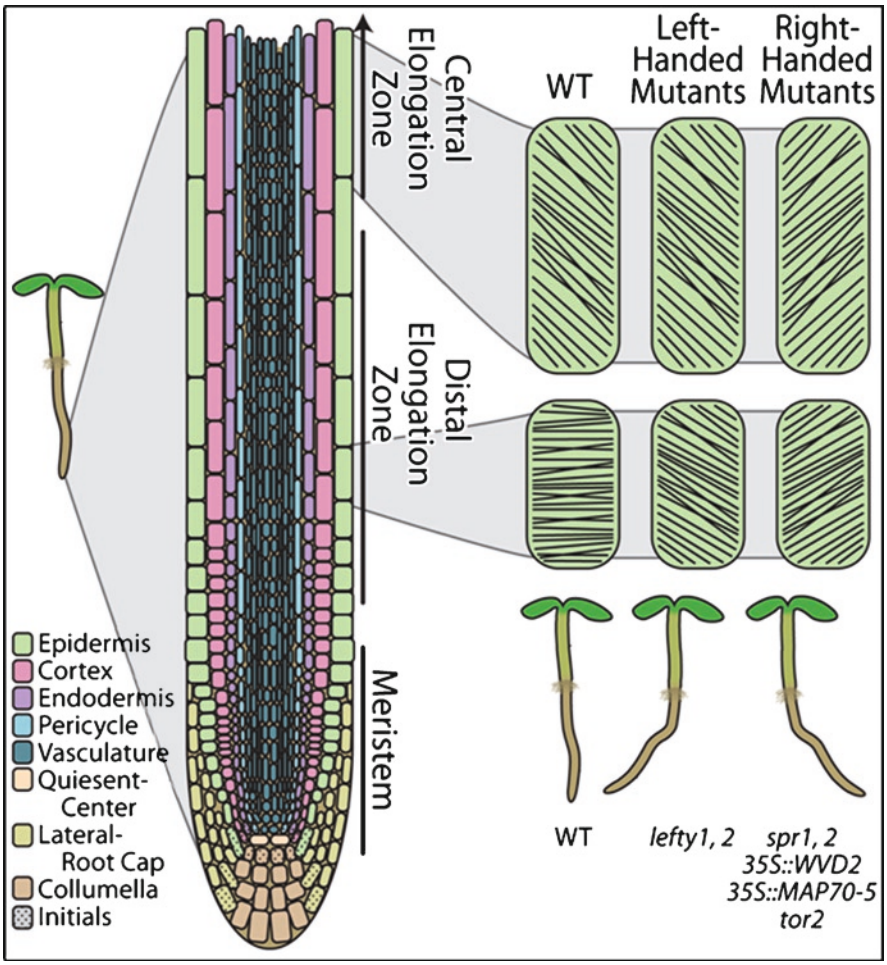

Fig. 14.1 Arabidopsis root tip, cortical microtubule arrangements, and helical growth. A schematic of a longitudinal section of the root tip highlights the different cell types along with the meristem and the distal and central elongation zones. Epidermal cells of the elongation zones are enhanced to show microtubule alignments in these sections for wild-type and selected helical growth mutants

surface triggered a wavy growth response that was reminiscent of that observed for *Arabidopsis thaliana* seedlings forced to grow on tilted hard-agar surfaces. In the Darwins' experiments, the path followed by the root tip was recorded by the trace left by the root on the black silt covering the glass plate [22].

The complex behavior displayed by Arabidopsis roots growing on hard surfaces has recently been used as a platform to identify genes that modulate a root's ability to perceive its environment. As discussed below, many of the mutations affecting such behaviors have uncovered genes that contribute either directly or indirectly to

the regulation of cortical microtubules dynamics and network organization in growing cells. This review focuses mainly on this aspect of the process. Before we discuss the contribution of cytoskeleton organization to growth behaviors, we will first describe the mechanisms that mediate overall root growth.

14.2 Roots Grow Using a Combination of Cell Division and Cell Expansion

14.2.1 Controlled Cell Division Contributes to Root Growth

Roots grow using a combination of controlled cell divisions in the apical meristem and regulated anisotropic cell expansion in the elongation zones (Fig. 14.1). In Arabidopsis, the primary root promeristem is organized in two layers of initial cells that surround a group of four quiescent center cells. The quiescent center cells emit a signal to the surrounding cells to maintain their identity as initials. Initial cells typically divide asymmetrically to regenerate themselves and produce a derivative that will ultimately differentiate into a defined cell type.

The outer circle of initials in the Arabidopsis promeristem gives rise to lateral root-cap and epidermal cells. Going inward within the basal layer of initials (toward the root-hypocotyl junction), a second group of initials produce the cortical and endodermal cell files that underlie the epidermis. The most internal groups of initials on the basal side produce vascular-tissue cells. On the apical side (closest to the root tip), a group of 12 initials divide to generate the columella cells of the cap, which will eventually differentiate into tip cells [94].

The collar of epidermis/lateral root cap initials follows specialized patterns of cell division. As mentioned above, these initials divide sequentially, first periclinally to generate a derivative that will lead to lateral root cap cells, then anticlinally to generate another derivative that will be added to the epidermis. These initials do not divide synchronously in the root promeristem. Instead, their divisions follow a spiral sequence that starts within the columella initials and produces a helical pattern of cell junctions along the meristem [7]. Wasteneys and Collins proposed that this helical pattern of cell divisions might be the basis for helical growth in wild type and mutant roots [83]. Compatible with this hypothesis, early studies showed that the wild type roots of many *Arabidopsis thaliana* accessions display left-handed helical growth when exposed to surfaces [68, 75]. Yet, the epidermal cell file rotation (CFR) that accompanies this helical growth behavior initiates at a site of the root that is basal to the site of initial divisions: pronounced CFR occurs at the basal side of the central elongation zone [68]. Therefore, if the spiral pattern of cell divisions occurring at the root epidermis were to be responsible for helical growth, this effect would have to be indirect. Another model places cell expansion, not division, as the causative process for production of helical growth. As discussed below, many lines of evidence suggest that the dynamic instability and/or organization of the cortical

microtubular network in expanding cells of roots might contribute to modulating this twisting behavior.

14.2.2 Regulated Cell Expansion Also Contributes to Root Growth

Cells laid down by the division of initials in the root promeristem continue to divide in the apical meristem, mainly in an anticlinal fashion, before expanding and differentiating into the distinct cell types that make up the mature section of a root. When transiting through the meristematic and transition zone of the root tip, cells expand both length-wise ("elongate") and radially. When they reach the elongation zones, these cells will expand anisotropically in a direction parallel to the root's axis of growth [6, 36, 37]. Regulation of cellular expansion in this region of the root is critical to the modulation of root growth direction. Tropic responses have been shown to involve a differential rate of cellular expansion on opposite sides of the elongation zone rather than differential cell divisions across the meristem (reviewed in [32]).

Anisotropic cell expansion is modulated in part by the organization of the cellulose microfibrils in the wall of expanding cells. These largely inextensible polymers are arranged transversely in the wall of root elongation zone cells, thereby restricting their anisotropic expansion in a perpendicular direction, along the long axis of the root (reviewed in [95]). The cellulose synthase complex that mediates cellulose synthesis at the surface of the cell follows a path that is parallel to the microtubules found at the cortex of these expanding cells [59]. It has been proposed that the organization and/or dynamic instability of cortical microtubules in expanding cells of roots dictate the patterns of cellulose microfibril deposition in the wall, thereby directing the patterns of cell expansion and shaping the root as well as guiding its growth ([46]; reviewed in [96, 21, 88]).

The cortical microtubules of interphase epidermal and lateral-cap cells form a regular network of transversely aligned microtubules throughout most of the elongation zone. This arrangement correlates with the unidirectional anisotropic cell expansion generally seen in this region of the root (reviewed in [32]). Eventually, as cells reach the basal side of the elongation zone, their cortical microtubules reorganize to form a right-handed helical array before returning to a more disorganized longitudinal array when the cell enters the mature zone of the root and stops elongating [97] (Fig. 14.1). The region at the basal side of the main elongation zone that displays a right-handed helical arrangement of cortical microtubules also corresponds to the site where left-handed helical growth movement initiates at the root tip, as illustrated by the rotation of epidermal cell files in this region ([56, 68]; see below).

Mutant studies have allowed plant biologists to identify some of the proteins involved in root cell microtubule dynamics, cell expansion, and overall growth behavior. Many identified mutants affect tubulin or proteins that bind to tubulin, implicating the cytoskeleton directly in any aberrant phenotypes. Another group of

mutants show defects in proteins that do not physically contact the microtubules but still impact their organization and/or dynamic properties. Besides mutant studies, natural genetic variation within species can be exploited to discover genes that lead to varied root growth among its members.

14.3 Mutations in Tubulin Genes Affect Microtubule Arrays and Root Skewing

As described above, a precise choreography exists for microtubule arrays as cells progress from the root's meristem to the mature zone. Altering microtubule orientations during this transition can lead to defects in cell expansion and aberrant helical growth. Changes in the structure of tubulin itself can be the culprit for faulty arrays. This is exactly the case with the mutants *lefty1* and *lefty2* of Arabidopsis, which contain single amino acid changes in two α-tubulin isoforms (TUA6 and TUA4, respectively) [79]. These mutants have right-handed microtubule arrays that extend well into the central elongation zone whereas the wild-type root has them only near the basal end of the same region. This correlates with an increase in the angle of leftward skewing over wild-type when grown on hard agar, as well as a left-handed epidermal cell file rotation. A similar phenotype can be seen when microtubule destabilizing drugs propyzamide and oryzaline are applied to wild-type roots, indicating that the altered α-tubulin in the *lefty* mutants leads to changes in microtubule dynamics. The *lefty1lefty2* double mutant has disordered arrays with a slight preference for right-handedness and a strong right-handed growth of epidermal cell files. Overall root growth is more impaired than in either single mutant due to the inability of the cells to expand anisotropically [1].

A later screen identified 32 mutants in several isoforms of both α- and β-tubulin [35]. The mutations in this collection occur at conserved residues in regions that are involved in three types of interactions necessary for constructing a microtubule. Some of the amino acid alterations affect the area where the α- and β-subunits come together to form dimers. Others are located at the interface where the αβ-tubulin dimers join to form higher-level structures, and the third category includes residues that link protofilaments. Some of the mutants show *lefty*-like microtubule arrays, cell file rotation, and root skewing behavior. Others show opposite, right-handed helical root growth and epidermal cell files as well as left-handed microtubule arrangement. There is a strong correlation between the angle of root skewing and the pitch of the microtubule array. Along with the shared phenotype of helical growth, several of the mutants show marked isotropic cell expansion, and some have altered microtubule dynamics.

Another Arabidopsis α-tubulin 4 mutant, *tortifolia2*, was recently studied in detail to gain a better understanding of helical growth and cortical microtubule dynamics [16]. The mutation results in an amino acid change in the region where one αβ-tubulin dimer bonds to a preceding dimer. This is the region where β-tubulin binds and hydrolyzes GTP. A group of plants with various mutations at this

interface have right-handed helical growth [35]. Therefore, the ability of the growing end to polymerize at normal rates is critical for proper microtubule orientation. *tor2* also has right-handed helical growth and radially expanded cells that show strong right-handed cell file rotation. The abnormal helical growth patterns for the *tor2* mutant arise during cell expansion, not during cell division, and even single *tor2* cells in culture showed a decreased ability to elongate and a tendency toward twisted morphology (this was also highly visible in the single-celled trichomes). Tracking cortical microtubules in *tor2* reveals that they are slow to both polymerize and depolymerize and spend more time in the pause state. Reduced microtubule dynamics make it difficult for a rapidly elongating cell to lay down the continuous pattern it needs to properly expand.

Eradication of γ-tubulin from plants also results in radial cell expansion and eventual seedling lethality [61]. γβ-tubulin is an essential component for microtubule organization in plant cells. Among other organ defects, seedlings carrying no functional copies of either Arabidopsis γ-tubulin gene had severe radial swelling in the meristematic region and disorganized microtubules in the elongation zone. Single mutants for either gene appeared wild-type.

Evidence from these tubulin mutant studies, particularly the detailed characterization of *tor2,* strongly support a role for anisotropic cell expansion in mediating helical growth. Again, careful analyses of the helical pattern of cell junctions along the *tor2* root meristem revealed a pattern of epidermis/lateral cap initial division that did not differ significantly from the wild type, but the mutant cells initiated a twist while expanding, independently of whether they were maintained within their native tissues or separated to grow in culture. The inherent ability of expanding cells to twist might be responsible for helical organ growth, at least in this mutant. It will be important to observe other tubulin mutants under these conditions to see if twisting arises during expansion rather than originating from altered cell division.

14.4 Mutations in Microtubule Associated Proteins Also Lead to Abnormal Root Development

Many protein families are able to associate with the cortical microtubules, and disrupting these interactions can alter root growth behavior. Possible outcomes include various combinations of abnormal cell expansion, disoriented microtubule arrays, and/or helical organ growth. The microtubule-associated proteins (MAPs) bind the microtubles of the cortical array in specific patterns and their localization allows them to play defined roles in array dynamics. Studies on families of plant MAPs are beginning to elucidate how their interactions with tubulins allow for proper cell expansion and organ growth. Tissues with rapidly elongating cells, like the root, are excellent systems for observing how various MAP mutations affect microtubule alignments.

A diverse group of MAPs has been found to associate with the fast growing plus-ends of cortical microtubules, at least in some tissues. SPIRAL1 (SPR1), SPIRAL2 (SPR2), MICROTUBULE ORGANIZATION 1 (MOR1)/MAP215,

END BINDING1 (EB1), and CLASP proteins all show this localization [3, 10, 40, 41, 72, 90]. MOR1/MAP215, CLASP, and EB1 proteins are conserved in eukaryotes and have been studied in animals and fungi as well as plants. SPR1 and SPR2 family proteins are plant-specific and may have roles specialized for organisms without distinct microtubule organizing centers.

Several of the plus-end MAP mutants show abnormal root skewing phenotypes when grown on hard agar surfaces. Roots of *spr1* and *spr2* (also known as *tor1*) have strong and slight right skews, respectively. The epidermal cell files of *spr1* roots are shaped into right-handed helices, but *spr2* cell files have wild-type handedness in the root despite visible cell file rotation in its aerial organs [26]. Members of a family of *SPIRAL1-LIKE* (*SP1L*) genes do not have skewing phenotypes as single mutants, but do affect skewing when combined as double or higher order mutants in a *spr1* background. The *sp1l* mutants lessen the skewing of *spr1*, and radial swelling is increased leading to disorderly cell files [54]. The decreased root skewing may be linked to a less organized cell layout. There is also a close homolog of *SPR2* in the Arabidopsis genome, *SPIRAL2-LIKE* (*SP2L*). Like the *sp1l* mutants, the *sp2l* mutant alone shows wild-type root skewing. However, an *sp2l* mutation in a *spr2* background increases rightward skewing over the *spr2* single mutant [90]. Also, a combination of *spr1* and *spr2* leads to increased right skewing, increased cell file twisting, and decreased anisotropic cell expansion [26]. The root phenotypes in this double mutant are markedly stronger than what would be expected if the *spr1* and *spr2* alleles were working only in an additive fashion.

Two other plus-end MAPs, *MOR1* and *EB1*, also show root skewing phenotypes when mutated, although their slant is leftward. In the temperature-sensitive *mor1* mutants, high temperatures lead to left skew in roots accompanied by left-handed cell file rotation and radial swelling of the cells [86]. Mutants in all three *EB1* genes grow with an increased leftward skew on tilted agar, and *eb1b-1* has a tendency to form loops with left-twisting epidermal cell files more often than wild-type [10]. The *eb1* mutants also have root phenotypes relating to altered thigmotropism and gravitropism. They elongate at the rate of wild-type but are delayed in producing a bending response after a touch- or gravity-induced stimulus [10]. Therefore, the skewing of *eb1* roots may be a case of helical growth due to abnormalities in differential side elongation instead of in cell file twisting, an idea put forth in [13].

In the various tubulin mutations, helical growth corresponded with helical arrays of opposite-handedness in the central elongation zone of the root. This is not always the case with the various MAP mutants. The cortical microtubules in the root elongation zone of *spr1* are dependent on growth conditions or possibly genetic background. The microtubules were oriented in left-handed arrays in the experiments of Furutani et al., whereas Sedbrook et al. reported that *spr1* arrays in this area were mostly transverse like the wild-type. In combinations of *spr1* with *sp1l* mutations, the microtubule arrays lack specific organization [54]. The epidermal microtubule arrays of *spr2* roots are wild-type even though in other organs, like the hypocotyl, there is a preference for left-handedness [26], and mutations in both *spr2* and *sp2l* increase the frequency of left-handed arrays [90]. The leftward skewing roots of *mor1* and *eb1* do not have helical arrays [10, 86]. However, the

arrays of *mor1* are disorganized, and the cortical microtubules are shorter than wild-type [86].

Many experiments have also investigated the interactions of the plus-end MAPs with microtubules on a molecular level and how the interactions affect microtubule dynamics. GFP-tagged SPR1 colocalizes with tubulin [53, 72], and the signal is enriched at microtubule plus-ends [72]. When the microtubule enters shrinkage phase, the SPR1 signal dissipates, so it seems SPR1 is encouraging growth at the plus end or discouraging disassembly [72].

In vivo, SPR2 decorates microtubules in both linear and concentrated patterns [15, 73], and there is often an enrichment of binding at the plus-end [90]. Both SPR2 and SP2L contain regions of HEAT repeats known to bind tubulin in yeast [15, 73, 90], and truncated constructs of *SPR2* containing the HEAT repeats of either the N- or C-terminus bind microtubules stabilized with taxol in vitro while the central region of the protein with no HEAT repeats does not [90]. Yao et al. also studied the effects of SPR2 on MT dynamics in some detail. In vitro, MTs incubated with SPR2 spent more time in the growth phase and less time in the pause state. So, SPR2 encourages the polymerization of tubulin, and its loss leads to an increase in the time spent in the pause phase in vivo.

MOR1 also contains stretches of HEAT repeats. In vivo studies of microtubule dynamics in the *mor1* mutant found that both growth and shrinkage were decreased, and the length of the pause state was extended [40]. Even at the permissive temperature, microtubule growth and shrinkage in *mor1* are abnormal, but the time devoted to those two aspects of dynamics and the pause state are wild-type. At higher temperatures, growth, shrinkage, and pause are abnormal in *mor1*, and microtubule arrays are very disordered [40]. Along with the evidence from *spr2* and possibly *tor2*, time spent in the pause state seems to have a large effect on array organization. The activities of MOR1 in the frequent remodeling of microtubule length allow for more microtubule encounters, which should contribute to the self-organization of the array (reviewed in [84]). Also in the *mor1* mutant, EB1 localization is shifted from the plus-end to discrete spots along the length of the microtubule, indicating MOR1 may contribute to normal binding of EB1 [40].

Another HEAT repeat containing MAP, CLASP, binds both to discrete, immobile sites along existing microtubules and to the plus-ends [3, 41]. More specifically, CLASP has an affinity for microtubules slightly basal to the site of EB1 binding [41]. Root length is decreased in *clasp-1* null seedlings. This phenotype is accompanied by shortened cells in the elongation zone [3, 41], and also lower cell numbers in this area [41]. Cells are radially expanded [3, 41] and contain significantly less cortical microtubules than the wild-type [3]. Microtubules are more likely to detach from the cortex in *clasp-1*, which is hypothesized to cause more frequent encounters and lead to the formation of parallel arrays [4].

Some plus-end binding MAPs have been shown to bind actin and actin-associated proteins as well as microtubules in mammals and Drosophila, but this is yet to be proven in plants (reviewed in [63]). Discovering the proteins involved in tubulin and actin crosstalk will be critical to understanding the synchronization of cytoskeleton structure, cell signaling, and trafficking in processes like cell

expansion. Mutations in plus-end binding MAPs may directly alter actin dynamics along with microtubule dynamics as proteins with the ability to bind both have been found in non-plant species. Formin proteins in Drosophila and other animals also have this capability (reviewed in [63]).

MAP-associated root morphology defects are not confined to mutations in proteins that bind microtubule plus-ends. Altered expression levels of another set of MAPs with a more general affinity for microtubules also lead to abnormal root growth behavior. Overexpression of WAVE DAMPENED 2 (WVD2) and MAP70-5 both result in rightward root skewing and epidermal cell file rotation [44, 91]. Interestingly, all organs of MAP70-5 overexpressors show right-handed helical growth, whereas in WVD2 overexpressors, the root and etiolated hypocotyl are right-handed while left-handed twisting is apparent in the petioles. Therefore, the effect of WVD2 on twisting is tissue-specific. Both overexpressors also result in stunted elongation of tissues. Reduced anisotropic cell expansion leads to swollen organs and cell bulging under some growth conditions. A group of WVD2 paralogs, WVD2-LIKE (WDL) 1–7, also have helical growth phenotypes when their expression levels are altered ([62, 91]; Jia and Masson, unpublished data).

The overexpression of WVD2 and MAP70-5 also leads to left-handed cortical microtubule arrays in the central elongation zone. WVD2, WDL1 through 7, and MAP70-5 proteins all share a common amino acid motif, "KLEEK," in a coiled coil domain, although the sequence similarity extends much further for WVD2 and the WDLs. The amino acid composition of the KLEEK domain in WVD2 and the WDLs is markedly similar to the C-terminus of TPX2. This region of TPX2 is also predicted to form a coiled-coil and has been implicated in microtubule nucleation in vertebrates [12]. In general, coiled-coil domains often function in protein–protein interactions and may facilitate these two MAP classes in binding tubulin [45] or some other component involved in microtubule array modeling.

MAP70-5, WVD2, and the WDLs colocalize with cortical microtubule arrays (Fig. 14.2 and [44, 62]; Jia and Masson, unpublished data), probably due to interactions at this coiled coil region. In vitro, MAP70-5 causes a decrease in number of microtubules but nearly doubles their length, suggesting it facilitates polymerization [44]. WVD2 can bundle microtubules in vitro, and in vivo, plants overexpressing WVD2 have an increase in the number of microtubule encounters that lead to parallel alignment of growth and a decrease in the number of crossover events [62].

Plants overexpressing another MAP, MAP18, have leftward root slanting and left-handed helical cell file rotation [81]. Decreased anisotropy and cell bulges are also

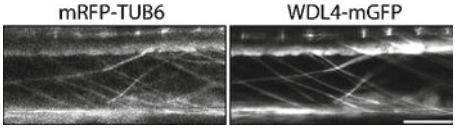

Fig. 14.2 WDL4 colocalizes with tubulin in vivo. WVD2-LIKE 4 fused to GFP and TUB6 fused to RFP were imaged in Arabidopsis root epidermal cells using confocal microscopy. Fusion of the two images shows strong overlap of signal. Scale bar is 10 μMz'

part of the phenotype. MAP18 colocalizes with microtubules in a punctate pattern. In vitro, tubulin incubated with MAP18 was less likely to polymerize into microtubules than tubulin in its absence. This suggests that MAP18 is an inhibitor of polymerization [81], the opposite of MAP70-5. Another group has found that MAP18 can also bind phosphatidylinositol phosphates [38]. Calcium competes with phosphatidylinositol phosphates for binding to the protein. It is unclear how all of these properties contribute to MAP18 function, but it could place MAP18 as an integrator of signaling between the cortical microtubule cytoskeleton and the plasma membrane.

A variety of mutant alleles in the Arabidopsis p60 subunit of katanin also have abnormal root growth. Katanins are conserved across eukaryotes and possess microtubule-severing capabilities. Mutants *botero* [9], *ectopic root hair 3* [85], *fragile fiber 2* [14], and *LUC super expressor 1* [11] all have disordered cortical microtubules. Their roots have radial swelling and impaired elongation. The disarray of microtubules and lessened ability to elongate may preclude the root from showing any helical growth tendencies. The studies on the katanins reveal that interruption of microtubule severing leads to deformed cells. Requirements for katanin levels appear strict, however, as overexpression of these genes in plants also leads to disordered microtubule arrays [76, 77]. Severing frequency is higher in cells with bundled microtubules [87].

Changes in the organization of γ-tubulin can also produce helical growth in Arabidopsis. The *spiral3* mutant has altered GCP2, a MAP that is part of the γ-tubulin complex which works to mark nucleation sites for new microtubules off of established ones. This mutant has rightward skewing and cell file rotation along with left-handed microtubule arrays. The dynamics of the microtubules do not appear to be affected, but nucleating angles are more random with an average slightly higher than that of wild-type. It is unclear why this leads to abnormal arrays, but possible causes include a change in the dynamics of microtubule encounters or a defective γ-tubulin template leading to microtubules that are themselves twisted [55].

All of these studies highlight that altering the rates at which microtubules grow and shrink, as well as how they behave when they encounter one another, have strong consequences for the form a cell takes. To exhibit wild-type cell expansion, microtubules need to be in constant flux between growth and shrinkage, as well as regulating the time they spend in pause between these states. These behaviors contribute to an ordered array, with the right amount of cortex attachment, bundling, and crossing over. Please refer to Chaps. 4 and 5 of this book for further information on microtubule dynamics.

14.5 Other Mutations that Alter Root Growth Behavior

Some mutations that result in defective root skewing affect genes that encode proteins that are not known to be associated directly with the microtubule cytoskeleton. Mutations in *SKU5* lead to increased rightward skew, exaggerated right-handed cell file rotation, and a slight decrease in root length [71]. SKU5 and 18 SKU5

SIMILAR (SKS) proteins are part of the multi-copper oxidase family. However, SKU5 retains only one copper ion binding center, and the SKS proteins all lack any intact copper binding centers. The SKU5 protein is anchored to the cell membrane via a GPI motif. Therefore, *SKU5* and its paralogs may be involved in enzymatic reactions at the cell wall, although it is unclear if they contribute to cell wall modification during cell expansion. Another GPI anchored protein, COBRA, functions in patterning of cell wall microfibrils [66], and several *COBRA* mutants have been isolated that are defective in anisotropic cell expansion [8, 66, 70]. *cob-1* and *cob-2* show increased root skewing on hard surfaces, as seen in *sku5*, but also have shortened, thickened roots that are unable to wave normally [92].

Similar to *cobra*, *ROOT HAIR DEFECTIVE 3* (*RHD3*) mutant roots lack waving on tilted agar surfaces, although they do not have an increase in skew over wild-type. The roots are also shorter and wider than wild-type, as are root hairs. Microtubule arrays are oriented normally in *rhd3*, but the span of the central elongation zone is significantly reduced [92]. *RHD3* codes for a protein that trafficks between the endoplasmic reticulum and the golgi apparatus [93]. It is required for proper growth of the cell wall and organization of the actin cytoskeleton [34], therefore RHD3 may have a role in directing components to the cell wall along the actin network that are necessary for normal root waving and skewing [82, 92]. The *erh2* mutant shows a similar root phenotype to *rhd3* and *cob* [92]. Excess lignin is deposited in the cell wall of this mutant [2] affecting wall rigidity. So, both RHD3 and ERH2 appear to be required for proper cell wall composition. Without them, root cells cannot elongate as they should, resulting in straight, short, and thickened roots that do not wave. In these mutants along with the *cob* mutants, it is possible the swollen root tips are unable to respond to the directional cues they receive when hitting the hard surface, or they are resistant to the buckling that is proposed in some waving models [49, 80, 92].

These studies where aberrant root growth behavior results from changes in cell wall composition highlight the links between the cytoskeleton, various signaling and trafficking pathways, and cellulose microfibril orientation. Cellulose microfibrils encase cells and restrict cell expansion to certain directions depending on their orientation. Parallel alignment between the cell wall microfibrils and plasma membrane-associated cortical microtubules was noticed many years ago [46], and led to models suggesting that the microtubules were responsible for guiding cellulose deposition. Now it has been shown that cellulose synthase complexes track along the cortical microtubules [59]. While disrupting microtubule arrays can alter cellulose deposition (first seen in [30]), partial removal of cellulose does not change array orientation, although inhibiting cellulose synthase complexes can disrupt the orientation of cortical microtubules [60]. Imaging of live cells is revealing how microfibril orientation changes in expanding plant cells [5], and also brings up new questions regarding how cellulose synthase movement is coupled with microtubule array dynamics [31]. Therefore, array dynamics are important not only for proper alignment along the plasma membrane but also for the docking of complexes like cellulose synthase and other contributors to cell wall composition. The co-alignment of microtubules and microfibrils is also affected by proteins that

can communicate between the two structures, and some of the root growth behavior mutants may prove to have defects in this aspect of cell expansion.

Many additional mutants have been studied that alter root skewing and/or waving and are linked to changes in auxin signaling pathways. The plant hormone auxin has multiple roles in plant growth and development, including cell division and expansion. In stems, auxin promotes cell elongation. Auxin signaling leads to the acidification of the apoplast (reviewed in [65]), which allows expansins to loosen the wall for cell growth powered by turgor pressure [20]. In the root, auxin impedes cell elongation, the opposite of its action in stems. Our understanding of the molecular cascade set off by auxin to inhibit cell expansion in roots has many gaps. However, it is clear from mutant studies that altering auxin's transport or localization that this hormone strongly contributes to root morphology, and both the actin and microtubule cytoskeletons appear to have roles.

AUX1 and PIN2 are auxin influx and efflux carriers, respectively. In *aux1* mutants, roots coil instead of maintaining wild-type skew and waving, although the coil follows normal skew direction [56]. This same phenotype is present in *axr4* [52, 74], which is compatible with results showing AXR4 is required to properly position AUX1 [23]. *pin2* mutants lack waving [18, 56] and also show an increased tendency to coil [18]. Vesicles containing AUX1 are not targeted properly in cells treated with actin disruptors [43], and auxin influx carriers PIN1 and PIN3 are also have abnormally distributed in roots with disorganized actin [25, 29]. For PIN2, targeting to the apical side of epidermal cells is affected when actin depolymerizing drugs are applied while distribution to the basal side is dependent on intact microtubules [42]. PIN1 targeting is similar in cells treated for extended periods with microtubule disruptors [42]. The exact role of tubulin and actin in directing auxin carriers is still controversial [28, 64], and pleiotropic effects of cytoskeleton disruption on cell expansion and vesicular trafficking lead to difficulties in the specific study of auxin positioning. However, growing evidence shows that auxin distribution is dependent on the cytoskeleton, and several pathways contribute to the proper quantity and localization of this crucial hormone. When these requirements are not met, abnormal root waving and skewing can arise.

Many additional mutants that do not directly affect auxin efflux or influx carriers still change auxin transport and signaling pathways, and through this alter root growth behavior. Two transcription factor mutants, *hy5* and *rha1*, both show reduced waving, although skewing is increased over wild-type in *hy5* and decreased in *rha1* [24] (Oyama et al. 1997). Mutations in two heterotrimeric G-protein subunits, AGB1 and XLG3, result in dampened waves and do not skew like wild-type on microtubule-interacting drugs [58]. *agb1* and *xlg3* mutants differentially express many auxin transport and signaling genes relative to wild type. These G-protein components may be acting as communication hubs between the interconnected pathways that affect root skewing and waving including, auxin, ethylene, and sugar [58]. Other mutants that have been proposed to indirectly affect auxin transport show increased waving over wild-type. These include *cle40*, *wag1*, *wag2*, *mdr1*, *mlo4*, and *mlo11* [19, 33, 47, 69, 89]. Similarly, the compressed waving in the *wav2* mutant may be due to an interaction with actin [50], thereby possibly changing auxin transport.

A few mutants have also been discovered that impact auxin synthesis and result in abnormal waving behavior. The production of ethylene is linked to the production of auxin, so changes in either pathway can alter root morphology. An excess of ethylene is present in the mutants *eto1-1* and *eto2-1*, which also lack wavy growth [92]. In contrast, mutants in several genes involved in tryptophan synthesis produce a compressed wave phenotype, possibly as a consequence of altered auxin synthesis through a tryptophan-dependent pathway [67].

Polarization of auxin carriers and localized auxin signaling are integral to the formation of root morphology and the ability of the root to alter its form when faced with environmental stimuli. Growing evidence suggests proper auxin localization is achieved using the actin and microtubule cytoskeletons in concert with a continual flux of endocytic cycling (reviewed in [27]). However, it remains unclear how auxin and other signaling pathways interact to produce waving patterns on surfaces.

14.6 Natural Variation in Root Growth Behavior

In several of the studies observing mutant skewing, it was also noted that skewing varies among the wild-type accessions of Arabidopsis. Colombia tends to grow vertically on tilted media, with only a minor skew to the left, while Landsberg *erecta* and Wassilewskija have a pronounced left skew [68]. Now that many different accessions from around the world are available to researchers, it is possible to expand our knowledge of the variation in Arabidopsis root growth behavior. A survey of roots grown on tilted media shows an assortment of skewing and waving behaviors in a variety of combinations (Vaughn and Masson, unpublished data, Fig. 14.3).

The skew of the roots ranges from vertical to left-handed. No accessions observed so far have had a mean right-handed skew on 1.5% agar, although many accessions will have individuals with a right skew. The strongest left skew is in the Cvi-1 accession, while several lines have little or no overall slant like Columbia. The other accessions fall in between in their degree of skewing. The overall tendency of Arabidopsis wild-type roots to display leftward skewing, if any, may be tied to the transient time their cortical microtubule arrays spend in right-handed helices, although several studies have reported exceptions to the correlation between both processes [13, 71]. The degree to which the roots are able to skew left would then be affected by modifiers in the genetic background. Genes affecting microtubule dynamics are strong candidates for these modifiers as indicated by the mutant studies.

Root waving also varies. Some accessions have a regular waving pattern, although the pattern differs among lines with respect to parameters such as period, amplitude, and smooth vs. kinked waves. Other accessions do not have a repeating wave pattern and instead tend to meander across the agar. Finally, a few lines typically grow rather straight. From mutant studies, loci affecting auxin distribution and signaling pathways are the most likely to contribute to the natural variation in waving patterns seen among accessions.

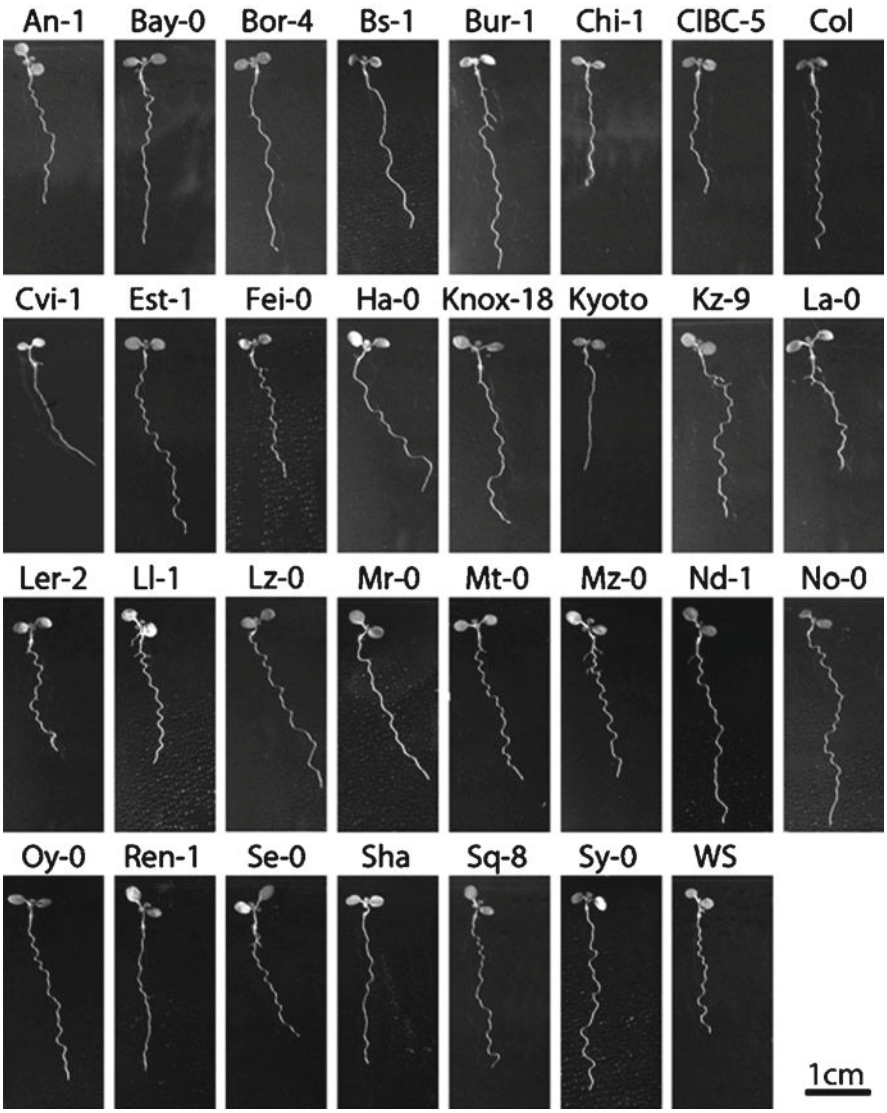

Fig. 14.3 Natural variation in Arabidopsis root waving and skewing. Several accessions of Arabidopsis were grown on ½ strength LSPS media plus 1.5% agar, first vertically for 3 days, then tilted back 30° for 2 more days. Pictures were taken from the back of the plate, through the media

Techniques like quantitative trait analysis and genome-wide association studies for root growth behaviors should reveal some of the molecular components involved in their regulation. Loci known to affect growth patterns will likely be identified, but new candidates may also emerge that enrich our understanding of all of the networks involved in regulating root morphology. Eventually, this genetic information

can be paired with our knowledge of the native soil conditions for different accessions to correlate growth behavior to adaptability in defined environments.

14.7 Closing Remarks

Understanding the molecular basis of root morphology on hard surfaces gives us insight into how these organs react to stimuli in their natural environments as well as intrinsic programs of root development. Through mutant studies, it is clear that cells require ordered cytoskeletons with proper dynamics to achieve normal growth behavior. Microtubules act as structural templates for cells and, together with actin networks, guide materials to the cell wall. Both cytoskeletons also contribute to the localization of auxin, a critical regulator of growth. There are still many gaps in our knowledge of the interactions between the cytoskeleton, vesicular trafficking, the cell wall, and the various signaling pathways that come together to design a root's shape. Along with traditional studies, using natural variation may also help us connect the components of these intertwined pathways and also bring insight into the ecological roles of diverse root architectures.

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References

1. Abe T, Thitamadee S, Hashimoto T (2004) Microtubule defects and cell morphogenesis in the *lefty1lefty2* tubulin mutant of *Arabidopsis thaliana*. Plant Cell Physiol 45(2):211–220
2. Houssain MA, Noh HN, Kim KI, Koh EJ, Wi SG, Bae HJ, Lee H, Hong, SW (2010) Mutation of the chitinase-like protein-encoding *AtCTL2* gene enhances lignin accumulation in dark-grown Arabidopsis seedlings. J Plant Physiol 167(8):650–658
3. Ambrose J, Shoji T, Kotzer A, Pighin J, Wasteneys G (2007) The Arabidopsis *CLASP* gene encodes a microtubule-associated protein involved in cell expansion and division. Plant Cell 19(9):2763–2775
4. Ambrose J, Wasteneys G (2008) CLASP modulates microtubule-cortex interaction during self-organization of acentrosomal microtubules. Mol Biol Cell 19(11):4730–4737
5. Anderson C, Carroll A, Akhmetova L, Somerville C (2010) Real-time imaging of cellulose reorientation during cell wall expansion in Arabidopsis roots. Plant Physiol 152:787–796
6. Baluska F, Volkmann D, Barlow P (1996) Specialized zones of development in roots: view from the cellular level. Plant Physiol 112:3–4
7. Baum S, Rost T (1996) Root apical organization in *Arabidopsis thaliana* 1. Root cap and protoderm. Protoplasma 192:178–188
8. Benfey P, Linstead P, Roberts K, Schiefelbein J, Hauser M, Aeschbacher R (1993) Root development in Arabidopsis: four mutants with dramatically altered root morphogenesis. Development 119:57–70

9. Bichet A, Desnos T, Turner S, Grandjean O, Höfte H (2001) *BOTERO1* is required for normal orientation of cortical microtubules and anisotropic cell expansion in Arabidopsis. Plant J 25(2):137–148
10. Bisgrove S, Lee Y, Liu B, Peters N, Kropf D (2008) The microtubule plus-end binding protein EB1 functions in root responses to touch and gravity signals in Arabidopsis. Plant Cell 20(2):396–410
11. Bouquin T, Mattsson O, Naested H, Foster R, Mundy J (2003) The Arabidopsis *lue1* mutant defines a katanin p60 ortholog involved in hormonal control of microtubule orientation during cell growth. J Cell Sci 116(pt 5):791–801
12. Brunet S, Sardon T, Zimmerman T, Wittmann T, Pepperkok R, Karsenti E, Vernos I (2004) Characterization of the TPX2 domains involved in microtubule nucleation and spindle assembly in *Xenopus* egg extracts. Mol Biol Cell 15(12):5318–5328
13. Buer C, Wasteneys G, Masle J (2003) Ethylene modulates root-wave responses in Arabidopsis. Plant Physiol 132:1085–1096
14. Burk D, Ye Z (2002) Alteration of oriented deposition of cellulose microfibrils by mutation of a katanin-like microtubule-severing protein. Plant Cell 14(9):2145–2160
15. Buschmann H, Fabri C, Hauptmann M, Hutzler P, Laux T, Lloyd C, Schäffner A (2004) Helical growth of the Arabidopsis mutant *tortifolia1* reveals a plant-specific microtubule-associated protein. Curr Biol 14(16):1515–1521
16. Buschmann H, Hauptmann M, Niessing D, Lloyd C, Schäffner A (2009) Helical growth of the Arabidopsis mutant *tortifolia2* does not depend on cell division patterns but involves handed twisting of isolated cells. Plant Cell 21(7):2090–2106
17. Cassab G (2008) Chapter 6: Other tropisms and their relationship to gravitropism. In: Gilroy S, Masson P (eds) Plant tropisms. Blackwell, Ames, pp 123–140
18. Chen R, Hilson P, Sedbrook J, Rosen E, Caspar T, Masson P (1998) The *Arabidopsis thaliana AGRAVITROPIC 1* gene encodes a component of the polar-auxin-transport efflux carrier. Proc Natl Acad Sci USA 95(25):15112–15117
19. Chen Z, Noir S, Kwaaitaal M, Hartmann H, Wu M, Mudgil Y, Sukumar P, Muday G, Panstruga R, Jones A (2009) Two seven-transmembrane domain MILDEW RESISTANCE LOCUS O proteins cofunction in Arabidopsis root thigmomorphogenesis. Plant Cell 21(7):1972–1991
20. Cosgrove D (2000) Loosening of plant cell walls by expansins. Nature 407(6802):321–326
21. Cyr R, Palevitz B (1995) Organization of cortical microtubules in plant cells. Curr Opin Cell Biol 7:65071
22. Darwin C (1880) The power of movement in plants. John Murray, London
23. Dharmasiri S, Swarup R, Mockaitis K, Dharmasiri N, Singh S, Kowalchyk M, Marchant A, Mills S, Sandberg G, Bennett M et al (2006) AXR4 is required for localization of the auxin influx facilitator AUX1. Science 312(5777):1218–1220
24. Fortunati A, Piconese S, Tassone P, Ferrari S, Migliaccio F (2008) A new mutant of Arabidopsis disturbed in its roots, right-handed slanting, and gravitropism defines a gene that encodes a heat-shock factor. J Exp Bot 59(6):1363–1374
25. Friml J, Wiśniewska J, Benková E, Mendgen K, Palme K (2002) Lateral relocation of auxin efflux regulator PIN3 mediates tropism in Arabidopsis. Nature 415(6873):806–809
26. Furutani I, Watanabe Y, Prieto R, Masukawa M, Suzuki K, Naoi K, Thitamadee S, Shikanai T, Hashimoto T (2000) The *SPIRAL* genes are required for directional control of cell elongation in *Arabidopsis thaliana*. Development 127(20):4443–4453
27. Gao X, Nagawa S, Wang G, Yang Z (2008) Cell polarity signaling: focus on polar auxin transport. Mol Plant 1(6):899–909
28. Geldner N (2009) Cell polarity in plants: a perspective on PINs. Curr Opin Plant Biol 12(1):42–48
29. Geldner N, Friml J, Stierhof Y, Jürgens G, Palme K (2001) Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. Nature 413(6854):425–428
30. Green P (1962) Mechanism for plant cellular morphogenesis. Science 138:1404–1405
31. Gutierrez R, Lindeboom J, Paredez A, Emons A, Ehrhardt D (2010) Arabidopsis cortical microtubules position cellulose synthase delivery to the plasma membrane and interact with cellulose synthase trafficking compartments. Nat Cell Biol 11(7):797–808

32. Harrison B, Morita M, Masson P, Tasaka M (2008) Chapter 2: Signal transduction in gravitropism. In: Gilroy S, Masson P (eds) Plant tropisms. Blackwell, Ames, pp 21–45
33. Hobe M, Müller R, Grünewald M, Brand U, Simon R (2003) Loss of CLE40, a protein functionally equivalent to the stem cell restricting signal CLV3, enhances root waving in Arabidopsis. Dev Genes Evol 213(8):371–381
34. Hu Y, Zhong R, Wr M, Ye Z (2003) The Arabidopsis *RHD3* gene is required for cell wall biosynthesis and actin organization. Planta 217(6):912–921
35. Ishida T, Kaneko Y, Iwano M, Hashimoto T (2007) Helical microtubule arrays in a collection of twisting tubulin mutants of *Arabidopsis thaliana*. Proc Natl Acad Sci USA 104(20):8544–8549
36. Ishikawa H, Evans M (1993) The role of the distal elongation zone in the response of maize roots to auxin and gravity. Plant Physiol 102:1203–1210
37. Ishikawa H, Evans M (1997) Novel software for analysis of root gravitropism: comparative response patterns of Arabidopsis wild-type and *axr1* seedlings. Plant Cell Environ 20:919–928
38. Kato M, Nagasaki-Takeuchi N, Ide Y, Maeshima M (2010) An Arabidopsis hydrophilic Ca2(+) -binding protein with a PEVK-rich domain, PCaP2, is associated with the plasma membrane and interacts with calmodulin and phosphatidylinositol phosphates. Plant Cell Physiol 51(3):366–379
39. Mirza JI (1987) The effects of light and gravity on the horizontal curvature of roots of gravitropic and agravitropic Arabidopsis thaliana L. Plant Physiol 83(1):118–120
40. Kawamura E, Wasteneys G (2008) *MOR1*, the *Arabidopsis thaliana* homologue of Xenopus *MAP215*, promotes rapid growth and shrinkage, and suppresses the pausing of microtubules in vivo. J Cell Sci 121(pt 24):4114–4123
41. Kirik V, Herrmann U, Parupalli C, Sedbrook J, Ehrhardt D, Hülskamp M (2007) CLASP localizes in two discrete patterns on cortical microtubules and is required for cell morphogenesis and cell division in Arabidopsis. J Cell Sci 120(pt 24):4416–4425
42. Kleine-Vehn J, Langowski L, Wiśniewska J, Dhonukshe P, Brewer P, Friml J (2008) Cellular and molecular requirements for polar PIN targeting and transcytosis in plants. Mol Plant 1(6):1056–1066
43. Kleine-Vehn J, Dhonukshe P, Swarup R, Bennett M, Friml J (2006) Subcellular trafficking of the Arabidopsis auxin influx carrier AUX1 uses a novel pathway distinct from PIN1. Plant Cell 18(11):3171–3181
44. Korolev A, Buschmann H, Doonan J, Lloyd C (2007) AtMAP70-5, a divergent member of the MAP70 family of microtubule-associated proteins, is required for anisotropic cell growth in Arabidopsis. J Cell Sci 120(pt 13):2241–2247
45. Korolev A, Chan J, Naldrett M, Doonan J, Lloyd C (2005) Identification of a novel family of 70 kDa microtubule-associated proteins in Arabidopsis cells. Plant J 42(4):547–555
46. Ledbetter M, Porter K (1963) A "microtubule" in plant cell fine structure. J Cell Bio 19:239–250
47. Lewis D, Miller N, Splitt B, Wu G, Spalding E (2007) Separating the roles of acropetal and basipetal auxin transport on gravitropism with mutations in two Arabidopsis *multidrug resistance-like* ABC transporter genes. Plant Cell 19(6):1838–1850
48. Massa G, Gilroy S (2003) Touch modulates gravity sensing to regulate the growth of primary roots of *Arabidopsis thaliana*. Plant J 33:435–445
49. Migliaccio F, Piconese S (2001) Spiralizations and tropisms in Arabidopsis roots. Trends Plant Sci 6(12):561–565
50. Mochizuki S, Harada A, Inada S, Sugimoto-Shirasu K, Stacey N, Wada T, Ishiguro S, Okada K, Sakai T (2005) The Arabidopsis WAVY GROWTH 2 protein modulates root bending in response to environmental stimuli. Plant Cell 17(2):537–547
51. Monshausen G, Swanson S, Gilroy S (2008) Touch sensing and thigmotropism. In: Gilroy S, Masson P (eds) Plant tropisms. Blackwell, Ames, pp 91–122
52. Mullen J, Turk E, Johnson K, Wolverton C, Ishikawa H, Simmons C, Söll D, Evans M (1998) Root-growth behavior of the Arabidopsis mutant *rgr1*. Roles of gravitropism and circumnutation in the waving/coiling phenomenon. Plant Physiol 118(4):1139–1145

53. Nakajima K, Furutani I, Tachimoto H, Matsubara H, Hashimoto T (2004) *SPIRAL1* encodes a plant-specific microtubule-localized protein required for directional control of rapidly expanding Arabidopsis cells. Plant Cell 16(5):1178–1190
54. Nakajima K, Kawamura T, Hashimoto T (2006) Role of the *SPIRAL1* gene family in anisotropic growth of *Arabidopsis thaliana*. Plant Cell Physiol 47(4):513–522
55. Nakamura M, Hashimoto T (2009) A mutation in the Arabidopsis gamma-tubulin-containing complex causes helical growth and abnormal microtubule branching. J Cell Sci 122(pt 13):2208–2217
56. Okada K, Shimura Y (1990) Reversible root tip rotation in Arabidopsis seedlings induced by obstacle-touching stimulus. Science 250(4978):274–276
57. Oyama T, et al. (1997) The Arabidopsis *HY5* gene encodes a bZIP protein that regulates stimulus-induced development of root and hypocotyl. Genes Dev 11(22):2983–2995
58. Pandey S, Monshausen G, Ding L, Assmann S (2008) Regulation of root-wave response by extra large and conventional G proteins in *Arabidopsis thaliana*. Plant J 55(2):311–322
59. Paredez A, Somerville C, Ehrhardt D (2006) Visualization of cellulose synthase demonstrates functional association with microtubules. Science 312:1491–1495
60. Paredez A, Persson S, Ehrhardt D, Somerville C (2010) Genetic evidence that cellulose synthase activity influences cortical microtubule array organization. Plant Physiol 147:1723–1734
61. Pastuglia M, Azimzadeh J, Goussot M, Camilleri C, Belcram K, Evrard J, Schmit A, Guerche P, Bouchez D (2006) Gamma-tubulin is essential for microtubule organization and development in Arabidopsis. Plant Cell 18(6):1412–1425
62. Perrin R, Wang Y, Yuen C, Will J, Masson P (2007) WVD2 is a novel microtubule-associated protein in *Arabidopsis thaliana*. Plant J 49(6):961–971
63. Petrásek J, Schwarzerová K (2009) Actin and microtubule cytoskeleton interactions. Curr Opin Plant Biol 12(6):728–734
64. Rahman A, Bannigan A, Sulaman W, Pechter P, Blancaflor E, Baskin T (2007) Auxin, actin and growth of the *Arabidopsis thaliana* primary root. Plant J 50(3):514–528
65. Rayle D, Cleland R (1992) The Acid Growth theory of auxin-induced cell elongation is alive and well. Plant Physiol 99(4):1271–1274
66. Roudier F, Fernandez A, Fujita M, Himmelspach R, Borner G, Schindelman G, Song S, Baskin T, Dupree P, Wasteneys G et al (2005) COBRA, an Arabidopsis extracellular glycosyl-phosphatidyl inositol-anchored protein, specifically controls highly anisotropic expansion through its involvement in cellulose microfibril orientation. Plant Cell 17(6):1749–1763
67. Rutherford R, Gallois P, Masson P (1998) Mutations in *Arabidopsis thaliana* genes involved in the tryptophan biosynthesis pathway affect root waving on tilted agar surfaces. Plant J 16(2):145–154
68. Rutherford R, Masson P (1996) *Arabidopsis thaliana sku* mutant seedlings show exaggerated surface-dependent alteration in root growth vector. Plant Physiol 111(4):987–998
69. Santner A, Watson J (2006) The WAG1 and WAG2 protein kinases negatively regulate root waving in Arabidopsis. Plant J 45(5):752–764
70. Schindelman G, Morikami A, Jung J, Baskin T, Carpita N, Derbyshire P, McCann M, Benfey P (2001) *COBRA* encodes a putative GPI-anchored protein, which is polarly localized and necessary for oriented cell expansion in Arabidopsis. Genes Dev 15(9):1115–1127
71. Sedbrook J, Carroll K, Hung K, Masson P, Somerville C (2002) The Arabidopsis *SKU5* gene encodes an extracellular glycosyl phosphatidylinositol-anchored glycoprotein involved in directional root growth. Plant Cell 14(7):1635–1648
72. Sedbrook J, Ehrhardt D, Fisher S, Scheible W, Somerville C (2004) The Arabidopsis *sku6/spiral1* gene encodes a plus end-localized microtubule-interacting protein involved in directional cell expansion. Plant Cell 16(6):1506–1520
73. Shoji T, Narita N, Hayashi K, Asada J, Hamada T, Sonobe S, Nakajima K, Hashimoto T (2004) Plant-specific microtubule-associated protein SPIRAL2 is required for anisotropic growth in Arabidopsis. Plant Physiol 136(4):3933–3944
74. Simmons C, Migliaccio F, Masson P, Caspar T, Soll D (1995) A novel root gravitropism mutant of *Arabidopsis thaliana* exhibiting altered auxin physiology. Physiol Plant 93:790–798
75. Simmons C, Soll D, Migliaccio F (1996) Circumnutation and gravitropism cause root waving in *Arabidopsis thaliana*. J Exp Bot 46:143–150

76. Stoppin-Mellet V, Gaillard J, Vantard M (2002) Functional evidence for in vitro microtubule severing by the plant katanin homologue. Biochem J 365(pt 2):337–342
77. Stoppin-Mellet V, Gaillard J, Vantard M (2006) Katanin's severing activity favors bundling of cortical microtubules in plants. Plant J 46(6):1009–1017
78. Takahashi N, Yamazaki Y, Kobayashi A, Higashitani A, Takahashi H (2003) Hydrotropism interacts with gravitropism by degrading amyloplasts in seedling roots of Arabidopsis and radish. Plant Physiol 132:805–810
79. Thitamadee S, Tuchihara K, Hashimoto T (2002) Microtubule basis for left-handed helical growth in Arabidopsis. Nature 417(6885):193–196
80. Thompson M, Holbrook N (2004) Root-gel interactions and the root waving behavior of Arabidopsis. Plant Physiol 135(3):1822–1837
81. Wang X, Zhu L, Liu B, Wang C, Jin L, Zhao Q, Yuan M (2007) Arabidopsis MICROTUBULE-ASSOCIATED PROTEIN18 functions in directional cell growth by destabilizing cortical microtubules. Plant Cell 19(3):877–889
82. Wasteneys G (2004) Progress in understanding the role of microtubules in plant cells. Curr Poin Plant Biol 7:651–660
83. Wasteneys G, Collings D (2004) Expanding beyond the great divide: the cytoskeleton and axial growth. In: Hussey P (ed) The plant cytoskeleton in cell differentiation and development. Blackwell, Oxford, pp 83–115
84. Wasteneys G, Ambrose J (2009) Spatial organization of plant cortical microtubules: close encounters of the 2D kind. Trends Cell Biol 19(2):62–71
85. Webb M, Jouannic S, Foreman J, Linstead P, Dolan L (2002) Cell specification in the Arabidopsis root epidermis requires the activity of ECTOPIC ROOT HAIR 3–a katanin-p60 protein. Development 129(1):123–131
86. Whittington A, Vugrek O, Wei K, Hasenbein N, Sugimoto K, Rashbrooke M, Wasteneys G (2001) MOR1 is essential for organizing cortical microtubules in plants. Nature 411(6837):610–613
87. Wightman R, Turner S (2007) Severing at sites of microtubule crossover contributes to microtubule alignment in cortical arrays. Plant J 52(4):742–751
88. Williamson R (1991) Orientation of cortical microtubules in interphase plant cells. Int Rev Cytol 129:135–206
89. Wu G, Lewis D, Spalding E (2007) Mutations in Arabidopsis *multidrug resistance-like* ABC transporters separate the roles of acropetal and basipetal auxin transport in lateral root development. Plant Cell 19(6):1826–1837
90. Yao M, Wakamatsu Y, Itoh T, Shoji T, Hashimoto T (2008) Arabidopsis SPIRAL2 promotes uninterrupted microtubule growth by suppressing the pause state of microtubule dynamics. J Cell Sci 121(pt 14):2372–2381
91. Yuen C, Pearlman R, Silo-Suh L, Hilson P, Carroll K, Masson P (2003) WVD2 and WDL1 modulate helical organ growth and anisotropic cell expansion in Arabidopsis. Plant Physiol 131(2):493–506
92. Yuen C, Sedbrook J, Perrin R, Carroll K, Masson P (2005) Loss-of-function mutations of *ROOT HAIR DEFECTIVE3* suppress root waving, skewing, and epidermal cell file rotation in Arabidopsis. Plant Physiol 138(2):701–714
93. Zheng H, Kunst L, Hawes C, Moore I (2004) A GFP-based assay reveals a role for RHD3 in transport between the endoplasmic reticulum and Golgi apparatus. Plant J 37(3):398–414
94. Dolan L, Janmaat K, Willemsen V, Linstead P, Poethig S, Roberts K, Scheres B (1993) Cellular organisation of the Arabidopsis thaliana root. Development 119(1):71–84
95. Szymanski DB (2009) Plant cells taking shape: new insights into cytoplasmic control. Curr Opin Plant Biol 12(6):735–744
96. Baskin TI (2001) On the alignment of cellulose microfibrils by cortical microtubules: a review and a model. Protoplasma 215(1–4):150–171
97. Sugimoto K, Williamson RE, Wasteneys GO (2000) New techniques enable comparative analysis of microtubule orientation, wall texture, and growth rate in intact roots of Arabidopsis. Plant Physiol 124(4):1493–1506

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