

A GLORIOUS HALF-CENTURY OF MICROTUBULES

The role of the cytoskeleton and associated proteins in determination of the plant cell division plane

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SUMMARY

In plants, as in all eukaryotic organisms, microtubule- and actin-filament based structures play fundamental roles during cell division. In addition to the mitotic spindle, plant cells have evolved a unique cytoskeletal structure that designates a specific division plane before the onset of mitosis via formation of a cortical band of microtubules and actin filaments called the preprophase band. During cytokinesis, a second plant-specific microtubule and actin filament structure called the phragmoplast directs vesicles to create the new cell wall. In response to intrinsic and extrinsic cues, many plant cells form a preprophase band in G₂, then the preprophase band recruits specific proteins to populate the cortical division site prior to disassembly of the preprophase band in prometaphase. These proteins are thought to act as a spatial reminder that actively guides the phragmoplast towards the cortical division site during cytokinesis. A number of proteins involved in determination and maintenance of the plane of cell division have been identified. Our current understanding of the molecular interactions of these proteins and their regulation of microtubules is incomplete, but advanced imaging techniques and computer simulations have validated some early concepts of division site determination.

Keywords: preprophase band, division plane, phragmoplast, cytokinesis, cytoskeleton, *Arabidopsis thaliana*, *Physcomitrella patens*, *Tradescantia virginiana*, maize.

INTRODUCTION

During the 19th century, Hofmeister, Sachs and Errera individually used direct observation of symmetric plant cell divisions to develop simple geometrical rules that predict the position of the division plane. Briefly, these rules state that cells divide perpendicular to the long axis of the cell (Hofmeister, 1863), that division produces two equal-sized daughter cells (Sachs 1878), and that the cell plate spans the minimum area necessary to divide the cell volume (Errera 1888). The use of more advanced microscopic techniques led to discovery of the mitotic spindle, an array of microtubules (MTs) that participates in chromosome segregation, and the phragmoplast, a plant-specific MT array that guides formation of the cell plate. The fibrous quality of the spindle and phragmoplast and the carbohydrate composition of the cell plate were observed using bright-field

microscopy on histologically stained cells, often pollen meiocytes, and recorded in drawings with exquisite detail (Strasburger, 1875; Timberlake, 1900; Yamaha, 1926; Inoué, 1953). Seminal work by Sinnott and Bloch described the alignment of pre-mitotic cytoplasmic strands with the eventual position of the cell plate (Sinnott and Bloch, 1940, 1941a; Sinnott and Bloch, 1941b). By application of electron microscopy (Yasui, 1939), the structure and composition of the spindle and phragmoplast were clarified, and the term 'microtubule' was invented to describe the tubular nature of spindle fibers (Ledbetter and Porter, 1963). Ultimately, the quest to identify a structure that predicts the division plane in plant cells was rewarded with the discovery of the preprophase band (PPB), a cortical array of MTs that forms during late G₂ and marks the cortical division site (CDS) that

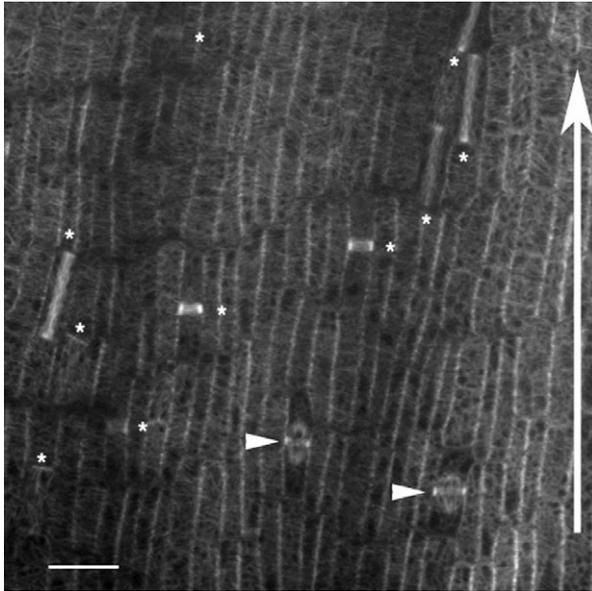


Figure 1. Longitudinal and transversely oriented cell divisions in a young maize leaf. Microtubule (MT) organization is visualized by YFP-tubulin (Mohanty *et al.*, 2009). For the majority of the pavement cells, interphase cortical MT arrays are oriented transverse to the leaf's longitudinal axis (indicated by the large white arrow). Various stages of PPB formation are visible in longitudinal and transverse orientations. Early (broad) PPBs are indicated by asterisks. Cells in prometaphase with pro-spindles and late (narrow) PPBs are indicated by arrowheads. The image is a maximum projection of a confocal Z-stack. Scale bar = 20 μm .

corresponds to the future site of cell-plate fusion (Pickett-Heaps and Northcote, 1966).

During PPB formation, MTs of mixed polarity are enriched at the cell equator (Vos *et al.*, 2004), and the cell cortex above and below this area is cleared of MTs (Figures 1 and 2). MT-associated proteins (MAPs) probably mediate the switch from the interphase cortical MT array, which covers the entire cell cortex, to the equatorial enrichment of MTs that is characteristic of the PPB. Preprophase band formation occurs via changes in the rates of MT polymerization and depolymerization, as well as selective MT stabilization and destabilization (Dhonukshe and Gadella, 2003; Vos *et al.*, 2004). In addition, components of the γ -tubulin ring complex (γ -TuRC) localize to the PPB and probably promote *de novo* MT nucleation (Liu *et al.*, 1994; Pastuglia *et al.*, 2006; Janski *et al.*, 2012). MTs in the PPB are organized into bundles, and these bundles contain high numbers of MTs that may persist in the same location for hours, similar to MT bundles observed in expanding epidermal pavement cells (Zhang *et al.*, 2011; Ambrose and Wasteneys, 2012). Recently, studies of cell division in the *Arabidopsis thaliana* leaf epidermis found that PPB formation is predominantly polar, with initiation and maturation beginning mostly on the cell cortex facing towards the inside of the leaf (Lucas and Sack, 2012), in agreement with earlier observations on polarized PPB formation (Gorst

et al., 1986; Hogan, 1988; Cleary, 2001). The PPB is thought to organize proteins and/or lipids as spatial markers that are maintained at the CDS after the PPB disassembles in prometaphase. These markers then guide the phragmoplast towards the CDS for accurate cell-plate insertion during cytokinesis (Figure 2). In this review, we summarize the current knowledge of MT-dependent and -independent molecular mechanisms involved in CDS selection, establishment and maintenance (Figure 3). Several excellent recent reviews provide more information regarding specialized asymmetric divisions (De Smet and Beeckman, 2011; Rasmussen *et al.*, 2011a; Facette and Smith, 2012; Jeong *et al.*, 2012; Lau and Bergmann, 2012).

WHAT DETERMINES WHERE THE PPB FORMS IN A CELL?

In the first half of the 20th century, much effort was devoted to describing the geometry of plant tissues. The geometrical organization of cells within these plant tissues was compared to that of soap foams, which, it was noted, use surface tension to maximize the volume enclosed by a minimal surface area (Lewis, 1926, 1928; Thompson, 1942; Matzke, 1945), as discussed by Lloyd (1991). Sinnott and Bloch (1940) described how a structure derived from fusion of transvacuolar cytoplasmic strands, the phragmosome, defines the future plane of cell division in pre-mitotic cells by supporting the central position of the nucleus (Sinnott and Bloch, 1940, 1941a,b). Laser ablation experiments showed that the cytoplasmic strands extending from the nucleus display spring-like properties, indicating that they are under tension (Hahne and Hoffmann, 1984; Goodbody *et al.*, 1991). Sinnott and Bloch (1941a) are also credited with suggesting that a cell usually places its division plane so that no more than three vertices are formed, although exceptions have also been described in normal development (Gunning *et al.*, 1978) and in response to wounding (Sinnott and Bloch, 1941b; Hardham and McCully, 1982). Eventually, Lloyd (1991) reconciled the 'soap foam' observations and geometric rules with cytoplasmic strand behavior, and suggested a mechanism whereby a cytoskeleton under tension senses and interprets cell geometry to select and establish a minimal division plane that avoids the creation of four-way junctions (Lloyd, 1991) (Figure 3b). Part of Lloyd's line of reasoning was recently validated by computer simulations (Besson and Dumais, 2011) showing that cytoplasmic strands, or more specifically MTs, reorganize into a finite number of configurations. The equilibrium configuration of a fixed pool of tense MTs favors short MTs, thus the probability distribution of MTs over time spans the shortest axis of the cell, biasing MT distribution and guiding PPB formation (Besson and Dumais, 2011) (Figure 3b). Asymmetric divisions may also be explained by the probability distribution of cytoskeletal structures when cell geometry serves as the polarity cue (Besson and Dumais, 2011).

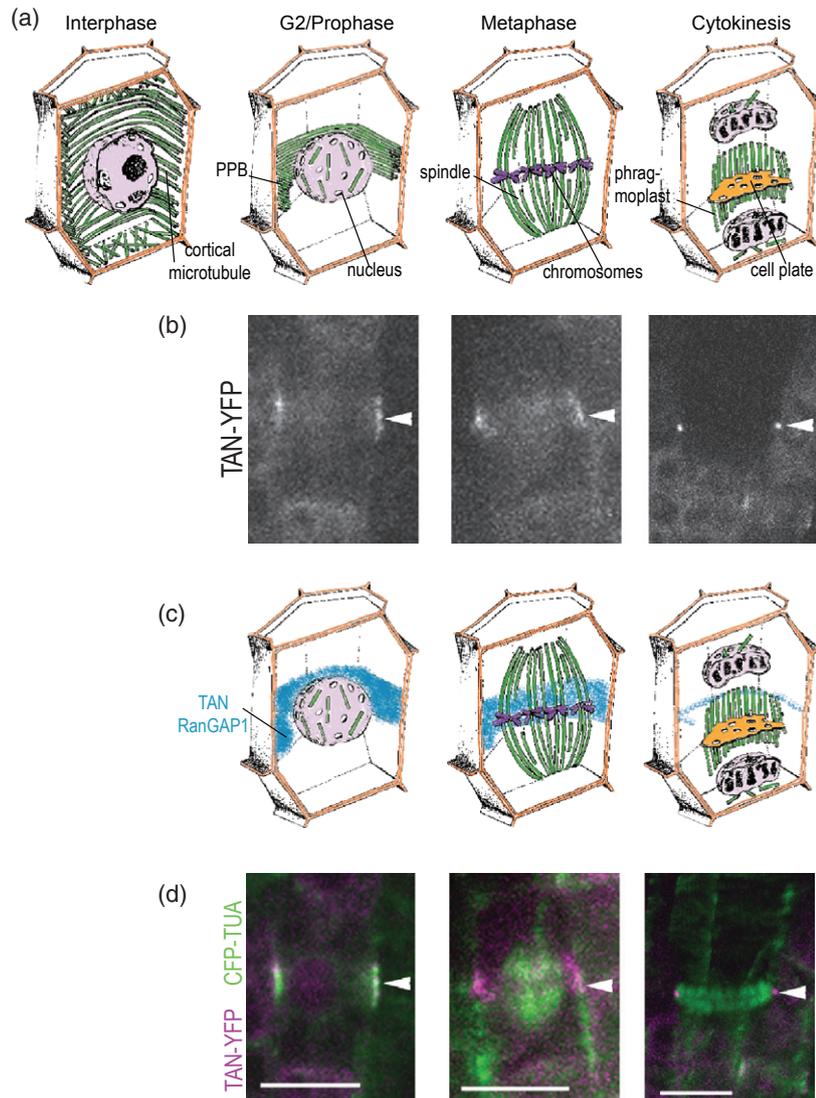


Figure 2. Spatial distribution of microtubules (MT) and the cortical division site marker TANGLED. (a) Schematic representation of cell-cycle stages and corresponding MT arrays (green). (b) Confocal micrographs of TANGLED (YFP-TAN) localization (arrowhead) in G₂/prophase, metaphase and cytokinesis in *A. thaliana* root meristem cells. (c) Schematic representation of the localization of YFP-TAN and RanGAP1 (blue) throughout mitosis. (d) Confocal micrographs of MT (green) and YFP-TAN localization (arrowhead) in G₂/prophase, metaphase and cytokinesis in *A. thaliana* root cells. The schematic representations in (a) and (c) were redrawn from Cyr (1994) and Ledbetter and Porter (1970) and modified. The micrographs in (b) and (d) were reproduced adapted from Rasmussen *et al.* (2011b) with permission from Journal of Cell Science.

In support of these observations and models, experiments showed that interphase MT arrays are oriented in response to tension, as application of mechanical stress leads to MT reorientation (Lintilhac and Vesecky, 1981; Hardham and McCully, 1982; Hush *et al.*, 1990). Computer simulations used to model the mechanical strain of internal forces exerted on cells in the *A. thaliana* shoot meristem found that the calculated stress maxima co-aligned with observed MT array orientations (Hamant *et al.*, 2008). Early observations showed that mechanical stress, in addition to altering the cortical MT array, also alters the division plane. In the case of exerted pressure, tension is

added and the divisions occur perpendicular to the applied stress, while, in the case of wounding, tension is released and division occurs parallel to the stress (wound) (Nemec, 1899; Kny, 1902). More specifically, it has repeatedly been demonstrated that realignment of interphase MTs after wounding leads to alterations in orientation of the PPB (Hush *et al.*, 1990; Hamant *et al.*, 2008). Thus, as with forces external to the plant tissue, it is likely that stress created by internal forces such as turgor pressure and cell geometry will affect the orientation of the interphase cortical MT array and the PPB, and therefore the location of the CDS. Actin may play a role in interpreting these stresses,

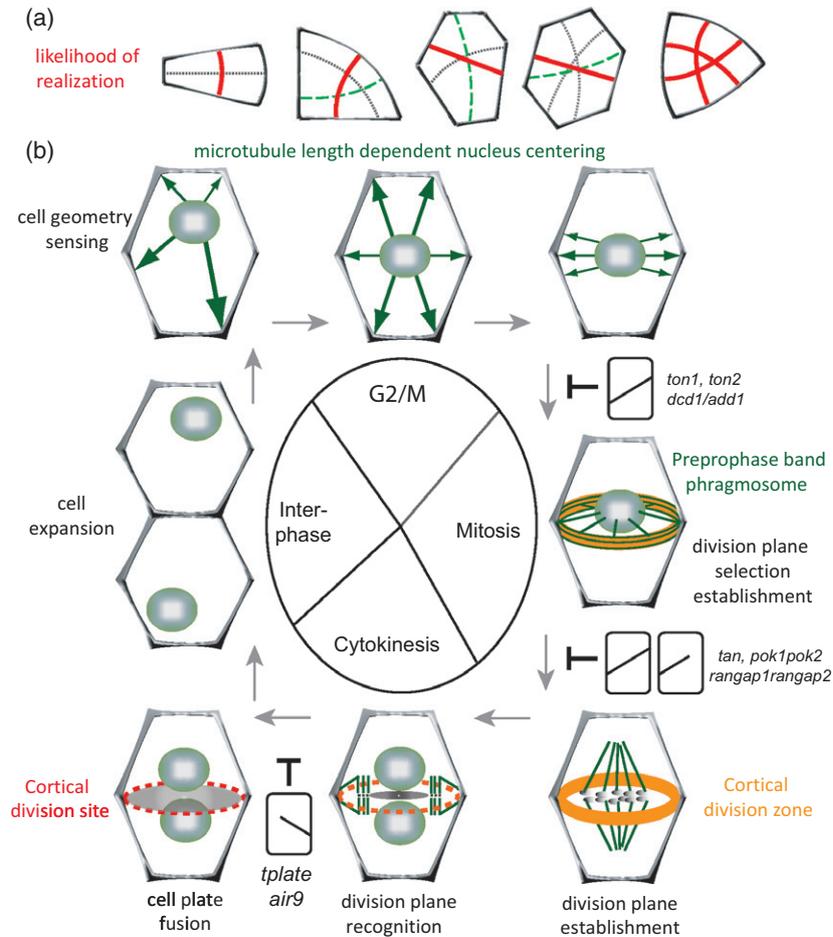


Figure 3. Cell division plane determination.

(a) Influence of cell geometry on division plane selection. Cells with one long and one short axis predominantly divide along the short axis (minimum division plane area). In triangular cells, potential division planes with similar area compete and are equally likely to be realized. Cells with complex shapes are less likely to divide along the shortest axis. The likelihood of realization is proportional to the difference in area of potential division planes. Division planes with highest probability of occurring are indicated by a continuous line. Less likely division planes are indicated by dashed and dotted lines.

(b) In pre-mitotic cells, MT length-dependent sensing of cell geometry aids in centering the nucleus and determining the shortest axis. Short MTs are favored according to the probability equilibrium, and thus MTs coalesce in the short distance bridging the nucleus with the cortex. Subsequently, the PPB (green oval) indicates the future site of cell-plate insertion at the cell cortex (CDS, yellow and red ovals). Throughout mitosis, proteins that are required for correct cell-plate positioning occupy the CDS. Mutants that lack PPBs or any of the CDS-resident proteins display cell-wall positioning defects, and in some instances form incomplete cell walls.

as the reorientation of division planes and cytoplasmic contents in vacuolated cells after wounding requires intact actin filaments (Schnepf and von Traiteur, 1973) that are present during interphase before the MT PPB forms (Panteris *et al.*, 2004).

It is still unclear whether the interphase MT array and the subsequently formed PPB independently sense and respond to these mechanical forces and other signals, or whether the orientation of the interphase MT array directly influences the orientation of the PPB that later forms in the same cell. A recent paper linked reorientation of the interphase MT cortical array to the subsequent position of the PPB via an auxin-dependent signaling cascade (Dhonukshe *et al.*, 2012). Expression of the PLETHORA (PLT) transcription factor causes stem cells to reorient from the default

anticlinal division plane to a periclinal one so that the stem cells can divide asymmetrically to form root cap cells (Dhonukshe *et al.*, 2012). One of the direct targets of PLT is the MT binding protein MAP65 (Dhonukshe *et al.*, 2012). Up-regulation of MAP65 changes the cellular distribution of the MT stabilization factor Cytoplasmic linker associated protein (CLASP). In plants, CLASP prevents edge-induced MT catastrophe at sharp cell edges (Ambrose *et al.*, 2011). In cells in which PLT is inactive, CLASP localizes to the lateral ends of the cell, facilitating maintenance of the interphase cortical MT array in the anticlinal direction. After PLT-dependent activation of MAP65, CLASP translocates to the apical and basal sides of the cell, resulting in a periclinal MT interphase array, and then a periclinal PPB. Thus, PPB formation is as an adaptive molecular mechanism that

allows cells to respond to external stresses or to developmental signals to readily adjust selection of the division plane.

ROLE OF THE PPB IN DIVISION PLANE DETERMINATION

There is compelling evidence that the PPB predicts the future site of cell-plate insertion in somatic cell divisions, as cells that completely lack PPBs due to genetic or pharmacological disruption of MTs show extreme disorder in division planes (Mineyuki *et al.*, 1991; Traas *et al.*, 1995; Camilleri *et al.*, 2002; Chan *et al.*, 2005; Vanstraelen *et al.*, 2006; Azimzadeh *et al.*, 2008; Wright *et al.*, 2009; Spinner *et al.*, 2010). However, there are numerous examples of plant species and cell types that divide in the absence of PPBs (Pickett-Heaps *et al.*, 1999). Proliferation of the starchy endosperm occurs without PPBs, and these cells are disordered, displaying random division planes (Sabelli and Larkins, 2009). Some cultured suspension cells, protoplasts and callus lack clear division plane orientation and also lack PPBs (Chan *et al.*, 2005). In contrast, in the green algae *Chara*, organized divisions occur without prior formation of a PPB (Pickett-Heaps, 1967; Jin and Hasenstein, 2008).

In vascular plants, endosperm cellularization and meiocyte development are examples of processes in which cells lack PPBs, but undergo well-ordered divisions. In the endosperm, radial MT arrays emanate from the nuclei to delimit 'nuclear cytoplasmic domains'. Mini-phragmoplasts form between these domains to complete cellularization (Brown and Lemmon, 2007). Meiocyte divisions also lack PPBs, and, like cellularization of the endosperm, a nuclear cytoplasmic domain is established and followed by phragmoplast-assisted formation of a cell plate (Otegui and Staehelin, 2004).

Preprophase bands are absent or incomplete in most non-vascular plant species (Pickett-Heaps *et al.*, 1999) or are observed only in certain cell types. For example, tip-growing protonema cells of some mosses divide without prior formation of PPBs, but cells in the leafy gametophores do form PPBs (Schmiedel *et al.*, 1981; Doonan *et al.*, 1985, 1987; Spinner *et al.*, 2010). A particularly interesting case of regulated division in the absence of a PPB occurs in vegetatively growing cells of *Physcomitrella turbinatum* and *Funaria hygrometrica*. The spindle and early phragmoplast form perpendicular to the cell's long axis, but subsequent rotation of the phragmoplast causes cell-plate attachment at the mother cell wall at an angle of approximately 40° (Jensen, 1981). Despite the lack of a PPB, the parental cell wall displays a localized thickening that predicts the site of cell-plate attachment. Inhibition of MTs using a low amount of colchicine prevents reorientation of the cell plate, but does not prevent its expansion to the cortex (Schmiedel *et al.*, 1981).

From an evolutionary viewpoint, the PPB is a derived structure that probably arose due to the tissue complexity

of higher plants. Pre-determined cell divisions ensure better control over the organization of cells, which are immobilized by surrounding walls and unable to migrate. Thus, it is not surprising that many proteins required for PPB formation or that associate with the PPB serve multiple functions in both mitosis and interphase. Many of these proteins alter MT dynamics and include MAP65, microtubule organization 1 protein (MOR1)/Gemini pollen 1, TONNEAU1 (TON1), TON2 and CLASP (Whittington *et al.*, 2001; Camilleri *et al.*, 2002; Hussey *et al.*, 2002; Twell *et al.*, 2002; Kawamura *et al.*, 2006; Ambrose *et al.*, 2007; Kirik *et al.*, 2007; Azimzadeh *et al.*, 2008). These proteins may have evolved specific roles during PPB function while still retaining their ancient functions in interphase, spindle or phragmoplast MT arrays. *Physcomitrella patens* TON1, a protein required for PPB formation, complements *A. thaliana ton1* mutants and *vice versa*, indicating a conserved function in PPB formation. However, in *P. patens* cells that do not form PPBs, TON1 does not have an obvious role in cell division, but instead modulates gravitropic responses, indicating an ancestral role for TON1 in MT-mediated gravitropic responses (Spinner *et al.*, 2010). A challenge for the future is to assess the PPB-specific and ancestral functions of these proteins separately.

PPB FORMATION

Although plant cells lack centrosomes and instead nucleate MTs from extant MTs as well as from the cell cortex and nuclear envelope, proteins required for PPB formation share homology with proteins that localize to animal centrosomes. This relationship suggests that MT nucleation may function through similar mechanisms in both plants and animals. In *A. thaliana*, TON1A and TON1B are tandem duplicated genes that encode proteins with similarity to the human centrosomal proteins fibroblast growth factor receptor 1 (FGFR1) oncogene partner (FOP) and oral facial digital 1 protein (Azimzadeh *et al.*, 2008). Deletion of these genes completely abolishes PPB formation in *A. thaliana*. TON1A/B localize to interphase cortical MT arrays and the PPB, and interact with *A. thaliana* CENTRIN, which is also homologous to a human centrosomal protein (Azimzadeh *et al.*, 2008). TON1 additionally interacts with some members of the TON1 recruiting motif (TRM) protein family, which consists of 34 proteins in *A. thaliana*. One of these proteins (TRM1) binds and recruits TON1 to MTs (Drevensek *et al.*, 2012). Three of the defining TRM motifs are also found in the human centrosome-associated protein 350 kDa (CAP350). In humans, CAP350 targets FOP, the human TON1 homolog, to the centrosome (Yan *et al.*, 2006).

A non-canonical Protein phosphatase type 2A (PP2A) phosphatase B" regulatory subunit is also required for PPB formation in both the dicot and monocot lineages. In dicots, this protein is encoded by *FASS/TON2* (Camilleri *et al.*, 2002), while in monocots, it is encoded by two closely

related homologs, *discordia1* (*dcd1*) and *alternate discordia1* (*add1*) (Wright *et al.*, 2009). Continuing the parallel between proteins needed for PPB formation and animal centrosome organization, the *Caenorhabditis elegans* homolog of this regulatory subunit is localized to centrosomes and is required for MT outgrowth (Schlitz *et al.*, 2007). Like the *ton1a/b* mutant, *fass* mutants in *A. thaliana* fail to form PPBs (McClinton and Sung, 1997), and the plants are morphologically abnormal due to an inability to correctly orient division planes (Traas *et al.*, 1995). Intriguingly, DCD1 and ADD1 localize to the PPB and remain localized at the CDS throughout metaphase, suggesting that the proteins may also play a role in initial maintenance of the CDS (Wright *et al.*, 2009). In interphase cells, FASS is cytosolic, and *fass* mutants display defects in MT organization characterized by a reduction in cortical MT density, higher rates of MT catastrophe, and MT networks that are less branched and more parallel (Kirik *et al.*, 2012). If FASS performs a similar role in the PPB, FASS may target the dephosphorylation activity of the PP2A complex to a protein that regulates MT stabilization or branching (Kirik *et al.*, 2012). Given the similarity between the *ton1a/b* and *fass* mutant phenotypes, it is possible that TON1 is target of the FASS phosphatase. Other observations also suggest that phosphorylation and dephosphorylation dynamically alter PPB stability. Microinjection of the cyclin-dependent kinase (CDK) inhibitor, ICK1, destabilizes the MT PPB (Wang *et al.*, 2003), and application of kinase inhibitors prevents both formation of the PPB and its destabilization (Shibaoka and Katsuta, 1992), while application of phosphatase inhibitors affects PPB formation (Ayaydin *et al.*, 2000).

In addition to *fass* and *ton1a/b*, other *A. thaliana* mutants have been identified with abnormal PPBs. *MOR1* is the *A. thaliana* homolog of *Xenopus* microtubule associated protein 215 kDa. XMAP215 *MOR1* is important for normal MT dynamics and PPB formation (Kawamura *et al.*, 2006; Kawamura and Wasteneys, 2008). *In vitro*, this MT polymerase increases both the growth and shrinkage rates of MTs. Analysis of a *mor1* temperature-sensitive point mutant confirmed this *in vitro* analysis, and suggested that *MOR1* also inhibits pause events, ensuring that MTs remain dynamic (Kawamura and Wasteneys, 2008). In *mor1* mutants, the cortical interphase array is disorganized and has short MTs (Whittington *et al.*, 2001). During mitosis, 50% of *mor1* cells fail to form a PPB, while spindles and phragmoplasts are disorganized and contain short MTs (Kawamura *et al.*, 2006).

Katanin is an evolutionarily conserved MT-severing protein complex. The *lue1* mutation affects the function of the p60 subunit of katanin. The *lue1* mutant has disorganized interphase cortical MT arrays (Bichet *et al.*, 2001; Burk *et al.*, 2001; Bouquin *et al.*, 2003) and oblique division planes were observed in the root (Panteris *et al.*, 2011) and shoot apical meristem (Uyttewaal *et al.*, 2012). In the root,

10% of early PPBs are disorganized and persist longer into prophase than wild-type PPBs (Panteris *et al.*, 2011). In a majority of the dividing root cells, the spindle was initially multipolar, but eventually became bipolar, and phragmoplasts undergoing expansion had longer MTs that remained abnormally connected to the nucleus. A more recent study suggests that proper katanin function is required to respond to mechanical forces generated by growth (Uyttewaal *et al.*, 2012). Thus, in the context of division plane selection, it appears that mutants with altered katanin function have lost their ability to translate cell geometry into the shortest division axis.

ACTIN PPB

In addition to containing MTs, the PPB also contains actin filaments. Intact MTs are critical for formation of the actin filament PPB, as application of MT-depolymerizing drugs prior to the end of G₂ entirely blocks formation of both the MT and actin components of the array (Palevitz, 1987; Vanstraelen *et al.*, 2006). The actin filament PPB appears to constrain the MT PPB, because actin depolymerization during preprophase/prophase leads to dramatic widening of the MT PPB and shifted division planes in asymmetrically dividing cells (Mineyuki and Palevitz, 1990) and symmetrically dividing cells (Eleftheriou and Palevitz, 1992; Liu and Palevitz, 1992). Timed and transient application of actin-depolymerizing drugs during preprophase and prophase also results in mis-oriented cell division planes in symmetrically dividing tobacco BY-2 cells (Sano *et al.*, 2005). Finally, mutants with reduced expression of *ACT7*, an actin isoform that is highly expressed in mitosis, have defects in division plane orientation. However, MT arrays have not been inspected in this mutant, so it is not clear how division plane orientation has been compromised (Gilliland *et al.*, 2003). These data suggest that the actin component of the PPB may play an important role in division plane determination in both asymmetrically and symmetrically dividing cells.

Overall, much remains to be learned about PPB formation in plant cells. While several proteins required for proper PPB formation have been identified, their interactions with each other are still unknown. Additionally, it is likely that more proteins required for PPB formation await discovery. Proteins that generally regulate MT dynamics often affect PPB formation, but predictive rules for the changes in MT dynamics that promote PPB formation have not yet been established. Finally, it is imperative that the relationship between interphase cortical MT arrays and PPB MT arrays be understood.

THE ROLE OF THE PPB IN SPINDLE ASSEMBLY AND THE ROLE OF THE SPINDLE IN DIVISION PLANE DETERMINATION

An additional proposed function of the PPB is its contribution to timely formation of a normal bipolar spindle. The

spindles of cells grown in culture that have no PPB or a double PPB are initially multipolar, although eventually a normal bipolar spindle is formed. It also takes longer for these cells to progress through metaphase (Chan *et al.*, 2005; Marcus *et al.*, 2005). Interestingly, once a bipolar spindle forms in BY-2 cells with two PPBs, it is the orientation of the spindle that predicts the future division plane (Yoneda *et al.*, 2005). Finally, MTs that connect the nucleus and PPB are important for proper spindle assembly and timely progression through the cell cycle (Ambrose and Cyr, 2008). Several mutants with abnormal spindles have been identified, and although some have severe defects in division plane orientation, others have no apparent defects. For example, mutations that eliminate the MT plus end-binding protein, EB1c, result in plants whose cells produce normal PPBs, but display defects in spindle orientation that lead to division plane defects. These defects are greatly enhanced by application of low concentrations of oryzalin, an MT-depolymerizing drug (Komaki *et al.*, 2010). In contrast, *A. thaliana* mutants lacking the closely related kinesin-like proteins, AKT1 or AKT5, display morphologically abnormal spindles, but have no apparent division plane defects (Marcus *et al.*, 2003; Ambrose *et al.*, 2005; Ambrose and Cyr, 2007).

Several examples show that spindle orientation does not determine the final division plane. Altering the position of the spindle by centrifugation does not necessarily lead to a defect in division plane orientation because the phragmoplast, which is derived from the central spindle, is often able to track back to the CDS established by the PPB (Ota, 1961). However, if the spindle position in a cell is significantly altered, the phragmoplast may not be able to track back successfully to the CDS (Ota, 1961; Galatis *et al.*, 1984; Marcus *et al.*, 2005). This suggests that formation of a spindle, the axis of which bisects and is perpendicular to the PPB, facilitates insertion of the cell plate at the CDS, although this positioning is not essential. Spindle movement from the ideal perpendicular position occurs naturally in many cell types and plants, suggesting that it may be the rule rather than the exception. For example, spindle rotation normally occurs in *Vicia faba* root meristem cells (Oud and Nanninga, 1992), *Tradescantia virginiana* cells (Palevitz, 1993), maize epidermal cells (Cleary and Smith, 1998), *A. thaliana* root cells (Panteris *et al.*, 2011) and cultured tobacco BY-2 cells (Rasmussen *et al.*, 2011b). Therefore, it seems unlikely that spindle orientation per se affects division plane orientation.

CDS-RESIDENT PROTEINS AND PHRAGMOPLAST GUIDANCE

The MT component of the phragmoplast is well characterized and consists of two cylinders of MTs with antiparallel polarity that overlap with their plus ends in the mid-zone, where the cell plate is synthesized by fusion of Golgi-derived vesicles (Jurgens, 2005). The phragmoplast pro-

gressively expands during cell-plate formation via MT depolymerization at the phragmoplast center and new polymerization on its outside edges. A recent paper used photobleaching and mathematical modeling to define the dynamics of phragmoplast MTs (Smertenko *et al.*, 2011). The polarity of the phragmoplast MTs is not generated by preferential nucleation (Asada *et al.*, 1991), but is instead derived from the pre-existing polarity of the spindle. Phragmoplast MTs show dynamic instability, with a majority polymerizing towards the cell plate (Smertenko *et al.*, 2011). Depolymerization is regulated by a mitogen-activated protein kinase cascade that prevents MAP65 cross-bridging activity at the mid-zone (Sasabe and Machida, 2012). It remains elusive how the phragmoplast and CDS communicate to achieve targeted fusion of the cell plate to the mother cell wall. MTs and actin filaments have been implicated as bridging structures between the phragmoplast and CDS (Dhonukshe *et al.*, 2005; Higaki *et al.*, 2008), but more work is required to clarify their role in CDS maintenance.

Several mutants have been identified that fail to promote proper guidance of the phragmoplast to the CDS established by the PPB. They exhibit aberrantly positioned cell walls, but do not appear to have defects in PPB or phragmoplast formation or cell-plate synthesis, suggesting that the problem lies in CDS establishment or recognition. Cells in the maize *tangled (tan)* mutant, whose name comes from its disordered pattern of leaf epidermal cells, are unable to properly orient division planes (Smith *et al.*, 1996). The epidermal pavement cells occasionally display mis-oriented PPBs, but have a much higher incidence of mis-oriented phragmoplasts, suggesting that TAN promotes phragmoplast guidance (Cleary and Smith, 1998). Similarly, *tan* mutants in *A. thaliana* have a related but milder defect in phragmoplast orientation, and no detectable defect in PPB orientation (Walker *et al.*, 2007). TAN is a highly basic protein that binds MTs *in vitro* (Smith *et al.*, 2001). A TAN-YFP fusion protein co-localizes with the PPB and remains at the CDS throughout mitosis, so TAN is a positive marker of the CDS (Walker *et al.*, 2007) (Figure 2) and fulfils the minimum requirements for the predicted 'molecular memory' of the division site (Murata and Wada, 1991; Mineyuki, 1999). Interestingly, TAN also localizes to the nucleolus in preprophase cells (Rasmussen *et al.*, 2011b), as does TORMOZ, another protein that is required for division plane determination (Griffith *et al.*, 2007). Although the MT arrays have not been analyzed in *toz* mutants, it is possible that a nuclear pathway may affect division plane orientation.

A localization analysis of the domains of the TAN protein revealed that TAN is not a static member of the CDS, and is instead recruited to the CDS by multiple independent mechanisms. The initial assembly of TAN at the CDS is dependent on the presence of a PPB, as TAN localization is abolished in the PPB-lacking *fass/ton2* mutants (Walker *et al.*, 2007). The PPB requirement was confirmed using transient and

timed MT depolymerization (Rasmussen *et al.*, 2011b). TAN's localization to the CDS during preprophase/prophase, and its later localization in telophase, are mediated by two distinct domains of the TAN protein that interact with different molecular players (Rasmussen *et al.*, 2011b).

Like TAN, Ran GTPase activating protein 1 (GFP-RanGAP1) localizes to the PPB and remains at the CDS after PPB breakdown (Xu *et al.*, 2008). GTPase-activating proteins stimulate the GTPase activity of their cognate GTPases, and this is thought to result in a decrease in GTPase activity. Depletion of RanGAP1 and a closely related protein, RanGAP2, leads to significant cytokinetic problems, including cell wall stubs and division plane orientation defects (Xu *et al.*, 2008), while complete loss of RanGAP1 and RanGAP2 results in female gametophyte lethality (Rodrigo-Peiris *et al.*, 2011). In addition to the CDS, RanGAP1 also localizes to the interphase nuclear envelope, kinetochores and the cell plate, suggesting that it has multiple functions during mitosis. In mammals, a gradient of Ran activity is involved in spindle formation. An attractive hypothesis suggests that RanGAPs are required for maintenance of a RAN gradient that regulates MT nucleation from the nuclear envelope as well as from the cell plate and CDS during cytokinesis (Van Damme, 2009). Although the MTs connecting the PPB and nucleus are bi-directional (Dhonukshe *et al.*, 2005), and it is likely that MTs nucleate from the PPB in early prophase, MT nucleation from the CDS has so far not been reported.

A pair of closely related kinesin motor proteins, the phragmoplast-orienting kinesins POK1 and POK2, are required for localization of TAN and RanGAP1 to the CDS during telophase. TAN and RanGAP1 disappear from the CDS in *pok1 pok2* mutants during metaphase, suggesting that POK function is required for TAN and RanGAP1 maintenance at the CDS or for maintenance of the CDS itself. In *A. thaliana pok1 pok2* double mutants, new cell walls in root meristems frequently display oblique orientations that are reminiscent of the *tan* mutant phenotype in maize. Defects in PPB location are observed, but are less abundant than defects in phragmoplast orientation (Muller *et al.*, 2006). These mis-oriented division planes probably result from absence of the appropriate reference molecules/structures at the CDS, which disrupts phragmoplast guidance. POKs, through their motor domains, may also act more directly at the phragmoplast to establish MT connections with the CDS (Dhonukshe *et al.*, 2005). Although no localization pattern has been reported for the POK proteins, a likely subcellular site of POK activity is the CDS itself, as for POK1's confirmed interactors, TAN and RanGAP1. The POKs are classified as kinesin-12s, suggesting MT plus end-directed motility or association, similar to kinesin-12a and kinesin-12b, which both associate with MT plus ends in the mid-zone region of the phragmoplast (Lee *et al.*, 2007; Ho *et al.*, 2011).

Although maintenance of the CDS and its recognition and translation into an accurately positioned cell wall are not essential for plant survival, they are critical for development of normal plant morphology. Cell plates synthesized in *pok1 pok2* and *tan* mutants fuse with the cortex at seemingly arbitrary sites, giving rise to dwarfed plants. Future research to identify TAN, POK1, POK2 and RanGAP1/2 interactors will provide more insight into factors regulating dynamic modification of the division site.

NEGATIVE CDS MARKERS

In addition to the positive CDS markers, several proteins are excluded from the CDS and are considered negative markers. From prometaphase through anaphase, actin is relocated away from the CDS, creating an actin-depleted zone (ADZ) (Mineyuki and Palevitz, 1990; Cleary *et al.*, 1992), which is flanked by a region that is enriched in actin filaments (Sano *et al.*, 2005). Panteris (2008) noted that actin filaments are not absent from the ADZ, but instead may be less abundant and therefore more difficult to visualize. Despite its striking localization, the purpose of the ADZ is still mysterious, although it may play a role in division plane orientation. A possible explanation for formation of an ADZ may be the need for a low-viscosity, gel-free cytoplasmic zone to curtail protein dynamicity and maintain CDS identity. A potential origin of the ADZ are the endocytotic vesicles that cluster near the PPB in late G₂ (Karahara *et al.*, 2009, 2010). Perhaps these endocytotic vesicles remove proteins that bind or modulate the activity of actin and other proteins from the CDS.

So far, only one other protein, the kinesin-like protein KINESIN CDKA;1-ASSOCIATED 1 (KCA1), shows a pattern reminiscent of the actin during mitosis. The absence of KCA1 at the CDS is referred to as the KCA1-depleted zone (KDZ). Unlike the ADZ, which is established in prometaphase, the KDZ appears during preprophase (G₂), and persists until the phragmoplast reaches the cortex (Vanstraelen *et al.*, 2006). Similar to the ADZ, the KDZ also requires an intact PPB in order to be established. KCA1 is predicted to encode a member of the kinesin-14 family, and has one close paralog, KCA2. Recently, *kca1 kca2* double mutants were isolated in *A. thaliana*, but they have no obvious MT defects, and the KCA1 and KCA2 proteins do not bind MTs *in vitro* (Suetsugu *et al.*, 2010). However, the authors did not directly assess division plane orientation in the *kca1 kca2* double mutant. Instead, they showed that KCA1 and KCA2 are required for actin-mediated chloroplast movement and anchorage of chloroplasts to the plasma membrane. KCA1 interacts with a host of other proteins, including a cyclin-dependent kinase (CDKA;1), which localizes to the PPB (Vanstraelen *et al.*, 2004), a geminivirus replication protein (Kong and Hanley-Bowdoin, 2002), and the MT-severing protein katanin (Bouquin *et al.*, 2003), suggesting potentially diverse roles for this protein. Further

insight may be obtained using *in vivo* co-localization techniques to determine when and where KCA1 interacts with its partners.

CELL-PLATE ANCHORING AND MATURATION

An additional challenge will be to determine how the phragmoplast mediates the connection between the developing cell plate and the mother cell wall at the CDS. It has been known for a long time that callose, a polysaccharide polymer that may be detected by staining cells with aniline blue, is observed in young cell plates, which later are enriched with cellulose (Fulcher *et al.*, 1976). Several proteins have been identified that have potential roles in cell-plate anchoring and maturation. The *A. thaliana* root-shoot-hypocotyl-defective (*rsh*) mutant has defects in cell-wall assembly and division plane orientation (Hall and Cannon, 2002). *RSH* encodes AtEXT3, an extensin protein that is localized to the cell wall and self-assembles to form a cross-linked network (Cannon *et al.*, 2008). As suggested by its localization to the cell plate and the mother cell wall, RSH may be involved in generating connections between the cell plate and the mother cell.

The adaptor/coatomer-like protein TPLATE is present at the growing edge of the cell plate, and appears at the CDS shortly before fusion of the cell plate with the parental wall. Subsequently, TPLATE is removed from the site of fusion, but remains at the CDS flanks and in the cell plate, possibly aiding in heterotypic vesicle fusion during cell-plate anchoring. TPLATE interacts with components of the clathrin-mediated endocytic machinery (Van Damme *et al.*, 2011), suggesting that TPLATE may be involved in endocytic events at the CDS that are necessary to maintain and narrow the CDS in preparation for cell-plate anchoring. Accumulation of callose depositions in *tplate* mutant pollen further suggests a role in cell-wall modification and maturation (Van Damme *et al.*, 2006).

The potential cell-plate maturation factor, AUXIN INDUCED IN ROOTS 9 (AIR9), initially co-localizes with the PPB, and then disappears from the CDS until late cytokinesis. AIR9 has two distinct domains that localize it to the division site during different phases of the cell cycle. One is the MT-binding domain, which co-localizes with the PPB, and the other domain localizes to the CDS as the cell plate reaches the cortex. After fusion of the cell plate and parental wall, AIR9 assumes a filamentous pattern at the CDS and extends into the cell plate before cortical MTs populate the plasma membrane (Buschmann *et al.*, 2006). Application of the herbicide chlorpropham causes branching of phragmoplasts and cell plates that frequently fuse at ectopic sites outside the CDS. Although the entire plasma membrane appears to be receptive for fusion with the cell plate, fusion outside the CDS prevents timely removal of callose, and AIR9 remains absent from ectopic cell-plate attachment sites (Buschmann *et al.*, 2006). Thus, AIR9 may be recruited specifically to the

late division site to promote cell-plate maturation. The *air9* mutation is lethal, which prevents a closer examination of AIR9 function during mitosis. Identification of partial loss-of-function alleles may help clarify roles for AIR9.

CONCLUSION

As the division plane is both established and executed by a series of MT-based cytoskeletal arrays, it is not surprising that genetic studies have revealed that MT-binding and MT-associated proteins are important for division plane orientation. However, a review of the localization, function and mutant phenotypes of these proteins highlights the challenge of uncoupling the general role that they play in MT array organization from more specific functions in division plane orientation. This issue may be mitigated by identification and analysis of weak alleles, although such alleles may not uncover the full range of phenotypes. Another problem in unraveling division plane orientation stems from the existence of groups of functionally redundant proteins arising from the expanded protein families and gene duplications that are common in plants. In these instances, mutations that remove one gene product do not always have an associated phenotype. A potential solution to the gene duplication problem is use of RNA-based silencing techniques to achieve simultaneous knockdown of two or more gene family members. Other challenges specific to studying the MT-binding proteins important in division plane orientation arise from the dense packing of MTs in mitotic arrays. Although many recent papers describe how loss of a particular protein affects MT dynamics in the interphase cortical array, where individual MTs and bundles are easily observed, it is challenging to make similar observations and measurements in the PPB, spindle or phragmoplast. Without these critical measurements, it is not clear whether these proteins modulate MT dynamics in mitotic and interphase cells in a similar manner.

Finally, it appears that the protein complexes that establish the CDS are different from those that maintain the CDS after disassembly of the PPB, suggesting that the CDS requires progressive modification and different molecular players throughout the cell cycle. Identification and characterization of the various sets of proteins required for establishment and maintenance of the CDS will clarify the dynamic process by which plant cells divide.

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