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FOCUS PAPER

Microtubule arrays and *Arabidopsis* stomatal development

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Abstract

Microtubule arrays in living cells were analysed during *Arabidopsis* stomatal development in order to more closely define stages in the pathway and contexts where intercellular signalling might operate. *Arabidopsis* stomata are patterned iteratively via the orientation of an asymmetric division in a cell located next to an existing stoma. It was found that preprophase bands of microtubules (PPBs) were correctly placed away from stomata and from two types of precursor cells. This suggests that all three cell types participate in an intercellular signalling pathway that orients the division site. These and other asymmetric divisions in the pathway were preceded by a polarized cytoplasm, with the PPB around the nucleus at one end, and the vacuole at the other. PPBs before symmetric divisions of guard mother cells (GMCs) were broader than those in asymmetric divisions, and the GMC division site was marked by unusual end-wall thickenings. This work identifies an accessible system for studying cytoskeletal function and provides a foundation for analysing the role of genes involved in stomatal development.

Key words: Asymmetric division, guard cell, microtubule, polarity, preprophase band, stomata.

Introduction

The stomatal pathway provides a convenient and fascinating system for studying cellular and molecular events in plant development. Examples include the regulation of asymmetric and symmetric cell division, the generation of a spacing pattern where stomata do not touch, a progression in cell fates, and a co-ordinated morphogenesis that produces two uniquely-shaped guard cells that surround the only regulated openings in the shoot epidermis.

As far as is known, the minimal one-celled separation between stomata in dicots and monocots arises via the placement of asymmetric division (Larkin *et al.*, 1997; Croxdale, 2000). In *Arabidopsis*, each asymmetric division produces a larger and a smaller cell, with the latter, the meristemoid, forming the stoma (Fig. 1A) (Nadeau and Sack, 2002). *Arabidopsis* asymmetric divisions are classified into three types based upon their spatial context, i.e. entry, amplifying, and spacing divisions (Fig. 1B). The latter occur in neighbour cells, meaning in cells adjacent to a stoma, meristemoid, or guard mother cell (GMC). Neighbour cell spacing divisions are oriented so that the new wall is not in contact with the pre-existing stoma, a process likely to involve cell–cell signalling (Geisler *et al.*, 2000).

Stomatal morphogenesis takes place after the symmetric division of the GMC (Fig. 1A, B). Each daughter cell develops a wall thickening that later separates to form the stomatal pore. The majority of angiosperms possess kidney-shaped guard cells. These cells possess a well-characterized cellulose array that radiates out from developing pore thickenings (Hepler and Palevitz, 1974).

The importance of microtubules for division orientation and cell shaping in stomatal development has been extensively studied (Pickett-Heaps and Northcote, 1966; Palevitz, 1981; Sack, 1987; Galatis and Apostolakos, 2004). The hypothesis that microtubules guide cellulose synthesis gained credence from early ultrastructural studies showing co-alignment between radial microtubule arrays and cellulose microfibrils (Hepler and Palevitz, 1974; Palevitz, 1981). Similarly, the idea that the preprophase band of microtubules (PPB) predicts the division site received strong initial support from the study of asymmetric division that produced the grass stomatal complex (Pickett-Heaps and Northcote, 1966; Smith, 2001).

PPBs and asymmetric division in the stomatal pathway have been studied in a few dicot species using electron

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Abbreviations: GFP, green fluorescent protein; GMC, guard mother cell; H2B::YFP, histone2B::yellow fluorescent protein; M, meristemoid; MMC, meristemoid mother cell; PPB, preprophase band of microtubules; TMM, TOO MANY MOUTHS.

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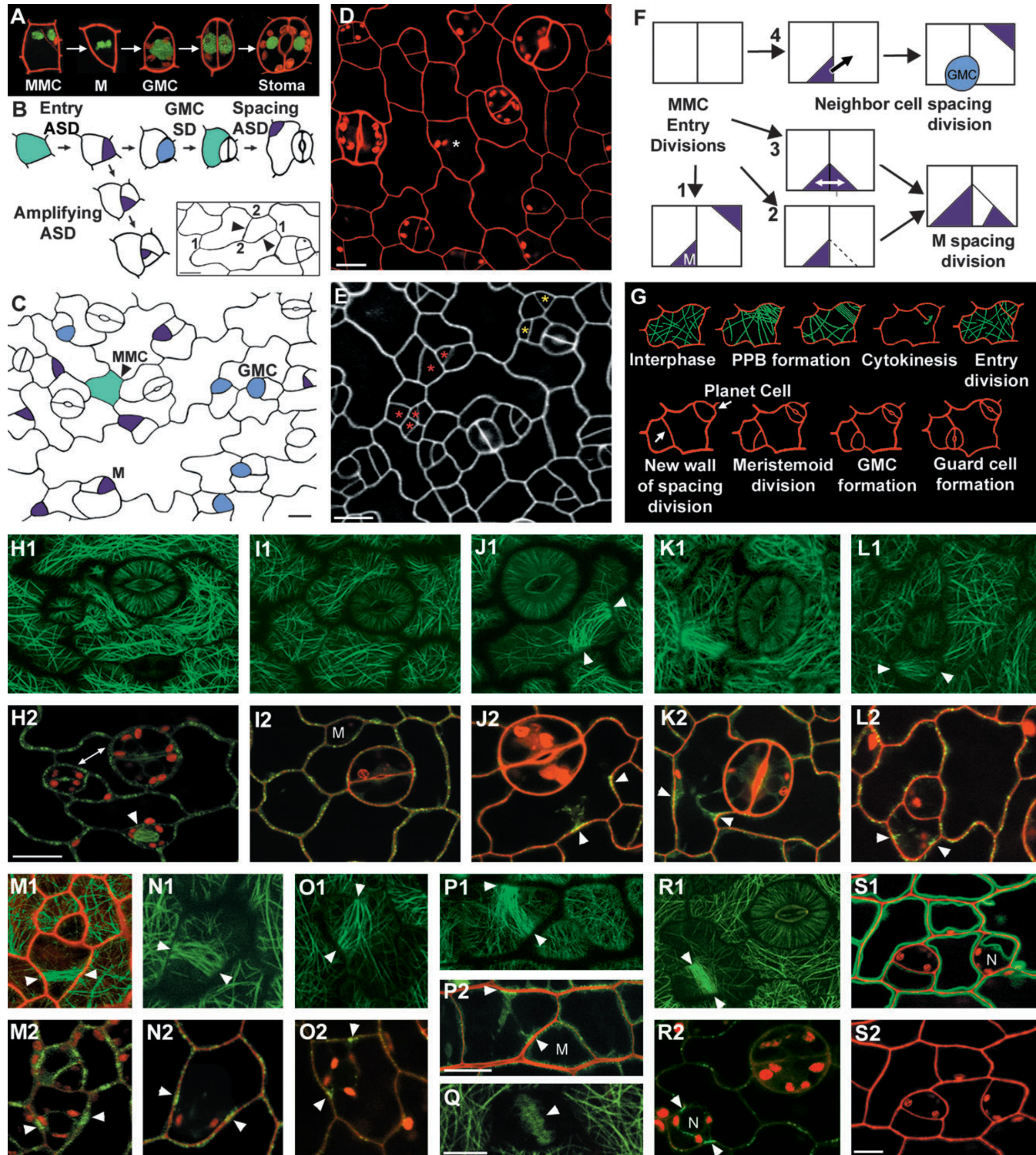

Fig. 1. Microtubules and asymmetric division. (A) Confocal micrographs showing key cell types in the stomatal pathway. Red cell wall fluorescence from propidium iodide. Chromatin visualized by H2B::YFP. The figure shows a meristemoid mother cell (MMC) and a triangular meristemoid (M) dividing asymmetrically (note incomplete cell wall in MMC and metaphase plate in M). The oval GMC contains a centrally located nucleus surrounded by autofluorescent chloroplasts. GMC division produces two cells that mature into two guard cells around a pore. (B) Drawing showing three types of asymmetric division (ASD), entry, amplifying, and spacing divisions, as well as the symmetric division (SD) of the GMC (blue). MMC (green) division marks entry into the pathway and produces a smaller cell, the meristemoid (purple). Cells next to guard cells often become an MMC and undergo a spacing division. Meristemoids usually divide unequally up to three times before converting into a GMC. Each division amplifies the number of cells capable of asymmetric division. Inset: Micrograph showing a meristemoid about to undergo an amplifying division in the plane marked by arrowheads (see PPB in same cell in Fig. 1N). Walls 2-2 and 1-1 denote a previous amplifying division and an entry division, respectively. Propidium iodide signal shown in black. (C) Tracing of developing epidermis showing cell types. Colour coding as in (B). Meristemoids are small and often triangular. GMCs are larger and rounder than meristemoids. An isolated MMC (arrowhead) is shown which is not in contact with a planet cell (guard cell, GMC, or meristemoid). Large pavement cells (bottom) do not divide. (D) Isolated MMC (asterisk) in a developing epidermal field. Same cell shown in (1O) with

microscopy (Galatis and Mitrakos, 1979; Galatis *et al.*, 1982; Zhao and Sack, 1999). Recent studies of the *Arabidopsis* epidermis have shown that rules governing asymmetric division depend upon spatial context and might vary in their dependence upon intercellular signalling (Geisler *et al.*, 2000). For example, entry asymmetric divisions are less predictably oriented than spacing divisions. Because these rules were derived from a study of the epidermal surface using dental impression material, it is important to assess whether they are supported by studies using intracellular division markers, such as the PPB that precedes cell division.

Finally, as far as is known, there is no published comprehensive, light microscopy study of microtubule arrays in dicot stomatal development. Existing light microscopy studies focus on lower plants and monocots, with most data on asymmetric division from grasses (Galatis and Apostolakos, 2004). These studies also used immunofluorescence, which is more time-consuming and probably more prone to produce artefacts than *in vivo* microtubule reporters.

A range of microtubule arrays present in living cells in the *Arabidopsis* stomatal pathway is characterized here. A green fluorescent protein (GFP) microtubule reporter was used that permits the detection of rich arrays and the acquisition of relatively large data sets to define the developmental stages more closely. In addition, descriptions are provided of the different types of asymmetric division in the pathway with respect to spatial context, cell type, cytology, and microtubule arrays. This contrasts with events surrounding the symmetric division of the guard mother cell.

Materials and methods

Plant material and cultivation

The *Arabidopsis thaliana* plants used were in a Col-0 background and were wild type except that they were homozygous for the *glabrous1* mutation since the absence of trichomes facilitates the observation of stomatal development. Seeds harbouring an α-tubulin translational fusion with GFP driven by a constitutive 35S promoter were provided by T Hashimoto (Ueda *et al.*, 1999). Seeds harbouring a 35S:H2B::Yellow Fluorescent Protein (YFP) gene fusion were provided by F Berger (Boisnard-Lorig *et al.*, 2001). Seeds harbouring GFP-tagged genes that mark the cell membrane (Q8) and the vacuolar membrane (Q5) were also used (Cutler *et al.*, 2000).

Seeds were planted aseptically on nutrient-supplemented 0.8% w/v agar. Seedlings were grown at 21–22 °C with a 16 h photoperiod (at 80–90 μmol $m^{-2} \cdot s^{-1}$). First and second leaves were dissected from 6–8-d-old plants.

Microscopy

To visualize cell walls, freshly dissected leaves were stained for 3–4 min in an aqueous solution containing 2 mg ml^{-1} propidium iodide. The leaves were then rinsed and mounted in distilled water. The abaxial side of the leaves was viewed with a ×100 oil objective (Nikon Plan Fluor NA 1.3) using a Nikon PCM 2000 confocal laser scanning microscope equipped with 488 nm argon and 543 nm helium–neon lasers. Images were captured using Simple PCI Software and managed with Adobe Photoshop. In some cases, the sensitivity of the photomultiplier tube was adjusted during image capture to enhance propidium iodide visualization, such as in cell plates and to reduce the background GFP signal from unpolymerized tubulin. Optical sections were typically captured at 0.3 μm intervals.

Results and discussion

In addition to the type of microtubule array, this analysis is concerned with when and where those arrays appear relative to events in the stomatal pathway. Although there are core elements of stomatal development and patterning in *Arabidopsis*, there is also flexibility in the pathway such as in the placement and number of asymmetric divisions. The types of asymmetric division, their spatial and temporal contexts, and how these relate to the distribution of

a PPB. (E) Unlike GMCs, two or even three meristemoids (red asterisks) can be found in direct contact in the young leaf epidermis. Correct spacing is re-established when asymmetric division in one meristemoid separates the meristemoids (yellow asterisks). (F) Comparison of pattern generation through divisions of MMCs and of meristemoids (purple). Simultaneous divisions of adjacent MMCs form meristemoids that are in contact (arrow 3) or are correctly spaced (arrow 1). Meristemoids in contact also result when one entry division is slightly later than the other (arrow 2; dashed line). In both cases, patterning is restored when one of the meristemoids divides away (bottom right). Arrow 4: signals from a single meristemoid (unlabelled arrow) orient spacing division in the neighbour cell. Double-headed white arrow (centre diagram) indicates presumed signalling between two adjacent meristemoids. (G) Drawing of events surrounding an entry MMC division (top) and a spacing MMC division in a neighbour cell (bottom). Microtubules (green) of the preprophase band (PPB) mark the division plane. (H–P, R–S). Pairs of optical sections (1, outer cell cortex; 2, closer to inside of leaf) for each field. Green, α-tubulin-GFP; two facing arrowheads, PPBs. Satellite meristemoid formation (spacing division) shown in (J–M). (H1, 2) Radial microtubule arrays of two stomata. Smaller stoma (left) formed after one spacing division (two-headed arrow in H2) and then one amplifying division. Spindle position (arrowhead H2) predicts polarized amplifying division of a meristemoid. (I1, 2) Satellite meristemoid (M). (J1, 2) A developing PPB (arrowheads) predicts the plane of a spacing division that will cut off a lobe of the neighbour cell. The PPB can be seen in both the cell cortex as well as deeper in the cell. (K1, 2) A more condensed PPB predicts division close to but still separated from the developing stoma. (L1, 2) Same as in (K) but with PPB placed away from GMC. (M1, 2) Positions of PPB (arrowheads), nucleus, and surrounding chloroplasts predict the plane of a spacing division in the largest of the neighbour cells next to a meristemoid. (N1, 2) PPB predicts plane of the amplifying division of the same meristemoid shown in Fig. 1B inset. (O1, 2) PPB indicates an entry division in the same MMC shown in (1D). (O2) Chloroplasts surround the nucleus which is already in the division plane. (P1, 2) PPB predicts MMC division that will produce meristemoids that are in contact (as in 1F, bottom centre). (Q) Phragmoplast (arrowhead) in meristemoid. Dark line in centre of phragmoplast is where the cell plate is forming. (R1, 2) Entry division. The nucleus (N) is surrounded by chloroplasts and microtubules which appear to connect the nucleus to the cortical PPB. (S1, 2) The cytoplasm in the meristemoid at the right (N, nucleus) is polarized prior to an amplifying division. The nucleus and surrounding chloroplasts are near the pointed end of the meristemoid with the vacuole (membrane visualized with Q5::GFP) at the other end. (S2) This is the same plane of optical section, but only the cell walls and the chloroplasts are visualized. A GMC is visible at the left. Scale bars in (B inset) (C–E), (P2)=10 μm. Bars in (H2), (Q), (S)=5 μm, with (H2) bar also representing magnifications of (H–O, R).

microtubules and other cytological markers are discussed first. Then a description is presented of how microtubule arrays define different stages of GMC and guard cell development with respect to what is already known and to future directions.

Classes of asymmetric division are defined by cell type and position

Asymmetric divisions in the *Arabidopsis* stomatal pathway roughly fall into three major functional classes: pathway entry, cellular amplification, and stomatal spacing. All three types produce or regenerate the smaller daughter cell, the meristemoid, but there are differences in when and where these divisions occur.

All *Arabidopsis* stomata originate from an entry asymmetric division. This division, by definition, takes place in the meristemoid mother cell (MMC), a precursor cell whose unequal division produces a meristemoid (Fig. 1B). Each meristemoid forms one GMC. Traits involved in recognizing MMCs are described in the section, ‘A polar PPB predicts MMC fate’.

A second major class of asymmetric divisions, so-called amplifying divisions, takes place in meristemoids. Meristemoids usually, but not always, divide asymmetrically one or more times. Each meristemoid division regenerates the meristemoid and also produces a larger daughter cell. Because these larger cells can, in turn, divide asymmetrically, meristemoid divisions amplify the pool of potential new stem cells (Fig. 1B). The shape of the meristemoid is partly determined by the number of amplifying divisions that have occurred (Galatis and Mitrakos, 1979). For example, a meristemoid with two straight walls probably divided twice (Fig. 1B inset). A meristemoid undergoing a symmetric division has never been observed.

The third class of asymmetric division is the spacing division that creates the one-celled separation of stomata. These divisions occur in neighbour cells, meaning cells next to a stoma, a GMC, or a meristemoid (Fig. 1B). Some neighbour cells enter the stomatal pathway by functioning as an MMC and dividing. The resulting smaller cell, a ‘satellite’ meristemoid, does not touch the pre-existing stoma or precursor.

The satellite meristemoid ‘orbits’ a ‘planet’ cell (the pre-existing stoma, GMC, or meristemoid). Spacing divisions probably involve intercellular signalling that conveys spatial information identifying the location of the planet cell. These signals are presumably used to orient the division plane so that the resulting satellite meristemoid is not in contact with the planet cell.

With respect to terminology, the categories of spacing and entry divisions overlap. All spacing divisions are entry divisions, but only those entry divisions that take place in neighbour cells are spacing divisions. Also, neighbour cells are defined solely by cell position without regard to cell lineage. Some neighbour cells and planet cells originate from the division of the same parent cell and are, therefore, clonal (Fig. 1B), whereas others are non-clonal (Geisler *et al.*, 2000).

PPB distribution consistent with intercellular signalling in spacing divisions

The location of the PPB predicts where the new cell wall will join the pre-existing wall of the parent cell. Thus the PPB marks the division site in the neighbour cell, just as it does in almost all cell divisions in plants (Mineyuki, 1999). The new wall becomes the side of the meristemoid that usually faces the planet cell.

PPBs in spacing divisions usually predict a division plane that will cut off either a corner of a neighbour cell that has angular and mostly straight walls, or a lobe in a neighbour cell that has more sinuous walls (Fig. 1J, O). Thus the initial shape of the resulting satellite meristemoid depends in part on the shape, age, and size of the neighbour cell as well as on the plane where division takes place (Fig. 1J–M, R). For example, in epidermal fields where mature stomata are not yet present, most cells are angular, many of the cells are small, and the planet cells are mostly meristemoids or GMCs (Fig. 2). In older stages, neighbour cells are adjacent to stomata, are larger, and have sinuous walls.

It was found that the PPB preceding a spacing division does not intersect the cell walls of guard cells, GMCs, and meristemoids (Fig. 1I–M). This supports the idea that all three types of planet cells participate in intercellular signalling that correctly orients the plane of the division site in a neighbour cell. This extends previous data derived from the dental impression method that was used to study the same cells through time. However, the impression method has limited time-resolution since divisions can only be detected after a new wall causes an indentation in the epidermal surface and since organ viability is threatened if impressions are taken more frequently than every 12–24 h. By contrast, PPB location in living tissue can be more closely correlated with planet cell type, allowing inferences about when intercellular signalling occurs. Thus these data from living cells provide more direct support for the hypothesis that all three types of planet cells are involved in stomatal patterning. Because PPBs were sometimes located very close to, but not touching, the planet cell (Fig. 1K), it is possible that signals from the planet cell create a zone in which the formation of the division site/PPB is prohibited.

A working model for how intercellular signalling might act is that planet cells broadcast spatial signals in the form of ligands that then bind to receptor complexes associated with the neighbour cell membrane (Nadeau and Sack, 2003). A possible component in this complex is TOO MANY MOUTHS (TMM), a leucine-rich repeat receptor-like protein (Nadeau and Sack, 2002). TMM might interact with leucine-rich repeat receptor-like kinases such as ERECTA (Shpak *et al.*, 2005). Ligand binding might then

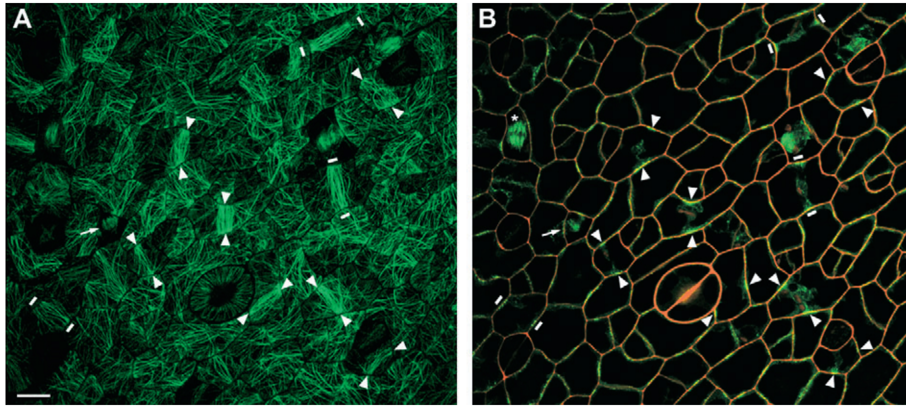

Fig. 2. Two optical sections of the same field from a developing leaf epidermis seen in the cell cortex (A) and deeper into the cell (B). Paired arrowheads in both planes of section indicate PPBs that predict correctly placed spacing divisions. Arrow (B, left) shows a phragmoplast of an asymmetric division. Asterisk (B, upper left) shows mitotic spindle in a probable meristemoid. PPBs that precede symmetric divisions in three pavement cells are indicated by the paired white bars. Scale bar in (A)=10 μm.

initiate a phosphorylation cascade via YODA, a MAPKKK (Bergmann *et al.*, 2004). These events might ultimately position the division site in the cell cortex away from the planet cell. Perhaps the PPB is prohibited from forming near the planet cell due to activated receptors; these might then influence the local phosphorylation status in the nearby cortex of the neighbour cell.

In addition to spacing divisions, PPBs were also found that predicted amplifying divisions of meristemoids (Fig. 1N), entry asymmetric divisions including in non-neighbour cells (Fig. 1O, P) (Zhao and Sack, 1999), and symmetric division of developing pavement cells (Fig. 2). The latter PPBs predicted the formation of daughter cells of roughly equal size, but not necessarily equal fate, as they can either differentiate into pavement cells or undergo an entry asymmetric division (Geisler *et al.*, 2000). Nothing is known about whether PPB position in the above divisions involves intercellular signalling, as is likely for spacing divisions, or whether cell intrinsic or mitosis-allocated factors determine the division plane.

Cytoplasmic polarities prior to asymmetric division

By the time that a polar-located PPB can be detected, other organelles are also asymmetrically distributed. The nucleus is usually found in the plane of the PPB and is often surrounded by microtubules (Fig. 1M2). Some of these microtubules connect the nucleus to the cortical PPB (Fig. 1J2, K2, R2). PPB-associated nuclei are surrounded by chloroplasts (Fig. 1L2, M2, N2, O2, R2). The vacuole becomes located at the other pole of the cell, a distribution that can readily be visualized using a GFP line that marks the vacuole membrane (Fig. 1S). These polarities were previously documented by electron microscopy for *Arabidopsis* and *Vigna* (Galatis and Mitrakos, 1979; Zhao and Sack, 1999).

The cytoplasmic polarities associated with the PPB are distinctive enough to predict a forthcoming meristemoid division, even when microtubules are not visualized (Fig. 1S). Amplifying divisions of meristemoids usually occur in an inward spiral (Fig. 1B including inset) (Serna *et al.*, 2002). A polar-located nucleus in a meristemoid is often in a position that would predict the next division plane in an inward spiral (Zhao and Sack, 1999).

Neighbour cells that undergo asymmetric spacing divisions vary in size (Fig. 1I–M, 2). As expected, larger neighbour cells show a strong cytoplasmic polarity associated with the PPB (data not shown). A PPB-associated cytoplasmic polarity was also detectable but was less dramatic in some smaller neighbour cells (Fig. 1M).

The majority of *Arabidopsis* neighbour cells in a developing leaf have a nucleus located away from (distal to) the planet cell (Geisler *et al.*, 2003). It is likely that only a fraction of neighbour cells with distal nuclei will undergo spacing divisions. This situation contrasts with developing stomatal complexes in some monocots, where an actin-mediated nuclear migration is tightly coupled to asymmetric division and predicts the location of the PPB (Smith, 2001). Another marker of the division site in larger, more vacuolated plant cells is a sheet of cytoplasm, the phragmosome, which coalesces in the plane where the PPB forms later (Kennard and Cleary, 1997; Mineyuki, 1999; Smith, 2001; Panteris *et al.*, 2004). With regard to *Arabidopsis* neighbour cells, it remains to be determined whether an actin-mediated nuclear migration to a distal position occurs, whether larger cells possess a phragmosome, and whether a sub-population of cells with distal nuclei are identifiably committed to a spacing division.

A polar PPB predicts MMC fate

As indicated, entry and spacing divisions occur in MMCs while amplifying divisions take place in meristemoids. In addition to predicting the location of an entry or spacing division, the PPB identifies cells committed to the MMC fate and to asymmetric division. Only a fraction of

neighbour cells enter the stomatal pathway and undergo a spacing division (Geisler *et al.*, 2000; Nadeau and Sack, 2002). Similarly, only selected cells in a developing leaf epidermis enter the stomatal pathway. Although large endopolyploid pavement cells are not likely to become MMCs (Geisler *et al.*, 2000), on the whole, neither cell size nor position by themselves predict whether a cell will undergo a spacing or entry division. Thus the presence of the PPB is the earliest criterion known to indicate an MMC fate (Fig. 1J–M, O, P). MMCs were previously identified based upon their division behaviour using the dental resin impression method (Geisler *et al.*, 2000).

Markers have not yet been identified for cells that are competent for, but not yet committed, to an entry division. Expression driven by the *TMM* promoter seems to mark division-competent cells in the stomatal pathway including, probably, MMCs. More broadly, TMM expression seems to mark a specialized stem cell compartment that functions in stomatal development (Nadeau and Sack, 2002, 2003). Whether early stages of TMM expression specifically mark entry MMCs remains to be determined.

Live cell imaging and timing of early pattern violations

The orientation of entry divisions appears to be unregulated in MMCs that do not touch a planet cell. Such MMCs are either adjacent to each other (Fig. 1F) or are isolated (Fig. 1C, D). The divisions of adjacent MMCs frequently result in pattern violations in the form of meristemoids in contact (Fig. 1E, F3) (Geisler *et al.*, 2000), indicating either the absence or the ineffectiveness of intercellular signalling in division orientation.

By contrast to adjacent MMCs, signalling does appear to take place between adjacent meristemoids (Fig. 1F3, white arrow). Support for this view comes from studies of the same cells through time showing that meristemoids that are in contact usually divide away from each other, thereby correcting the pattern violation (Fig. 1F3) (Sachs, 1991; Geisler *et al.*, 2000). Also, meristemoids that are in contact are not uncommon in developing leaves of *Arabidopsis* (Fig. 1E) and in other dicots (Galatis and Mitrakos, 1979), whereas stomata that are in contact are rare.

Live cell imaging was used to analyse the behaviour of adjacent MMCs with respect to the timing and placement of their respective entry divisions. Some divisions occurred simultaneously, producing either correctly placed meristemoids or meristemoids that were in contact (Fig. 1F, arrows 1 and 3). In other cases, the timing of one MMC division was delayed relative to the other, with the PPB predicting a division plane that would place the new meristemoid in contact with the older one (dashed line, Fig. 1F2, P). Cases were also found where three successive asymmetric divisions of adjacent cells caused three meristemoids to form that were in contact (data not shown). These observations suggest that spacing errors are likely to occur not just when entry divisions occur simultaneously, but when they take place in close succession.

These results suggest that the difference between spacing divisions in neighbour cells and those in adjacent meristemoids can become blurred depending upon the time interval between the asymmetric divisions of two MMCs that are in contact. If this delay is relatively long, then the lagging MMC probably undergoes a spacing division in which the first-formed meristemoid functions as a planet cell and signals a correct division orientation (Fig. 1F arrow 4, M). If the delay is brief, then the extent of signalling might be insufficient to prevent meristemoids from forming that are in contact (Fig. 1F arrow 2). These predictions should be testable by studying the behaviour of MMCs that are in contact in a larger sample of living cells. Again, visualization of microtubules and cytokinesis should allow the relative timing of divisions to be more directly evaluated than using the dental impression method.

GMC arrays, symmetric division, and the initiation of stomatal morphogenesis

The microtubule arrays discussed above are involved in asymmetric divisions and in shaping meristemoids. The arrays present during GMC and stomatal development relate primarily to symmetric division and to stomatal morphogenesis (Fig. 3M).

After a meristemoid stops undergoing amplifying divisions, it converts into a GMC. *Arabidopsis* meristemoids tend to be triangular or partially angular (Fig. 3A), whereas GMCs are more oval, or at least have some convex anticlinal cell walls in surface view (Fig. 3C) (Nadeau and Sack, 2002). Although microtubules might play a role in the change from a triangular to an oval shape, both interphase meristemoids, as well as early GMCs, show comparable mesh-like arrays in the outer cell cortex (Fig. 3A, B).

Later, oval-shaped GMCs display an astral microtubule array which, in turn, gives way to a predominantly longitudinal and broad set of microtubules (Fig. 3C, D). This broad array constricts slightly and becomes more prominent as the PPB forms (Fig. 3E). However, the GMC PPB does not get as narrow as in meristemoids or MMCs. PPB formation seems to coincide with the development of wall thickenings located at the two ends of the GMC (Fig. 3E2). The placement of both the PPB and the end-wall thickenings predict a symmetric division. The PPB disappears by metaphase, and then the mitotic spindle aligns with the end-wall thickenings (Fig. 3F). Cytokinesis starts in the cell centre and proceeds until the cell plate meets the outer walls, bisecting the end-wall thickenings (Fig. 3G). In *Arabidopsis*, neither the anaphase microtubule array nor the cell plate rotate, events that have been shown to occur in onion GMCs (Palevitz, 1986). The end-wall thickenings are of interest for several reasons.

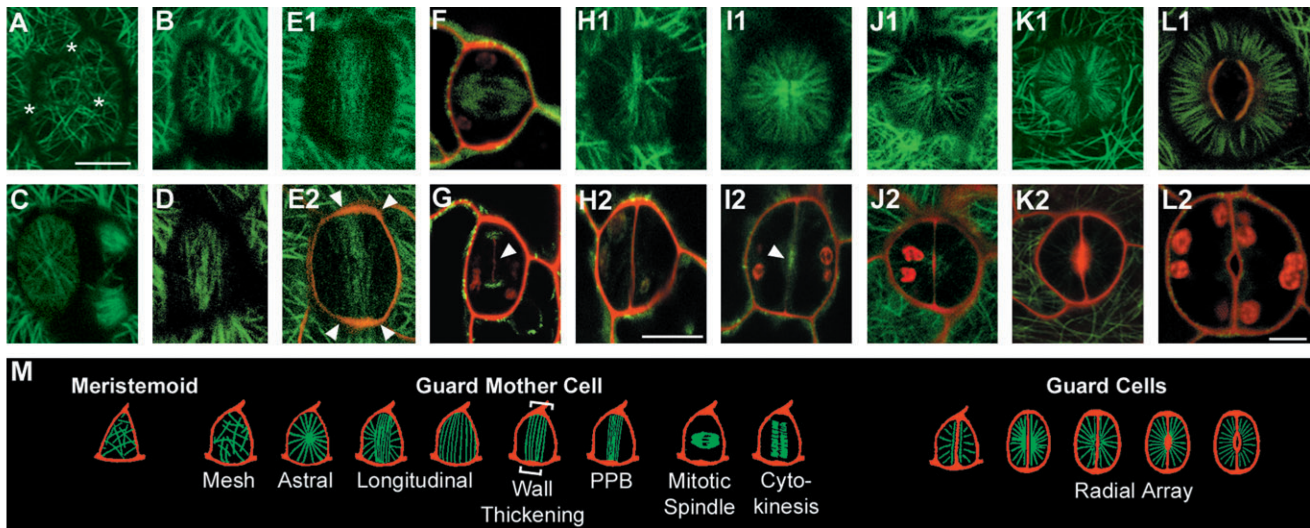

Fig. 3. Microtubule arrays in successive stages of meristemoid, guard mother cell, and stomatal development. Pairs of optical sections in the outer cell cortex (1) and deeper in the cell (2). (A) Triangular meristemoid (located between three asterisks) with a mesh-like microtubule array. (B) Mesh-like array in a young GMC (note slightly angular wall at top right). (C) Astral array in GMC that has a more oval shape. The phragmoplast in the neighbour cell (right) indicates a spacing division. (D) Microtubules aligned along the long axis of the GMC. (E1, 2) GMC with PPB and end-wall thickenings (arrowheads). (F) Metaphase spindle in GMC is aligned with the end-wall thickenings. (G) GMC with a phragmoplast at the ends of the growing cell plate (arrowhead). (H1, 2) Recently-formed guard cells with nascent radial array. Some cortical microtubules are parallel to the new wall which is thin and not yet straight. (I1, 2) Radial arrays in each guard cell focus on where the pore thickening will later develop (arrowhead). (J1, 2) Radial arrays have enlarged and the pore thickening is now visible. (K1, 2) Later stage. (L1, 2) Mature stoma. Chloroplasts are typically located around the nucleus (not shown) which is adjacent to the pore. (M) Drawing summarizing microtubule arrays (green) present during GMC and stomatal development. Cells shown at equivalent size to emphasize differences in arrays. Bar in (A)=5 μm for (A–G); bar in (H2)=10 μm for (H–K); bar in (L2)=10 μm for (L1, 2).

First, they persist after GMC cytokinesis and then thicken during stomatal morphogenesis. They are known to be present in *Arabidopsis* and in legume GMCs (Galatis *et al.*, 1982; Zhao and Sack, 1999; Galatis and Apostolakos, 2004), but not in GMCs of other taxa such as ferns (Apostolakos *et al.*, 1997) and grasses (Galatis and Apostolakos, 2004). It is not known whether such GMC thickenings are widely present in dicots. One function of the thickenings might be to contribute to the mechanics of stomatal opening and closing (Galatis and Apostolakos, 2004). For example, these thickenings might restrict expansion at the ends of the stoma, but promote bowing out along the sides.

Second, since end-wall thickenings initiate in the GMC, stomatal morphogenesis begins before the guard cells form. This scenario is also supported by the oval shape of the GMC which anticipates the shape of the stoma in paradermal view.

Third, like the PPB in GMCs, these wall thickenings predict the site of cell plate fusion with the parent GMC wall and thus can be considered to be part of the division site (Zhao and Sack, 1999). GMCs are the only plant cell type known to have wall thickenings in addition to the PPB at the division site (Galatis and Apostolakos, 2004).

Fourth, the relative timing of PPB formation and end-wall thickening has not been precisely established. However, it is possible that the GMC PPB, in addition to revealing the division site, also directs localized wall synthesis. Differential wall thickening often coincides with local aggregations of microtubules in plant cells, such as in the radial microtubule array described below for stomata. Some early observers suggested that the PPB generally guides local wall deposition in many types of plant cells (Packard and Stack, 1976; Mineyuki, 1999), but only GMCs are known to produce a local wall thickening when the PPB is present.

Fifth, these thickenings are useful for analysing mutant phenotypes since the thickenings can readily be visualized by light microscopy and are a stage-specific GMC marker (Fig. 3E) (Galatis *et al.*, 1982; Lai *et al.*, 2005). An example is that *four lips* mutants form stomata that are in contact by inducing a persistent GMC fate, as shown, in part by the reiteration of wall thickenings in GMC daughter cells. Also, the timing of expression driven by the *FOUR LIPS* promoter coincides with wall-thickening formation and GMC division, suggesting that this MYB transcription factor normally limits the symmetric division of the GMC to one.

As far as is known, GMCs are a universal feature of stomatal development (Sack and Paolillo, 1985; Galatis and Apostolakos, 2004). This comparison of *Arabidopsis* GMC arrays to other taxa is limited here to ferns and grasses since the development of dicot GMCs has not otherwise been studied by light microscopy. Fern GMCs display very robust astral-like arrays that are probably related to specialized wall constrictions and thickenings (Apostolakos *et al.*, 1997). *Selaginella* GMCs possess unusual arrays likely to function specifically in duplicating and allocating the single chloroplast during cytokinesis (Cleary *et al.*,

1992). Grass GMCs have interphase microtubule bands of microtubules that form before and are perpendicular to the subsequent PPB (Galatis and Apostolakos, 2004). None of the above GMC arrays were found in *Arabidopsis*. However, some GMC arrays found in monocot GMCs resemble those in *Arabidopsis* such as the astral array and the wide PPB (Mineyuki *et al.*, 1989).

These data raise questions about the functions of *Arabidopsis* GMC arrays, such as whether the astral array helps generate the oval cell shape, why the PPB is less condensed in the GMC than in other cell types, whether PPB breadth affects the precision of where the new cell wall is placed, and whether the PPB also functions in the synthesis of the end wall thickenings.

Guard cell radial arrays from start to finish

Kidney-shaped guard cells contain a striking and unusual array of radially-oriented microtubules that converge near the central rim of the stomatal pore (Hepler and Palevitz, 1974; Palevitz, 1981; Sack, 1987; Galatis and Apostolakos, 2004). The assembly of this array starts just after GMC division (Fig. 3H). This array becomes prominent before any thickenings in the new wall can be detected, and the locations of the array foci predict where pore wall swelling will occur later (Fig. 3I). Each array mirrors the other in developing, adjacent guard cells (Fig. 3I1). The focus of each array is connected to microtubules that traverse the depth of the guard cell (Fig. 3I2). Radial arrays are still present during pore opening and stomatal maturation (Fig. 3J–L).

These observations are consistent with previous ultrastructural and immunofluorescence data on microtubules in developing dicot and monocot stomata (Galatis and Mitrakos, 1980; Palevitz, 1981; Marc *et al.*, 1989). The radial co-alignment of cellulose in developing stomata might result from microtubules positioning cellulose synthase complexes, although the relationship between the two structures might be more complex (Wasteneys, 2004). The presence of radially organized microtubules in mature as well as developing stomata (Fig. 3L) suggests that this array has roles in addition to orienting cellulose.

By visualizing microtubules in living cells it has been possible to define the stages and events in the stomatal pathway more closely than by using dental impression methods. These results establish a baseline for analysing events in the pathway such as the development of intracellular polarities, the intercellular signalling that affects stomatal spacing and distribution, the development and function of the division site, and the co-ordinated morphogenesis of the stoma. This approach also provides an accessible and rich framework for testing the function of cytoskeletal-related proteins and of genes tied to the stomatal pathway by expression or phenotype.

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