

ULTRASTRUCTURE OF STOMATAL DEVELOPMENT IN *ARABIDOPSIS* (BRASSICACEAE) LEAVES¹

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Stomatal development was studied in wild-type *Arabidopsis* leaves using light and electron microscopy. Development involves three successive types of stomatal precursor cells: meristemoid mother cells, meristemoids, and guard mother cells (GMCs). The first two types divide asymmetrically, whereas GMCs divide symmetrically. Analysis of cell wall patterns indicates that meristemoids can divide asymmetrically a variable number of times. Before meristemoid division, the nucleus and a preprophase band of microtubules become located on one side of the cell, and the vacuole on the other. Meristemoids are often triangular in shape and have evenly thickened walls. GMCs can be detected by their roughly oval shape, increased starch accumulation, and wall thickenings on opposite ends of the cells. Because these features are also found in developing stomata, stomatal differentiation begins in GMCs. The wall thickenings mark the division site in the GMC since they overlie a preprophase band of microtubules and occur where the cell plate fuses with the parent cell wall. Stomatal differentiation in *Arabidopsis* resembles that of other genera with kidney-shaped guard cells. This identification of stages in stomatal development in wild-type *Arabidopsis* provides a foundation for the analysis of relevant genes and of mutants defective in stomatal patterning, cell specification, and differentiation.

Key words: *Arabidopsis*; asymmetric division; Brassicaceae; differentiation; division site; guard cell; meristemoid; stomate.

The formation and distribution of stomata are integral to epidermal and leaf architecture and to photosynthesis and crop productivity. Stomata are also an important system for studying plant cell patterning, partly because their development involves such key processes as asymmetric divisions that are involved in stomatal spacing and in specifying cell fate (Galatis and Mitrakos, 1979; Horvitz and Herskowitz, 1992; Sachs and Novoplansky, 1993; Sylvester, Smith, and Freeling, 1996; Larkin et al., 1997; Croxdale, 1998).

Stomatal development and differentiation have been described (reviewed in Palevitz, 1981; Sack, 1987; Larkin et al., 1997), but there are few cytological studies of the development of the kidney-shaped stomata of dicotyledons (e.g., Landré, 1972; Galatis and Mitrakos, 1979, 1980; Galatis et al., 1982). Moreover with the emergence of *Arabidopsis* as a valuable genetic system for identifying genes involved in cell patterning and development and with the identification of several stomatal mutants in *Arabidopsis*, it is important to identify key ultrastructural events and stages leading to the formation and differentiation of wild-type stomata (Yang and Sack, 1995; Larkin et al., 1997; Schiefelbein, Masucci, and Wang, 1997; Geisler, Yang, and Sack, 1998).

Ultrastructural data in the literature on *Arabidopsis* stomata are confined to a few images of mature cells (Hippe-Sanwald, 1993; Lascève, Leymarie, and Vavasseur, 1997). Aspects of the distribution and timing of stomatal formation in *Arabidopsis* are known (Pyke, Marrison and Leech, 1991; Telfer and Poethig, 1994; Berger et al.,

1998), but only preliminary cytological data have been published on precursor cells and on stomatal development in this plant (Yang and Sack, 1995; Serna and Fennell, 1997). Here we describe, using light and electron microscopy, the types of divisions of stomatal precursor cells and the major morphologically detectible stages that characterize stomatal development in *Arabidopsis*.

MATERIALS AND METHODS

Plant material—Seeds of *Arabidopsis thaliana*, (Brassicaceae), Columbia ecotype, were planted in plastic pots filled with peat, perlite, and vermiculite (Promix). Plants were grown under continuous light from “Cool White” fluorescent lamps (General Electric Co.) at a light intensity of $\sim 150 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ and a room temperature of $\sim 23^\circ\text{C}$. Unless otherwise noted, all observations were derived from examination of the first pair of leaves, which are subopposite.

Microscopy—Differential interference contrast microscopy was used to examine hand-cut sections of fresh leaves. Possible division patterns from fixed tissues were reconstructed as in Paliwal (1967) and Landré (1972) using leaves from 3- to 21-d-old seedlings that were cleared according to Geisler, Yang, and Sack (1998). Cleared material was also used to calculate cell size, which was estimated by tracing the paradermal outline of cells of different stages from a bright-field videomicroscope image onto an acetate overlay on a video monitor. The outlines were digitally scanned and areas were determined for 60 cells of each stage using NIH image analysis software.

For transmission electron microscopy, leaf pieces from 5- to 15-d-old seedlings were fixed in 3% (v/v) glutaraldehyde in 75 mmol/L phosphate buffer at 4°C for 6–8 h. The tissue was then rinsed, postfixed in 2% (w/v) osmium tetroxide for at least 3 h, dehydrated in an acetone series, and embedded in Spurr's epoxy resin. Thin sections cut with a diamond knife were stained with aqueous uranyl acetate and lead acetate and examined at 60 kV with a CM-12 Phillips electron microscope.

RESULTS

Overview of cell types and divisions leading to stomatal differentiation—The scheme of stomatal development shown in Fig. 1 is based upon the examination

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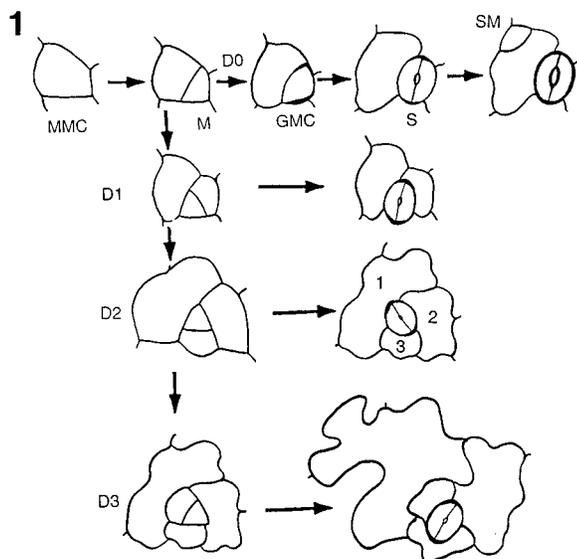


Fig. 1. Summary of key cell types and some of the patterns of divisions in stomatal initiation and development in *Arabidopsis* leaves. Asymmetric division of meristemoid mother cell (MMC) produces a meristemoid (M), which converts into a guard mother cell (GMC). A symmetrical division of the GMC connecting to end wall thickenings produces two guard cells forming the stomate (S). The meristemoid may divide asymmetrically one to three times (D1 to D3) before converting into a GMC, but this conversion also appears to occur without a division of the meristemoid (D0). Some stomatal complexes are anisocytic with three neighbor cells of different sizes (D2, at right, cells numbered 1–3). Neighbor cells can also divide asymmetrically producing a satellite meristemoid (SM).

of many leaf regions at various stages and on the analysis of patterns of cell walls in different developing stomatal complexes. Stomatal development involves the sequential activities of three different precursor cells. The first stomatal precursor cell is termed a meristemoid mother cell (MMC). The MMC divides asymmetrically to produce a meristemoid, the second type of precursor cell in the pathway. Meristemoids, the smaller cell product of the asymmetric division, are often triangular in shape in paradermal section. Extrapolations from the size of cells and the arrangement of cell walls surrounding meristemoids suggest that meristemoids can divide 0–3 times (abbreviated D0 to D3 in Fig. 1). Each division of a meristemoid results in a larger cell (a potential neighbor cell) and a smaller cell product, which can function again as meristemoid. The third type of stomatal precursor cell, the guard mother cell (GMC), arises directly from a meristemoid. GMCs are more rounded and are often oval in shape. The GMC divides symmetrically producing two cells that develop into guard cells that form the stomate.

Some epidermal cells adjacent to stomata (neighbor cells) divide asymmetrically to produce a precursor cell termed a satellite meristemoid (SM) here, but referred to as a secondary meristemoid by Landré (1972). The SM also can divide asymmetrically one or more times before becoming a GMC. With respect to timing, *Arabidopsis* stomata develop in a mosaic fashion, and several stages can often be seen side by side (Figs. 2–3).

Some stomata are surrounded by one smaller neighbor cell and two other larger neighbor cells (Fig. 1, D2), an

anisocytic type of stomatal complex that is characteristic of the Brassicaceae (Metcalf and Chalk, 1950). One way that this pattern appears to arise is from meristemoids that divide twice (D2, Fig. 1); thus, these D2 complexes are produced by a total of three asymmetric divisions including that of the MMC.

Meristemoid formation and cytology—The earliest cytological event detected in stomatal precursor cell formation was the polarization of cytoplasm in the meristemoid mother cell. Prior to the asymmetric division of the MMC, the nucleus and most of the cytoplasm become located on one side of the cell near the apparent division site, the future location of cell plate fusion with the parent wall (Fig. 4). The division site in MMCs appeared marked by a weak preprophase band of microtubules (PPB) consisting of a single layer (Figs. 4–6). The cell wall at the division site has the same thickness as elsewhere in the cell.

MMCs were found in regions devoid of stomata as well as in cells next to stomata. Asymmetric divisions of the latter type of MMC produced satellite meristemoids. These divisions are oriented such that the satellite meristemoid (and the GMC it produces) is placed away from the already existing stomate (Figs. 1,7).

Meristemoids are recognizable as the smaller cell product of an asymmetric division (Fig. 8). When first formed, meristemoids are densely cytoplasmic with few vacuoles and with the nucleus in the center of the cell (Fig. 8). In contrast, the larger cell product of the asymmetric division has a large central vacuole and the cytoplasm is confined to a thin peripheral layer (Fig. 8). Meristemoid plastids lack starch and have a poorly developed thylakoid system (Fig. 9). In paradermal sections, the meristemoid often appears triangular in outline, although one or more walls may be slightly bowed and other shapes can be found. The cell walls of the meristemoid are thin and of uniform width. Meristemoids (identified as roughly triangular cells in the light microscope) have a mean paradermal area of 40.1 ± 1.1 (SE) μm^2 with a mean length of $\sim 9 \mu\text{m}$.

Older meristemoids that appear to be premitotic display a polar distribution of cell contents with a vacuole at one end, and the nucleus and a preponderance of cytoplasm at the other end (meristemoid “b” in Figs. 2 and 9). Relatively few meristemoids were found at this stage in fixed and sectioned material. In several polarized meristemoids, a weak preprophase band of microtubules (PPB) appeared to be present in the form of a mostly single layer of aggregated cortical microtubules on both sides of the nucleus (Figs. 9–11). This band was located in a position that would predict the formation of a smaller triangular cell and a larger sister cell. No cell wall thickenings were detected at the apparent division site.

The large vacuole eventually becomes allocated to the larger daughter cell of the asymmetric division of the meristemoid. In some divisions, there was not a great disparity in the sizes of the two daughter cells (e.g., Fig. 12). Even in these cases, there usually was a difference in the shape and in the extent of vacuolation of the two daughter cells, the triangular cell, presumably the regenerated meristemoid, being less vacuolated.

It is likely that the positions of some of the asymmetric

divisions of meristemoids are spatially regulated. The two meristemoids shown in Fig. 2 appear to be part of what might have become anisocytic stomatal complexes. Meristemoid "a" is surrounded by three vacuolated cells whose outer walls (away from the meristemoid) are almost continuous, a pattern consistent with the possibility that the meristemoid arose from the division of a single MMC and then divided twice (a D2 meristemoid, see Fig. 1). If so, then the larger cell products from the two asymmetric divisions of the meristemoid were placed to the outside of the cell lineage. An earlier stage of such a polar placement may be shown in meristemoid "b" in Fig. 2. If this meristemoid were also part of an anisocytic complex, then the polarity of its probable second division would have been positioned such that the vacuole would be to the outside; a subsequent division would thus have positioned the larger cell product, the neighbor cell, to the outside, producing an anisocytic stomatal cell complex and thus surrounding the future stomate with three neighbor cells produced from the same MMC (see Fig. 1). However, not all stomatal complexes are anisocytic, e.g., some complexes contain four cells (Fig. 2, top left). It is difficult to reconstruct a likely history of divisions from cell wall patterns in some stomatal complexes.

Guard mother cell development and division—After the meristemoid has finished dividing, it converts into a guard mother cell, the final stomatal precursor (Fig. 1). Young GMCs can be distinguished morphologically from meristemoids by their increased accumulation of starch in plastids, by their larger vacuoles, and by their more rounded shape (compare Fig. 13 with Figs. 3, 7, 14). Mature guard mother cells that are oval in shape are about twice the paradermal area of meristemoids and have a mean area of $74.3 \pm 2.0 \mu\text{m}^2$ with mean lengths and maximal widths of 11.4 ± 0.2 and $9.4 \pm 0.2 \mu\text{m}$, respectively.

A prominent and distinctive feature of mature GMCs is the presence of two cell wall thickenings at opposite ends of the cell (Figs. 15–17). These are ~ 0.2 – $0.4 \mu\text{m}$ thick and 2.7 – $3.4 \mu\text{m}$ long. The rest of the cell wall is typically 0.05 – $0.09 \mu\text{m}$ thick. Some thickenings span cell junctions or corners (Fig. 15, lower left), and other thickenings are entirely located between cell corners (Fig. 15, upper right). In GMCs that are somewhat elongated, these thickenings are usually perpendicular to the long axis as seen in paradermal section (Figs. 14–15), and this position at the ends of the cell is often maintained during GMC expansion.

Prior to and during early wall thickening, microtubules, often as a single cortical layer, are present on opposite sides of the GMC, either under new, developing thickenings (Fig. 14) or in locations where presumably the thickenings subsequently arise. Cortical microtubules are less frequent elsewhere in the GMC at these stages. During GMC expansion the wall thickenings increase in width (Figs. 14–15), a widening accompanied by the formation of a PPB with several layers of microtubules located opposite the thickenings (Figs. 15–17).

During GMC telophase, the reforming nuclei and the cell plate are aligned at right angles to the wall thickenings (Fig. 18). Both early and late cell plates are sometimes slightly curved or zigzag-shaped. The cell plate fuses

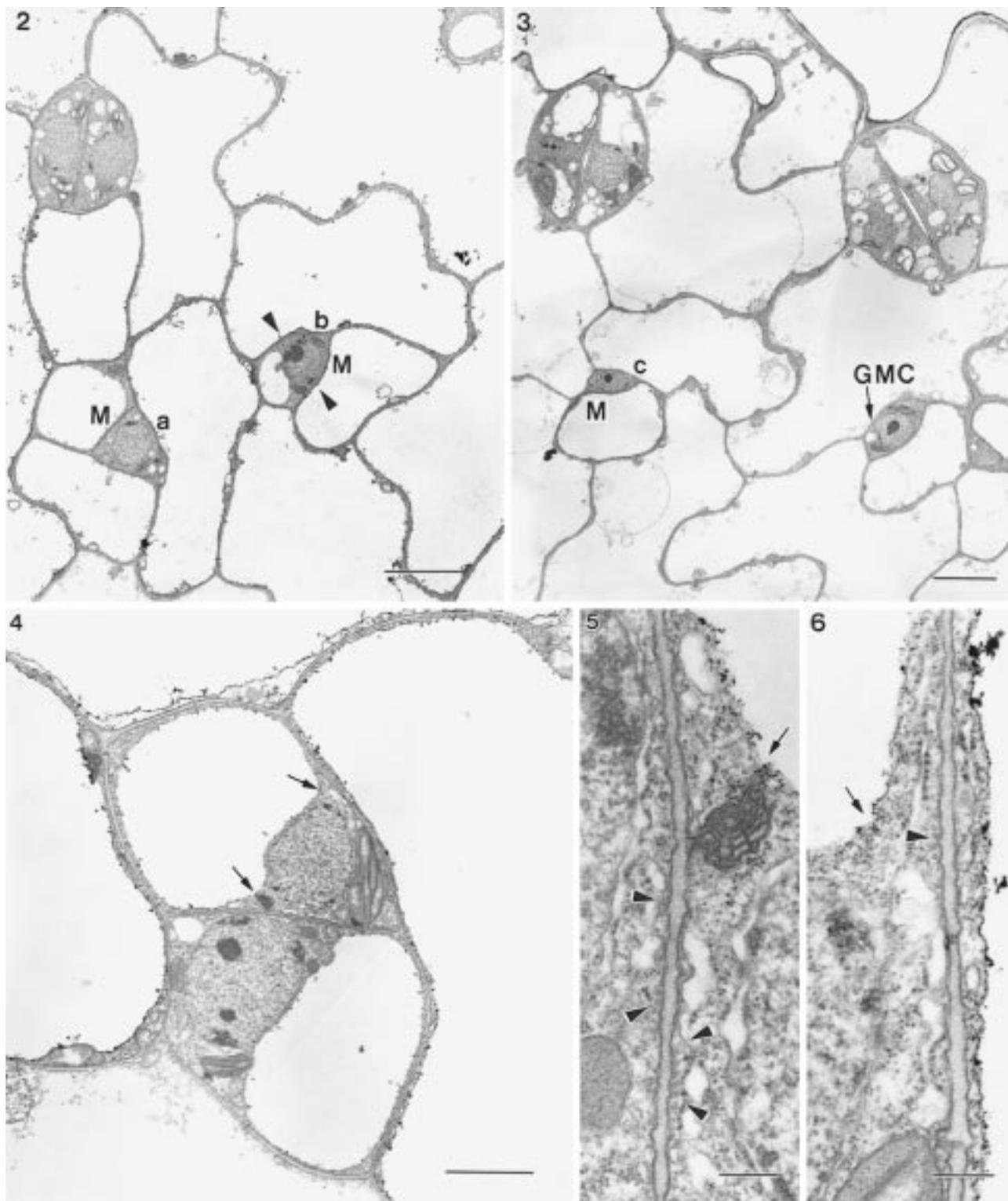
with the wall thickenings and frequently bisects them (Figs. 19–20). The wall thickenings are still evident in developing stomata (Figs. 20–23). Thus, the wall thickenings mark the location where the cell plate will fuse with the parent cell wall. This division site is placed such that cytokinesis is symmetrical and produces two guard cells of equal size and fate.

Guard cell and stomatal development—Following cytokinesis, both guard cells expand along their long and short axes, and each guard cell becomes approximately semicircular in paradermal section (Figs. 21–23). The new cell wall, the ventral wall, is often somewhat undulate, but it soon becomes straight (Figs. 20–21). Plasmodesmata are located throughout all cell walls in newly formed guard cells.

The pore develops from the ventral wall. Early on, microtubules are abundant along the length of the ventral wall. Portions of the middle lamella between the two ventral walls become electron lucent. Then a lens-shaped thickening develops at the mid-length of the ventral wall (Figs. 21–22) where the pore eventually forms. Anticlinally oriented cortical microtubules are concentrated around the growing pore thickening. The middle lamella at the pore site becomes electron dense (Fig. 22). The pore is created by a separation at the middle lamella that appears to involve the stretching of electron dense material (Fig. 23). In the mature stomate, the surface of the pore is somewhat undulate, often in a complementary fashion on opposite sides of the pore as if these ribs and grooves might fit together upon full closure (Fig. 24). Pore opening results in the attainment of the typical kidney- or bean-shaped guard cell (Fig. 25). Parts of the separating pore wall can sometimes be seen as strands connecting the two sides of the pore (arrowhead in Fig. 26).

In addition to the pore site, other parts of the wall thicken differentially (Figs. 26–28). The guard cell walls are especially thick under the outer ledge (protuberance) that faces the atmosphere and at the part of the pore wall and the inner periclinal wall that face the substomatal chamber. The wall is also thickened where the outer periclinal wall joins the neighbor cell. The wall is thinner between this junction and the ledge-associated thickening. The anticlinal wall between the guard cell and the neighbor cell (the dorsal wall) is even and thin throughout. The cell wall displays layers of varying electron densities, especially in the thickened regions and at the cuticularized ledges. The ledges extend to the ends of the pore (along the length) where they never separate (Fig. 28). These form enclosed air spaces at both ends of the pore. During wall thickening, the plasmodesmata become truncated and embedded in wall material. The wall thickenings found in the GMC usually become obscured after pore formation due to the overall growth of the anticlinal walls (Figs. 24–25).

Various cytoplasmic changes accompany stomatal maturation. Starch accumulation increases strongly after the pore opens (Figs. 23–25). Vacuole enlargement occurs prior to and during pore formation (Figs. 21–24). In mature (functional) stomata, large vacuoles are located at both ends of the guard cell, and the nucleus is usually located opposite the pore and closer to the inner parad-



Figs. 2–6. Electron micrographs of stomatal precursor cells. 2, 3. Low magnification paradermal views of the developing abaxial epidermis of the same leaf from a 10-d-old *Arabidopsis* seedling. Different stages of stomatal development are present in the same fields. Note the small size and the densely cytoplasmic content of meristemoids (M, labeled a–c in Figs. 2–3), and of a guard mother cell (GMC in Fig. 3) compared with other epidermal cells that are highly vacuolated. Meristemoids “a” and “b” (Fig. 2) would probably have developed into anisocytic stomatal complexes after two divisions of each meristemoid. The proposed second division is complete for meristemoid “a” but not yet complete for meristemoid “b,” which would probably have divided in the plane shown by the two arrowheads. A non-anisocytic stomatal complex can be seen with four neighbor cells (Fig. 2, top left). Bars = 5 μ m. 4. Two apparent meristemoid mother cells in contact. Each cell has a polarized distribution of cell contents with most of the cytoplasm and the nucleus in the smaller corner of each cell and the larger vacuole and parietal cytoplasm occupying the remainder of each cell. The bottom and top arrows indicate the regions shown at higher magnification in Figs. 5 and 6, respectively,

ermal wall (towards the substomatal cavity) of the guard cell (Figs. 24–27).

Mature stomata are on average $22.2 \pm 0.2 \mu\text{m}$ wide and $15.6 \pm 0.2 \mu\text{m}$ long. The mean paradermal area of mature stomata $243.8 \pm 3.9 \mu\text{m}^2$ (including the pore), is three times larger than the GMC and about six times larger than the meristemoid.

DISCUSSION

This study represents the first ultrastructural description of the cytological stages of precursor cell and stomatal development in *Arabidopsis*. It reveals key events such as cell polarities involved in the formation of meristemoids, unique wall thickenings that identify the division site in guard mother cells, and the identification of other markers of different stages of development.

Importance of asymmetric divisions—*Arabidopsis* meristemoids form in an asymmetric division, and meristemoids themselves divide asymmetrically. These divisions are asymmetric in cell fate and also usually in cell size. Premitotic meristemoids also display an asymmetric distribution of the nucleus, cytoplasm, vacuoles, and PPB. Similar polarities have been found in meristemoids in the Fabaceae and in the formation of stomatal subsidiary cells in monocots (Galatis and Mitrakos, 1979; Galatis et al., 1982; Cho and Wick, 1989; Kennard and Cleary, 1997). These premitotic polarities probably function in positioning the future cell wall and in distributing cell contents asymmetrically upon division.

However, some stomatal meristemoid divisions in *Vigna* are reported to be equal in the distribution of vacuoles and in the size of the daughter cells (Fig. 28 in Galatis and Mitrakos, 1979). And there are many examples in non-plant systems of asymmetric divisions producing daughter cells of different fate but with the same size (e.g., Horvitz and Herskowitz, 1992). But in *Arabidopsis* most meristemoid divisions were cytologically polar and unequal in the size of the products. Even in those few cases where the sizes were almost equal, there were still differences in vacuole distribution and in the shape of the daughter cells. The key event in all asymmetric divisions may be the unequal distribution of molecular determinants specifying cell fate (e.g., Rhyu, Jan, and Jan, 1994). It remains to be determined whether the cytoplasmic polarities found in *Arabidopsis* meristemoids are necessary for the allocation of cell fate determinants.

A similar issue concerns the importance of the preprophase band of microtubules in stomatal formation. McClinton and Sung (1997) found that stomata are present in the *fass (tonneau)* mutant of *Arabidopsis*, which does not form a PPB. Assuming that PPBs were also absent from stomatal precursors, then either these asymmetric divisions can occur without PPBs or stomatal formation can bypass asymmetric divisions.

The PPBs found in asymmetric divisions of wild-type *Arabidopsis* stomatal precursor cells seem weaker than in

the symmetric division of the GMC, whereas in legumes no such difference was reported (Galatis et al., 1982). This difference in *Arabidopsis* might result from the period of maximal microtubule accumulation being relatively short, from a fixation protocol that could be sub-optimal for microtubules, or it could be representative for *Arabidopsis*. While PPBs have previously been described in *Arabidopsis*, to our knowledge this has only been at the light microscope level (e.g., McClinton and Sung, 1997).

Asymmetric divisions and formation of stomatal complex—It is likely that the asymmetric divisions that produce the anisocytic stomatal complex in *Arabidopsis* meristemoids are positionally regulated. The anisocytic stomatal complex consists of three neighbor cells of which one is much smaller than the other two. This type of complex is representative for the Brassicaceae and is found in *Arabidopsis* (Figs. 1–3; Metcalfe and Chalk 1950; Paliwal, 1967; Pant and Kidwai, 1967; Landré, 1972; Telfer and Poethig, 1994; Yang and Sack, 1995; Serna and Fenoll, 1997). Analysis of the arrangement of cell walls suggests that anisocytic complexes can be formed by two divisions of the meristemoid (Fig. 1, D2) and that the premitotic polarity and division site for the second division are positioned so that the larger cell product is placed to the outside (Fig. 2).

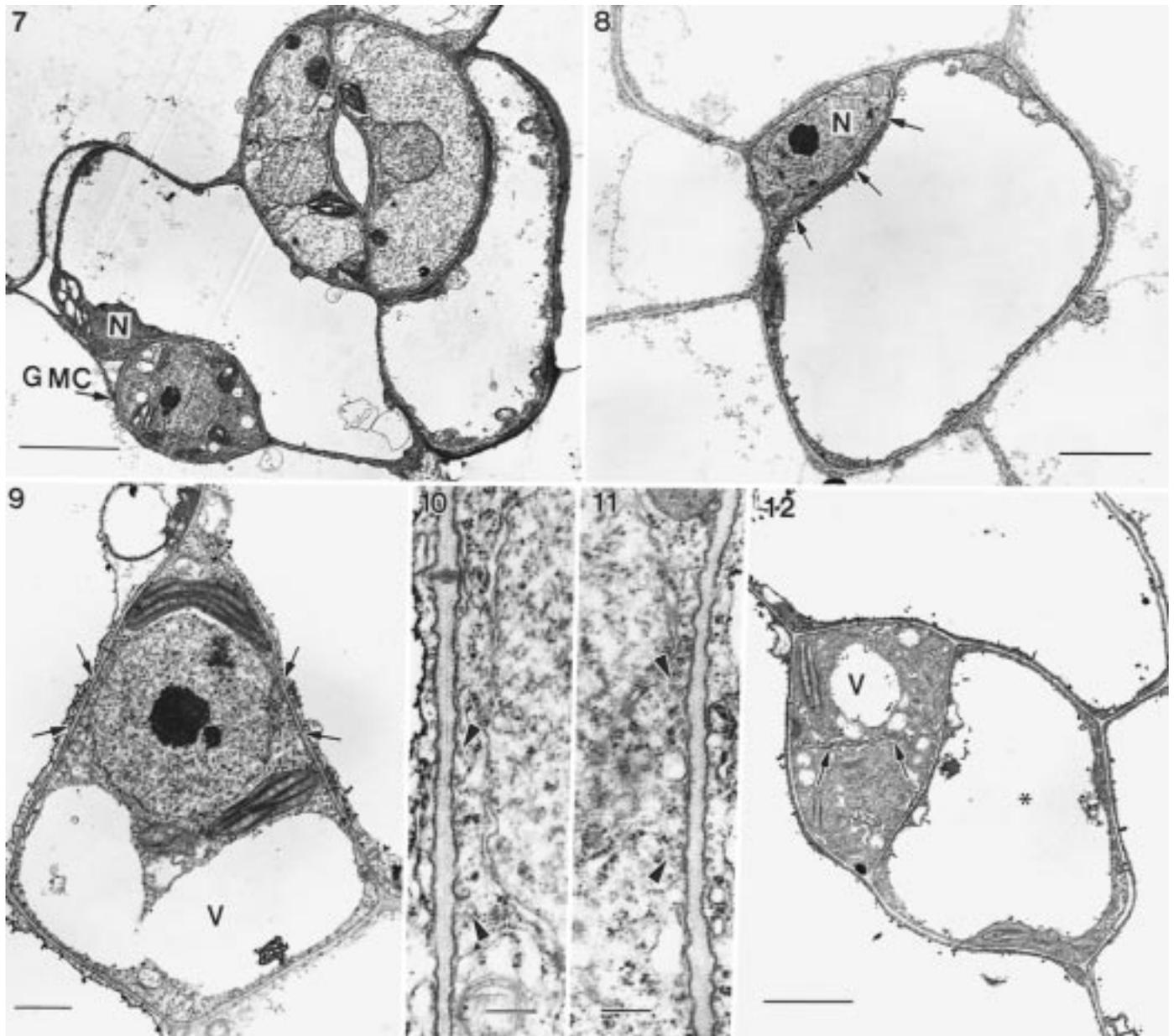
But meristemoids appear to divide a variable number of times, and many stomatal complexes are not anisocytic (this study; Landré, 1972; Galatis and Mitrakos, 1979; Yang and Sack, 1995; Serna and Fenoll, 1997). Berger et al. (1998) also found evidence for a variable number of divisions leading up to stomatal formation in *Arabidopsis* hypocotyls. Given this variability, a more direct description of meristemoid behavior and stomatal patterning will require studying the same cells through time, e.g., using dental resin replicas (Sachs and Novoplansky, 1993).

Meristemoid and guard mother cell markers—Stage-specific markers for different precursor cells are valuable for the cytological, molecular, and genetic analysis of stomatal development and patterning. Meristemoids are distinguishable as small, mostly triangular cells. Newly formed meristemoids are densely cytoplasmic with a central nucleus. In premitotic meristemoids (and MMCs), the nucleus, cytoplasm, vacuoles, and PPB are distributed asymmetrically. Guard mother cells are larger, more rounded, and contain more starch than meristemoids, and GMCs also have wall thickenings at opposite ends of the cell.

Although GMCs originate from meristemoids, no cytological events accompanying this conversion were identified. Cells starting to convert to GMCs may express genes involved in the specification and differentiation of the GMC. If so, these “pre-GMCs” might look like newly formed meristemoids, but not premitotic meristemoids.

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and also roughly indicate the positions of apparent preprophase bands of microtubules. Bar = $2 \mu\text{m}$. **5, 6.** For orientation, the arrows are in approximately the same positions as in Fig. 4. The arrowheads indicate possible preprophase bands. Bars = $0.25 \mu\text{m}$.

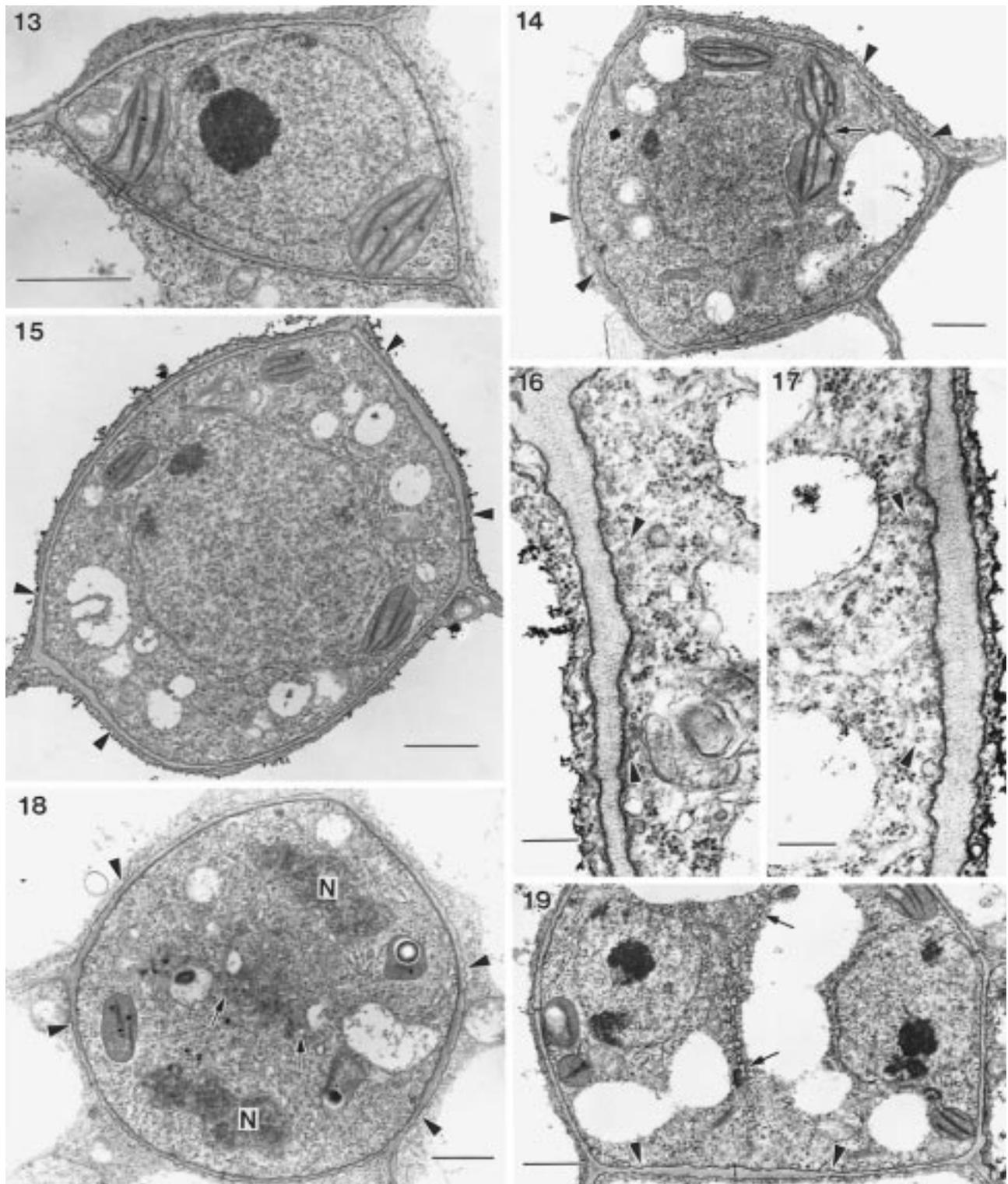


Figs. 7–12. Meristemoid stages. **7.** Stomate and guard mother cell (GMC) separated by intervening larger epidermal cell (N = nucleus in latter cell). It is likely that the GMC and intervening cell were produced by an asymmetric division of the neighbor cell of the stomate. The resultant satellite meristemoid converted directly into a GMC without dividing. Bar = 5 μm . **8.** Apparent newly formed meristemoid (almost filled with a nucleus, N) and a larger sister cell. The arrows indicate the recently formed separating wall. Bar = 2 μm . **9.** Polar distribution of cell contents (note vacuole, V, at one end) in a meristemoid prior to a probable asymmetric division. The arrows mark the limits of the preprophase band of microtubules shown at higher magnifications in Figs. 10–11. Bar = 1 μm . **10, 11.** PPB between arrowheads. Bars 0.2 μm . **12.** Late cytokinesis (arrows indicate cell plate) in a meristemoid. The asymmetry of the division is marked by differences in daughter cell shape and vacuolation (V). Note the triangular shape and absence of large vacuoles in the meristemoid (cell containing arrows). The arrangement of the cell walls suggests that both daughter cells derived from a small cell product of a previous asymmetric division, which also produced a larger cell (asterisk). Bar = 2 μm .

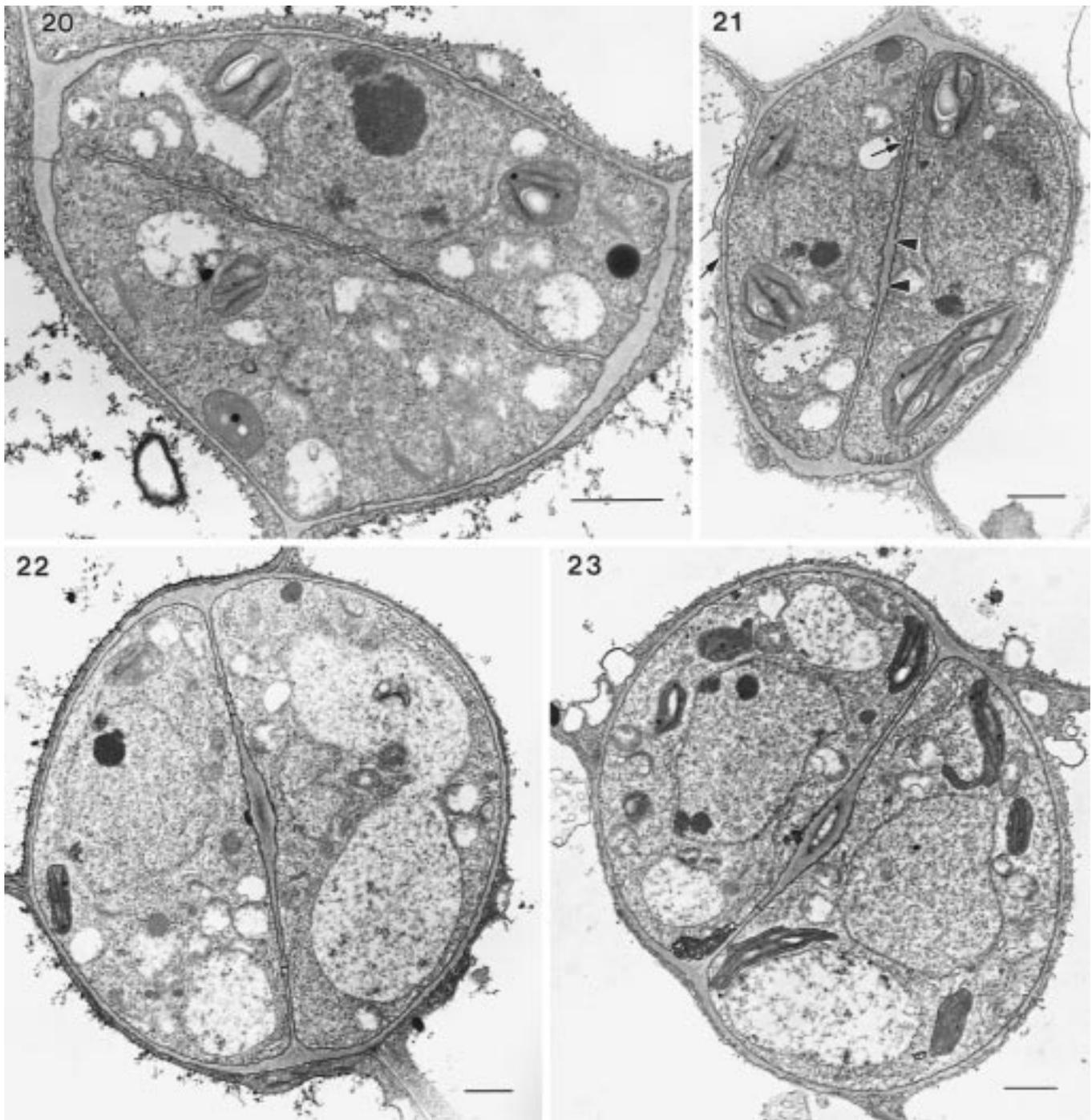
Wall thickenings mark the GMC division site—*Arabidopsis* guard mother cells have specialized wall thickenings at both ends that are later intercepted by the fusion of the cell plate. Thus, these thickenings are a morphological manifestation of the division site in addition to the spatially-associated PPB (Galatis et al., 1982; Mineyuki and Gunning, 1990).

Such thickenings have also been found in GMCs in 20

members of the Fabaceae (Galatis and Mitrakos, 1979; Galatis et al., 1982), but have not been described in GMCs from other families. There has been no systematic study to determine which taxa have GMCs with thickenings, but they appear to be absent from mosses, ferns and grasses (Galatis, 1982; Sack and Paolillo, 1985; Apostolakos, Panteris, and Galatis, 1997). These thickenings are also absent from stomatal meristemoids in *Ar-*



Figs. 13–19. Guard mother cell development. **13.** Early interphase (nonpolar) meristemoid. Bar = 1 μm . **14.** A differentiating GMC with the start of wall thickenings that mark the division site (arrowheads). Note the increase in vacuolation (compare with Fig. 13). The chloroplast at the upper right appears to be dividing. Bar = 1 μm . **15.** Later stage of GMC development. The cell is more elongated, and the wall thickenings (arrowheads) at both ends are more pronounced. Bar = 1 μm . **16, 17.** Enlargements of each end of cell depicted in Fig. 15 showing a well-developed PPB (arrowheads) especially in Fig. 17. This band is located approximately in the middle of the end wall thickenings. Bars = 0.2 μm . **18.** GMC in telophase with reforming nuclei (N) and an early cell plate (arrows) aligned towards the cell wall thickenings (arrowheads). Note starch grain in plastid at right center. Bar = 1 μm . **19.** Late stage of GMC cytokinesis. The cell plate (arrows) is close to fusing with the division site wall thickening (located between arrowheads). Bar = 1 μm .

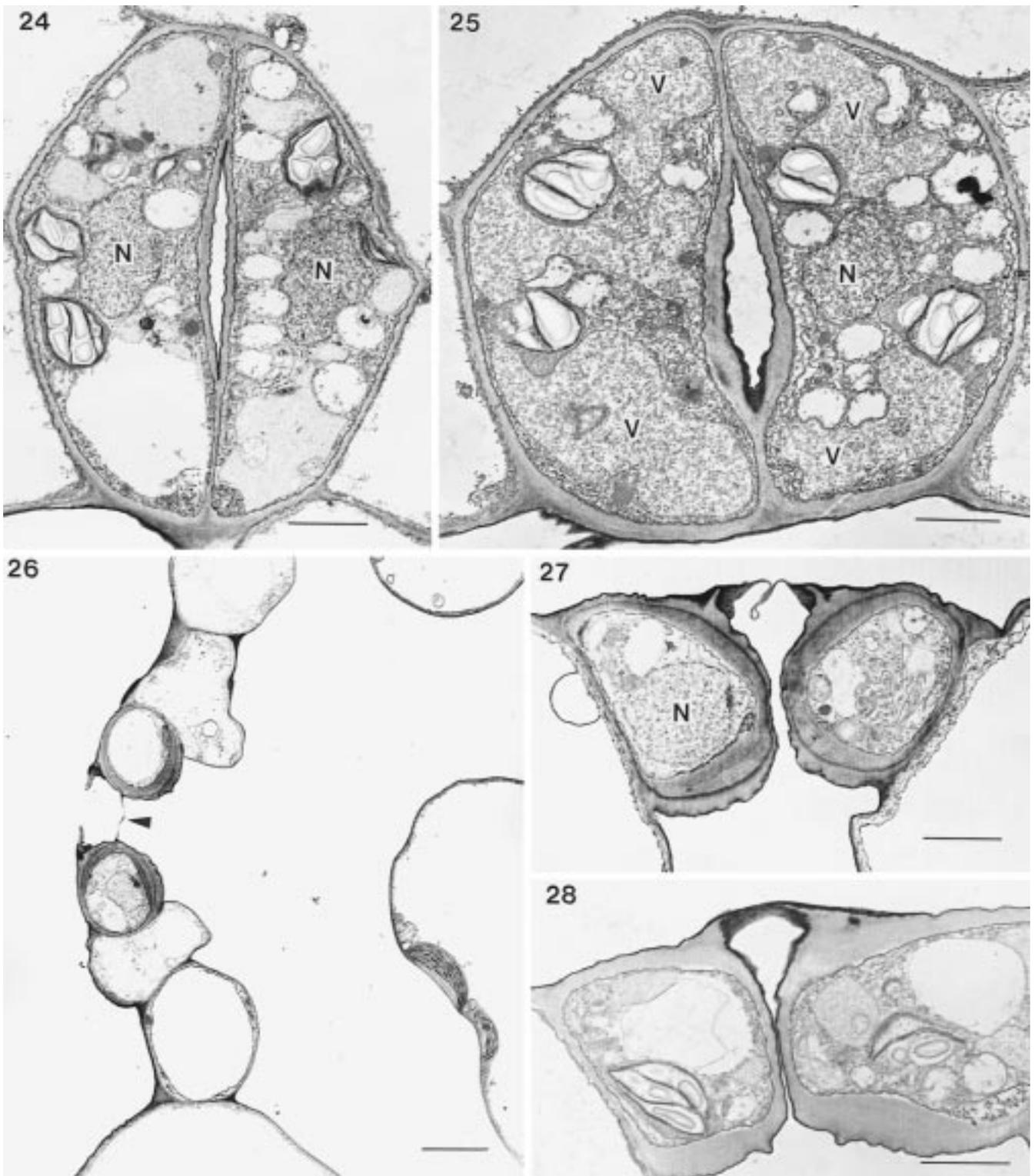


Figs. 20–23. Stages of stomatal development. **20.** Late stage of GMC cytokinesis, as in Fig. 19. **21.** Beginning of swelling of future pore site, which has a middle lamella that is slightly electron-lucent in some regions (arrowheads). Note plasmodesmata (arrows) and starch accumulation. Bar = 1 μm . **22.** Lens-shaped pore site thickening along mid-length of ventral wall with electron-dense middle lamella. Note increased vacuolation and semicircular shape of guard cells. The wall thickenings formed in the GMC are still detectible. Bar = 1 μm . **23.** Pore separation. Bar = 1 μm .

abidopsis and in legumes (this study; Galatis and Mitra-kos, 1979; Galatis et al., 1982). Although Packard and Stack (1976) reported the presence of wall thickenings near the PPB in root cells of *Allium* undergoing symmetric divisions, thickenings were not unequivocally illustrated in their report and, if present, are not as thick or as specialized as in GMCs. To our knowledge, clear

division site wall thickenings have not been described in any cell type except guard mother cells, suggesting that they are a unique feature of GMCs in some dicots.

The function of these wall thickenings is not known. The division site is thought to contain unknown factors that are responsible for guiding the edges of the cell plate to the correct site of insertion and fusion at the parent



Figs. 24–28. Later stages of stomatal differentiation. **24.** Stomate with newly formed pore. Note the complementary, interdigitating undulations on both surfaces of the pore. The nuclei (N) are located near the pore. Bar = 2 μm . **25.** Mature stomate. The GMC-derived wall thickenings are no longer distinct, as many parts of the anticlinal walls have thickened. Note extensive starch accumulation and wall layering. The vacuoles (V) are located at the ends of the guard cells. Bar = 2 μm . **26.** Low magnification electron micrograph of the abaxial epidermis showing the substomatal cavity. The cells adjacent to the guard cells are not as deep as epidermal cells located farther away. The wall strand stretching across the pore (arrowhead) indicates that the pore was still in the process of separation. Bar = 5 μm . **27.** Cross section of stomate. The cell walls are differentially thickened with the thickest walls near the pore and the thinnest between the guard cell and the neighbor cell. Note the wall layering. **28.** Cross section close to the end of the pore where the ledges stay attached. Bar = 2 μm .

cell wall, and other factors that promote the maturation of the new cell wall (Mineyuki and Gunning, 1990). Although the GMC wall thickenings predict where subsequent cell plate fusion will occur, so do PPBs in all cell types in which they occur. PPBs are also present in *Arabidopsis* and legume GMCs (Galatis and Mitrakos, 1979; Galatis et al., 1982). Thus, the GMC wall thickenings may play no direct role in the insertion of the cell plate.

Galatis et al. (1982) suggested that these thickenings set up mechanical forces that promote the splitting of the stomatal pore during development. This possibility is supported by the observation that after pore opening, the end wall thickenings often cease to be distinguishable in legumes and in *Arabidopsis*. Another hypothesis is that the end wall thickenings act earlier in stomatal differentiation by retarding the local expansion of the GMC; this could promote the elongation of the young GMC and the attainment of an oval shape. If so, this would be one more example, along with the initiation of starch accumulation and vacuole enlargement, of stomatal differentiation actually beginning during the guard mother cell phase.

Stomatal differentiation—The events involved in the development and differentiation of the *Arabidopsis* stomate are comparable to those described using electron microscopy for other genera with kidney-shaped guard cells (e.g., Landré, 1972; Galatis and Mitrakos, 1980; Sack, 1987).

The cell biological mechanisms controlling the attainment of the complex architecture of mature stomata are unknown. Although the developmental relationship between the cytoskeleton and the shaping of GMCs and guard cells has not yet been described at a light microscope level in *Arabidopsis* as it has for other genera (e.g., Marc, Mineyuki, and Palevitz, 1989; Apostolakis, Panteris, and Galatis, 1997), *Arabidopsis* stomata do contain a characteristic radial microtubule array that emanates from the cell cortex at developing and mature stomatal pores (L. Zhao and J. Nadeau, Ohio State University, unpublished data; Wasteneys, Willingale-Theune, and Menzel, 1997). But fundamental questions remain about stomatal differentiation, such as how the pore site thickens in a coordinated fashion across two cells, how pore separation is localized and initiated (the only anticlinal walls in the epidermis that do so), how the complex distribution of wall thickenings throughout the guard cell is programmed, and what controls the distinct distribution of cell components (central nucleus, polar vacuoles). A comparable ignorance characterizes the possible role of these and other features, such as guard cell wall layering, in stomatal functioning.

Clearly, stomatal development involves an intricate series of events, some of which are variable, such as the number of asymmetric divisions, and others which are invariant, especially those related to guard cell differentiation. These data on stomatal development will provide a foundation for the analysis of the phenotypes of various stomatal mutants in *Arabidopsis* that affect stomatal patterning, guard mother cell cytokinesis, stomatal differentiation, and stomatal function and signal transduction (Armstrong et al., 1995; Yang and Sack, 1995; Larkin et al., 1997). A coordinated cytological and molecular anal-

ysis of stomatal development and functioning should provide major new opportunities for understanding the mechanisms underlying these processes in an important and fascinating cell type.

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