 ©2002 American Society of Plant Biologists

First published on September 30, 2002: e0066. doi: 10.1199/tab.0066

Stomatal Development in Arabidopsis

Jeanette A. Nadeau and Fred D. Sack[1]

Department of Plant Biology, Ohio State University, 1735 Neil Avenue, Columbus, Ohio 43210
[1] Corresponding author: e-mail: sack.1@osu.edu; fax 614-292-6345; telephone: 614-292-0896

ABSTRACT

Stomata consist of two guard cells around a pore and act as turgor-operated valves for gas exchange. Arabidopsis stomata develop from one or more asymmetric divisions followed by the symmetric division of the guard mother cell. Stomatal number is partly a function of the availability of smaller epidermal cells that are competent to divide asymmetrically. Stomata are spaced apart from each other by at least one neighbor cell. Pattern generation may involve cell-cell signaling that transmits spatial cues used to orient specific classes of asymmetric divisions. TOO MANY MOUTHS may function in receiving or transducing these cues to orient asymmetric divisions. TMM also is a negative or positive regulator of entry into the stomatal pathway, with the direction of the response dependent on organ and location. STOMATAL DENSITY AND DISTRIBUTION1 is a negative regulator of stomatal formation throughout the shoot and encodes a processing protease that may function in intercellular communication. FOUR LIPS apparently controls the number symmetric divisions at the guard mother cell stage. In some organs, such as the hypocotyl, the placement of stomata may be coordinated with internal features and involves genes that also regulate root hair and trichome formation. Other mutations affect guard cell morphogenesis, cytokinesis, and stomatal number in response to carbon dioxide concentration. The molecular analysis of stomatal development promises advances in understanding intercellular signaling, the control of the plane and polarity of asymmetric division, the specification of cell fate, and the regulation of cell differentiation and shape.

INTRODUCTION

Stomata are not only central to plant productivity, but represent an accessible model system for studying cell patterning and specification. Recent progress in Arabidopsis indicates that complex pathways regulate stomatal development and that intercellular communication may guide patterning and stomatal initiation. First we review events that form stomata such as the initiation and regulation of precursor cells, the generation of the spacing pattern, and morphogenesis. Then we discuss the genes and mutations known to affect stomatal initiation, density, patterning, and differentiation.

STRUCTURE AND SIGNIFICANCE OF STOMATA

Stomata are specialized epidermal structures that act as turgor-operated valves for gas exchange (Schroeder et al., 2001). The term “stoma” denotes a mouth in Greek. Just as a mouth contains an opening surrounded by lips, the stoma consists of two guard cells that surround a pore (see list of definitions in appendix; Figure 1). Guard cells are kidney-shaped in dicots.

The stomatal pore connects intercellular spaces inside the plant to the atmosphere (Figure 2). This continuity is critical for plant survival because it allows carbon dioxide to reach mesophyll chloroplasts for photosynthetic fixation. Regulation of pore width restricts water loss. Stomatal opening and closing is controlled by environmental and plant parameters such as water stress and is mediated through complex signal transduction pathways (Schroeder et al., 2001). Several Arabidopsis stomatal

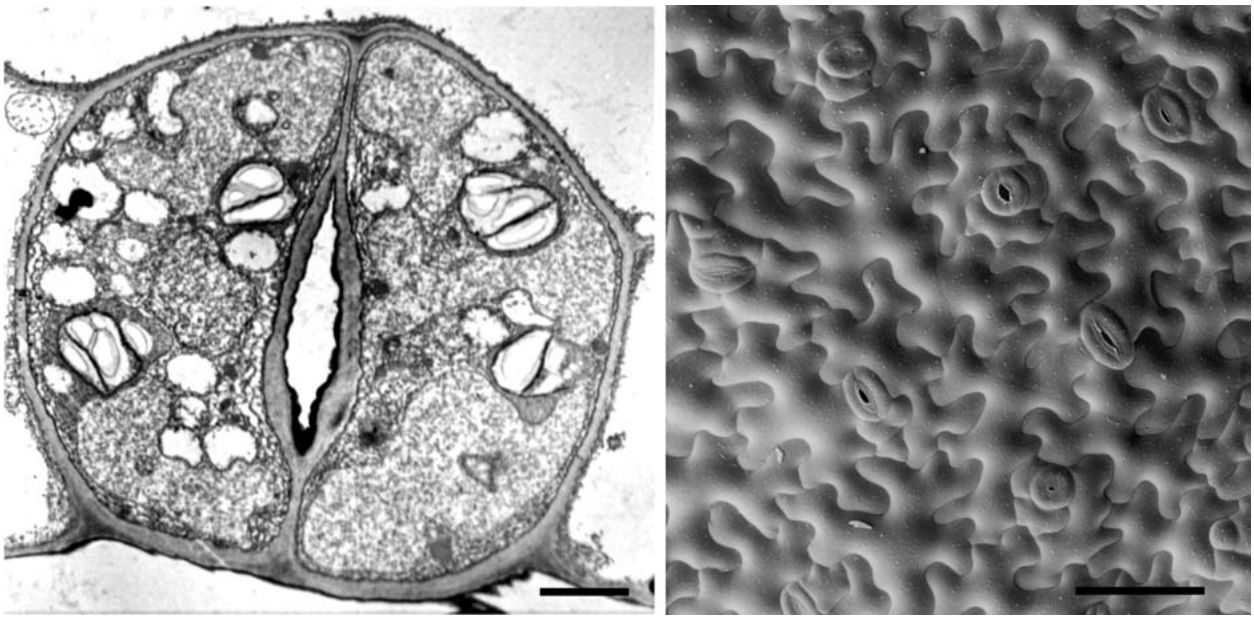

Figure 1. Morphology and Distribution of Arabidopsis Stomata.
(Left) Two kidney-shaped guard cells surround a pore. Transmission electron micrograph from Zhao and Sack (1999).
(Right) Cryo-scanning electron micrograph of maturing epidermis from a cotyledon. The larger, non-stomatal cells are pavement cells that are shaped like pieces of a jigsaw puzzle. Bars = 2 μm (left) and 30 μm (right).

mutants have been isolated that affect these pathways.

Stomatal opening occurs when ions from the surrounding apoplast are imported into guard cells. The resulting increase in hydrostatic pressure reforms the guard cell in three dimensions so that the pore widens (Franks and Farquhar, 2001). The cells that surround the stoma contribute indirectly to stomatal movement by ion exchange. Thus, neighbor cells – cells adjacent to guard cells - are functionally part of a stomatal complex.

The evolution of stomata was a central event in the movement of plants onto land because it allowed gas exchange while limiting desiccation. Stomata are critical for biosphere and crop productivity and for the food chain. The identification of the genes that specify stomatal formation and development should enable phylogenetic studies and the manipulation of crop traits to improve productivity and stress tolerance.

Arabidopsis stomata are also valuable for studying cell development. Their relatively accessible epidermal location has facilitated the isolation of mutants using microscopy-based screens, as well as phenotypic analysis using impression techniques that record the behavior of the same cells through time. Stomatal development displays novel features compared to that of trichomes and root hairs, such as a progression of precursor cells and patterning through asymmetric divisions (Larkin et al., 1997).

DISTRIBUTION AND PATTERNING

Stomatal number varies in different organs of Arabidopsis. Stomatal distribution can also be confined to specific domains such as the sides of the petiole or away from the very edge of leaf. Despite these variations, stomata are present in the mature epidermis of all shoot organs except petals and stamen filaments (Bowman, 1994; Sessions et al., 1997; Geisler et al., 1998).

One-celled spacing pattern. A consistent feature of patterning is that stomata are separated from each other by at least one cell (Sachs, 1991). In Arabidopsis, as well as in other species, the frequency of stomata in contact is much lower than would be found in a random distribution (Figures 1 and 3; Korn, 1972; Sachs, 1978, 1991; Geisler et al., 2000). The stoma-free zone reduces the overlap between gaseous diffusion shells from nearby stomata. This increases the efficiency of each stoma, avoids unnecessary evaporation, and establishes an optimal ratio between CO_2 uptake and photosynthetic capacity. The minimal one-celled spacing pattern also ensures the presence of neighbor cells for ion exchange.

Higher order spacing patterns. In some cases, different stomatal densities result from higher order patterns. For example, more cells separate adaxial (upper epidermis) than abaxial (lower) stomata in Arabidopsis leaves and cotyledons (Serna and Fenoll, 2000a; Geisler et al., 2000; Geisler and Sack, 2002). Variations in spacing may opti-

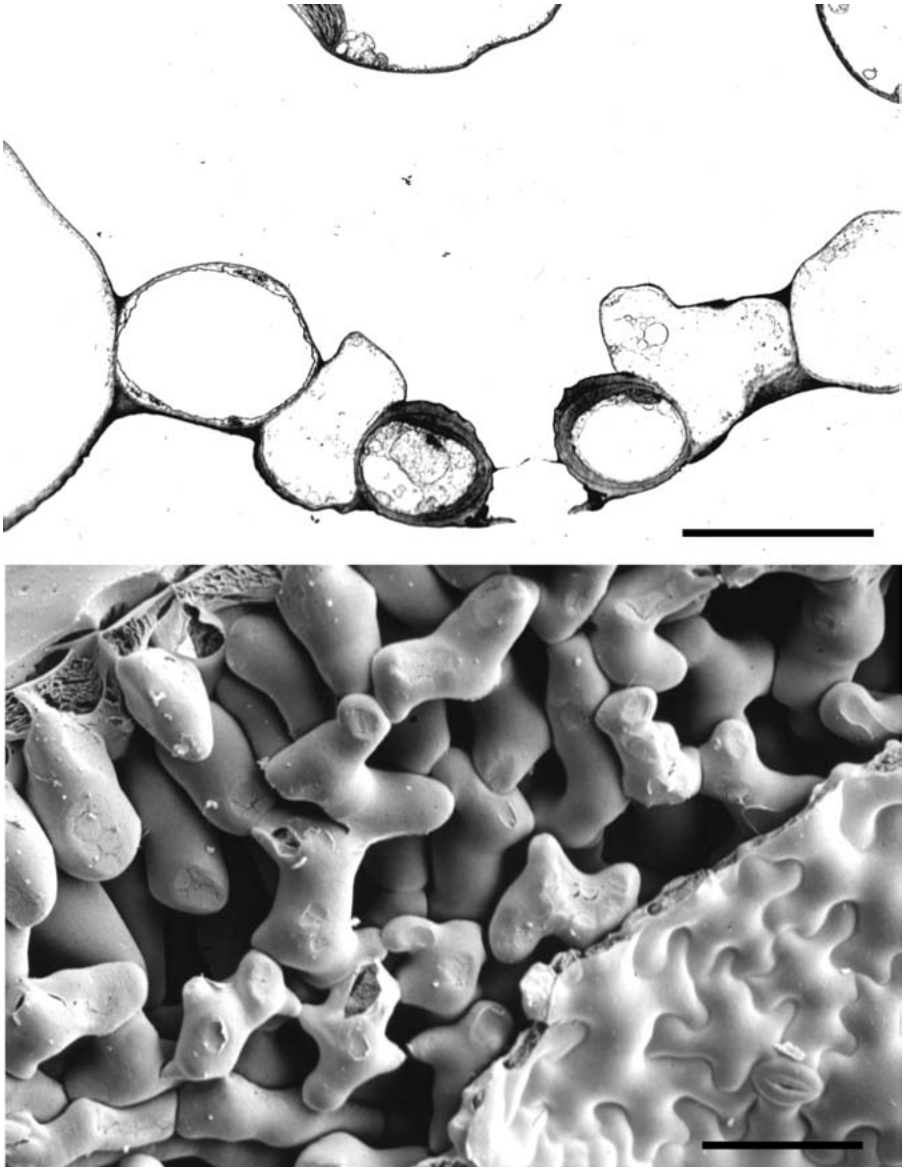

Figure 2. The Stomatal Pore Connects the Atmosphere and Air Spaces within the Leaf.
(Top) Cross section through pore and substomatal cavity. Transmission electron micrograph from Zhao and Sack (1999).
(Bottom) Cryo-scanning electron micrograph showing abaxial epidermis with one stoma at lower right, spongy mesophyll cells at center, and palisade mesophyll cells at upper left. Bars = 10 μm (top) and 40 μm (bottom).

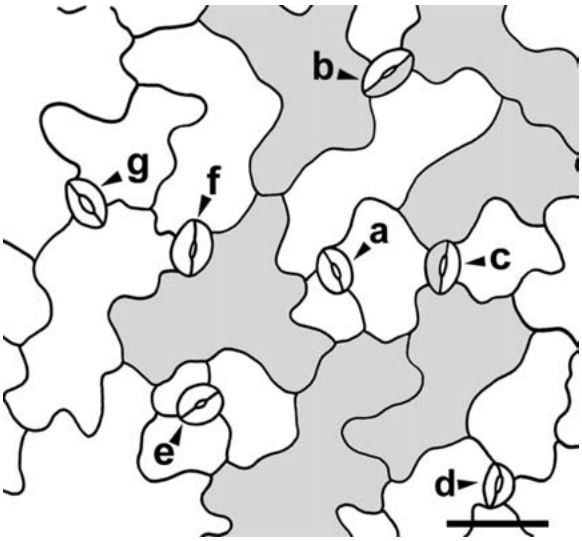

Figure 3. Spacing Pattern and Anisocytic Stomatal Complexes in Arabidopsis.
Stomata are surrounded by non-stomatal epidermal cells. Thus guard cells are excluded from the first ring of cells (non-shaded cells around stoma "a"). The second ring can include guard cells (e.g. shaded guard cells in stomata "b" and "c"). Many stomatal complexes are anisocytic (a, e, g). Others are not because they have more than three neighbor cells (b, c, d), or the neighbor cells are comparable in size (f). Tracing (by Matt Geisler) from the abaxial epidermis of a mature cotyledon. Bar = 40 μm.

mize the ratios between CO_2 uptake, evaporation, and internal leaf temperature. The presence of some adaxial stomata would allow evaporative cooling (as well as gas uptake), while higher densities might permit too much water loss.

Modified stomata. Arabidopsis also has modified stomata such as in the teeth of leaves (hydathodes), in anthers, and in nectaries at the base of stamens (Figure 4; Bowman and Smyth, 1999; Baum et al., 2001). Modified stomata may not function in gas exchange; some may release fluids such as excess water (guttation) or nectar (Davis and Gunning, 1993). Some modified stomata in wild-type Arabidopsis occur in contact (Figure 4) and may be patterned differently than normal stomata.

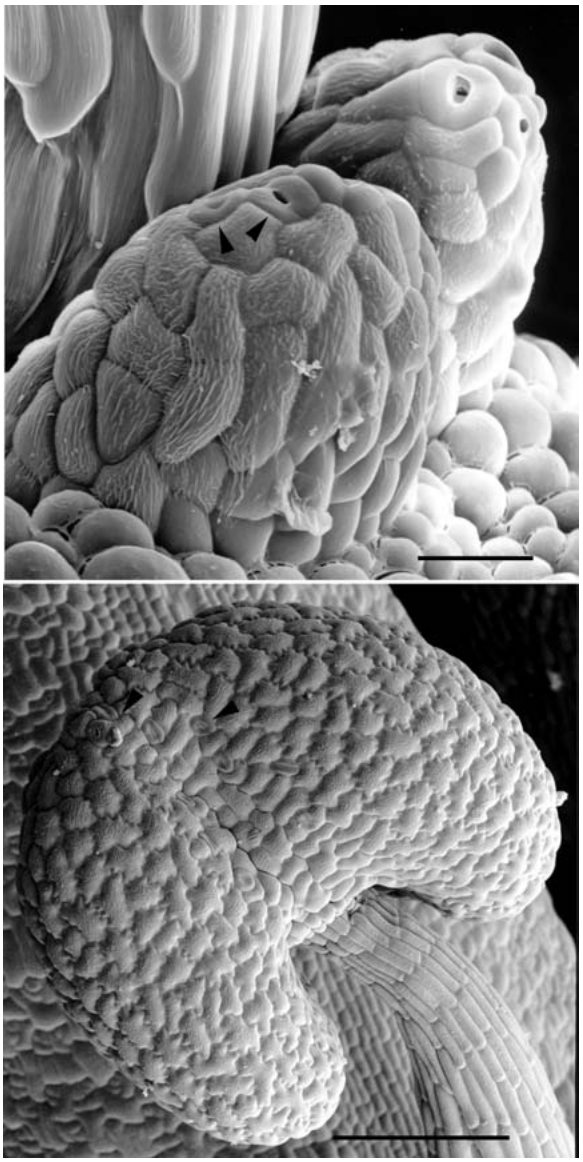

Figure 4. Specialized Stomata in Wild Type Arabidopsis
Cryo-scanning electron micrographs showing stomata at the tips of nectaries (top) and in the abaxial epidermis of the anther (bottom). Arrowheads indicate stomata, some of which are in contact with each other, even in the wild type. Anther micrograph from Kim Findlay. Bars = 30 μm (top) and 100 μm (bottom).

STOMATAL PRECURSORS

Understanding the cell types and stages of stomatal development provides a foundation for explaining the mechanisms of patterning, the regulation of stomatal density, and the functions of proteins involved in stomatal development. Because dicot stomatal development is variable in some features, it has proven helpful to analyze development by studying the behavior of cells through time using various impression methods (Sachs, 1991; Sachs and Kagan, 1993). The following description of precursor cells and of stomatal development in Arabidopsis is based upon the dental resin impression method, as well as cytological studies (Yang and Sack, 1995; Zhao and Sack, 1999; Berger and Altmann, 2000; Geisler et al., 2000).

Stomatal development in Arabidopsis invariably requires three different precursor cells (Figures 5-9), the meristemoid mother cell (MMC), the meristemoid, and the guard mother cell (GMC).

MMCs. The first precursor cell is the first cell in the stomatal pathway to divide asymmetrically. This division produces a smaller, usually triangular meristemoid and a larger sister cell (Figure 5). As far as is known, all Arabidopsis stomata are produced by at least one asymmetric division, that of the MMC. MMCs that are committed to this division can be recognized by their polarized cytoplasm with the nucleus and preprophase band of microtubules located at one pole, and a vacuole at the other (Zhao and Sack, 1999). MMCs typically originate from relatively small epidermal cells that do not have very sinuous cell walls.

Meristemoids. Some newly formed meristemoids convert directly into guard mother cells, the third and final precursor. Other meristemoids divide one to three times asymmetrically before conversion. As in the asymmetric divisions of MMCs, meristemoids that are about to divide can be recognized by their polarized cytoplasm (Figure 9; Galatis and Mitrakos, 1979). Each time a meristemoid divides, it regenerates a meristemoid and a larger sister cell. In this way, a meristemoid can be considered a "stem" cell. We were not able to predict the number of times a meristemoid will divide by its size, position, or any other parameter that can be detected by the dental resin method. Thus, the simplest assumption is that all meristemoids have the same potential for divisions, and that the factors that regulate the number of divisions need to be determined. It is, however, useful to separate out one class of meristemoids, satellite meristemoids, based on where they form. Satellite meristemoids originate from MMCs that are located next to a pre-existing stoma or precursor and, as will be discussed, are central to the generation of the spacing pattern.

GMCs. Guard mother cells divide symmetrically to produce two guard cells. The GMC division site, the location of cell plate fusion with the parental cell wall, is marked by a preprophase band of microtubules, a marker found in almost all types of cells (Zhao and Sack, 1999 Smith, 2001). In Arabidopsis GMCs, the division site is also marked by wall thickenings at opposite ends of the cell (Figure 9; Zhao and Sack, 1999). While similar thickenings have been found in GMCs in the Fabaceae (Galatis and Mitrakos, 1979; Galatis et al., 1982), they have not been found in any other cell type. It is not clear whether these thickenings function in guiding cytokinesis and/or whether they help the GMC attain an oval shape.

STOMATAL MORPHOGENESIS

GMC and stoma. The final stage of stomatal development involves the differentiation of the stoma itself. Differentiation in Arabidopsis is comparable to that described for other dicots (Galatis and Mitrakos, 1980; Sack, 1987; Zhao and Sack, 1999). Stomatal morphogenesis actually begins in the GMC. The oval outline of the stoma is first established in the GMC, perhaps by the end wall thickenings that may restrict local elongation of the cell wall (Figure 9). Other features of mature stomata, such as starch accumulation and vacuolar enlargement also initiate in the GMC, and then continue until the stoma matures.

Stomatal pore. Pore formation starts with a lens-shaped thickening located at the middle of the ventral wall (the new wall formed in the GMC; Figure 9). This thickening may be organized by radial arrays of microtubules that have gamma-tubulin at their focus (Marc, 1997). It is striking that each guard cell develops with a mirror-like symmetry with respect to the other. For example, the radial microtubule arrays and the wall thickenings in each guard cell are precisely opposite each other (Wasteneys et al., 1997). After the pore thickening reaches a critical stage, the anticlinal walls separate in the region of the thickening. This creates the stomatal pore. Obviously, the location of wall separation is regulated spatially, a trait under strong selection pressure because a separation elsewhere could result in desiccation.

Wall specialization. Stomata have an elaborate architecture. In Arabidopsis, there are distinct differences in the thickness of the different cell walls in the mature guard cell (Zhao and Sack, 1999). In addition, cellulose is deposited in arrays that radiate out from the pore, arrays that presumably result from microtubule-directed deposition. The collective distributions of cellulose, wall thickenings, and hinges presumably translate changes in hydrostatic pres-

sure into pore opening and closing. The elimination of plasmodesmata during stomatal development helps maintain turgor by making each guard cell an island of symplasm in the epidermis.

STOMATAL PATHWAY

This overview shows that each precursor cell is capable of division, and that changes in fate are progressive. Although meristemoids sometimes do not divide, MMCs and GMCs invariably do so. Each type of precursor cell has a different fate, shape, identity and behavior. However, each step in the pathway advances towards the terminal events of stomatal specification and differentiation.

As stated, the pathway begins with the selection and asymmetric division of the MMC. This also initiates the stomatal cell lineage, which is a series of clonally related cells derived from the MMC.

Although differentiation terminates the stomatal pathway from the meristemoid, the larger daughter cells produced by asymmetric divisions may follow three different fates (Figure 10). First, they may differentiate into pavement cells, large jigsaw puzzle-shaped cells that undergo endoreduplication (Melaragno et al., 1993). Second, they may become MMCs and start a new stomatal lineage. Third, they may divide symmetrically and produce two cells that can independently follow one of these three fates. One consequence of asymmetric divisions in the stomatal pathway is that these divisions produce about half of all pavement cells and perhaps up to three-quarters of all epidermal cells in leaves.

GENERATION AND TYPES OF STOMATAL COMPLEXES

A stomatal complex can be defined as the stoma plus adjacent epidermal cells. In many species, a set number and arrangement of cells surround each stoma. Stomatal

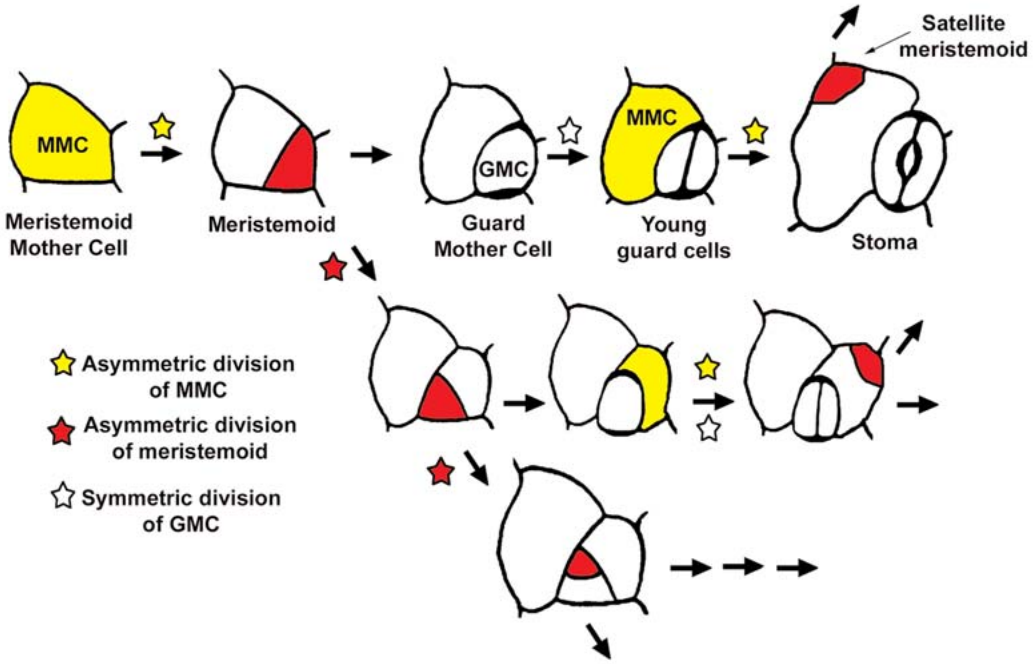

Figure 5. Diagram Showing Key Cell Types and Divisions in Arabidopsis Stomatal Development.
The first asymmetric division (in a MMC) produces a meristemoid. Meristemoids can divide before converting into a guard mother cell (GMC). GMCs divide symmetrically producing two guard cells. Note that the process can reiterate; neighbor cells can re-enter the pathway by becoming MMCs and dividing asymmetrically to produce satellite meristemoids. The plane of division is oriented so that the new meristemoid is placed away from the pre-existing stoma or precursor cell. Yellow, meristemoid mother cells (MMCs); red, meristemoids.

complexes can be distinct taxonomically and developmentally (Esau, 1977). Here we address whether the Arabidopsis stomatal complex is predictable in the position, number, and origin of neighbor cells. This analysis also relates to patterning in the stomatal pathway.

Anisocytic complexes. Arabidopsis and other members of the Brassicaceae exhibit anisocytic complexes where the stoma is surrounded by three cells, one of which is smaller (Figures 5 and 9B; Metcalfe and Chalk, 1950; Pant and Kidwai, 1967). This arrangement can result from three consecutive asymmetric divisions, one of the MMC and two of the meristemoid (Berger and Altmann, 2000; Geisler et al., 2000). Because the divisions are arranged in an inward spiral, the larger daughter cells become located to the outside; as a result they completely surround the stoma and their relative size correlates with their age. Here the complex is monoclonal because all five cells (three neighbor cells plus two guard cells) originated from the same MMC. Anisocytic complexes make up 40-66% of all complexes in leaves and cotyledons (Geisler et al., 2000; Serna and Fenoll, 2000a).

The inward spiral of asymmetric divisions can occur in a clockwise (Figure 5) or in a counterclockwise direction (Serna et al., 2002). Moreover, the angle of the symmetric division of the GMC is often roughly parallel to that of the last asymmetric division of the meristemoid (Galatis and Mitrakos, 1979; Serna et al., 2002). Nothing is known in this system about how division planes are positioned with respect to each other over four sequential divisions.

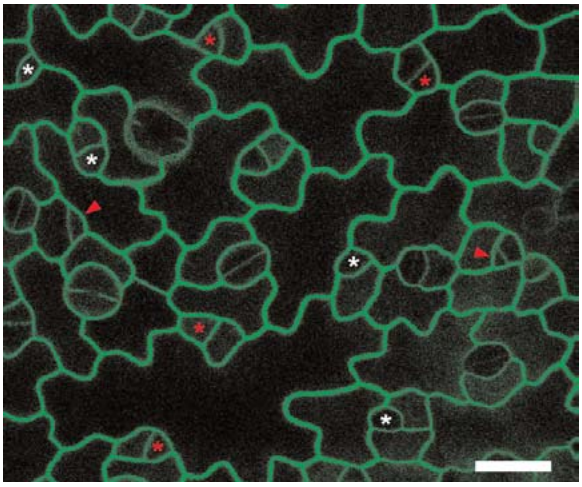

Figure 6. Living Abaxial Epidermis from Developing Leaf Showing Asynchrony in Stomatal Development and Different Cell Types.
Confocal scanning laser micrograph of green fluorescent protein line that labels cell membrane ("Q8" from Cutler et al., 2000). Because the cell walls are thin, the two cell membranes appear as one line at this magnification. GMCs (white asterisks) tend to have convex walls, whereas meristemoids (red asterisks and arrowheads) are more triangular. The stomatal pore is not visible because of the sharp curvature of the pore wall or the plane of the optical section. Bar = 20 μm.

Complexes other than anisocytic. Other complexes in Arabidopsis are not anisocytic either because the stomata are surrounded by 2, 4 or 5 neighbor cells, or because the three cells are of equal size, or because two complexes share one neighbor cell (Figure 3; Geisler et al., 2000; Serna and Fenoll, 2000a). Using the dental resin impression method, we found that this variability results in part from how many times meristemoids divide (Figure 5; Geisler et al., 2000). We estimated that about a third of all meristemoids in a lineage divide once or not at all before converting to a GMC. The resulting complexes would contain at least one non-clonal neighbor cell and would thus be polyclonal. Other estimates of the number of polyclonal complexes are lower (13 to 23%) and were derived from the analysis of transposon-induced sectors marked by GUS-staining (Larkin et al., 1996; Serna and Fenoll, 2000a; Serna et al., 2002). Thus, about 13-34% of Arabidopsis stomatal complexes are polyclonal, and 33-60% are not anisocytic.

These findings show that unlike the one-celled stomatal spacing pattern, the anisocytic pattern is not obligate. Because not all cells adjacent to stomata are distinct morphologically, a requirement for the term "subsidiary cell" (Esau, 1977), we refer to these cells generally as neighbor cells. More importantly, the existence of polyclonal complexes has implications for how the stomatal spacing pattern is generated (see below).

GENERATION OF SPACING PATTERN

Hypotheses for spacing. As mentioned the central feature of patterning is that stomata are separated by at least one intervening cell. Several hypotheses have been proposed to explain how this pattern is generated in dicotyledons (Figure 11; Sachs, 1979, 1991; Sylvester et al., 1996; Larkin et al., 1997; Croxdale, 2000).

The classical lateral inhibition hypothesis invokes the presence of an inhibitory field around developing stomata that prevents new stomata from forming in neighbor cells (Bünning, 1953; Korn, 1993, 1994). This implies that a neighbor cell should be prohibited from functioning as an MMC.

In the cell lineage hypothesis, a series of stereotyped asymmetric divisions creates a monoclonal boundary of neighbor cells (Sachs, 1991; Larkin et al., 1997; Serna and

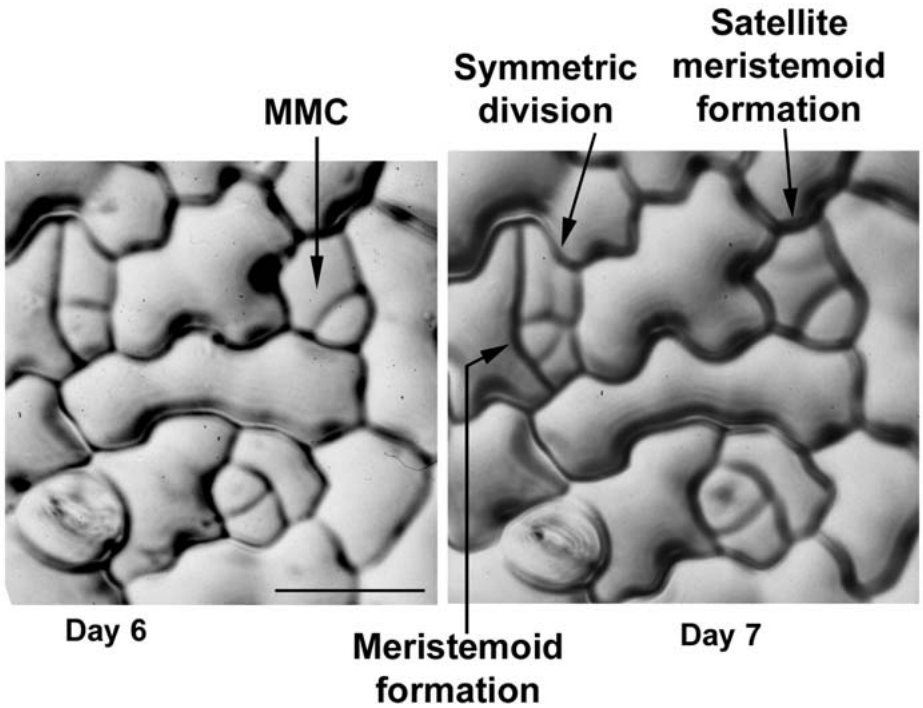

Figure 7. Example of Dental Resin Data.
Bright-field light micrographs of nail polish replicas of dental impressions showing the same field of cells over a 24-hour period (6-7 days after germination). Abaxial epidermis of cotyledon. The MMC (left) divided asymmetrically producing a satellite meristemoid (right). Another neighbor cell divided symmetrically. Figure from Matt Geisler. Bar = 25 μm.

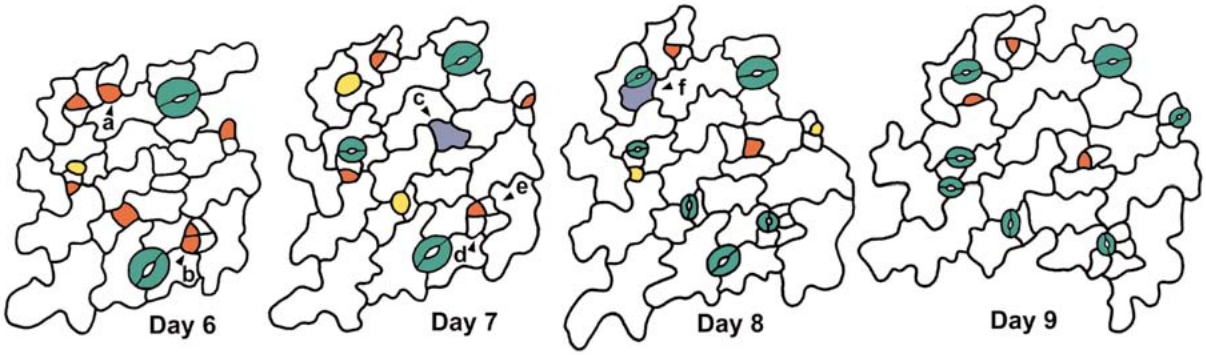

Figure 8. Key Events in Stomatal Development Shown in Dental Impression Series.
The abaxial epidermis of a single cotyledon is shown through time. Cells "c" (day 7) and "f" (day 8) are MMCs because they divided asymmetrically to produce meristemoids. The initial asymmetric division of MMC "f" took place next to a pre-existing stoma and produced a satellite meristemoid. Both MMCs arose from smaller, less sinuous cells. One smaller cell ("e", day 7) divided symmetrically by day 8. Meristemoid "a" (day 6) divided twice asymmetrically in an inward spiral. Two apparent meristemoids are adjacent (day 6, lower right); the upper one formed a stoma but the lower meristemoid did not progress in development ("b" and "d"). From Geisler et al. (2000).
Yellow, meristemoid mother cells (MMCs); red, meristemoids; blue, guard mother cells; green, stomata.

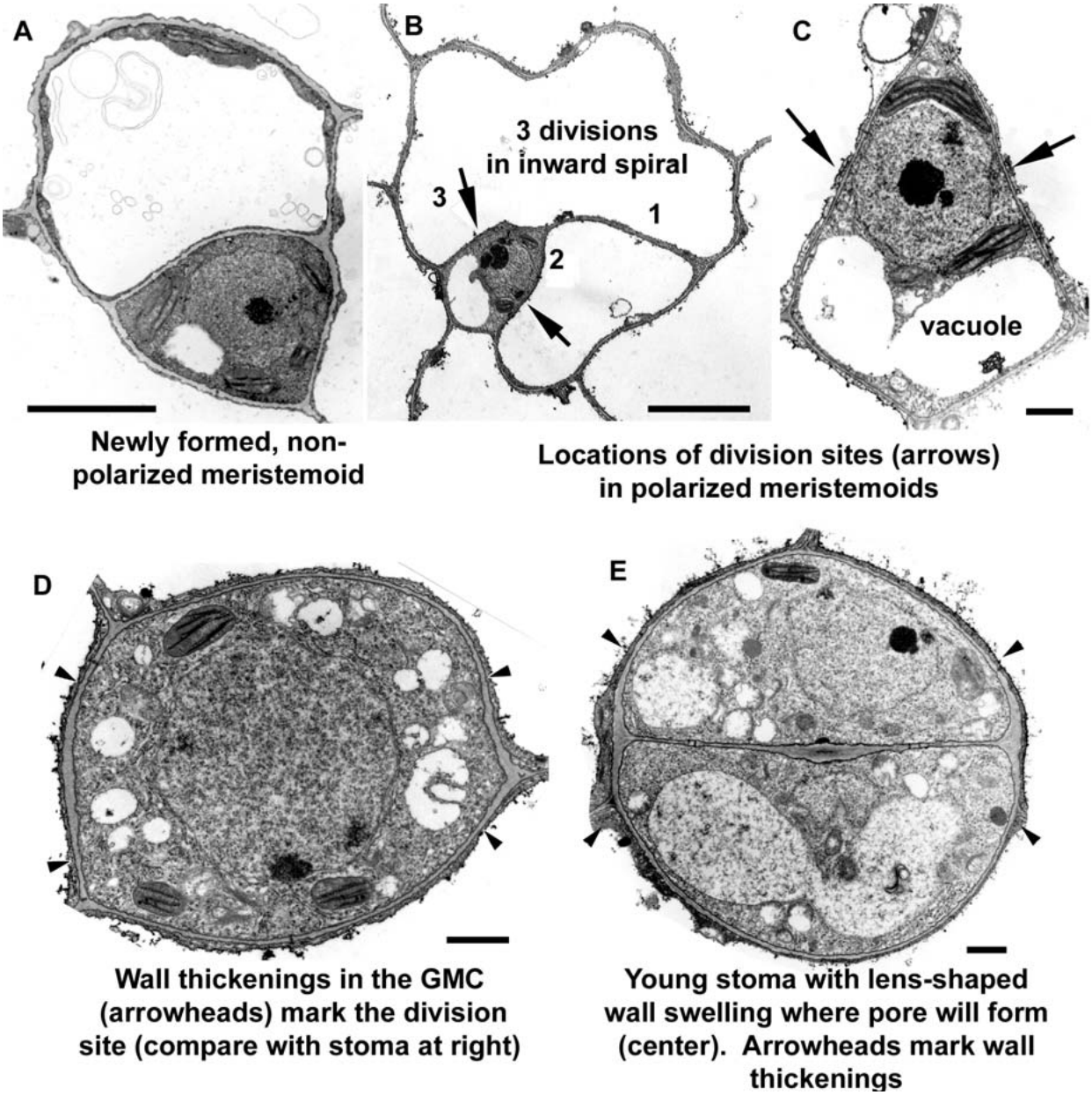

Figure 9. Asymmetric and Symmetric Divisions.
(A) A newly formed meristemoid is not polarized cytologically and is much smaller than its sister cell.
(B) The meristemoid shown was produced by two previous asymmetric divisions (1 & 2). It is now polarized prior to a likely third asymmetric division (arrows). The series of divisions occurs in an inward spiral.
(C) A higher magnification of a polarized meristemoid with an asymmetrically placed nucleus. This nucleus is close to the future site of division as marked by a preprophase band of microtubules (shown in Zhao and Sack, 1999).
(D) The division site in GMCs is marked by wall thickenings before symmetric division.
(E) The GMC division site wall thickenings are still visible in a developing stoma (arrowheads).
Adapted from Zhao and Sack (1999). Bars = 3 μm (A), 5 μm (B), 1 μm (C-E).

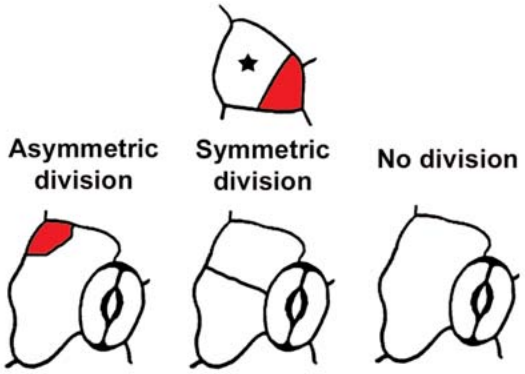

Figure 10. Fates of Sister Cell to Meristemoid.
The same asymmetric division that produces a meristemoid (red) also produces a larger sister cell (*). This cell is plastic in fate. It can divide asymmetrically to produce a satellite meristemoid. It can divide symmetrically to produce two new neighbor cells that in turn have the same plasticity in cell fate. It may stay relatively small and not divide, or it may enlarge and differentiate into a pavement cell (latter not shown).

Fenoll, 2000a; Serna et al., 2002). According to this hypothesis, the placement of neighbor cells is generated within the cell lineage and does not require communication with surrounding cells. This is thought to prevent the formation of stomata in contact by generating intervening cells.

A third view, based on study of the same cells through time, is that stomata are mostly randomly placed, but oriented divisions correct occasional patterning mistakes (Sachs, 1991; Sachs and Kagan, 1993; Sachs and Novoplansky, 1993). For example, Sachs and co-workers found that when meristemoids formed in contact, one meristemoid divided away, behavior thought to result from cell signaling.

Stomata are spaced by the placement of satellite meristemoids. We studied the development of many Arabidopsis stomata using the dental resin impression method (Geisler et al., 2000). This made it possible to analyze how Arabidopsis stomata are patterned. We found that many neighbor cells become MMCs, including cells adjacent to a stoma, a GMC or a meristemoid. The asymmetric division of the neighbor cell MMC is invariably oriented so that the new meristemoid, the satellite meristemoid, is placed away from the pre-existing stoma/precursor (Figure 12).

This is a central event in stomatal patterning in Arabidopsis in the abaxial leaf epidermis. The majority of stomata in these tissues originate from satellite meristemoids. Satellite meristemoid placement creates the one-cell spacing around this precursor and maintains the space around the pre-existing stoma or precursor so that both become patterned.

It is likely that cell signaling influences the orientation of the asymmetric divisions that produce satellite meristemoids. Satellite meristemoids were correctly placed regardless of whether the new MMC was or was not clonally related to the adjacent stoma/precursor. This argues against the idea that division orientation requires the inheritance of mitosis allocated factors which organize the site of subsequent divisions as is found in yeast budding (Chen et al., 2000). The simplest explanation is that the neighbor cell receives signals from the pre-existing stoma or precursor, positional information that is used to select the location of the division site.

Relationship to other patterning hypotheses. Our results support Sachs' (1991) concept that the positions of the first generation of stomata are generally not regulated with respect to each other. We found that MMCs formed in contact with each other with a frequency predicted by chance (Figures 10 and 13; Geisler et al., 2000). This suggests that the first precursor cells are randomly placed and that MMCs do not laterally inhibit each other.

As noted, Sachs (1991) suggested that oriented divisions could correct patterning mistakes. We also found that when meristemoids were in contact, that either or both divided away (Geisler et al., 2000). While this contributes to stomatal patterning, we found it occurs less frequently in Arabidopsis than satellite meristemoid formation, and thus it is a secondary mechanism of patterning.

Bünning's (1953) formulation of the lateral inhibition hypothesis (Figure 11) implies that the presence of a stoma prevents adjacent cells from becoming stomata. Lateral

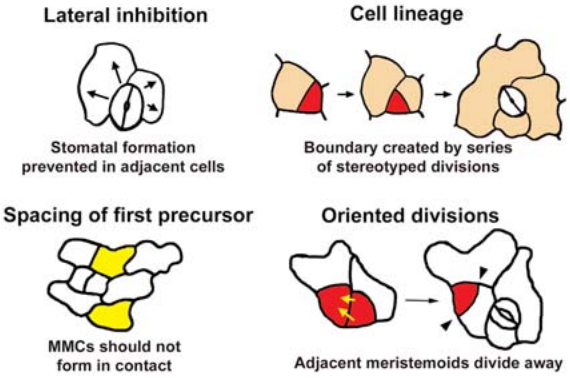

Figure 11. Hypotheses for the Generation of the Stomatal One-Celled Spacing Pattern.
Hypotheses tested by analysis of dental resin impression series (Geisler et al., 2000).

inhibition usually means that one cell prevents an adjacent cell from assuming a particular fate (Appel et al., 2001). But the only way that a neighbor cell can form a stoma is by functioning as an MMC, a frequent event in Arabidopsis. Thus, in this sense, patterning does not involve lateral inhibition. However, the presence of a stoma or precursor does seem to inhibit the formation of a meristemoid nearby. But the mechanism is the control of the placement of the MMC division site, not the fate of the neighbor cell itself.

Our data suggest that cell lineage does not generate the one-celled spacing pattern in the leaf epidermis. First, the lineage hypothesis doesn't provide a mechanism for how stomata in polyclonal complexes are spaced. Up to a third of all complexes are polyclonal in the abaxial epidermis of the first leaf and cotyledon (Geisler et al., 2000). The cell lineage hypothesis as formulated fails to explain why the non-clonal cells in polyclonal complexes are not stomata.

Second, even when a monoclonal complex is produced, neighbor cells do not provide a boundary or buffer, because many divide and produce stomata. It is not the existence of the neighbor cell itself, but the correct placement of the satellite meristemoid that establishes the minimal one-celled spacing pattern.

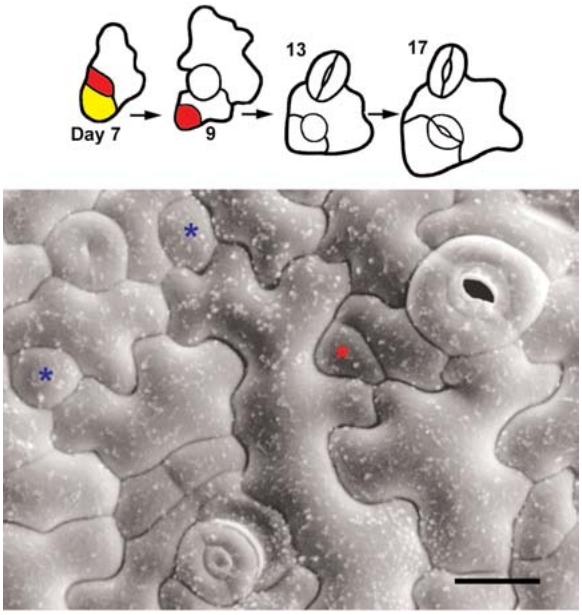

Figure 12. Satellite Meristemoid Formation.
(Top) Dental resin series showing asymmetric division of an MMC (yellow).
(Bottom) Cryo-scanning electron micrograph. Asterisks show a satellite meristemoid (red), or GMCs produced by satellite meristemoids (blue). Successive stages of pore development are shown at the upper left and lower center. Note that five of the six existing and future stomata shown are patterned by satellite meristemoid placement. Cells located next to two stomata and/or precursors (such as the cell to the right of the red asterisk) usually do not divide. Adapted from Geisler et al. (2000). Bar (bottom) = 10 μm.

Third, even when adjacent complexes are each monoclonal (Serna and Fenoll, 2000a; Serna et al., 2002), it remains to be demonstrated that a series of stereotyped divisions within a cell lineage actually establishes the one-cell spacing pattern. The key question is when in development the separation between meristemoids is first generated. A single asymmetric division can be sufficient to create a one-celled spacing such as in the placement of the satellite meristemoid or in the division of meristemoids in contact. In addition, many meristemoids are correctly spaced by chance (Geisler et al., 2000). In all three cases, subsequent asymmetric divisions in a lineage would increase the number of cells between stomata but would not affect the already established minimal one-celled spacing. Although cell lineage may not generate the one-celled spacing pattern, divisions within a cell lineage are crucial for establishing the position, arrangement and higher order spacing of stomata and other epidermal cells.

Cell position and events in the stomatal pathway. Some events in the stomatal pathway seem independent of cell position. For example, MMCs can form in contact, and the divisions of these MMCs appear to be randomly oriented (Figure 13A-E). It is unclear whether other events require positional cues communicated via intercellular signaling. For example, the divisions of meristemoids in an inward spiral could result from an endogenous patterning program such as through the allocation of spatial cues through mitosis.

Other events seem to require the communication of information about adjacent cell position. Examples mentioned include the orientation of asymmetric divisions in neighbor cells and in adjacent meristemoids (Figure 13). Cell signaling also appears to regulate the fate of cells in two specific positions in the stomatal pathway in Arabidopsis (Figure 13 H and I; Geisler et al., 2000). First, when meristemoids accidentally form in contact, sometimes one meristemoid arrests and does not divide or become a GMC. Second, cells next to any combination of two stomata, meristemoids, and GMCs seem to be prohibited from dividing asymmetrically. Neither event seems to play a major role in stomatal patterning (Geisler et al., 2000).

In domains that have fewer stomata and satellite meristemoids, such as the adaxial epidermis, spacing may rely more upon chance or minor spacing mechanisms. But even in the adaxial epidermis, we estimate that the majority of all stomata are patterned by satellite meristemoid placement (Geisler and Sack, 2002).

In summary, some stomata are placed by chance. The

remainder is spaced largely by oriented asymmetric divisions of neighbor cells. Other position-dependent events play either a secondary role or are not directly involved in generating the minimal one-celled spacing pattern.

REGULATION OF STOMATAL NUMBER

Many factors influence stomatal number such as environmental and developmental signals. For example, light and CO_2 concentration regulate the stomatal index which is defined as the ratio of stomata to all epidermal cells (Woodward and Kelly, 1995; Tang and Liang, 2000; Brownlee, 2001). Signals that influence stomatal number must ultimately regulate the specification of the first precursor cell in the stomatal pathway, the MMC. Genes involved in this regulation are reviewed later. Here the importance of cell position, ploidy and size are discussed.

Cell competence and selection as MMCs. Stomatal formation appears prohibited in several positions. In some cases, such as around trichomes, this prohibition occurs before asymmetric divisions start in the developing leaf

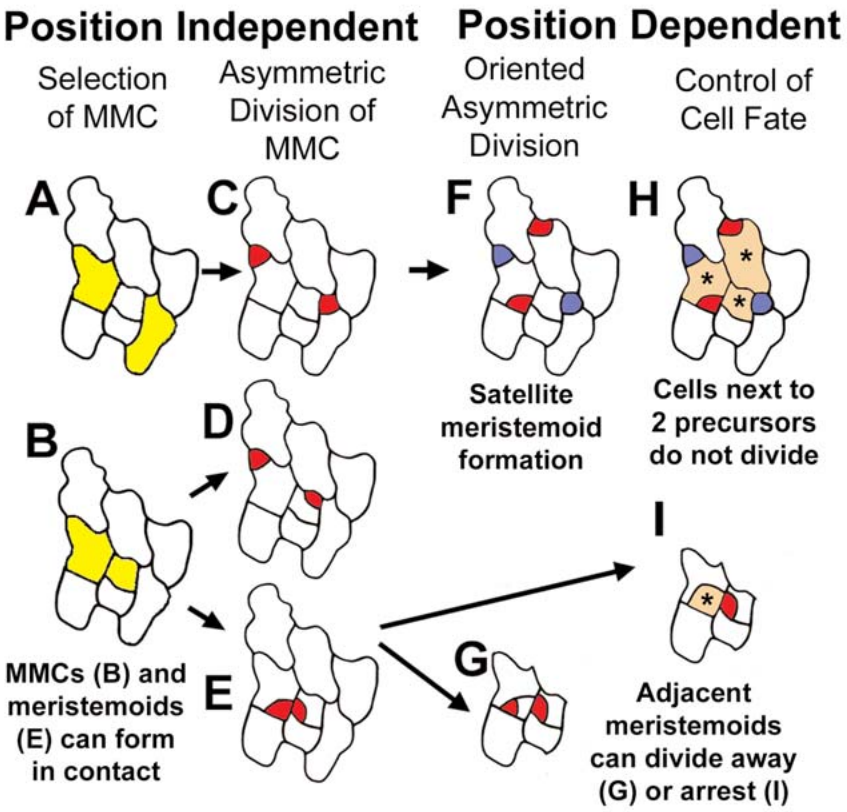

Figure 13. Position Independent and Dependent Events in the Stomatal Pathway
(A) and **(B)** The placement of the first precursor cell, the MMC, appears to be random so that MMCs sometimes form in contact (**B**).
(C) The plane of asymmetric division in separated MMCs also seems to be random.
(D) and **(E)** Divisions of MMCs in contact are randomly oriented and can produce meristemoids in contact.
(F) The orientation of asymmetric divisions next to a single stoma or precursor is position-sensitive and patterns most stomata.
(G) Asymmetric divisions of adjacent meristemoids can be oriented and thus space stomata.
(H) Cells next to two stomata or precursors (asterisks) usually do not divide asymmetrically.
(I) The arrest of an adjacent meristemoid (asterisk) spaces some stomata.
Adapted from Geisler et al. (2000).
Yellow, meristemoid mother cells (MMCs); red, meristemoids; blue, guard mother cell (GMCs); tan, cells whose fate is affected by their position.

(Larkin et al., 1997; Martin and Glover, 1998; Glover and Martin, 2000). In other cases, specific cells become prohibited even in regions actively forming stomata. For example, we found that cells next to two stomata and/or precursors in any combination usually do not divide asymmetrically (such as the cell to the right of red asterisk in Figure 12 bottom; Geisler et al. 2000).

The differentiation of pavement cells also removes cells from the pool of those competent to form MMCs. Large, sinuous cells that are known to undergo endoreduplication have not been observed to divide asymmetrically (Melaragno et al., 1993; Geisler et al., 2000; Glover, 2000). Conversely, smaller less wavy cells with 2C levels of DNA are most likely to be division competent and form MMCs (Donnelly et al., 1999; Geisler et al., 2000). Such cells are abundant in the young, developing epidermis. Later in leaf development, smaller cells are mostly located next to stomata (Figure 14). Thus the generalized capacity of the protoderm to initiate a stomatal lineage later becomes progressively restricted to small, less sinuous cells. Multiple generations of stomata can develop over the course of leaf development when sister cells to satellite meristemoids themselves divide asymmetrically and so on (Landré, 1972; Berger and Altmann, 2000).

Other small cells arise from symmetric divisions. Symmetric divisions produce cells of comparable size and developmental potential (Geisler et al., 2000). Symmetric divisions occur in neighbor cells (Figures 7 and 10) or in cells removed from stomata or their precursors. The pool of cells competent to form stomata is filled by asymmetric and symmetric divisions, and drained by cell enlargement and differentiation. Stomatal number is therefore partly a function of the balance between cell proliferation and cell differentiation.

In summary, stomatal number is regulated by the availability of division competent cells and by how many of those cells are induced to divide asymmetrically. Many of the cells capable of forming MMCs originate from prior asymmetric divisions in the stomatal pathway.

Stomatal index. This ratio depends upon how many stomata and other cells arise during leaf development. MMC divisions directly increase the number of both stomata and epidermal cells. Symmetric divisions of epidermal cells and asymmetric divisions of meristemoids *directly* increase only the number of epidermal cells. Thus, the stomatal index may be modulated by the differential regulation of these three types of divisions, a possibility that has not been studied. Such an analysis could identify the mechanisms by which environmental parameters influence plant productivity.

Longitudinal gradients in organs. In developing Arabidopsis leaves most stomata form and mature in a roughly tip to base gradient (Pyke et al., 1991; Donnelly et al., 1999). Successive waves of stomatal initiation, satellite meristemoid formation, and generalized cell enlargement start at the tip and proceed basipetally.

Grasses also display a tip-to-base gradient of stomatal formation. But the gradient in stomatal development is predictable in time and space within cell files. Meristemoids appear to be absent in grasses (Larkin et al., 1997). Stomatal number in monocots is controlled partly by the production of new regions of the leaf blade. In Arabidopsis leaves the initial longitudinal gradient in stomatal formation is much less strictly defined and is disrupted by mosaic growth. Stomatal formation is asynchronous and variable in the number of asymmetric divisions. Stomata are produced iteratively and progressively including in regions that are otherwise mature (Figures 12 and 14).

Relatively little is known about stomatal development in Arabidopsis stems and other cylindrical organs such as petioles, carpels and pedicels. In hypocotyls, a series of longitudinal and transverse divisions seems to create a pool of cells competent to form MMCs (Berger et al., 1998b). In stems and hypocotyls stomata appear to mature acropetally (Nadeau and Sack, unpublished). These observations imply that longitudinal waves of competence to form MMCs pass through developing leaves and stems.

COORDINATION WITH INTERNAL FEATURES

Because the stomatal pore regulates gas exchange between the atmosphere and subjacent tissues, it might be expected that position-dependent signals would coordinate the placement of stomata and underlying features. This section reviews two lines of evidence relating to such a position-dependent coordination.

Leaves. There seems to be no obligate relationship between the placement of stomata and substomatal cavities in dicot leaves. In one study, 13% of mature stomata were located directly over a mesophyll cell rather than a substomatal cavity (Sachs, 1979). Stomatal precursor cells also can be positioned over mesophyll cells instead of cell junctions (Figure 15; reviewed in Sachs, 1978).

Serna and Fenoll (2000a) analyzed whether stomatal placement is coordinated with internal tissues in Arabidopsis. Because substomatal cavities are small or absent in the adaxial side of the leaf, they used junctions rather than cavities between mesophyll cells for scoring. Over 90% of stomata and meristemoids were located over cell junctions. This percentage differed significantly from their estimate of a randomly generated placement. However, meristemoids only form at cell edges, a feature that would differ from a randomly generated control. Also,

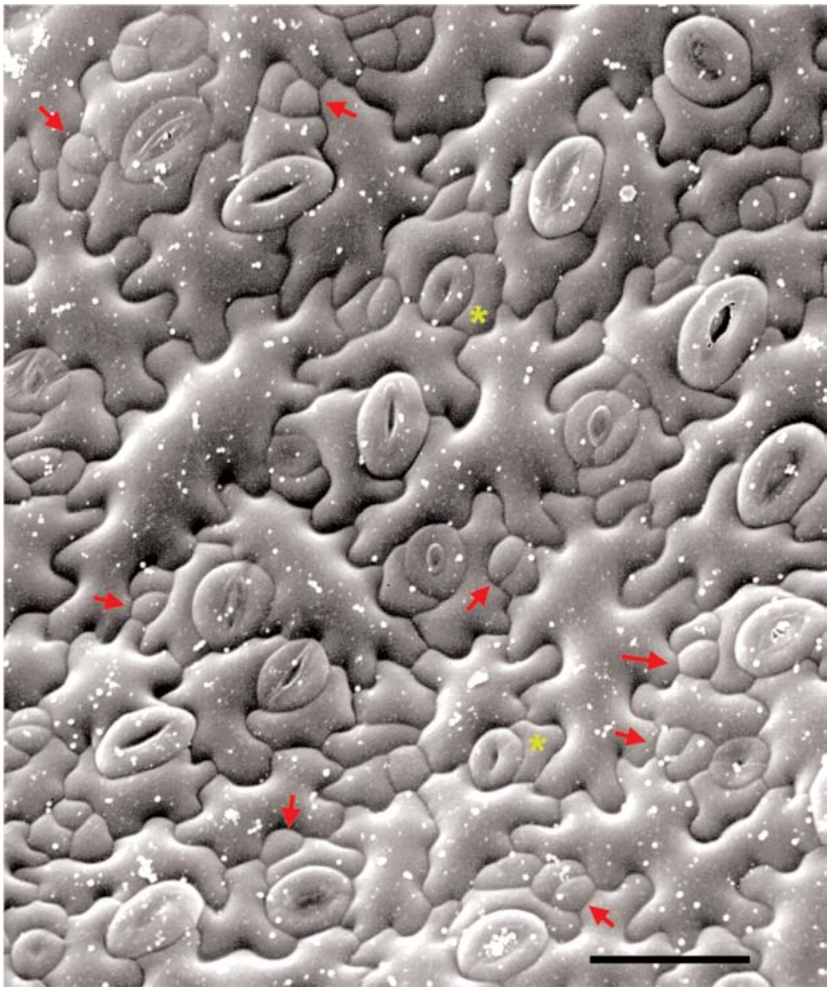

Figure 14. Many Satellite Meristemoids Are Produced During Leaf Development.
Cryo-scanning electron micrograph of the abaxial epidermis of a cotyledon. The red arrows indicate satellite meristemoids. Some of the satellites have divided asymmetrically. The yellow asterisks mark small neighbor cells; such cells usually remain division competent (compare with Figure 10). Adapted from Geisler et al. (2000). Bar = 30 µm.

epidermal cell outlines may be positionally out of register with the cells below (Figure 15). Using these parameters, it would be valuable to confirm that the distribution over cell junctions is non-random. If so, then radial signaling may take place between these two cell layers. One scenario could be that a signal from mesophyll cells could help orient asymmetric divisions so that meristemoids tend to form over cell junctions (Serna and Fenoll, 2000a).

Hypocotyls. Hypocotyl stomata are present in files of short, flattened cells and excluded from files of cells that are elongated and bulging (Gendreau et al., 1997). The protruding epidermal cells are located over the periclinal wall of a single underlying cortical cell. The files that produce stomata are above anticlinal walls (Schiefelbein 2002; Berger et al., 1998b; Hung et al., 1998). Thus, the competence to form MMCs is restricted in a position-dependent manner to hypocotyl cells that are radially aligned with subjacent landmarks.

A similar mechanism controls root hair formation and considerably more is known about root hair patterning. Root hairs form in cells (H cells) located over anticlinal cortical cell walls rather than in cells (N cells) over periclinal walls (Schiefelbein, 2000, 2002). The signal that coordinately establishes root hairs over anticlinal walls is unknown. The cellular pattern is set up in the embryo and is maintained in derivatives of the root apical meristem (Berger et al., 1998a). These authors killed subjacent root cortical cells using laser ablation before the fate of recent epidermal derivatives was fixed so that the derivatives no longer contacted living cortical cells. The isolated derivatives continued to express markers appropriate to their position relative to underlying cells. These data argue against the need for continuous communication between cell layers, and are consistent with the possibility that the outer cell walls of dead cortical cells retain some factor that maintains the fate of the overlying epidermal cells (Berger et al., 1998a; Benfey, 1999).

The formation of stomata and root hairs over anticlinal cortical walls is a striking example of the conservation of a radial patterning mechanism. It places stomata over future intercellular spaces and root hairs over the shortest route to the xylem. This conservation involves common molecular pathways, the subject of the next section.

"ROOT HAIR" GENES AND MMC FORMATION IN HYPOCOTYLS

It is appropriate that this discussion of the genes regulating stomatal development begins with genes that ultimately control the first step in the pathway, the creation of small cells that are capable of forming MMCs. Loci known to affect both root hair and stomatal patterning include *GL2, TTG*, and *WER* (see model in Schiefelbein 2002; Berger et al., 1998b; Hung et al., 1998; Schiefelbein, 2000).

In roots, these three gene products block the formation of root hairs in N cells. Mutations in all three result in ectopic root hairs in N cells. GL2, a homeodomain protein, is preferentially expressed in non-hair files. The expression of *GL2* is positively regulated by WER, a myb protein, and by TTG, a WD-40 protein. Both maintain the higher relative expression levels of *GL2* in N cells (Schiefelbein, 2000). These data are consistent with a model whereby the expression of *GL2* in N cells negatively regulates root hair specification (Lee and Schiefelbein, 1999; Schiefelbein, 2000, 2002). Conversely, reduced *GL2* expression in H cells allows hairs to form.

In addition, TTG may bind to a basic Helix-Loop-Helix transcriptional activator. When this complex is bound to WER, it may promote *GL2* expression in N cells. In H cells,

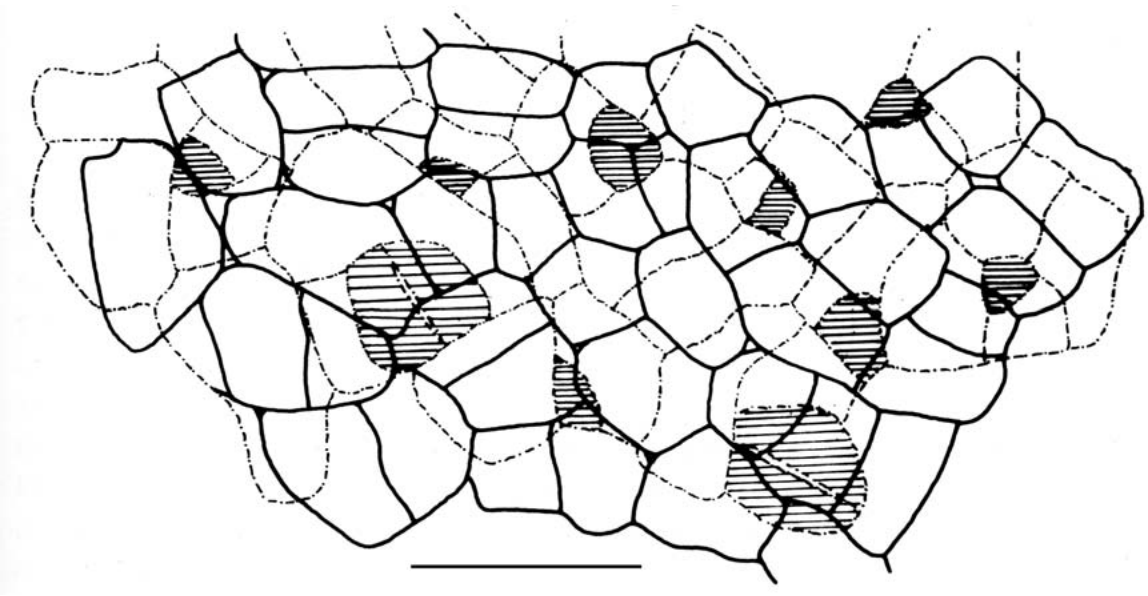

Figure 15. Stomatal Precursor Cell Placement with Respect to the Mesophyll.
Tracing from a pea stipule showing epidermal cells (broken lines), stomata and their precursors (parallel hatching), and the subjacent mesophyll (solid lines). Figure from Sachs (1978) who did not find a consistent spatial relationship between stomatal precursor cell placement and the anticlinal walls of the mesophyll. Bar = 30 μm.

TTG-bHLH may bind to CPC, a myb protein. This complex is hypothesized to repress *GL2* expression because CPC lacks an activation domain. This model is supported by the findings that a 35S::R transgene (a maize bHLH) in a *ttg* mutant background restores a non-hair fate to N cells and represses hair formation in H cells (Galway et al., 1994; Schiefelbein, 2000, 2002).

In hypocotyls, *GL2* and *WER* are preferentially expressed in protruding cells. Mutations in *GL2*, *TTG*, and *WER* exhibit ectopic stomata in *GL2*-expressing cells (Hung et al., 1998; Lee and Schiefelbein, 1999). In wild-type hypocotyls, 2-5% of all stomata were found to be ectopic. In contrast 12-27% were ectopic in *gl2, ttg* and *wer* mutants or when the R transgene was introduced under the control of the 35S promoter (Berger et al., 1998b; Hung et al., 1998; Lee and Schiefelbein, 1999). The *gl2* and *ttg* mutations had no effect on stomatal density or on relative cell length in the normal stomatal files, but 35S::R reduced the total number of stomata by 80%.

GL2, WER, TTG, and the 35S::R transgene all negatively regulate stomatal formation in cells that normally express *GL2*. A model for how these genes function in the hypocotyl is comparable to their position-dependent effects in roots (Berger et al., 1998b; Hung et al., 1998). Thus, ectopic stomata may form because (1) GL2 is not functional in *gl2* mutants, (2) *GL2* is not expressed when either TTG and WER, which positively regulate *GL2* expression, are non-functional, or (3) the 35S::R transgene disrupts the differential expression of *GL2* (Hung et al., 1998; Lee and Schiefelbein, 1999; Schiefelbein 2002).

The effects of the same *ttg* and *gl2* alleles appear to be more severe in roots than in hypocotyls. These mutations cause almost all non-hair cells to form ectopic root hairs (Galway et al., 1994; Masucci and Schiefelbein, 1996). In contrast, relative few ectopic stomata are formed in mutant hypocotyls. This difference in apparent severity might be related to the findings that wild-type hypocotyls have relatively few stomata and that the protruding cells files can be disrupted by intervening smaller cells (Berger et al., 1998b; Gendreau et al., 1997).

GL2 may negatively regulate stomatal formation by specifying a protruding cell fate. These cells are larger, and divide considerably less than the non-*GL2* expressing cells (Gendreau et al., 1997; Hung et al., 1998; Berger et al., 1998b). Protruding cells probably undergo endoreduplication because half of all hypocotyl cells do so and because endoreduplication can be proportional to cell size (Gendreau et al., 1998). These cells are probably removed from the pool of cells competent to divide. Thus, GL2 may block MMC formation by promoting an alternate cell fate in a position-dependent manner. Whereas in roots, almost all non-*GL2* expressing cells form hairs, in hypocotyls only a few stomata form per file. This suggests that the absence of *GL2* expression only results in MMC and stomatal formation in a subset of cells.

The finding that genes that affect root hairs also affect stomatal patterning provides a new context for studying the functions of these genes. It will be interesting to see whether additional loci that affect root hair patterning, such as *CPC*, show a stomatal phenotype, and whether any Arabidopsis bHLH homologues of the maize R gene (Payne et al., 2000) is expressed in stomatal-forming files. Also, many of the root hair mutations seem to affect lateral roots, not just the primary, embryonic root (Schiefelbein, personal communication). This invites a determination of whether these genes influence stomatal patterning in the shoot, not just in embryonic tissues such as the hypocotyl.

TOO MANY MOUTHS (*TMM*)

While GL2, TTG, and WER affect the distribution and patterning of stomata in the hypocotyl, the minimal one-celled spacing pattern is unaffected. In contrast, mutations in several loci have been shown to disrupt the fundamental spacing pattern by allowing stomata to form in contact. The best described of these loci are *TOO MANY MOUTHS, STOMATAL DENSITY AND DISTRIBUTION1*, and *FOUR LIPS*.

Clusters result from disruption of the number, plane and polarity of asymmetric divisions. TMM is essential for the establishment of stomatal patterning (Yang and Sack, 1995; Larkin et al., 1997). A major *tmm* phenotype is the presence of clusters of adjacent stomata in leaves and cotyledons (Figure 16). Stomata in clusters are arranged at different angles with respect to each other, and the stomata vary in size and stage of development. These traits suggest that the clusters develop progressively and iteratively.

Analysis of cluster development using both dental resin impressions and static images shows that *tmm* disrupts several spacing mechanisms (Figure 17; Geisler et al., 2000).

First, instead of satellite meristemoids being placed away from pre-existing stomata, some *tmm* meristemoids form in contact with the stoma (Figure 17; Yang and Sack, 1995). These ectopic satellite meristemoids result from the misorientation of the plane of asymmetric divisions in neighbor cells. Analysis of division angles shows that *tmm* randomizes the division plane. This appears to be the major defect resulting in cluster formation.

Second, *tmm* disrupts a secondary stomatal spacing mechanism, the oriented divisions of meristemoids in contact (Figures 11 and 13; Geisler et al., 2000). Unlike the wild type, *tmm* meristemoids fail to divide away from each

other.

Third, stomatal clusters also develop from alterations in the frequencies of cell divisions. Satellite meristemoids, like all meristemoids, can divide asymmetrically, divisions that often add to the separation between the pre-existing stoma and the new one. However, *tmm* meristemoids divide fewer times compared to the wild type resulting in an acceleration of when meristemoids convert into GMCs. This reduces the likelihood that meristemoid divisions will correct patterning mistakes.

Fourth, clusters form partly from an overproduction of satellite meristemoids. While the number of asymmetric divisions of meristemoids is reduced in *tmm*, the number of neighbor cells that divide asymmetrically increases. Thus, TMM acts as a negative regulator of asymmetric divisions in neighbor cells, and a positive regulator of divisions in meristemoids. The consequence of disrupting this regulation is an overproduction of satellite meristemoids, many of which are misplaced and which may not divide.

Fifth, cells that do not divide in the wild type do so in *tmm*. Cells located between two stomata or precursors appear to be prohibited from dividing asymmetrically in the wild type. The asymmetric divisions of these cells in *tmm* combined with randomized division planes produces more stomata in contact (Figure 17). Thus, cluster formation also results from the disruption of the position-dependent control of cell fate.

Sixth, *tmm* also occasionally alters the fates of daughter cells of divisions that normally would be asymmetric. Usually *tmm* asymmetric divisions are clearly polar meaning that they produce daughter cells of different sizes and fates. However, sometimes both products of a division in a neighbor cell develop into stomata. This shows that TMM is also necessary for the polarity of the asymmetric division, and that *tmm* may disrupt the segregation of different fate factors.

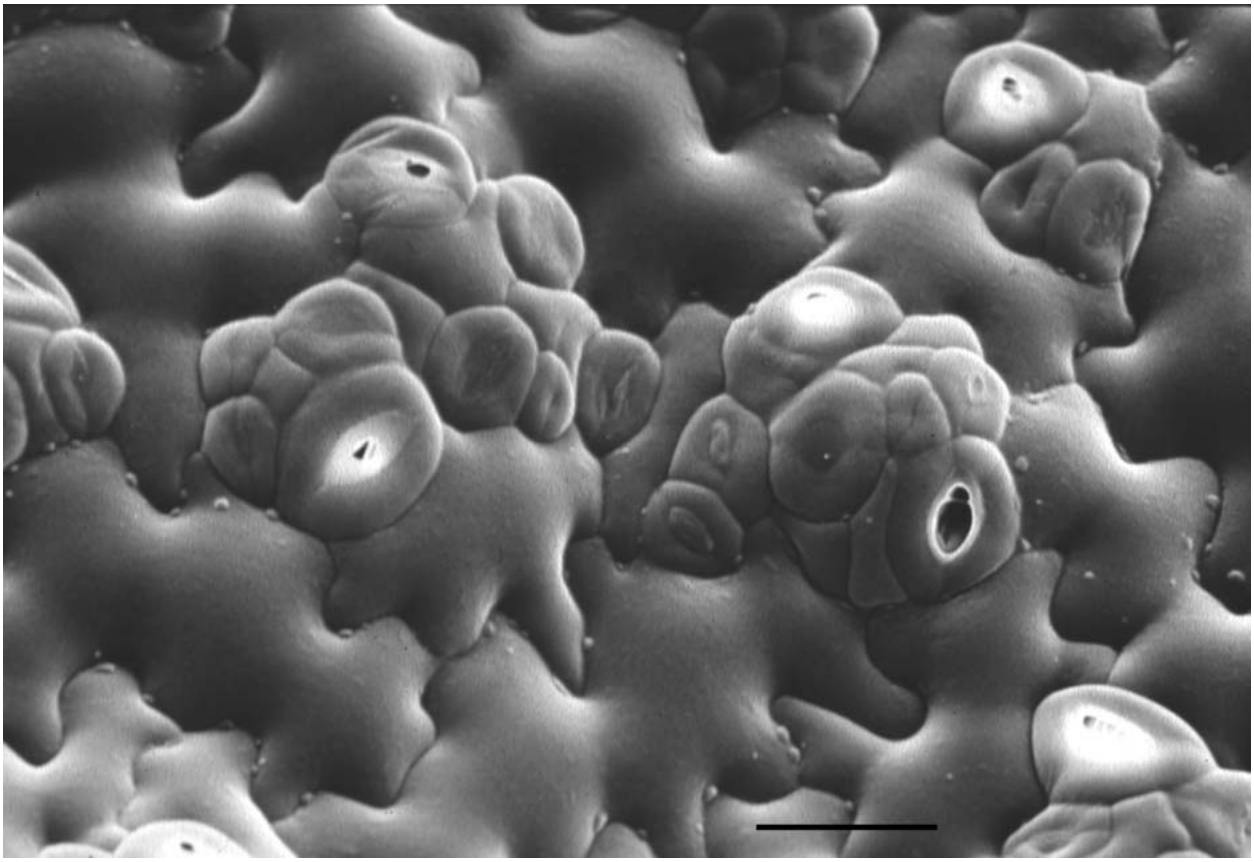

Figure 16. Stomatal Clusters in *too many mouths*
Cryo-scanning electron micrograph of *tmm* abaxial cotyledon epidermis. Bar = 15 µm.

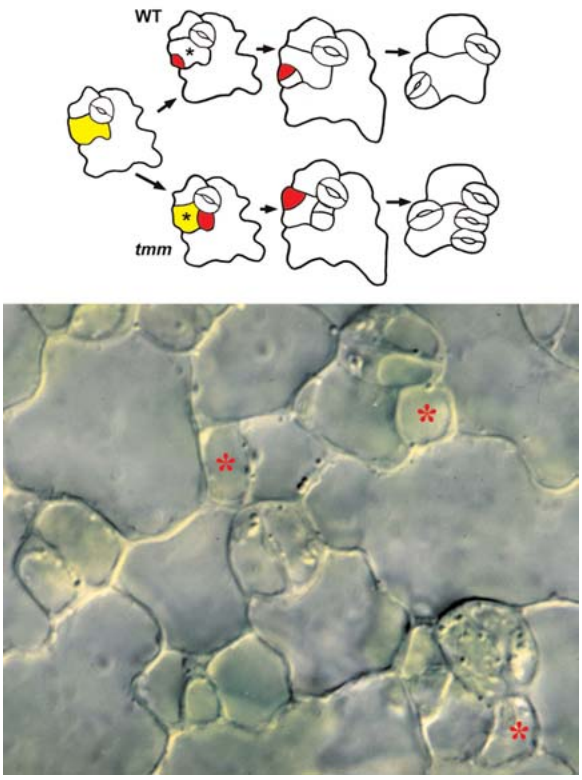

Figure 17. Developmental Basis of Stomatal Cluster Formation in *tmm*
(Top) Dental resin series from *tmm* (bottom row) illustrating that stomatal clusters result from several defects including (1) the randomization of the orientation of asymmetric division, (2) cells (*) next to two stomata and/or precursors normally do not divide but do in *tmm*, and (3) sometimes both products of division develop into GMCs (lower right). Top row shows cartoon of what would have happened in wild type.
(Bottom) Differential interference contrast micrograph of *tmm* epidermis showing new meristemoids (red asterisks) that are in contact with developing stomata. Bar = 10 µm.

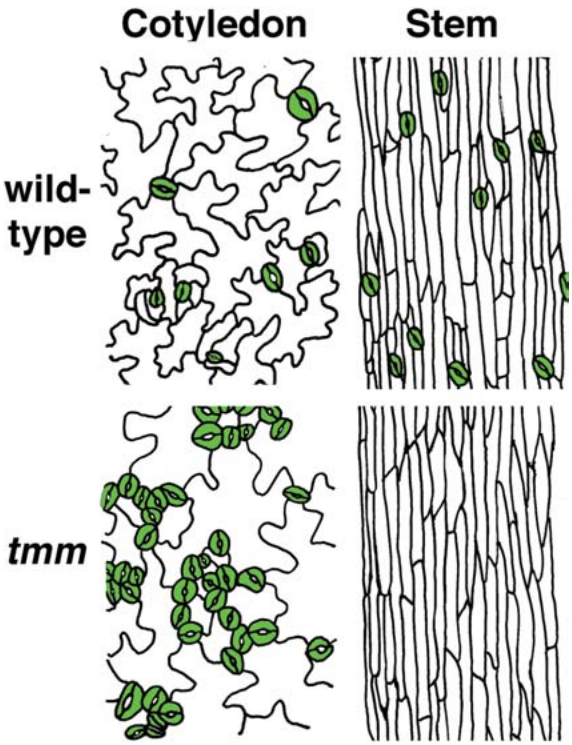

Figure 18. Opposite Stomatal Phenotypes in *tmm*
tmm has clustered stomata in cotyledons and leaves but no stomata in stems. Tracings from cleared tissue.

Thus, stomatal clusters result from alterations in the orientation and polarity of asymmetric divisions, from disruptions in cell fate, and from defects in the regulation of division frequency. Many of these events probably require cell-cell signaling to communicate spatial cues. Both *tmm* alleles are recessive and are presumed to be loss-of-function mutations. We hypothesize that TMM functions in an intercellular communication pathway that receives and conveys information about the identity and location of nearby cells. Positional cues may play several roles. These may orient and fix the plane of asymmetric divisions in neighbor cells. They could be required for the correct distribution of cell fate determinants that result in the asymmetric fates of daughter cells. Also spatial signals may guide the divisions of meristemoids in contact, and ensure that cells adjacent to two stomata or precursor cells do not divide. Thus, TMM may help interpret spatial signals so as to regulate the number, plane and polarity of asymmetric divisions. Further study of TMM may provide access to genes and events that regulate the placement of asymmetric divisions in plants.

TMM is a negative or positive regulator of entry into the stomatal pathway. In addition to a role in stomatal patterning, TMM is also a key regulator of stomatal production. *tmm* stems have virtually no stomata (Yang and Sack, 1995; Geisler et al., 1998). Thus *tmm* has two completely opposite stomatal phenotypes, clusters and the elimination of stomata.

Sometimes these phenotypes are found in separate organs. As mentioned, leaves have clusters and stems lack stomata (Figure 18). Other organs display both phenotypes. The abaxial sepal epidermis has clusters, but no stomata are present in the adaxial epidermis (Figure 19; Geisler et al., 1998). The most striking case is in the flower stalk (pedicel) where clusters are present at the floral end,

stomata are absent at the stem end, and there is a gradient in between (Figure 20).

TMM is thus essential for stomatal formation in some contexts, and a negative regulator in others (Figure 21). TMM appears to control how many cells are induced to divide asymmetrically (to function as MMCs). The direction of that regulation depends upon organ and domain. Thus, TMM is a major determinant of stomatal formation as well as patterning. It remains to be seen whether these are separate functions of the same protein, or whether they result from the same key function such as regulation of the frequency and execution of asymmetric divisions.

The diversity of *tmm* phenotypes suggests that TMM interacts with partners in a molecular complex or pathway. In addition, cues conveying tissue identity and position may signal the direction of the interaction thereby increasing or decreasing the number of MMCs as appropriate to location.

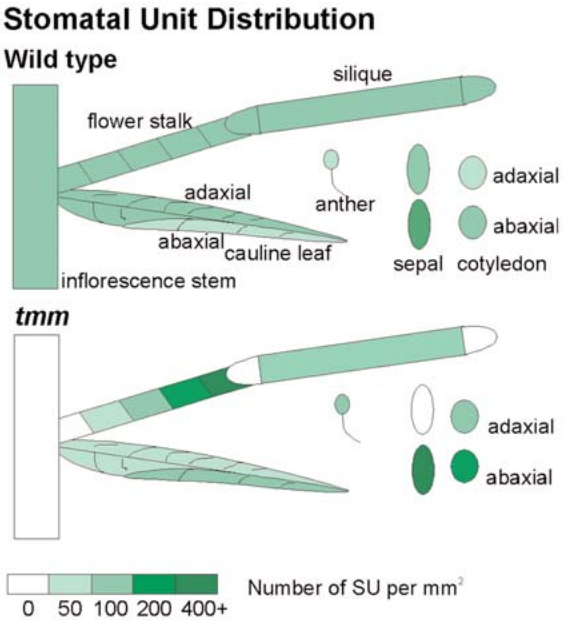

Figure 19. *tmm* Alters Stomatal Production in an Organ-Specific Manner

Diagram showing the distribution of stomata in the reproductive shoot of wild type (top) and *tmm* (bottom). The stomatal density is more or less uniform throughout the stem and floral organs in the wild type. In *tmm,* stomata are absent from the stem, the base of the flower stalk, the ends of the silique and the adaxial sepal. The abaxial *tmm* sepal has more stomata than the wild type. A stomatal unit is defined as a single stoma or a cluster of stomata in contact. The density of stomatal units is shown in color coding at the bottom left. Adapted from Geisler et al. (1998).

STOMATAL DENSITY AND DISTRIBUTION1 (*SDD1*)

The *sdd1* mutation dramatically increases stomatal number, index, and density throughout the shoot (Figure 22 top; Berger and Altmann, 2000; Von Groll and Altmann, 2001). Three defects contribute to this increase (Figure 22 bottom; Berger and Altmann, 2000). First, more protodermal cells enter the stomatal pathway (function as MMCs) in *sdd1-1*. Second, more satellite stomata are produced in succession (multiple generations of satellite meristemoids). Third, meristemoids divide fewer times; the stomatal index increases when fewer daughter cells are available to form pavement cells.

sdd1 also disrupts the primary mechanism of stomatal patterning in that it disorients asymmetric divisions in neighbor cells; this produces ectopic satellite meristemoids and stomatal clusters (Figure 22 bottom). No data are available on whether SDD1 is also required for a secondary patterning mechanism, the ability of adjacent meristemoids to divide away from each other.

TMM and SDD1 seem to regulate many of the same events in leaves (Figure 21). Both affect the orientation of asymmetric divisions, both function as positive regulators of meristemoid division, and both are negative regulators of MMC/stomatal formation (Berger and Altmann, 2000; Geisler et al., 2000). Also, both prevent cells adjacent to two stomata from dividing (Figure 22 bottom; Geisler et al., 2000). But it is not clear whether TMM and SDD1 restrict stomatal number by the same mechanisms. For example, data are not yet available as to whether *tmm*, like *sdd1*, increases MMC formation from protodermal cells and permits extra generations of satellite meristemoids (Berger and Altmann, 2000). Moreover, the phenotypes of *sdd1* and *tmm* are quite distinct (Figure 22 top). Leaves of both mutants have many more stomata than the wild type. But *sdd1* has many more correctly patterned stomata than *tmm* and many fewer stomatal clusters. Where present, *sdd1* clusters tend to be smaller than in *tmm*. These data suggest that SDD1 is less important for the one-celled spacing pattern than TMM, but more important for determining stomatal density.

sdd1 displays roughly the same phenotype throughout the plant whereas *tmm* has opposite stomatal phenotypes in different organs. Thus, SDD1 is negative regulator of entry into the stomatal pathway throughout the shoot, whereas TMM can be a negative or positive regulator depending upon context.

SDD1 encodes a subtilisin-like serine protease that may function as a processing protease in developmental signaling (Berger and Altmann, 2000). *SDD1* is expressed in meristemoids and GMCs, and SDD1 has a signal peptide (Von Groll and Altmann, 2001). Thus SDD1 may process

Figure 20. Gradient in Stomatal Phenotypes in *tmm* Flower Stalks
Cleared and stained flower stalks of wild type (left) and *tmm* (right). Dark blue ovals are stomata. Note the even distribution of stomata in the wild type. In contrast, *tmm* displays a gradient in phenotype from excessive stomata, many in stomatal clusters at the floral end (top) to the elimination of stomata at the base (bottom). Reprinted from Geisler et al. (1998). Bar = 200 μm

some signal either in the endomembrane system of GMCs and meristemoids or outside these cells. This signal may inhibit some neighbor cells from dividing asymmetrically. If SDD1 also negatively regulates stomatal initiation in fields of cells devoid of any stomatal precursor, then this communication may take place over larger distances. In contrast, SDD1 may positively regulate the divisions of meristemoids, the same cells in which it is expressed.

The phenotypes of *tmm* and *sdd1* and the molecular identity of *SDD1* support the idea that there is a balance between cell proliferation and differentiation. One balance point might be between the induction of asymmetric divisions that cause entry into the stomatal pathway and the specification of a pavement cell fate for neighbor cells. Another might be between the induction of meristemoid divisions and the conversion of the meristemoid into a GMC. It is likely that these set points are responsive to intercellular communication.

This balance might also function in patterning. The disruption of signaling in cells selected to divide could cause abnormal division planes. The analysis of SDD1 and TMM should help define cell signaling pathways required for balancing cell proliferation and differentiation, for orienting asymmetric divisions, and for specifying cell fate.

FOUR LIPS (*FLP*)

four lips mutants have clusters of laterally-aligned stomata in direct contact (Yang and Sack, 1995; Larkin et al., 1997). In some alleles, clusters consist primarily of 4 guard cells. Larger clusters are present in other alleles. All alleles exhibit unpaired guard cells that either form in isolation, or in contact with pairs of guard cells. Most cell walls in stomatal clusters are parallel but other angles are found as well. Large clusters contain mature and developing stomata, but also other cells of uncertain identity. Only a subset of stomata is clustered (Figure 23). The proportion of clusters to normal stomata is roughly similar in different plants of the same allele.

Stomatal clusters are found throughout the plant in all places where stomata are found in the wild type (Geisler et al., 1998). In *flp-1*, clusters are less frequent in cylindrical organs such as stems, flower stalks, and siliques, than in dorsi-ventral ones such as rosette leaves.

No other defects have been found in *flp*. The *flp-1* allele does not appear to affect the total number of meristemoids or pavement cells that are produced (Yang and Sack, 1995; Geisler et al., 1998). Although guard cell development can be arrested in clusters, stomatal morphogenesis is not seriously affected. *flp* plants are healthy, and trichome and root hair development appear normal.

The arrangement of cell walls in clusters is consistent with the hypothesis that most or all cells derive from the same parent cell. Dental resin impressions of developing clusters show that clusters originate from a single guard

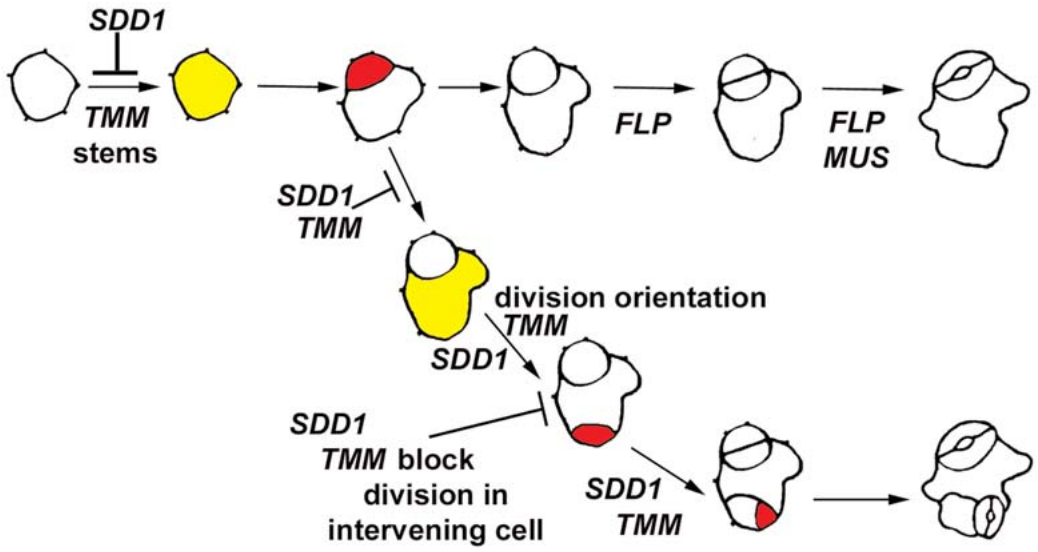

Figure 21. Model for Where Gene Products Act During Stomatal Development
Summary of major events disrupted by different mutations. Yellow represents the selection of an MMC fate. Meristemoids are red. Negative regulation is indicated by "T"-shaped lines, positive regulation is indicated when just the gene abbreviation is shown.

mother cell. As in the wild type, *flp* GMCs usually divide symmetrically. In some *flp* stomata, the two cell products differentiate into guard cells as indicated by the development of a lens-shaped pore thickening. This scenario leads to the development of normal unclustered stomata. But in other cases, the two daughter cells fail to develop pore thickenings and they divide again indicating that these cells do not differentiate normally as guard cells.

These data suggest that the *four lips* patterning defect results from a reiteration of the guard mother cell program in the stomatal cell lineage. The longer the GMC program persists, the larger the cluster. GMC persistence might be caused by a failure to positively regulate guard cell identity or to negatively regulate guard mother cell identity in progeny cells, or by a defect in exiting from the cell cycle. FLP is therefore required to limit the number of GMC divisions to one and may act at the intersection of the regulation of the cell cycle and cell specification.

Whereas stomatal clusters in *tmm* result partly from excess asymmetric divisions, *flp* clusters develop mostly from excess symmetric divisions. Based on cluster phenotypes, it is likely that FLP acts later in the stomatal pathway than TMM (Figure 21). It is not clear whether there is any direct interaction between TMM and FLP. *tmm* is epistatic to *flp-1* in many organs. For example, stems of the double mutant lack stomata as in *tmm* (Geisler et al., 1998). Stomatal clusters are smaller in the double mutant than would be predicted if the phenotypes of each mutant were simply additive. But this apparent interaction between the mutations might instead be due to packing constraints. *tmm* clusters are tightly grouped. Perhaps this density restricts the numbers of extra GMCs that form in the double mutant.

Finally, while *flp* clearly affects the one celled spacing pattern, data to date suggest that it does not disrupt this pattern by misplacing precursor cells. Instead, pattern violations appear to result from cell duplications late in the pathway. Thus, FLP may function as a negative regulator of cell division at the GMC to guard cell transition.

HIGH CARBON DIOXIDE (*HIC*)

The many influences of the environment on stomatal density indicate that these signals operate through complex molecular pathways that modulate stomatal number. One such locus is *HIGH CARBON DIOXIDE* (*HIC*). Wild type Arabidopsis plants show a decrease in stomatal number when exposed to abnormally high concentrations of carbon dioxide (Woodward and Kelly, 1995; Gray et al., 2000). This decrease depends on the CO_2 concentration around mature rather than developing leaves suggesting that older leaves send a signal that inhibits stomatal formation in

developing leaves (Brownlee, 2001; Lake et al., 2001; Lake et al., 2002). In contrast to the wild type, the *hic* mutant exhibits an increase in stomata in elevated CO_2 (Gray et al., 2000). High CO_2 increases both the stomatal density and the stomatal index. Stomatal patterning and development are unaffected regardless of the CO_2 level. Besides the conditional response to high CO_2 no other phenotype has been reported for *hic*.

hic was isolated as a promoter trap line that exhibits -*glucuronidase* (*GUS*) staining in stomata. The *GUS* gene inserted in the 3' untranslated region of the HIC gene that is also disrupted in the open reading frame. *HIC* encodes

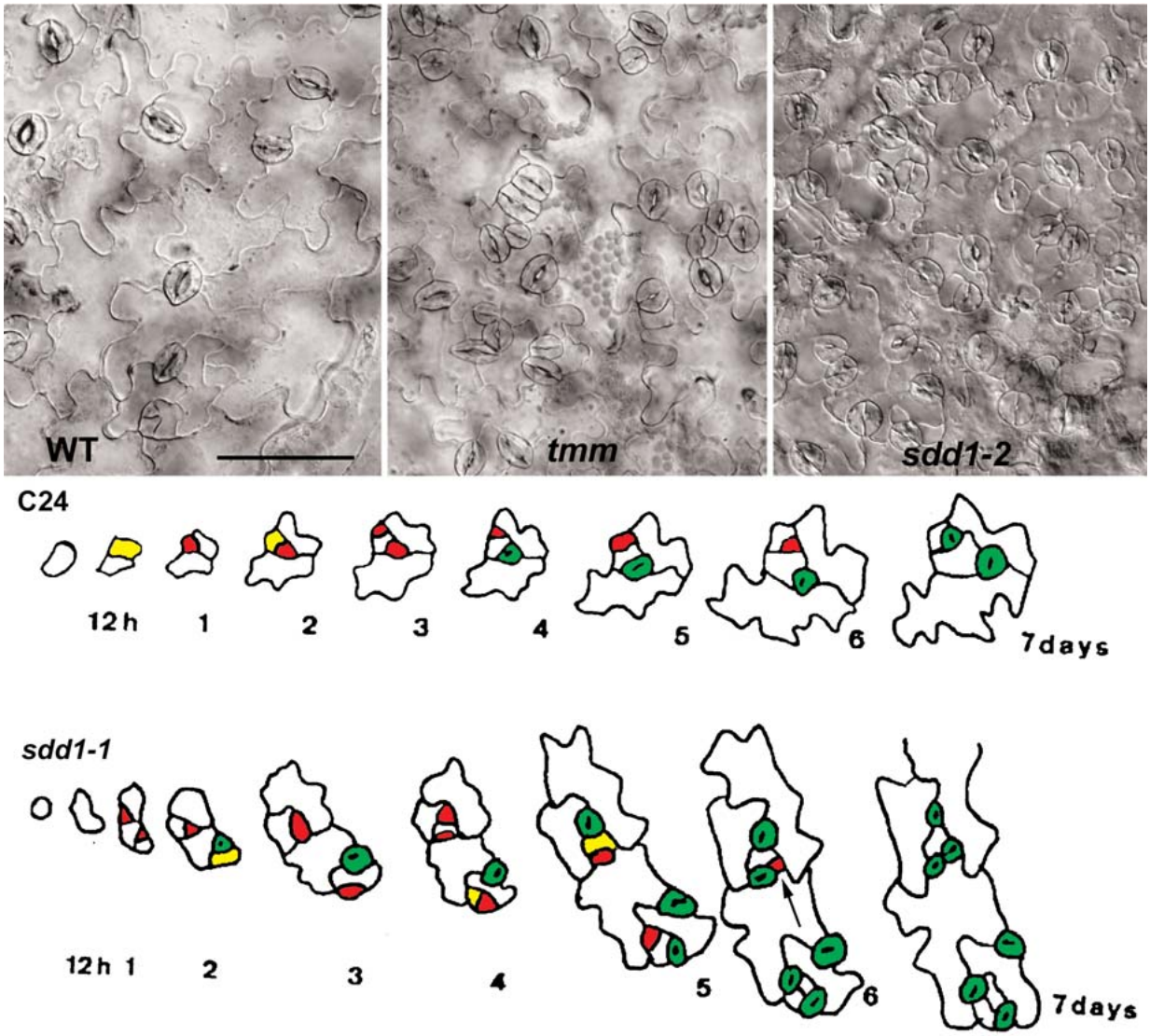

Figure 22. *sdd1* has a Higher Stomatal Density but Fewer Clusters than *tmm*
(Top) Micrographs from leaves of wild type (left), *tmm* (middle), and *sdd1-2* (right). *sdd1-2* has a higher stomatal density but fewer stomata in contact than *tmm*. Bar at left = 50 µm for all three micrographs.
(Bottom) Developmental basis of *sdd1-1* phenotype. Adapted from dental resin series from Berger and Altmann (2000). Wild type (C24 ecotype) at top, *sdd1-1* at bottom. *sdd1* produces extra generations of meristemoids. One stomatal cluster develops from an ectopic satellite meristemoid (arrow, day 6). However, most extra stomata are correctly spaced.
Yellow, meristemoid mother cells (MMCs); red, meristemoids; green, stomata.

a putative 3-keto acyl coenzyme A synthase, a fatty acid elongase that may be involved in the synthesis of very long chain fatty acids such as waxes and cutin. HIC appears to function in a signal transduction pathway that negatively regulates stomatal number in response to an increase in CO_2. It is hypothesized to affect the movement of a repressor through guard cell walls, perhaps by altering wall permeability via very long chain lipids (Gray et al. 2000). Mutations in other loci, such as those affecting jasmonate, ethylene and abscisic acid signaling, also show defects in regulating stomatal number in response to high CO_2 (Lake et al., 2002).

This initial work with *HIC* raises several intriguing issues related to CO_2 concentration. Because no abnormal *hic* phenotype was found at ambient CO_2 levels, HIC may only function when CO_2 levels are high. One alternative is that redundancy may prevent the detection of a *hic* phenotype and function at ambient CO_2. Perhaps HIC-related pathways evolved in response to severe fluctuations in atmospheric CO_2 concentrations during the evolution of land plants.

Other issues concern development and signaling. It will be important to determine whether *HIC* is expressed in stomatal precursors as well as in guard cells. As discussed MMCs arise next to precursor cells, not just stomata. A repressor that acted only around stomata would regulate only a subset of MMCs. Also, HIC would be expected to act in developing leaves because this is a strategic stage to control stomatal density and because young leaves are apparently receive long-distance CO_2 signals. But HIC

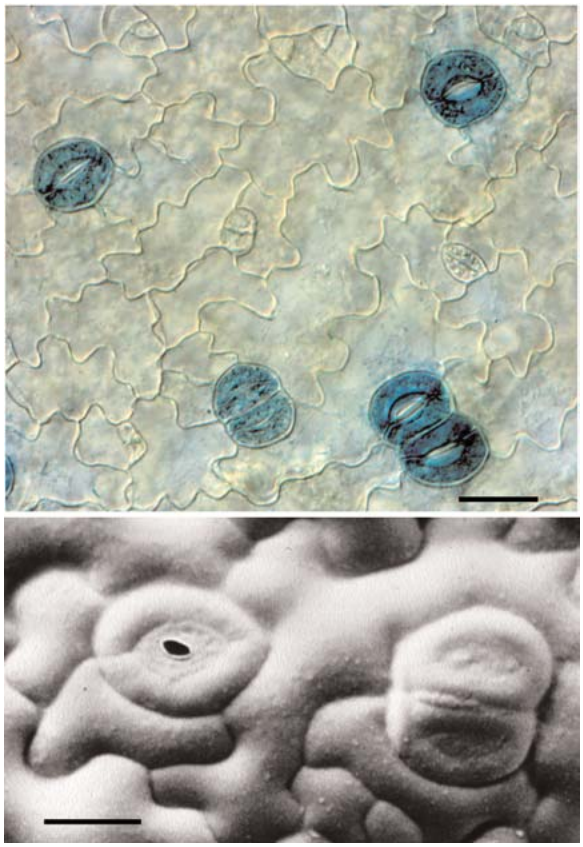

Figure 23. Paired Stomata in *four lips*
(Top) *flp-1* displays both unclustered and clustered stomata. Stomata visualized using KAT::GUS staining (promoter from Rebecca Hirsch and Michael Sussman, methods as in Larkin et al. 1997).
(Bottom) Cryo-scanning electron micrograph showing paired stomata at right. Bars = 200 μm (top) and 10 μm (bottom).

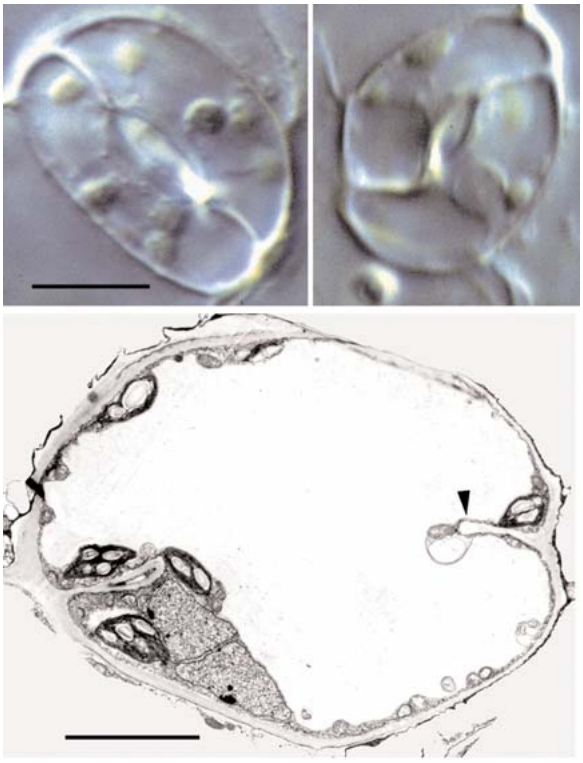

Figure 24. Phenotypes of *mus* and *cyd1*
(Top) Differential interference contrast optics micrographs of wild type (left) and *mustaches* (right) stomata. In *mus*, the pore wall and the shape of the guard cells are skewed with respect to each other. Wild type guard cells are bilaterally symmetric.
(Bottom) Transmission electron micrograph of *cyd1* stoma with incomplete cytokinesis as shown by wall stubs at both end. The left wall stub has a small stomatal pore that is lacking in the stub on the right side (arrowhead). From Yang et al. (1999). Both bars = 5 μm.

TABLE 1: ARABIDOPSIS GENES WITH MUTATIONS KNOWN TO AFFECT STOMATAL DEVELOPMENT

LOCUS	SYMBOL	MUTANT PHENOTYPE	IDENTITY (ACCESSION NO.)	REFERENCES
GLABRA2	*GL2*	ectopic stomata in hypocotyl	Homeodomain leucine zipper (NC003070)	Hung et al. (1998)
WEREWOLF	*WER*	ectopic stomata in hypocotyl	MYB transcription factor (AF126399)	Lee and Schiefelbein (1999); Schiefelbein (2000), (2002)
TRANSPARENT TESTA GLABRA	*TTG*	ectopic stomata in hypocotyl	WD 40 repeats (AJ133743)	Berger et al. (1998b)
FOUR LIPS	*FLP*	stomatal clusters (laterally-aligned)	-	Yang and Sack (1995); Larkin et al. (1997); Geisler et al. (1998)
TOO MANY MOUTHS	*TMM*	stomatal clusters in leaves; no stomata in stems	-	Yang and Sack (1995); Larkin et al., (1997); Geisler et al. (1998, 2000)
STOMATAL DENSITY AND DISTRIBUTION1/NC 003070	*SDD1*	higher stomatal density; some clusters	Subtilisin processing protease (NC003070)	Berger and Altmann (2000); Von Groll and Altmann (2001)
CONSTITUTIVE PHOTOMORPHOGENESIS 10	*COP10*	stomatal clusters	-	Wei et al. (1994); Schwechheimer and Deng (2000); Serna and Fenoll (2000b)
CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM	*CPD*	stomatal clusters	Cytochrome P450 (brassinosteroid synthesis) (X87368)	Szekeres et al. (1996)
HIGH CARBON DIOXIDE	*HIC*	increased stomatal density in high CO_2	Putative fatty acid elongase (NP 182195)	Gray et al. (2000)
AUXIN RESISTANT 2	*AXR2*	decreased stomatal index in stems	Auxin inducible gene (AT3G23050)	Timpte et al. (1994); Nagpal et al. (2000)
CYTOKINESIS DEFECTIVE 1	*CYD1*	stomatal differentiation without cytokinesis	-	Yang et al. (1999)
MUSTACHES	*MUS*	misshapen stomatal pores and guard cells	-	Nadeau and Sack (1997)

might also function in perceiving the CO_2 concentration in older leaves and in transmitting that signal to younger leaves. If so, then it will be critical to work out how a fatty acid elongase influences long-distance signaling.

OTHER MUTANTS

Mutants known to affect stomatal development to varying degrees are shown in Table 1. Like the mutants discussed previously (*flp*, *hic*, *sdd1*, and *tmm*), *mustaches* appears to only affect stomatal development. *mus* has abnormally-shaped guard cells, and absent or skewed stomatal pores and thus seems to regulate stomatal morphogenesis (Figure 24 top; Nadeau and Sack, 1997).

Other mutations shown in Table 1 are pleiotropic. Some, such as *ectopic root hair3* (Schneider et al., 1997), *gl2*, *wer*, and *ttg* affect the patterning of several different cell types. Others affect basic signaling processes that also control stomatal development. For example, mutations at some loci that cause constitutive photomorphogenesis in the dark such as *cpd*, and *cop10* also display stomatal clusters (Szekeres et al., 1996; Serna and Fenoll, 2000b). *axr2,* which is auxin resistant, affects the stomatal index in stems (Timpte et al., 1994).

cyd1 exhibits cytokinesis defects throughout the shoot, but these defects are particularly noticeable in GMCs because their divisions are so stereotyped (Figure 24 bottom; Yang et al., 1999). Some mature stomata lack any dividing wall. Others have wall stubs with or without pores. The resulting cells display cytological traits typical of stomata and express appropriate markers indicating that aspects of stomatal differentiation do not require cytokinesis of the GMC. The incomplete walls are correctly placed opposite the division site wall thickenings of the GMC indicating that it is the execution, not the placement of cytokinesis that is defective.

OVERVIEW AND FUTURE DIRECTIONS

Although the events and genes involved in stomatal development are just beginning to be described, what we have learned reveals a pathway that is fascinating and complex. The minimal one-celled spacing apparently results from cell communication that orients specific asymmetric divisions (Figure 21). TMM is required for the orientation of these divisions and for regulating the fate of various cells in a position-dependent manner. TMM seems to function as a negative or positive regulator of entry into the pathway depending upon the organ and domain. SDD1 is a negative regulator of stomatal formation throughout the plant. Although *tmm* and *sdd1* disrupt some of the same events, their phenotypes are distinct. FLP may limit GMC divisions and MUS affects stomatal differentiation; thus both probably act downstream of TMM and SDD1 in the

developmental pathway.

Much remains to be clarified such as the molecular and cell biological mechanisms that control the position and type of division, the specification of different precursors, and the progressive morphogenesis of the guard cell. Little is known about the roles of environmental cues such as the photoreceptors and downstream targets that influence stomatal number and how environmental stresses such as high relative humidity and mechanical perturbation disrupt stomatal patterning (Serna and Fenoll, 1997). In contrast to stomatal movement where the participation of growth regulators is well established, the roles of hormones in controlling stomatal density are not yet defined.

Of course, characterization of molecular identities and expression patterns of existing loci will allow pathways and additional players to be determined. Of key interest is whether cell communication in the pathway relies upon a peptide signal, e.g. one released by the action of SDD1, a processing protease. Similarly, the nature and extent of communication between the epidermis and subjacent layers in stomatal patterning deserves careful analysis.

Many more loci must function in stomatal development than have been found to date. There are at least about five times as many genes known to affect the development of root hairs or trichomes than stomata (Schiefelbein 2002; Marks, 2002). The detection of stomatal mutants is relatively labor intensive, and thus the screen is probably far from saturated. For example, TMM probably interacts with other factors that regulate whether it functions positively or negatively in stomatal formation. And the elaborate and unique architecture of the guard cell presumably requires more than MUS for morphogenesis. It remains to be seen whether stomatal development requires novel types of genes and molecular pathways and/or whether it uses members of known gene families in new contexts. Regardless, given the complexity and importance of stomatal development, this field is likely to inform and flourish for years to come.

ACKNOWLEDGMENTS

Thanks to Tsuyoshi Nakagawa, Matt Geisler, Liming Zhao, Kim Findlay, and Lien Lai for sharing ideas and figures. Supported by grants from NSF and USDA.

APPENDIX: STOMATAL TERMINOOGY RELEVANT TO ARABIDOPSIS

Stoma (pl. stomata; also stomate and stomates): an epidermal structure consisting of two guard cells that surround a pore whose width is actively regulated.

Guard cell: A kidney-shaped cell that changes shape with alterations in turgor thus affecting pore width. Wild type guard cells are paired and face each other.

Stomatal complex: The stoma plus adjacent, surrounding epidermal cells.

Neighbor cell: an epidermal cell in contact with a guard cell or precursor.

Subsidiary cell: a neighbor cell that is morphologically distinct from other epidermal cells.

Anisocytic complex: a stomatal complex common to the Brassicaceae which includes Arabidopsis; the stoma is surrounded by three subsidiary cells, one of which is much smaller.

Sub-stomatal cavity: the airspace in the mesophyll under the stomatal pore.

Stomatal lineage: starts with the selection and division of an MMC. The formation of an MMC from a cell (larger sister cell to a meristemoid) that was produced by an earlier lineage can either be considered the start of a new lineage, or a continuation of the older one, depending on context and author.

Clonal vs. non-clonal neighbor cell: A neighbor cell that does or does not share a common cell lineage with the adjacent stoma, meristemoid or GMC.

Monoclonal vs. polyclonal: Complexes in which all neighbor cells derive from the same cell lineage as the central stoma are considered to be monoclonal.

Asymmetric division: each daughter cell has a different fate; the asymmetric divisions of MMCs and meristemoids are also asymmetric in cell size.

Symmetric division: The two daughter cells have the same fate and usually size. The symmetric division of smaller epidermal cells produces two cells of equal developmental potential.

Meristemoid mother cell (MMC): a relatively small epidermal cell that has entered the stomatal pathway by becoming committed to divide asymmetrically. Division produces a smaller cell that becomes a meristemoid. The larger daughter cell can become a pavement cell or an MMC.

Meristemoid: A small often triangular cell that can divide asymmetrically. Asymmetric division regenerates the meristemoid and produces a larger daughter cell. Some meristemoids do not divide. Meristemoids eventually convert into guard mother cells.

Satellite meristemoid: Similar to other meristemoids except that they form in neighbor cells i.e. the neighbor cell becomes an MMC. The orientation of the MMC division is regulated so that the satellite meristemoid is located away from the pre-existing stoma/precursor. This is a major mechanism of stomatal patterning.

Guard mother cell (GMC): The terminal precursor that divides symmetrically and produces two guard cells.

Pavement cells: Generic, relatively large, jigsaw puzzle-shaped epidermal cells including some neighbor cells.

Small less sinuous epidermal cells: These cells can divide symmetrically or asymmetrically or differentiate as a pavement cell. They are found next to stomata and in the protoderm.

Stomatal density: Number of stomata per unit area.

Stomatal index: Number of stomata divided by total number of epidermal cells including stomata.

REFERENCES

Appel, B., Givan, L.A., and Eisen, J.S. (2001). Delta-Notch signaling and lateral inhibition in zebrafish spinal cord development. BMC Devel. Biol. **1,** 13.

Baum, S.F., Eshed, Y., and Bowman, J.L. (2001). The Arabidopsis nectary is an ABC-independent floral structure. Development **128,** 4657-4667.

Benfey, P.N. (1999). Is the shoot a root with a view? Curr. Opin. Plant Biol. **2,** 39-43.

Berger, D., and Altmann, T. (2000). A subtilisin-like serine protease involved in the regulation of stomatal density and distribution in *Arabidopsis thaliana*. Genes Devel. **14,** 1119-1131.

Berger, F., Haseloff, J., Schiefelbein, J., and Dolan, L. (1998a). Positional information in root epidermis is defined during embryogenesis and acts in domains with strict boundaries. Curr. Biol. **8,** 421-430.

Berger, F., Linstead, P., Dolan, L., and Haseloff, J. (1998b). Stomata patterning on the hypocotyl of *Arabidopsis thaliana* is controlled by genes involved in the control of root epidermis patterning. Devel. Biol. **194,** 226-234.

Bowman, J.L. (1994). *Arabidopsis*: an atlas of morphology and development (NY: Springer-Verlag).

Bowman, J.L., and Smyth, D.R. (1999). CRABS CLAW, a gene that regulates carpel and nectary development in Arabidopsis, encodes a novel protein with zinc finger and helix-loop- helix domains. Development **126,** 2387-2396.

Brownlee, C. (2001). The long and the short of stomatal density signals. Trends Pl. Sci. **6,** 441-442.

Bünning, E. (1953). Entwicklungs- und Bewegungsphysiologie der Pflanze (Berlin: Springer-Verlag).

Chen, T., Hiroko, T., Chaudhuri, A., Inose, F., Lord, M., Tanaka, S., Chant, J., and Fujita, A. (2000). Multigenerational cortical inheritance of the Rax2 protein in orienting polarity and division in yeast. Science **290,** 1975-1978.

Croxdale, J.L. (2000). Stomatal patterning in angiosperms. Am. J. Bot. **87,** 1069-1080.

Cutler, S.R., Ehrhardt, D.W., Griffitts, J.S., and Somerville, C.R. (2000). Random GFP::cDNA fusions enable visualization of subcellular structures in cells of Arabidopsis at a high frequency. Proc. Natl. Acad. Sci. **97,** 3718-3723.

Davis, A.R., and Gunning, B.E.S. (1993). The modified stomata of the floral nectary of *Vicia faba* L. 3. Physiological aspects, including comparisons with foliar stomata. Bot. Acta **106,** 241-253.

Donnelly, P.M., Bonetta, D., Tsukaya, H., Dengler, R.E., and Dengler, N.G. (1999). Cell cycling and cell enlargement in developing leaves of *Arabidopsis*. Devel. Biol. **215,** 407-419.

Esau, K. (1977). Anatomy of Seed Plants (New York: John Wiley).

Franks, P.J., and Farquhar, G.D. (2001). The effect of exogenous abscisic acid on stomatal development, stomatal mechanics, and leaf gas exchange in *Tradescantia virginiana*. Plant Physiol. **125,** 935-942.

Galatis, B., and Mitrakos, K. (1979). On the differential divisions and preprophase microtubule bands involved in the development of stomata of *Vigna sinensis* L. J Cell Sci **37,** 11-37.

Galatis, B., and Mitrakos, K. (1980). The ultrastructural cytology of the differentiating guard cells of *Vigna sinensis*. Am. J. Bot. **67,** 1243-1261.

Galatis, B., Apostolakos, P., Katsaros, C., and Loukari, H. (1982). Pre-prophase microtubule band and local wall thickening in guard cell mother cells of some Leguminosae. Ann. Bot. **50,** 779-791.

Galway, M.E., Masucci, J.D., Lloyd, A.M., Walbot, V., Davis, R.W., and Schiefelbein, J.W. (1994). The *TTG* gene is required to specify epidermal cell fate and cell patterning in the *Arabidopsis* root. Devel. Biol. **166,** 740-754.

Geisler, M., Yang, M., and Sack, F.D. (1998). Divergent regulation of stomatal initiation and patterning in organ and suborgan regions of the *Arabidopsis* mutants *too many mouths* and *four lips*. Planta **205,** 522-530.

Geisler, M., Nadeau, J., and Sack, F.D. (2000). Oriented asymmetric divisions that generate the stomatal spacing pattern in *Arabidopsis* are disrupted by the *too many mouths* mutation. Plant Cell **12,** 2075-2086.

Geisler, M., and Sack, F.D. (2002). Variable timing of the developmental progression in the stomatal pathway in *Arabidopsis* cotyledons. New Phytol. **153,** 469-476.

Gendreau, E., Traas, J., Desnos, T., Grandjean, O., Caboche, M., and Hofte, H. (1997). Cellular basis of hypocotyl growth in *Arabidopsis thaliana*. Plant Physiol. **114,** 295-305.

Gendreau, E., Hofte, H., Grandjean, O., Brown, S., and Traas, J. (1998). Phytochrome controls the number of endoreduplication cycles in the *Arabidopsis thaliana* hypocotyl. Plant J. **13,** 221-230.

Glover, B.J. (2000). Differentiation in plant epidermal cells. J. Exp. Bot. **51,** 497-505.

Glover, B.J., and Martin, C. (2000). Specification of epidermal cell morphology. Adv. Bot. Res. **31,** 193-217.

Gray, J.E., Holroyd, G.H., van der Lee, F.M., Bahrami, A.R., Sijmons, P.C., Woodward, F.I., Schuch, W., and Hetherington, A.M. (2000). The HIC signalling pathway links CO2 perception to stomatal development. Nature **408,** 713-716.

Hung, C.Y., Lin, Y., Zhang, M., Pollock, S., Marks, M.D., and Schiefelbein, J. (1998). A common position-dependent mechanism controls cell-type patterning and GLABRA2 regulation in the root and hypocotyl epidermis of *Arabidopsis*. Plant Physiol. **117,** 73-84.

Korn, R.W. (1972). Arrangement of stomata on the leaves of *Pelargonium zonale* and *Sedum stahlii*. Ann. Bot. **36,** 325-333.

Korn, R.W. (1993). Evidence for stomatal patterning by inhibition. Int. J. Plant Sci. **154,** 367-377.

Korn, R.W. (1994). Pattern formation in the epidermis through inhibition of immediately adjacent cells by pattern elements. Protoplasma **180,** 145-152.

Lake, J.A., Quick, W.P., Beerling, D.J., and Woodward, F.I. (2001). Plant development. Signals from mature to new leaves. Nature **411,** 154.

Lake, J.A., Woodward, F.I., and Quick, W.P. (2002). Long-distance CO2 signalling in plants. J. Exp. Bot. **53,** 183-193.

Landré, P. (1972). Origine et développemente des épidermes cotylédonaires et foliares de la moutarde (*Sinapis alba* L.). Différenciation ultrastructurale des stomates. Ann. Sci. Nat. Bot. Biol. Veg. **12,** 247-322.

Larkin, J.C., Young, N., Prigge, M., and Marks, M.D. (1996). The control of trichome spacing and number in *Arabidopsis*. Development **122,** 997-1005.

Larkin, J.C., Marks, M.D., Nadeau, J., and Sack, F. (1997). Epidermal cell fate and patterning in leaves. Plant Cell **9,** 1109-1120.

Lee, M.M., and Schiefelbein, J. (1999). WEREWOLF, a MYB-related protein in Arabidopsis, is a position-dependent regulator of epidermal cell patterning. Cell **99,** 473-483.

Marc, J. (1997). Microtubule-organizing centres in plants. Trends Pl. Sci. **2,** 223-230.

Martin, C., and Glover, B.J. (1998). Cellular differentiation in the shoot epidermis. Curr. Opin. Plant Biol. **1,** 511-519.

Masucci, J.D., and Schiefelbein, J.W. (1996). Hormones act downstream of TTG and GL2 to promote root hair outgrowth during epidermis development in the Arabidopsis root. Plant Cell **8,** 1505-1517.

Melaragno, J.E., Mehrotra, B.M., and Coleman, A.W. (1993). Relationship between endopolyploidy and cell size in epidermal tissue of *Arabidopsis*. Plant Cell **5,** 1661-1668.

Metcalfe, C.R., and Chalk, L. (1950). Anatomy of Dicotyledons. (Oxford: Clarendon Press).

Nadeau, J.A., and Sack, F.D. (1997). The *Arabidopsis* mutant *mustaches* is defective in guard cell shape and pore formation. International Arabidopsis Meeting, Abstract 3:49.

Nagpal, P., Walker, L.M., Young, J.C., Sonawala, A., Timpte, C., Estelle, M., and Reed, J.W. (2000). AXR2 encodes a member of the Aux/IAA protein family. Plant Physiol **123,** 563-574.

Pant, D.D., and Kidwai, P.F. (1967). Development of stomata in some Cruciferae. Ann. Bot. **31,** 513-521.

Payne, C.T., Zhang, F., and Lloyd, A.M. (2000). GL3 encodes a bHLH protein that regulates trichome development in Arabidopsis through interaction with GL1 and TTG1. Genetics **156,** 1349-1362.

Pyke, K., Marrison, J.L., and Leech, R.M. (1991). Temporal and spatial development of the cells of the expanding first leaf of *Arabidopsis thaliana* (L.) Heynh. J. Exp. Bot. **42,** 1407-1416.

Sachs, T. (1978). The development of spacing patterns in the leaf epidermis. Pp., 161-183 *In*: The Clonal Basis of Development, ed. S. Subtelny and I. M. Sussex (New York. Academic Press).

Sachs, T. (1979). Cellular interactions in the development of stomatal patterns in *Vinca major* L. Ann. Bot **43,** 693-700.

Sachs, T. (1991). Pattern Formation in Plant Tissues (New York: Cambridge University Press).

Sachs, T., and Kagan, M.L. (1993). Variable development and cellular patterning in the epidermis of *Ruscus hypoglossum*. Ann. Bot. **71,** 237-243.

Sachs, T., and Novoplansky, N. (1993). The development and patterning of stomata and glands in the epidermis of *Peperomia*. New Phytol. **123,** 567-574.

Sack, F.D. (1987). The development and structure of stomata. Pp., 59-89 *In*: Stomatal Function, ed. E. Zeiger, G. D. Farquhar and I. R. Cowan (Stanford. Stanford University Press).

Schiefelbein, J.W. (2000). Constructing a plant cell. The genetic control of root hair development. Plant Physiol. **124,** 1525-1531.

Schneider, K., Wells, B., Dolan, L., and Roberts, K. (1997). Structural and genetic analysis of epidermal cell differentiation in Arabidopsis primary roots. Development **124,** 1789-1798.

Schroeder, J.I., Allen, G.J., Hugouvieux, V., Kwak, J.M., and Waner, D. (2001). Guard cell signal transduction. Annu. Rev. Plant Physiol. Plant Molec. Biol. **52,** 627-658.

Schwechheimer, C., and Deng, X.W. (2000). The COP/DET/FUS proteins-regulators of eukaryotic growth and development. Sem. Cell Devel. Biol. **11,** 495-503.

Serna, L., and Fenoll, C. (1997). Tracing the ontogeny of stomatal clusters in Arabidopsis with molecular markers. Plant J. **12,** 747-755.

Serna, L., and Fenoll, C. (2000a). Stomatal development and patterning in *Arabidopsis* leaves. Physiol. Plant. **109,** 351-358.

Serna, L., and Fenoll, C. (2000b). Stomatal development in *Arabidopsis*: How to make a functional pattern. Trends Pl. Sci. **5,** 458-460.

Serna, L., Torres-Contreras, J., and Fenoll, C. (2002). Clonal analysis of stomatal development and patterning in *Arabidopsis* leaves. Devel. Biol. **241,** 24-33.

Sessions, A., Nemhauser, J.L., McColl, A., Roe, J.L., Feldmann, K.A., and Zambryski, P.C. (1997). ETTIN patterns the *Arabidopsis* floral meristem and reproductive organs. Development **124,** 4481-4491.

Smith, L.G. (2001). Plant cell division: Building walls in the right places. Nat. Rev. Mol. Cell Biol. **2,** 33-39.

Sylvester, A.W., Smith, L., and Freeling, M. (1996). Acquisition of identity in the developing leaf. Annu. Rev. Cell Devel. Biol. **12,** 257-304.

Szekeres, M., Nemeth, K., Koncz-Kalman, Z., Mathur, J., Kauschmann, A., Altmann, T., Redei, G.P., Nagy, F., Schell, J., and Koncz, C. (1996). Brassinosteroids rescue the deficiency of CYP90, a cytochrome P450, controlling cell elongation and de-etiolation in Arabidopsis. Cell **85,** 171-182.

Tang, Y., and Liang, N. (2000). Characterization of the photosynthetic induction response in a *Populus* species with stomata barely responding to light changes. Tree Physiol. **20,** 969-976.

Timpte, C., Wilson, A.K., and Estelle, M. (1994). The *axr2-1* mutation of *Arabidopsis thaliana* is a gain-of-function mutation that disrupts an early step in auxin response. Genetics **138,** 1239-1249.

von Groll, U., and Altmann, T. (2001). Stomatal cell biology. Curr. Opin. Plant Biol. **4,** 555-560.

Wasteneys, G.O., WillingaleTheune, J., and Menzel, D. (1997). Freeze shattering: a simple and effective method for permeabilizing higher plant cell walls. J. Microscopy **188,** 51-61.

Wei, N., Kwok, S.F., von Arnim, A.G., Lee, A., McNellis, T.W., Piekos, B., and Deng, X.-W. (1994). Arabidopsis *COP8*, *COP10*, and *COP11* genes are involved in repression of photomorphogenic development in darkness. Plant Cell **6,** 629-643.

Woodward, F.I., and Kelly, C.K. (1995). The influence of CO2 concentration on stomatal density. New Phytol. **131,** 311-327.

Yang, M., and Sack, F.D. (1995). The *too many mouths* and *four lips* mutations affect stomatal production in Arabidopsis. Plant Cell **7,** 2227-2239.

Yang, M., Nadeau, J.A., Zhao, L.M., and Sack, F.D. (1999). Characterization of a cytokinesis defective (*cyd1*) mutant of Arabidopsis. J. Exp. Bot. **50,** 1437-1446.

Zhao, L., and Sack, F.D. (1999). Ultrastructure of stomatal development in Arabidopsis (Brassicaceae) leaves. Am. J. Bot. **86,** 929-939.