Stomatal Development

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Abstract

Stomata are cellular epidermal valves in plants central to gas exchange and biosphere productivity. The pathways controlling their formation are best understood for *Arabidopsis thaliana* where stomata are produced through a series of divisions in a dispersed stem cell compartment. The stomatal pathway is an accessible system for analyzing core developmental processes including position-dependent patterning via intercellular signaling and the regulation of the balance between proliferation and cell specification. This review synthesizes what is known about the mechanisms and genes underlying stomatal development. We contrast the functions of genes that act earlier in the pathway, including receptors, kinases, and proteases, with those that act later in the cell lineage. In addition, we discuss the relationships between environmental signals, stomatal development genes, and the capacity for controlling shoot gas exchange.

Contents

INTRODUCTION

Stomata are epidermal valves that are essential for plant survival because they control the entry of carbon dioxide assimilated in photosynthesis and optimize water use efficiency (58, 63). Collectively, these valves enhance plant performance and influence global water and carbon cycles. Each stoma originates through a series of divisions that generate the stomatal spacing pattern, control stomatal frequency, and produce the two guard cells of the valve (49). The importance of these divisions is underscored by the finding that all genes shown to act during stomatal development function by regulating division, with different sets of genes controlling different transitions in the progression toward terminal cell fates. This review focuses on *Arabidopsis thaliana*, the source of most currently known "stomatal" genes.

Major research efforts have elucidated the signal transduction pathways that control the movement of mature stomata, especially in response to abiotic stresses (66) at the whole-plant, canopy, and global levels. For developmental biology, this technically accessible epidermal system is valuable for understanding how plant cells are specified and patterned and how these events are integrated with specific division modes. For example, relatively little is known about how division polarities are generated and oriented in plants compared with animals and yeast, but the stomatal pathway is a promising system for studying these questions.

Meristemoid: intermediate precursor cell that undergoes one or more amplifying asymmetric divisions that regenerate the meristemoid and increase the number of larger daughter cells capable of founding the next generation in the lineage; converts into a GMC

Stomatal Cell Lineage

Stomata are produced by a dedicated and specialized cell lineage (24, 49, 50, 84). This lineage is prevalent in the developing shoot epidermis (such as in young leaves) and is inactive after epidermal maturation. The lineage starts with an asymmetric division and ends with a symmetric one (**Figure 1**). The former division marks pathway entry and produces a small stomatal precursor cell, the meristemoid. The latter produces the two cells of the stoma. Between these events, the meristemoid converts into a guard mother cell (GMC), the end-stage precursor cell. Meristemoids usually divide asymmetrically several times, whereas GMCs divide just once symmetrically. Thus, a stoma is produced after a series of cell fate changes with each precursor cell undergoing a specific cell division program.

Each asymmetric division produces a meristemoid and a larger sister cell. The latter can divide or become a pavement cell, the generic type of cell in the epidermis. Asymmetric divisions in the lineage can be grouped by context and function into three types. Entry divisions occur in division-competent postprotodermal cells called meristemoid mother cells (MMCs) and

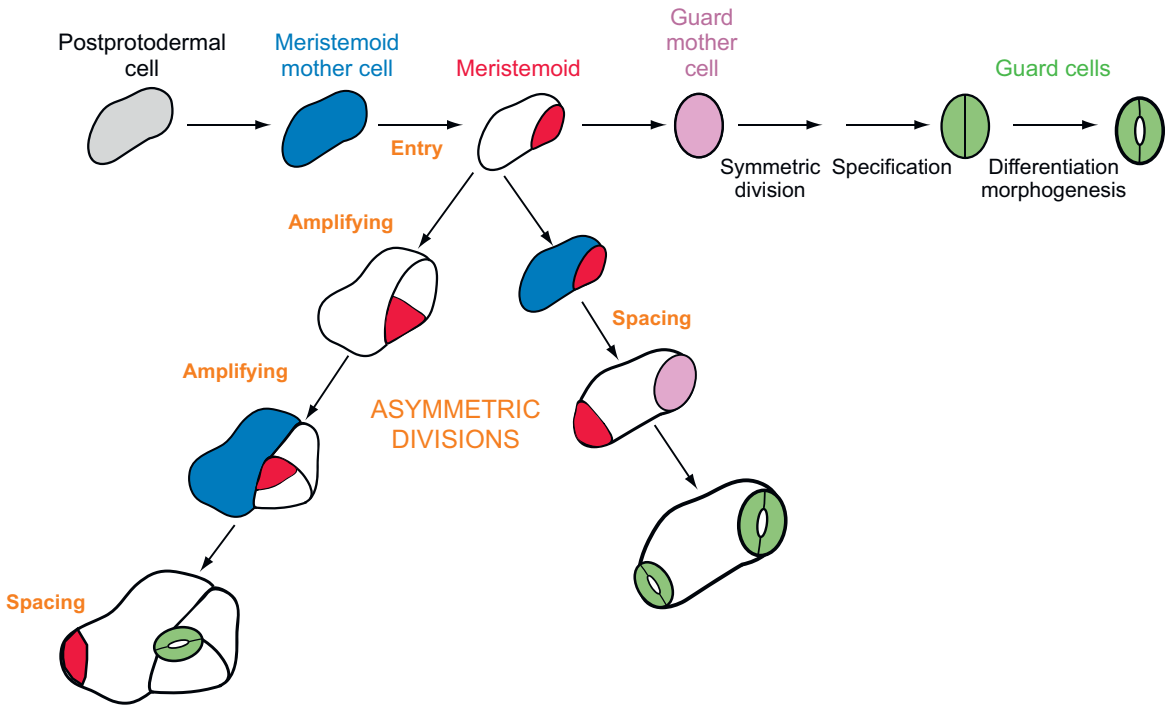

Figure 1

Diagram of key stages and divisions in *Arabidopsis thaliana* stomatal development. Protodermal cells in the epidermis are converted into meristemoid mother cells (MMCs) through an unknown process. MMCs undergo an asymmetric entry division to create a meristemoid. Meristemoids may undergo additional asymmetric amplifying divisions, or convert into a guard mother cell (GMC). The GMC will divide a single time, symmetrically, to form the two guard cells. Later, morphogenesis and pore formation create the mature stoma. The division process is reiterative. Cells next to meristemoids, GMCs, and guard cells can become MMCs and undergo spacing divisions to create new meristemoids. The plane of this division is oriented so that the new meristemoid is placed away from the preexisting stoma or precursor cell.

initiate the stomatal lineage. Amplifying divisions occur in meristemoids and increase the number of larger daughter cells, thereby increasing the epidermal cell total. The meristemoid is regenerated in each amplifying division. Spacing divisions take place in cells next to a stoma or precursor, and establish the one-cell spacing pattern in which stomata do not directly contact each other. Both entry and spacing divisions produce new meristemoids and thus directly increase the total number of stomata formed. Amplifying divisions increase stomatal number indirectly by producing larger daughter cells that undergo spacing divisions. The net effect of all these divisions is an increase in the number of pavement cells as well as stomata. Output estimates suggest that most of the epidermal cells in a leaf are generated by the stomatal lineage (24).

The number of stomata produced depends on the frequency of the different types of asymmetric divisions. If no larger daughter cells divide then only one stoma will be produced per lineage even if there are many amplifying divisions. If larger daughter cells do divide (via spacing divisions) then the initial entry division can lead to many generations of stomata (2, 26). These variations provide a developmental mechanism to generate the observed differences in stomatal number between different parts of a plant (for example, between the adaxial and abaxial leaf

Guard mother cell (GMC): last precursor cell in the lineage; it divides symmetrically, producing the two guard cells of a stoma

Pavement cell: the general or ground type of epidermal cell; often produced by asymmetric divisions in the stomatal pathway

epidermis, or between stems and leaves), between plants in bright and shaded environments, and among different taxa (25, 26, 62).

The attributes and timing of asymmetric and symmetric divisions provide a rough framework for analyzing the function of genes during stomatal development. Earlier-acting genes largely control asymmetric divisions and thus cell proliferation, patterning, and stomatal number. Later-acting genes function at or after the terminal symmetric division in processes such as stopping cell proliferation and promoting stomatal morphogenesis. Genes that act in mature stomata are considered elsewhere in this volume (66).

Meristemoid mother cell (MMC): earliest precursor cell that founds the cell lineage by undergoing an entry asymmetric division

Spacing division: an asymmetric division in a neighbor cell that establishes the one-celled stomatal spacing pattern when the new wall is placed away from the stoma or precursor cell

Neighbor cell: an epidermal cell adjacent to a stoma, meristemoid, or GMC

Patterning

Although stomatal density varies, virtually all stomata are separated by at least one intervening cell (24, 60). This one-celled spacing is probably adaptive in generating solute reservoirs between stomata, in minimizing mechanical interference between adjacent valves, and in optimizing the distribution of gas diffusion shells. The spacing pattern arises when a new asymmetric division takes place next to an existing stoma (24). Here the division is oriented so that the new wall is placed away from the stoma, resulting in the larger daughter cell—not the new meristemoid—contacting the stoma.

Patterning likely involves the transmission of spatial cues from the stoma to the adjacent cell that are used to correctly orient the plane of the spacing division. Patterning is unlikely to require mitosis-allocated factors because stomata are correctly spaced even when the cells involved are clonally unrelated. Intercellular signaling probably occurs through the free space of the cell wall (apoplasm) because mature stomata can signal even though they lack plasmodesmata. In addition to the divisions occurring next to stomata, divisions next to stomatal precursors (meristemoids and GMCs) are also correctly oriented, suggesting that these cells can also broadcast spatial cues (24, 43). The earliest marker of a cell about to undergo a spacing division is the appearance of a preprophase band of microtubules surrounding a nucleus that is located away from a neighboring stoma or precursor cell (43).

Many tissues are patterned by interactions between differentiated cells and their immature neighbors (21). Some plant epidermal cells, like trichomes and root hairs, are probably patterned by lateral inhibition (38). The stomatal spacing mechanism differs from lateral inhibition because the neighbor cell is not prevented from acquiring a particular cell fate (49). Neither is stomatal spacing due to the generation of a border of surrounding nonstomatal cells via a series of stereotyped asymmetric divisions. In fact, the neighbors, far from providing a "boundary," are more likely to produce stomata than other epidermal cells. Instead, spacing appears to result from cell-cell signaling that orients the plane of asymmetric division in cells situated next to a stoma or precursor.

A secondary spacing module generates the "anisocytic" arrangement of cells around the stoma typical of the Brassicaceae (including *Arabidopsis)* (54, 55). Here three successive asymmetric divisions form an inward spiral (23, 65). Although this pattern is common in *Arabidopsis*, it is, in contrast to the one-cell spacing pattern, not invariant. Because these spiral divisions all take place in same-cell lineage, the mechanism could involve the control of wall placement by landmarks deposited during successive mitoses—a process similar to that used in yeast bud site selection.

EARLY-ACTING GENES

Perhaps the most dramatic recent progress in the field of stomatal development has been the identification of genes that control the production and spacing of *Arabidopsis* stomata. These genes, which comprise putative receptors, a processing protease, and a kinase, act primarily by modulating the number and placement of asymmetric divisions in the stomatal cell lineage.

Receptor-Mediated Cell-Cell Signaling and Pattern Formation

Mutations in the *TOO MANY MOUTHS (TMM)* gene lead to alterations in all types of asymmetric divisions in the stomatal lineage. *tmm* mutants form excess stomata in leaves (see **Figure 2**) indicating that the normal role of *TMM* is to repress divisions. *tmm-1* mutants fail to orient the asymmetry of spacing divisions, fail to inhibit asymmetric divisions in cells adjacent to two or more stomata or their precursor cells, and have a reduced number of amplifying divisions, which leads to the premature conversion of meristemoids into GMCs (24). The first two of these *tmm-1* phenotypes appear to arise from a failure of neighbor cells to respond to positional cues.

TMM encodes a putative cell-surface receptor that is expressed in meristemoids, in GMCs, and in sister cells of the asymmetric divisions that create meristemoids (49). The protein also appears in cells likely to undergo entry division. *TMM* expression is absent from fully differentiated guard cells and from pavement cells. This expression pattern is consistent with TMM normally being required for stomatal lineage cells to perceive and respond to signals that control the number and orientation of spacing divisions.

Receptor-mediated signal transduction in *Arabidopsis* follows the logic of animal signaling, but does not employ the receptor tyrosine kinases typical of animals. In *Arabidopsis*, more than 200 receptor-like kinases share a common structure of leucine-rich extracellular domains, a single transmembrane domain, and a cytoplasmic kinase (LRR-RLKs) (67, 76). TMM is a member of a related class of proteins that contains extracellular LRRs and a transmembrane domain, but lacks the kinase domain (LRR-RLPs) (48). Based on studies of the shoot meristem size control genes *CLAVATA1 (CLV1)* and *CLAVATA2 (CLV2)* that encode an LRR-RLK and LRR-RLP, respectively, it was hypothesized that LRR-RLPs require an LRR-RLK to participate in signal transduction (16).

ERECTA (ER) is an LRR-RLK required for diverse processes including growth and development, as well as responses to biotic and abiotic stresses (39, 68). Many processes affected by loss of ER function are united in their requirement for cell proliferation. Expression of a form of ER missing the kinase domain (ER-Δkinase) yields growth phenotypes more severe than in *er* null mutants, suggesting that the truncated ER protein acts as a dominant negative, probably by forming unproductive heterodimers with other LRR-RLKs (69). Likely partners are *ER's* closest homologs *ERECTA-LIKE 1 (ERL1)* and *ERECTA-LIKE 2 (ERL2)*, which can each partially rescue the *er* growth phenotype when driven by the *ER* promoter (68). Under normal growth conditions neither *erl1* or *erl2* single mutants nor the *erl1;erl2* double mutant has any obvious growth phenotype, but *er;er1;er2* triple mutants are dwarfed and sterile (68).

Examination of the *er;erl1;erl2* shoot epidermis reveals striking stomatal overproliferation and spacing defects (70). These phenotypes, like the growth phenotype, reveal functional redundancy. For example, only the triple mutant shows stomatal spacing defects, suggesting that the activity of any ER-family member on its own is sufficient for pattern generation. However, ER, ERL1, and ERL2 have subtly different roles in epidermal development. Meristemoid differentiation, for example, is consistently inhibited by ERL1 and is often promoted by ER. ER also appears to function in repressing entry divisions. The contrasting phenotypes of different double and triple *ER*-family mutant combinations highlight different genetic requirements during various stages of stomatal development.

TMM and ER-family members control asymmetric divisions in stomatal development. *TMM* and the *ER*-family also have overlapping domains of gene expression. ER is rather broadly expressed, but *TMM*, *ERL1*, and *ERL2* all show overlapping expression patterns in aerial organs (48, 70). LRR-RLKs are surmised to act as homodimers

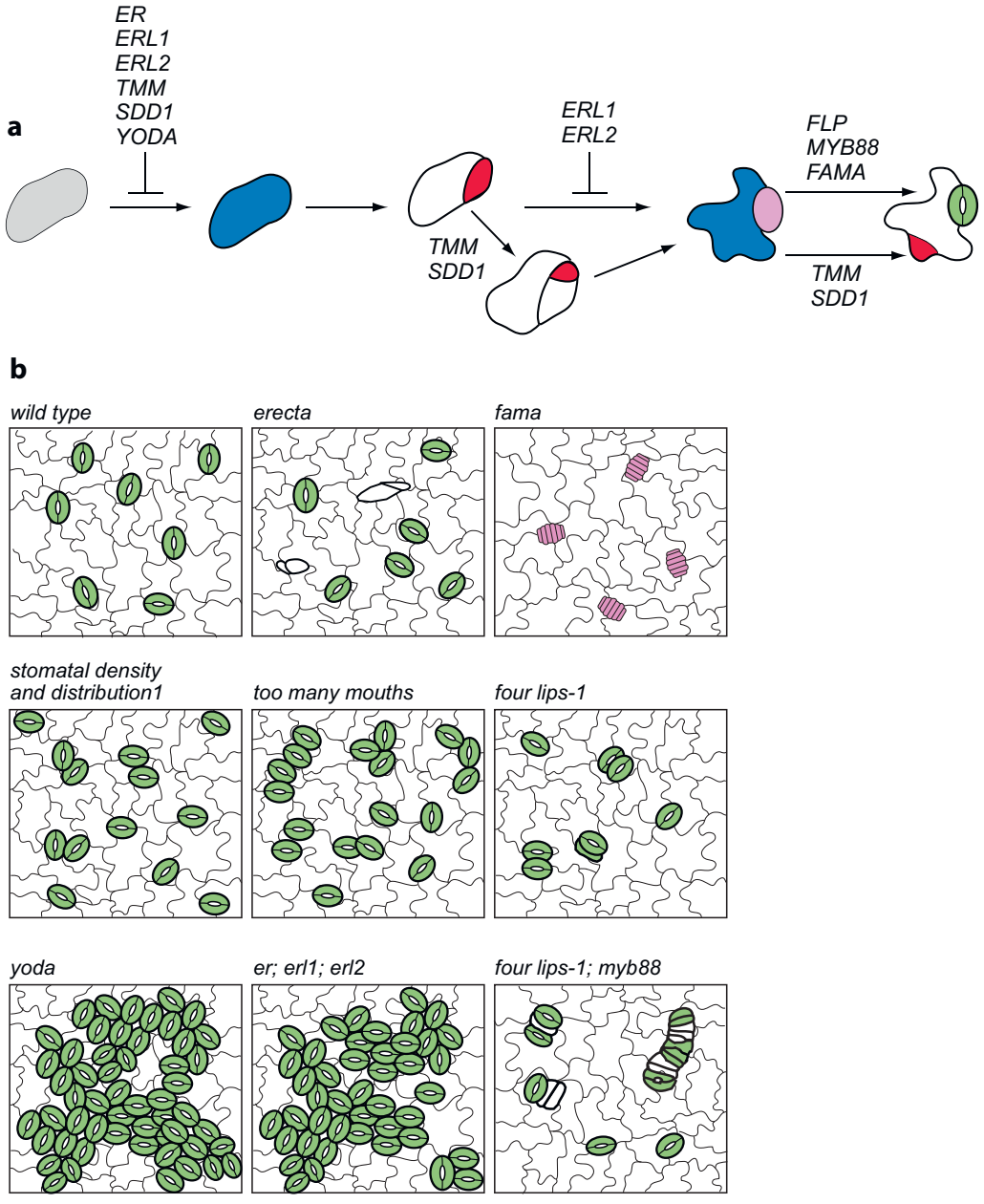

Figure 2

Genetic control of stomatal development. (*a*) Summary of the major stomatal development stages annotated with the presumed point of action of genes. Negative regulation is indicated by T-shaped lines; positive regulation is indicated when just the gene abbreviation is shown. Note that with the current markers, it is not possible to determine whether mutations affect the transition from protodermal cell to meristemoid mother cell (MMC), or the ability of a MMC to divide asymmetrically. (*b*) Diagrams of terminal leaf phenotypes in stomatal mutants indicating the typical number and arrangement of stomata (*green*) or terminal cell type (*pink* for guard mother cells). White cells in *erecta* and *flp;myb88* panels represent cells of indeterminate identity.

or heterodimers with other LRR-RLKs or LRR-RLPs. To work as a complex, TMM and the ER-family proteins must also have overlapping subcellular localizations. TMM-GFP appears to be expressed in both the cell membrane and the endoplasmic reticulum of stomatal lineage cells (48). The localization of the ER-family proteins has yet to be determined.

Genetic data from the CLV signaling system suggest that the association of an RLP with its partner RLK serves to activate the complex (32, 73). However, TMM has contrasting functions in different organs that suggest that if TMM does form a complex with the ER-family, it cannot consistently serve as an activator. In stems, *TMM* has an opposite mutant phenotype to that of the *ER*-family and the published genetic interactions do not distinguish whether TMM is independent of or in the same complex as any member of the ER-family. In siliques, a neomorphic phenotype (failure of stomatal lineage cells to differentiate into guard cells) appears in plants doubly mutant for *TMM* and *ER*. Neomorphism is not expected when two proteins are exclusive partners, but it can arise when two proteins compete for a common partner. In this specific case, ERL1 was proposed as a possible target of TMM inhibition (via the formation of inactive heterodimers) (70). To be consistent with the epistatic relationships among *ER*, *ERL1*, and *TMM*, the "ERL1 target" model requires there to be exquisite control on the levels of each receptor in silique stomatal lineage cells. Quantitative data on TMM and ER-family protein levels and dosage studies using heterozygotes and mild overexpression would allow this interaction model to be tested more rigorously. Other, currently unidentified, partners might also be involved in the tissue-specific regulation of stomatal formation.

Signaling Through a MAP Kinase Cascade

Regardless of whether the *ER*-family and *TMM* participate in shared signaling complexes, loss of these proteins leads to changes in multiple cellular behaviors including altered gene expression and division plane determination. Intracellular signaling is required to transduce signals from the cell periphery to nuclear and cytoplasmic targets. The signaling cascades downstream of plant LRR-RLKs are diverse and not easily predicted by the sequence of the receptors. For example, two well-studied pathways employing LRR-RLKs—brassinosteroid signaling and response to bacterial pathogens—utilize widely conserved, but distinct intracellular signaling cascades (1, 22, 77).

A mitogen-activated protein (MAP) kinase signaling pathway has been implicated in the control of cell division and cell fate during stomatal development (3). Loss-of-function mutations in the MAP kinase kinase kinase (MAPKKK) gene *YODA* profoundly alter stomatal density and spacing. The phenotype of the *yoda* mutant is similar to the *er;erl1;erl2* triple mutant and includes a severe reduction in overall plant height and internode length as well as excess production of guard cells (3, 70). Cells in the epidermis of *yoda* cotyledons exhibit excessive entry divisions, fail to orient spacing divisions and fail to prevent division of neighbor cells that contact two cells of the stomatal lineage. Asymmetry of cell fates is also compromised in *yoda;* often both daughters of a stomatal lineage cell division become stomata without the obvious production of an intervening cell.

YODA is a member of a class of MAPKKKs that possess a long N-terminal extension with negative regulatory activity (44). Expression of N-terminally deleted YODA (*CA-YODA)* results in dose-dependent effects on stomatal development—in the strongest lines, no stomata are produced (3). The block in stomatal development occurs early and results in the production of a leaf epidermis composed entirely of pavement cells and occasional trichomes. *CA-YODA/+* is capable of suppressing the *tmm-1* mutant phenotype, consistent with YODA acting downstream in a common signaling pathway, but

it cannot be ruled out that YODA participates in an independent and parallel signaling pathway in young epidermal cells. A physical or biochemical connection between the YODA MAPKK kinase and the stomatal regulators at the membrane will be needed to answer this question, but obtaining this information may be challenging. Even in the best-studied *Arabidopsis* LRR-RLK/MAPK pathways, it is not clear whether the LRR-RLK directly phosphorylates the MAPKKK or whether phosphorylation requires an intermediary protein or protein complex (1).

MAP kinase cascades are organized into a core module of three protein kinases consisting of a MAPKKK, a MAP kinase kinase (MKK), and a MAP kinase (MPK). Transmission of a signal to downstream targets is achieved by sequential phosphorylation and activation of the core MAP kinase components. One or more MKKs and MPKs are predicted to act downstream of *YODA*. Finding the distinct downstream kinases is complicated by the fact that multiple, interdependent, MAP kinase pathways are at work in *Arabidopsis* cells. The *Arabidopsis* genome is predicted to contain 20 MEKK1/STE11 class MAPKKKs, 10 MAPKK genes, and 20 MAPK genes (28). Despite this gene family expansion, which might provide the raw material for specialization, several lines of evidence suggest that the specific outcomes of *Arabidopsis* MAPK signaling do not arise from the use of dedicated kinases for each biological event. For example, redundant kinase pairs MKK4/5 and MPK3/6 are required for biological processes as diverse as immune recognition (1) and responses to hormones and ozone (42). No single MKK or MPK mutant has been reported to have a significant effect on stomatal development suggesting overlapping or compensatory functions for MPKs and MKKs in this biological process. It will be intriguing to see whether, like with the ER-family kinases, mutations in multiple MKK or MPK genes reveal roles in stomatal development.

Receptor-Ligand Interactions

Stomatal receptors may induce cellular responses by activating a MAP kinase cascade, but how are they themselves activated? Animal receptor tyrosine kinases dimerize, and ligand binding induces phosphorylation of the receptors and activation of downstream signaling pathways. Plant LRR-RLKs probably share some of these activities based on genetic and protein interaction studies. Evidence from the brassinosteroid perception pathway suggests that plant LRR-RLKs may not need a ligand to dimerize (59). Therefore, physical interactions among ER-family members or between this trio and *TMM* could be ligand-independent. Yet even if it is possible to activate LRR-RLKs without a ligand, stomatal development and patterning require positional information. TMM localization in the cell membrane of stomatal lineage cells is uniform (49). It is difficult to imagine how TMM (or any receptor) could instruct cell division orientation unless its activation was restricted to only a small part of the cell periphery. A simple way to selectively activate a receptor is to provide a spatially restricted ligand from a neighboring cell.

LRR-RLKs respond to a diverse set of ligands including steroids [brassinosteroid (35)], small secreted proteins [CLV3 (20)], and exogenous peptides [flagellin (42)]. Indirect evidence for a protein-based signal involved in stomatal development comes from analysis of the subtilisin protease *STOMATAL DENSITY AND DISTRIBUTION 1 (SDD1)*. *sdd1* mutants undergo excessive entry divisions and fewer amplification divisions and can fail to orient spacing divisions (2). SDD1 appears to be secreted from the cell and is expressed in meristemoids and GMCs (78). Overexpression of *SDD1* represses stomatal divisions and also causes arrest of meristemoids and GMCs. Although *sdd1* mutants increase the stomatal index and misorient spacing divisions, the overall stomatal phenotypes of *sdd1* are distinct from those in *tmm*, *yoda*, and the *er*-family. However, the *SDD1*

overexpression phenotype depends on a functional *TMM* (78), and loss of *SDD1* function can be dominantly suppressed by *CA-YODA* (3). All of these data point to interdependent relationships among known early-acting stomatal genes, but also indicate that placing them in a single linear pathway is likely to be oversimplistic.

Stomatal Pattern by Negative Regulation

All of these earlier-acting genes are broadly required for the number, distribution, and patterning of stomata. However, the cellular mechanisms by which these genes control stomatal development may vary. For example, TMM and SDD1 directly generate pattern by orienting spacing divisions (2, 24), whereas patterning defects may be a secondary consequence of the cell fate transformations in *YODA* mutants (3). The genes discussed in this section encode proteins that are predicted to act in inter- and intracellular signaling. Moreover, they all act essentially as negative regulators of stomatal formation. While this review was in press, several transcription factors were identified that actively promote stomatal development at the entry, proliferation, and differentiation stages (44a, 52a, 57a). The regulatory relationships between these proteins and the negative regulators discussed here have yet to be determined.

Dispersed and Specialized Stem Cell Compartment

The stomatal cell lineage can be considered a specialized stem cell compartment (50). Like multipotent stem cells, the lineage produces only a few mature cell types (guard cells and pavement cells), and the stem cell population can be renewed when a larger daughter cell produced by asymmetric division undergoes an entry division. This compartment is marked by TMM expression, which is found in both daughter cells produced by all asymmetric divisions in the lineage. ERL1 and ERL2 expression overlap with that of TMM, but these genes are also more widely expressed (70).

A unifying way to think about the *tmm*, *sdd1*, *yoda*, *er*, *erl1*, and *erl2* phenotypes is to look at the relative and combinatorial effects of these genes in forming and/or maintaining the stem cell–like compartment. Compartment size increases when cells undergo entry and amplifying divisions and decreases when cells terminally differentiate. How protodermal cells are chosen to enter this compartment is unknown, and cells so chosen cannot be distinguished by size or location. The above genes mostly restrict compartment size, for example by limiting the number of entry divisions (**Figure 2**). But many of these genes also positively regulate compartment size by promoting amplifying divisions. Thus, the number of stomata produced depends on the net integration of genetic and signaling inputs at each developmental node in the pathway.

Although TMM is expressed in this specialized compartment, it is not required for the specification of the cell types in it. Instead, it seems to receive signals that modulate the placement and number of divisions in the compartment in a cell-type and cell-position appropriate manner. This stem cell/TMM-marked compartment exists in a spatially dispersed and temporally transient developmental window in the postprotodermal shoot epidermis (48). The compartment is critical for leaf development because many epidermal cells are produced in the stomatal cell lineage. This function was graphically demonstrated when the *TMM* promoter was used to drive the expression of a cyclin-dependent kinase inhibitor (KRP1), which blocks cell cycle progression, resulting in a severe reduction in asymmetric divisions and in much smaller leaves (80).

The stomatal stem cell compartment differs from apical meristems, which are coherent and perpetually active groups of stem cells that produce the progenitors of all shoot and root cells (79). Bünning recognized the similar but more restricted division potential of the

stomatal compartment by calling the distinctive precursor cell a meristemoid instead of a meristem (8). Other dispersed self-renewing populations of cells such as the procambium and the cork cambium (which produce the primary vascular tissues and the bark, respectively) act outside the apical meristems. Study of the stomatal pathway should contribute to understanding how all such dispersed and specialized stem cell compartments help build the plant.

LATE-ACTING GENES

After asymmetric divisions initiate and populate the cell lineage and establish the spacing pattern, the developmental program changes to symmetric division and terminal differentiation. Genes known to act during these later stages help end cell proliferation, execute GMC cytokinesis, and regulate the timing of guard cell specification and differentiation.

Ending Cell Proliferation

A common feature of eukaryotic cell lineages is that precursor cells divide a limited number of times and then differentiate into specialized cell types (41, 75). The symmetric division of the GMC is the last division in the stomatal cell lineage. The resulting daughter cells mature into guard cells that withdraw from the cell cycle either in G_1 or G_0 (46). The relative timing of cell cycle withdrawal and guard cell specification has not been established. However, ending cell cycling before terminal differentiation appears to be tightly regulated and adaptive for valve function because stomata in virtually all plant taxa consist of just two cells.

Three putative transcriptional regulators, FOUR LIPS (FLP), MYB88, and FAMA, restrict cell cycling at the end of the stomatal lineage (36). Loss-of-function mutations in *FLP* induce clusters of laterally aligned cells that are clonal in origin. These clusters result from the reiteration of a GMC program in daughter cells that would usually differentiate directly into guard cells. The excess divisions delay rather than block stomatal specification because clusters contain normal and arrested stomata. *FLP* encodes an R2R3 MYB protein whose expression starts before GMC mitosis and is downregulated as guard cells differentiate. FLP limits GMCs to one symmetric division and promotes a timely transition to terminal differentiation. As a putative transcription factor, FLP could halt proliferation directly by regulating the expression of cell cycle genes, and/or indirectly by promoting the developmental transition to a terminal cell fate.

MYB88 and *FLP* (*MYB124*) are paralogs (36). The genes overlap in function and expression. *myb88* mutants do not have a phenotype alone, but enhance the stomatal cluster phenotype of *flp*, and extra copies of the *MYB88* genomic region complement *flp*. These data hint at a possible gene dosage mechanism where a threshold level of FLP and/or MYB88 controls the number of symmetric divisions at the end of the cell lineage.

Mutations in *FAMA*, which encodes a basic helix-loop-helix (bHLH) protein, cause clusters reminiscent of severe *flp* alleles and of *flp/myb88* double mutants, suggesting that *FAMA* also limits symmetric divisions at the end of the stomatal cell lineage (3). However, unlike cells in *flp* clusters, those in *fama* lack cytological traits characteristic of guard cells, revealing that *FAMA* is also required for proper cell specification and differentiation. FAMA is also sufficent to promote at least partial guard cell identity because FAMA overexpression or misexpression in ectopic domains causes cells to take on a guard cell morphology and express molecular markers of guard cell identity (52a).

The specification of other epidermal cell types, such as trichomes and root hairs, involves the physical interaction of R-like bHLH proteins with R2R3 MYB proteins to form a complex that transcriptionally activates cell fate factors (4, 57). The phenotypes of *FLP*, *MYB88*, and *FAMA* mutants and the genes' overlapping expression

patterns in the late stages of stomatal development suggest that they might act in the same transcriptional complex to limit divisions and to promote guard cell specification. However, tests of both genetic and physical interactions between FAMA and FLP/MYB88 indicate that these particular bHLH and MYB proteins work independently (52a). FLP/MYB88 might have a primary role in cell division that feeds back on differentiation, whereas FAMA is likely to be responsible for differentiation and indirectly for cell cycle control (52a). In addition, FAMA is not an R-like bHLH protein, and FLP/MYB88 lacks the amino acid signature implicated in MYB binding to such bHLH proteins (29, 85). Whereas early-acting genes directly establish stomatal patterning via asymmetric division, *FLP*, *MYB88*, and *FAMA* act only indirectly in patterning by preventing extra symmetric divisions in correctly positioned GMCs.

Division and Differentiation: Independent and Coordinated

The final stages of stomatal development comprise GMC cytokinesis, guard cell specification and differentiation, and stomatal morphogenesis. The latter includes the elaboration of pore thickenings in each guard cell followed by the controlled separation of their walls to form the opening of the valve.

These events are normally coordinated in time and space, but many of them can be uncoupled artificially. Because the GMC division is stereotyped, cytokinetic defects can be readily identified when division is mutationally blocked (5, 19, 31, 71, 72, 83). These studies show that guard cell specification, differentiation, and morphogenesis can all continue without proper GMC cytokinesis (31). Specification markers include *KAT1* (potassium channel) gene expression and microtubule arrays that radiate out from the future pore site (22, 43, 51, 53). GMCs with defective division still acquire a guard cell identity (19, 74). In the absence of GMC cytokinesis, wall deposition and secretion can continue, but are redirected as evidenced by the presence of abnormal swellings in the center of the wall facing the atmosphere (6, 71, 83). Single cells can even become kidney shaped in the absence of GMC division, such as when FAMA or a dominant negative version of *CYCLIN-DEPENDENT KINASE B1;1* (*CDKB1;1*) is overexpressed (6, 52a).

Excess divisions, as well as no division, can also permit guard cell morphogenesis. Extra symmetric divisions in *flp* GMCs still allow the formation of normally shaped, although ectopic, stomata (36). Stomatal morphogenesis can also tolerate an abnormal extra division in guard cells as shown by the formation of four-celled stomata in dark-grown cucumber hypocotyls transiently exposed to ethylene and red light (33, 34).

That these processes can be artificially separated underscores the temporal and spatial coordination normally needed for stomatal morphogenesis. Some guard cell differentiation can take place without cell division, but forming a functional valve requires that equal-sized daughter cells be produced and specified at the same time. The beautiful and adaptive mirror-like symmetry of the stoma might arise cell autonomously, or it might use signaling between developing guard cells to orchestrate wall deposition and pore formation.

Cell Cycle Regulators

Stomatal lineage proteins like TMM, FLP, and FAMA presumably regulate division by interacting directly or indirectly with the cell cycle machinery. The identities of most such cell cycle regulators are unknown because loss-of-function phenotypes are often uninformative due to lethality or redundancy and because gene overexpression has mostly not produced a dramatic stomatal phenotype (7, 15, 18).

Three classes of cell cycle regulators have been implicated in the stomatal pathway so far. *CDKB1;1* positively regulates stomatal production in addition to promoting GMC mitosis and cytokinesis as described above (6, 7).

The second class includes *CDT1* and *CDC6*, which regulate the licensing of origins of replication. Both are normally expressed in stomatal precursor cells, and their overexpression increases stomatal density twofold, suggesting that stomatal fate acquisition is normally kept in check by regulating the number of cells permitted to initiate DNA replication (11). The third class includes the RETINOBLASTOMA RELATED (RBR) protein, which represses the activity of the heterodimeric transcription factor complex E2F-DP. The number of asymmetric divisions in the stomatal lineage increases strongly when E2Fa is overexpressed as well as when RBR is inducibly inactivated (13, 14, 56). RBR inactivation by virus-induced gene silencing also results in *tmm*-like stomatal clusters (56), raising the possibility that TMM restricts asymmetric divisions via the RBR, E2F, and DP pathway. RBR was recently shown to play a major role in maintaining stem-cell competence in root apical meristems (81), highlighting some common molecular requirements among the self-renewing cell populations in plants.

Based on their phase-specific expression pattern, other cell cycle genes likely act in stomatal pathway, including *CDKA;1* (CDC2a) and *CYCLINA2;2*, *2;3*, and *B1;1* (formerly cyca1At) (6, 30, 64). All of these are also expressed outside the stomatal pathway; there are no reports of a cell cycle regulator whose expression is restricted to the stomata lineage. Of particular interest will be determining how stomatal developmental regulators work with the cell cycle machinery. One could imagine that cell cycle regulators are regulated transcriptionally by FLP and FAMA, or posttranslationally by phosphorylation of kinases downstream of YODA or the ER-family. More broadly, the stomatal system is valuable for studying cell cycles in multicellular development because division behavior and gene expression can be visualized in living tissues and because the pathway includes a rich sampling of division types and events.

INFLUENCE OF ENVIRONMENT

The experimental focus on the "developmental" genes that act within the epidermis to control cell identity and division behavior will identify many of the signaling pathways and cell autonomous factors required for stomatal development and pattern. However, interactions with underlying tissues and the environment also influence the final density and distribution of stomata. These long-range signals could act by modulating the activity of genes like *TMM*, *YODA*, *SDD1*, and the *ER*-family or they could impinge directly on the cell cycle machinery and other downstream targets. In this section we examine the nature of the environmental response including potential signals and possible connections between regulation at the levels of development and physiology.

Inputs from Old to New

Paleontologists and ecophysiologists have long noticed a correlation between stomatal density and environmental parameters such as the levels of humidity, light, and carbon dioxide (CO_2). A strong inverse correlation between stomatal density and atmospheric [CO_2] (10) was observed in preserved and fossil plant specimens and this correlation was used to retrospectively estimate global [CO_2] (10) over the past 450 million years (82). Changes in stomatal density can also occur over much shorter timescales. *Arabidopsis* plants of the Col ecotype grown at double the normal [CO_2] produce fewer stomata per unit area than siblings grown at ambient [CO_2] (37). This response depends on the activity of the *HIC1* gene, which encodes an enzyme required for the synthesis of the very long chain fatty acids that are components of the cuticle (27). Interestingly, the selective application of high [CO_2] to mature leaves causes newly formed leaves to exhibit a decrease in stomatal density similar to leaves of plants grown continuously at high [CO_2]. However, directly exposing only developing (as opposed to mature)

leaves to high [CO_2] has no effect on stomatal density (37). This suggests that the environmental stimulus and stomatal response are spatially distinct and, consequently, plants require long-range signals to transmit environmental information.

Precedent for systemic signaling comes from studies on a wide variety of plant behaviors including flowering, pathogen and herbivore responses, and inhibition of lateral branching. Structurally diverse molecules can serve as the signals in these events, including proteins, peptides, and phytohormones. Many of the classical plant hormones [including ethylene, abscisic acid (ABA), gibberellins, and cytokinins] mediate plant responses to the environment, including regulation of stomatal opening. Some recent evidence points to a role for these hormones in regulating stomatal development—both gibberellins and ethylene promote cell divisions, leading to stomatal formation in hypocotyls (33, 61)—but these studies do not distinguish local vs. long-range effects.

Subsequent studies on long-range signals in maize, *Arabidopsis*, and poplar suggest that environmental perception by old leaves and response by new leaves is universal, but the details of the response can vary among species (12, 17, 47). Several cellular mechanisms could account for changes in stomatal density including altered expansion of pavement cells, changes in the number of entry and amplifying divisions in the stomatal lineage, and the arrest or dedifferentiation of meristemoids or GMCs. Combining environmental treatments with developmental methods such as lineage tracing could reveal which steps and which genes in the stomatal pathway are targets of environmental regulation. Some evidence already points to the expansion of pavement cells and the division of stomatal lineage cells being under independent control (47).

As much as environmental studies would benefit from careful examination of development, so would the understanding of developmental genes benefit from testing their response to environmental change. This has been done to some extent with the *SDD1* gene. *sdd1* plants have increased stomatal density in ambient conditions, a phenotype that could reflect either a developmental defect or an inability to correctly sense environmental signals (similar to *hic1*). When tested for response to light intensity changes, *sdd1* mutants responded similarly to wild type, suggesting that the circuit that controls light responses is still intact in *sdd1* (62). Whether *sdd1* is deficient in response to other environmental parameters, or whether other stomatal genes like *TMM* and *ER* mediate both developmental and environmental responses, remains to be tested.

Stomatal Development and Physiology

Mutations in stomatal development genes can affect the physiology of the entire plant. *sdd1* plants, with their higher stomatal density, can assimilate 30% more carbon than wild-type plants when transferred to high light (62). Conversely, *35S::SDD1* plants with reduced stomatal density fare worse than wild type in similar assays (9). *ERECTA* also has a major effect on transpiration efficiency (45). Transpiration efficiency is the ratio of carbon fixation to water loss and requires coordination between photosynthesis and transpiration and is therefore closely related to stomatal activity.

The independent identification of ER as a factor influencing both stomatal development and transpiration efficiency raises the question of how this single protein might affect these two processes. ER's role in development could be completely independent from its role in transpiration efficiency. Because plant LRR-RLKs sit at the top of signaling cascades with many potential targets and because the RLKS can homo- and heterodimerize (40, 52), ER might act with one set of proteins in stomatal development and a different set in coordinating transpiration and photosynthesis. Alternatively, the effect

of *ER* on transpiration efficiency could be an indirect consequence of *ER*'s developmental roles (such as altered stomatal density, plant height, and leaf thickness). Identifying partners and downstream targets of ER through protein interactions or genetic screens may reveal how diverse plant activities are mechanistically coupled to ER function.

SUMMARY POINTS

1. Stomata are produced through a stereotyped series of asymmetric and symmetric cell divisions within a dispersed stem cell compartment. The activity of this compartment is a major source of cells that build the *Arabidopsis* leaf epidermis.
2. Stomata are spaced via intercellular signaling pathways that appear to involve several types of receptors and a MAPK phosphorylation cascade.
3. Transcription factors help end proliferation in the stomatal lineage and promote timely cell differentiation.
4. Stomatal development represents a tractable system for analyzing the cell and molecular biology of division site selection, cytokinesis, and cell cycle progression and withdrawal.
5. Just as they influence the functioning of mature stomata, environmental signals also regulate the development of the stomatal lineage.

FUTURE ISSUES

1. Despite the progress described, the genes needed for stomatal specification and morphogenesis are mostly unknown. Sensitized genetic screens and genome-based analysis to target genes expressed in the stomatal lineage were very recently used to identify genes involved in promoting pathway entry and in "counting" proliferative divisions of meristemoids (44a, 52a, 57a), and these approaches hold promise for identifying the complete network of stomatal regulatory genes.
2. Uncertainties about signal transduction and other regulatory pathways might be resolved by biochemically characterizing the interactions and activities among known genes and by identifying their transcriptional and signaling targets. This, in combination with new gene discovery in *Arabidopsis*, should generate a core molecular and biochemical framework for understanding stomatal development. Identifying these components and relationships will enable testing the extent to which these players and pathways are conserved in the plant kingdom.
3. The relationships between asymmetric divisions and cell fate are still poorly understood for plants. The stomatal lineage is a promising system for revealing the mechanisms of cell polarity, intercellular signaling, and division site selection in plants. This pathway is also favorable for revealing relationships between cell specification and the context-specific regulation of the cell cycle machinery in a green cell lineage.
4. The roles of plant growth regulators and environmental signals in regulating stomatal number and development are largely unexplored.

5. Despite the complexity of events in the stomatal pathway, these events are visually accessible on the leaf surface. In *Arabidopsis*, the fate decision can be reduced to a binary choice between pavement and guard cell. These traits and progress to date bode well for learning about how intrinsic and extrinsic factors combinatorially affect a developmental decision.

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36. The first in-depth description of transcription factors required for proper stomatal formation.

37. Using a cuvette system, the authors found that stomatal density is influenced by an environmental signal perceived in mature leaves and transmitted to new leaves.

44a. The authors identify a bHLH related to FAMA that is required for the asymmetric entry divisions that establish the stomatal lineage.

45. Using Δ13C discrimination as a method for estimating transpiration efficiency, the authors identified QTLs between *Arabidopsis* accessions and mapped a major QTL to ERECTA.

48. Identifies TMM as a receptor-like protein expressed in the endomembrane system of stomatal lineage cells.

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57a. The authors identify a bHLH related to FAMA that is required for terminating amplifying divisions and promoting the formation of stomata.

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78. *SDD1* is expressed in stomatal precursor cells, and *SDD1* overexpression reduces stomatal formation (opposite phenotype to *sdd1*).

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Errata

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