Mechanisms of Stomatal Development

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Abstract

The main route for CO_2 and water vapor exchange between a plant and the environment is through small pores called stomata. The accessibility of stomata and predictable division series that characterize their development provides an excellent system to address fundamental questions in biology. Stomatal cell-state transition and specification are regulated by a suite of transcription factors controlled by positional signaling via peptide ligands and transmembrane receptors. Downstream effectors include several members of the core cell-cycle genes. Environmentally induced signals are integrated into this essential developmental program to modulate stomatal development or function in response to changes in the abiotic environment. In addition, the recent identification of premitotic polarly localized proteins from both *Arabidopsis* and maize has laid a foundation for the future understanding of intrinsic cell polarity in plants. This review highlights the mechanisms of stomatal development through characterization of genes controlling cell-fate specification, cell polarity, cell division, and cell-cell communication during stomatal development and discusses the genetic framework linking these molecular processes with the correct spacing, density, and differentiation of stomata.

Contents

INTRODUCTION

Stomata are microscopic pores on the epidermal surface of land plants, providing the principal route for release of water vapor and gas between the plant and the environment. The extent of this exchange is controlled by an adjustment of the stomatal aperture via the coordinated turgor-driven movements of two specialized guard cells (GCs) that surround the intervening pore. The number and distribution of stomata also affect gas exchange and are closely regulated and coordinated with cell growth and division, while preserving a level of plasticity to respond to ever-changing environmental conditions. The processes leading to the production of properly spaced stomata incorporate several events fundamental to the generation of cellular diversity in multicellular organisms including coordinated signaling among cell types, asymmetric division, cell-fate specification, as well as the establishment and maintenance of stem-cell populations. This review focuses on current advances that define the emerging genetic and cellular interaction network controlling these basic processes during stomatal development and further discusses how the external environment influences such programs. For a list of genes discussed in this review, see **Table 1**.

CELL-STATE TRANSITIONS

Stomatal Development in *Arabidopsis*

In the model plant *Arabidopsis thaliana*, the mechanism driving the production of a mature

Table 1 Genes regulating stomatal development and patterning

Gene name	AGI	Symbol	Gene description	Reference(s)
Stomatal differentiation genes				
SPEECHLESS	*At5g53210*	*SPCH*	bHLH protein	67, 80
MUTE	*At3g06120*	*MUTE*	bHLH protein	67, 80
FAMA	*At3g24140*	*FAMA*	bHLH protein	75
SCREAM/ICE1	*At3g26744*	*SCRM*	bHLH protein	47
SCREAM2	*At1g12860*	*SCRM2*	bHLH protein	47
FOUR LIPS	*At1g14350*	*FLP*	R2R3 MYB protein	54, 107
MYB88	*At2g02820*	*MYB88*	R2R3 MYB protein	54, 107
Stomatal patterning genes				
YODA	*At1g63700*	*YDA*	MAPKKK	5, 59
MKK4/MKK5	*At1g51660* *At3g21220*	*MKK4* *MKK5*	MAPKK	102
MKK7/MKK9	*At1g18350* *At1g73500*	*MKK7* *MKK9*	MAPKK	58
MPK3/MPK6	*At3g45640* *At2g43790*	*MPK3* *MPK6*	MAPK	102
ERECTA	*At2g26330*	*ER*	Leucine-rich-repeat receptor kinase	93
ERECTA-LIKE1	*At5g62230*	*ERL1*	Leucine-rich-repeat receptor kinase	93
ERECTA-LIKE2	*At5g07180*	*ERL2*	Leucine-rich-repeat receptor kinase	93
TOO MANY MOUTHS	*At1g80080*	*TMM*	Leucine-rich-repeat receptor protein	7, 29, 110
STOMATAL DENSITY AND DISTRIBUTION1	*At1g04110*	*SDD1*	Subtilisin-like protease	4, 101
EPIDERMAL PATTERNING FACTOR1	*At2g20875*	*EPF1*	Cysteine-rich-secreted peptide	39
EPIDERMAL PATTERNING FACTOR2	*At1g34245*	*EPF2*	Cysteine-rich-secreted peptide	40, 44
STOMAGEN/EPF-LIKE9	*At4g12970*	*EPFL9*	Cysteine-rich-secreted peptide	95
CHALLAH/EPF-LIKE6	*At2g30370*	*CHAL* *EPFL6*	Cysteine-rich-secreted peptide	1
Intrinsic polarity genes				
BREAKING OF ASYMMETRY IN THE STOMATAL LINEAGE	*At5g60880*	*BASL*	Novel protein	25
POLAR LOCALIZATION DURING ASYMMETRIC DIVISION AND REDISTRIBUTION	*At4g31805*	*POLAR*	Novel protein	61
PANGLOSS1	N/A	*PAN1*	Leucine-rich-repeat receptor protein	14, 28
Mitosis and cytokinesis genes				
CYCLIN DEPENDENT KINASE B1;1	*At3g54180*	*CDKB1;1*	Cyclin-dependent kinase	11
CYCLIN D4;2	*At5g10440*	*CYCD4;2*	Cyclin	53
CYCLIN A2 gene family	*At5g25380* *At5g11300* *At1g15570* *At1g80370*	*CYCA2;1* *CYCA2;2* *CYCA2;3* *CYCA2;4*	Cyclin	100
CYTOKINESIS DEFECTIVE 1	Unknown	*CYD1*	Not cloned	109

(*Continued*)

Table 1 (*Continued*)

Gene name	AGI	Symbol	Gene description	Reference(s)
STOMATAL CYTOKINESIS DEFECTIVE 1	*At1g49040*	*SCD1*	DENN-WD40 protein	26
GLUCAN SYNTHASE-LIKE 8/CHORUS	*At2g36850*	*GSL8/CHOR*	Callose synthase	19, 37
Environmental control genes				
CRYPTOCHROME	*At4g08920* *At1g04400*	*CRY1* *CRY2*	Blue-light photoreceptor	48
PHYTOCHROME	*At1g09570* *At2g18790*	*PHYA* *PHYB*	Red/far-red photoreceptor	8, 16, 48
CONSTITUTIVE PHOTOMORPHOGENIC 1	At2g32950	*COP1*	E3 ubiquitin ligase	48
HIGH CARBON DIOXIDE	*At2g46720*	*HIC1*	3-keto acyl coenzyme A synthase	35

Meristemoid: transient cell state of the stomatal lineage, capable of reiterative asymmetric divisions prior to transition into a GMC

Meristemoid mother cell (MMC): stomatal-lineage founder cell that undergoes the first asymmetric division to enter the lineage

Entry division: an asymmetric division that initiates stomatal cell lineages, producing a meristemoid and its sister SLGC

Stomatal-lineage ground cell (SLGC): the larger cell produced through MMC or meristemoid asymmetric division

Spacing division: an SLGC asymmetric division that is oriented away from a neighboring stoma (or GMC)

stoma relies on a series of cell divisions and successive cell-state transitions, in which each transitional state is marked by dramatic changes in morphology, transcript accumulation, and protein localization (25, 29, 79, 113). The stomatal lineage begins with a subset of protodermal cells that undergo a cellular transition to become meristemoid mother cells (MMCs) (**Figure 1**). An MMC initiates the stomatal lineage through an asymmetric entry division to produce a small triangular cell called a meristemoid and a larger sister cell called a stomatal-lineage ground cell (SLGC). An SLGC can terminally differentiate into a lobed pavement cell; alternatively, SLGCs can initiate an asymmetric spacing division to produce a satellite meristemoid that is always oriented away from an existing stomatal precursor. The oriented divisions of SLGCs are regulated by cell-cell signaling components that ensure stomata develop at least one cell apart from one another via the so-called one-cell spacing rule (29, 39, 40, 44, 93) (**Table 1**). Both primary and satellite meristemoids have stem cell–like activity in which the meristemoid divides repeatedly (generally up to three divisions) to regenerate a meristemoid and increase the total number of SLGCs produced by a single lineage. These repetitive amplifying divisions create the main source of both pavement cells and stomata on the epidermal surface (29). After a variable number of amplifying divisions, a meristemoid loses its ability to reiterate asymmetric division and undergoes a cell-state transition to produce a guard mother cell (GMC), the final stomatal precursor. The GMC transition is accompanied by a distinct change in cell shape and cell-division capacity. In contrast to the asymmetric divisions of the triangular meristemoid, the round GMC divides once symmetrically to yield two cells, which concurrently proceed through a final cell-state transition to form highly specialized GCs that work in concert to control the size of the stomatal opening. Mature GCs are terminally differentiated and do not divide further.

Stomatal Cell-Fate Specification: Basic Helix-Loop-Helix Proteins

The basic helix-loop-helix (bHLH) protein family is an evolutionarily ancient group of transcription factors that often work in cascades to specify cellular identity (33, 71, 83, 104). The bHLH domain consists of 50–60 amino acids composed of two functionally distinct regions, basic and HLH, involved in DNA binding and protein dimerization, respectively. Two groups of bHLH proteins have been identified as positive regulators of stomatal-lineage progression (47, 67, 75, 80) (**Table 1**). SPEECHLESS (SPCH), MUTE, and FAMA represent the first group and encode closely related paralogs belonging to subgroup Ia (81) that share non-bHLH conserved motifs and act sequentially to promote cellular transition during stomatal

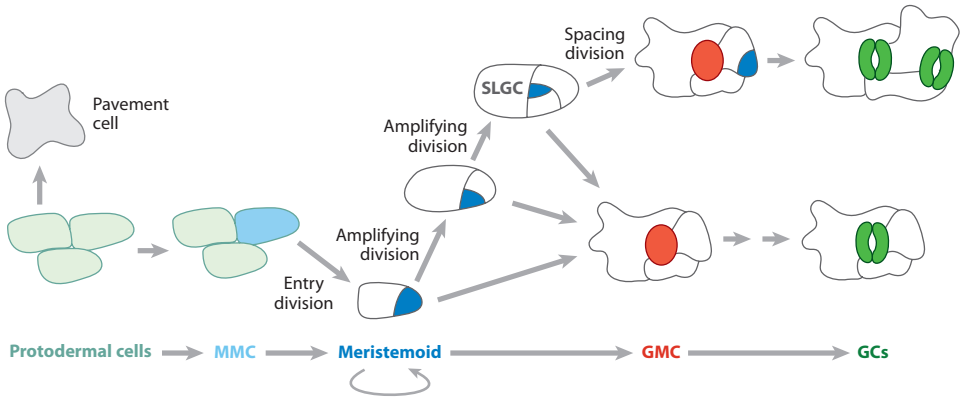


Figure 1

Diagram of cell-state transitions of the stomatal lineage in *Arabidopsis*. A subset of protodermal cells (*light green*) undergoes a transition to become a meristemoid mother cell (MMC) (*light blue*). Other protodermal cells differentiate into pavement cells. MMCs divide asymmetrically to enter the stomatal lineage and create a meristemoid (*dark blue*) and a stomatal-lineage ground cell (SLGC). SLGCs can differentiate into a pavement cell or undergo a spacing division to form a satellite meristemoid (*dark blue*) oriented away from an existing stomatal precursor. All meristemoids proceed thorough a limited number of asymmetric amplifying divisions and eventually transition into a guard mother cell (GMC) (*red*). The GMC divides once symmetrically to produce two equally sized guard cells (GCs).

development (67, 75, 80). Despite similarities among these three bHLH proteins, distinguishing features of each gene likely restrict their roles during development, as they are unable to substitute functionally for one another (66, 67). For instance, SPCH possesses an extra domain that is a target for phosphorylation (59). Two additional bHLH proteins, SCREAM (SCRM) and SCRM2, belong to subgroup IIIb and have partially redundant function to coordinate the activities of SPCH, MUTE, and FAMA through preferential heterodimerization (47, 81). These five genes combinatorially form a regulatory cascade driving cell-state transition during the stomatal lineage.

Key Switch Basic Helix-Loop-Helix Proteins: SPCH, MUTE, and FAMA

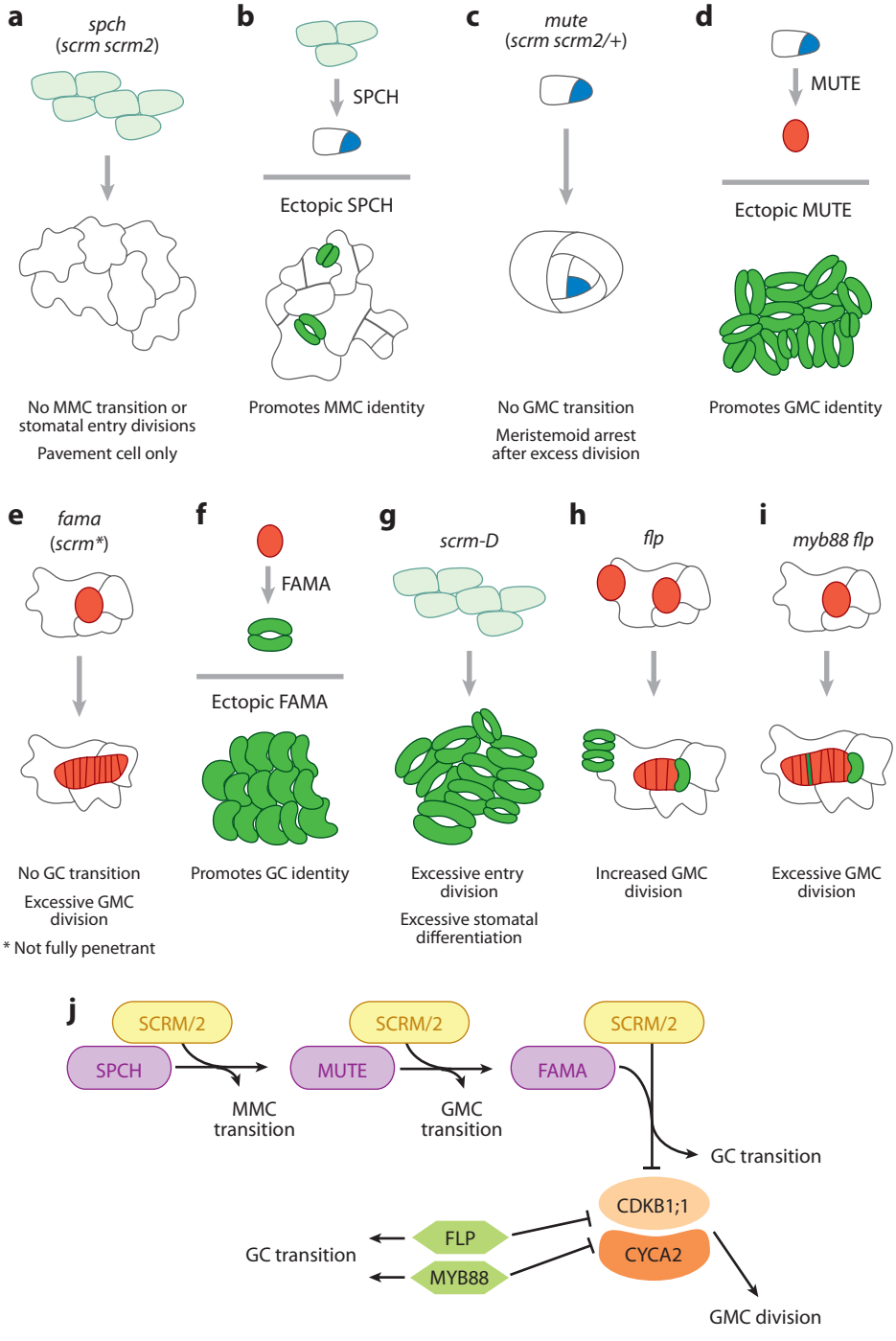
The initial transition of a protodermal cell to an MMC and the resulting entry division requires the activity of SPCH. As a result of the complete lack of stomatal-lineage initiation, *spch* loss-of-function mutants produce an epidermis composed solely of interlocking pavement cells (**Figure 2*a***). No known stomatal-lineage-specific markers are expressed in *spch*, indicating that the phenotype is not caused by the loss of stomatal-lineage identity after initiation (67, 80). Consistently, overexpression of *SPCH* causes excessive entry into the stomatal lineage, resulting in an epidermis of highly divided cells that express stomatal-lineage-specific markers (67, 80) (**Figure 2*b***). In weak *spch-2* mutants, entry divisions are initiated, but the resulting meristemoids undergo significantly fewer amplifying divisions compared with wild type, implicating a role for *SPCH* in maintenance of meristemoid stem-cell activity (67). The expression pattern of *SPCH* is consistent with a dual role, as it is expressed broadly across the protoderm early in development and then becomes restricted to stomatal-lineage cells (67, 80).

The bHLH protein MUTE is required for the termination of meristemoid asymmetric division and promotion of GMC cell-state transition. No stomata are produced in *mute*; instead, meristemoids divide excessively in an inward-spiral pattern and eventually arrest,

One-cell spacing rule: a rule that prohibits the differentiation of two adjacent cells into stomata; proposed on the basis of observations

Amplifying division: an asymmetric division of a meristemoid to produce surrounding nonstomatal cells while renewing the meristemoid

Guard mother cell (GMC): stomatal precursor that divides to produce two equally sized GCs of the stoma



presumably owing to a physical size restriction for further division (80) (**Figure 2*c***). *MUTE* promoter activity is restricted to a subpopulation of late-stage meristemoids, which presumably marks cells that will undergo GMC transition. Unrestricted *MUTE* expression using the ubiquitous *CaMV 35S* promoter results in all epidermis cells becoming stomata (78, 80) (**Figure 2*d***). The results from *MUTE* overexpression analysis suggest that all aboveground epidermal cells are capable of stomatal differentiation and that the stomatal lineage does not precondition cells to respond to *MUTE*. The mechanism that underlies the variable timing of *MUTE* expression in a subset of meristemoids remains unclear; however, cell-cell signaling is likely involved, because disruption of signaling components can cause an extension or reduction of meristemoid asymmetric division (7, 80, 112).

The final transition from GMC to GC is controlled, in part, by *FAMA* and is divided into two separable events: symmetric cell division of the GMC and GC transition. *FAMA* negatively controls GMC cell division and positively controls GC transition (75). Loss of *FAMA* function results in excessive GMC divisions and lack of GC differentiation, resulting in the production of long parallel rows of cells that do not display GC-specific markers or hallmark GC morphology (**Figure 2*e***). Consistent with its positive role in GC differentiation, ectopic expression of *FAMA* results in the production of unpaired GCs that line up like fish scales across the entire epidermis (75) (**Figure 2*f***). *FAMA* overexpression can also cause cells located in the mesophyll to express GC-specific markers, confirming the idea that the stomatal lineage per se is not a prerequisite for stomatal differentiation (75). Two additional bHLH proteins, bHLH071 (subgroup Ia) and bHLH093 (subgroup IIIb), strongly interact with FAMA, as demonstrated in yeast two-hybrid assays. However, no clear role in stomatal development has been determined (75).

Integrators of Three Cell-State Transitions: SCREAMs

In contrast to SPCH, MUTE, and FAMA, two partially redundant paralogous bHLH proteins, SCRM and SCRM2, are broadly expressed throughout the stomatal lineage and function to promote all three cell-state transitions (47). Double-knockout *scrm scrm2* plants phenocopy the *spch* epidermal defect, whereas successive loss of *SCRM* and *SCRM2* produces *fama*- and *mute*-like phenotypes (**Figure 2*a*,*c*,*e***). Consistent with promoting stomatal transitions, a gain-of-function mutation (*scrm-D*) located in a conserved "KRAAM"

Figure 2

Epidermal phenotypes due to altered activities of transcription factor genes regulating stomatal cell-state transitions. Cell types and colors are as described in **Figure 1**. (*a*) *spch* and *scrm scrm2* double mutants produce identical phenotypes consisting of pavement cells only. (*b*) Ectopic *SPEECHLESS* (*SPCH*) induces stomatal entry divisions producing a highly divided epidermis. (*c*) *mute* and *scrm scrm2/+* plants produce similar phenotypes resulting in meristemoid arrest. (*d*) *MUTE* overexpression causes all epidermal cells to become guard mother cells (GMCs) and differentiate into stomata. (*e*) *fama* and *scrm* both display additional GMC divisions, although only *fama* produces an absolute block in guard-cell (GC) transition. (*f*) *FAMA* overexpression results in the production of single GCs, consistent with its role in inhibiting GMC division. (*g*) A gain-of-function *scrm-D* mutation produces an epidermis composed solely of stomata, similar to *MUTE* overexpression. (*h*) *flp* mutants produce an increase in GMC division and delay GC transition. (*i*) *myb88 flp* double mutants enhance the *flp* phenotype. (*j*) Diagram of the coordinated actions of the stomatal differentiation genes. *SPCH* and *SCRM/2* direct meristemoid mother cell (MMC) transition, *MUTE* and *SCRM/2* direct the GMC transition, and *FAMA* and *SCRM/2* promote the GC transition. CDKB1;1 and the CYCA2 family control GMC division. FAMA, FLP, and MYB88 directly bind the *CDKB1;2* promoter. FLP and MYB88 bind directly to the *CYCA2;3* promoter. FLP and MYB88 delay GC transition but are not absolutely required for GC differentiation.

domain of either SCRM or SCRM2 mimics a *MUTE* overexpression phenotype (**Figure 2*g***). Both SCRM and SCRM2 preferentially interact with SPCH, MUTE, and FAMA in yeast and in plants, and the *scrm-D* mutation results in higher affinity binding to SPCH (47). The current model suggests that the mutation in the KRAAM domain stabilizes SCRM/SPCH heterodimerization, resulting in enhanced activation of stomatal-lineage entry. The specific mechanism underlying heterodimer stabilization is unclear; however, interference with regulatory serine phosphorylation sites has been suggested (20, 47).

CELL DIVISION AND CYTOKINESIS

Core Cell-Cycle Machinery and Control of Stomatal Differentiation

Cell division is fundamental to the production and appropriate spacing of stomata across the epidermal surface and plays a critical role in the creation of daughter cells with distinctive cell fates (25, 31). How does a developmental program leading to stomatal differentiation intersect with the core cell-cycle machinery? Recent studies revealed direct connections of transcription factors specifying stomatal differentiation to the cell-cycle machinery, specifically at the late stages of stomatal differentiation (**Table 1**).

Two functionally redundant R2R3 MYB transcription factors, FOUR LIPS (FLP) and MYB88, negatively regulate GMC cell division and delay the timing of the final stomatal transition from GMC to GCs (54, 107, 110). A *myb88* mutation produces no phenotype as a single mutation (54). In weak *flp* mutants, two adjacent stomata (i.e., four lips) oriented in parallel to one another are produced owing to an additional symmetric division of the GMC prior to differentiation (**Figure 2*b***). In *flp myb88* double mutants, both division and differentiation are more severely impacted, causing an extension of GMC division resembling a *fama*-like phenotype. However, GC differentiation takes place to some extent (**Figure 2*i***). The strong phenotypic resemblance and similar expression pattern between *fama* and *flp myb88* prompted the idea that these proteins physically interact to promote the GC transition. However, neither FLP or MYB88 have recognized sites necessary for bHLH/MYB binding (6, 54, 114), and no physical interaction between these components has been established (75), suggesting that FAMA and FLP/MYB88 proteins act independently and converge on the GMC division node (**Figure 2*j***).

A mechanistic understanding of both FLP/MYB88 and FAMA targets has been clarified through the negative regulation of *CYCLIN DEPENDENT KINASE B1;1* (*CDKB1;1*) via direct promoter binding (38, 107). Further support of this mechanism comes from the observation that the additional GMC divisions characteristic of the *flp myb88* double mutant are dependent on CDKB1;1 and CDKB1;2 function (107) (**Figure 2*j***). A dominant-negative form of CDKB1;1 has been independently shown to specifically restrict GMC division (11), most likely through the combined interference with *CDKB1;1* and *CDKB1;2* (107). CDKB1;1 can functionally interact with CYCA2;3 (12), and recent analysis of combinatorial mutants of *CYCA2* genes revealed a role for this family of cyclins in promoting GMC division (100). *CDKB1;1* works synergistically with the *CYCA2* genes, as a *cdkb1;1 cycA2;2 cycA2;3 cycA2;4* quadruple mutant produced significantly more single GCs than the *cycA2;2 cycA2;3 cycA2;4* triple mutant alone (100). Consistently, the *CYCA2* genes were required for the *flp myb88* phenotype but not the *fama* phenotype, and both FLP and MYB88 bind directly to the *CYCA2;3* promoter (100) (**Figure 2*j***).

The intersection of the cell-cycle machinery and earlier steps of stomatal development are less clearly understood. Retinoblastoma protein (Rb) acts as a negative regulator of the G1-to-S transition of the cell cycle by associating with E2F/DP transcription factors in diverse organisms (10, 87). The conditional downregulation of the *Arabidopsis Rb-RELATED* (*RBR*) gene results in overproliferation of meristemoids, with an increased number of stomatal-lineage cells

expressing the early stomatal-lineage markers, *TOO MANY MOUTHS* (*TMM*) and *SPCH* (9). This indicates that restricting mitotic activity is critical for proper stomatal density and distribution. Whether SPCH acts as a transcription activator or repressor is not known; it will be interesting see whether *RBR* acts downstream of *SPCH*.

D-type cyclins control the onset of cell division in response to mitotic signals (99). In hypocotyls, loss-of-function mutations in *CYCD4* genes reduce the number of cells in stomata-producing cell files compared with wild type (53). Conversely, *CYCD4;2* overexpression confers excessive divisions in stomata-producing cell files of hypocotyls that express stomatal-lineage markers. These findings suggest *SPCH* may regulate *CYCD4;2* to drive initiation of stomatal-lineage divisions specifically in hypocotyls. Plants possess a large number of cyclins and CDKs (99), many of which may act redundantly. Identification of transcriptional targets of SPCH may reveal the direct connection between cell division and stomatal-lineage initiation.

Some upstream signaling components enforcing stomatal patterns (described below), the ERECTA family [ERECTA, ERECTA-LIKE1 (ERL1), and ERL2] and YODA, affect cell proliferation outside of the stomatal lineages: *erecta erl1 erl2* triple and *yoda* mutant plants are extremely dwarfed with incomplete flower differentiation (65, 92). Mechanistic understanding of how these signaling components tie into cell proliferation will provide a broader view of cell-division control during plant development.

Cytokinesis and Regulation of Cellular Integrity

Some genes involved in cytokinesis produce a range of stomatal defects (**Table 1**). *GLUCAN SYNTHASE 8* (*GSL8*)/*CHORUS* (*CHOR*) is important for callose deposition at cell plates and plasmodesmata, and it has recently been implicated in restricting the movements of cell-fate determinants or other regulatory proteins during stomatal development (19, 37, 97). *gsl8/chor* mutants display an increase in the number of entry divisions and small stomatal clusters, thereby highlighting the importance of cell autonomy during division (19, 37, 97). *STOMATAL CYTOKINESIS-DEFECTIVE1* (*SCD1*) disrupts the final division of the GMC. *SCD1* encodes a protein with a highly conserved DENN domain, which is critical for SCD1 function (26). Defective *scd-1* GCs display typical GC markers, are binucleate, and produce a hanging-pore structure indicating the phenotype is the result of cytokinesis, not GC-fate specification. *CYTOKINESIS DEFECTIVE 1* (*CYD1*), which has not been cloned, produces similar cytokinesis phenotypes to those described for *SCD1* (109).

Subsidiary cell: a cell type found in a stomatal complex of some plant species (e.g., grasses) specialized to supply water and ions for GC function

CELL-CELL SIGNALS PATTERNING STOMATA

One-Cell Spacing Rule and Potential Signaling Components

Regardless of the differences in the developmental origin of stomatal complexes, across species, stomata are formed such that each stoma is separated by at least one cell (77, 86). This one-cell spacing rule is likely an adaptive trait of land plants to promote efficient stomatal opening and closure, which requires rapid water and ion (such as K^+ and Cl^-) exchange between GCs and neighboring nonstomatal cells (e.g., subsidiary cells). In *Arabidopsis*, which forms anisocytic stomatal complexes (90), the spacing division of an SLGC is oriented away from the preexisting stoma (**Figure 1**). However, correction of misplaced meristemoids can occur later in the stomatal cell lineage, where two meristemoids formed right next to each other will most often divide away from each other (73). These observations point to the presence of short-distance signals that specify proper stomatal distribution.

A search for *Arabidopsis* mutants that violate the one-cell spacing rule revealed putative

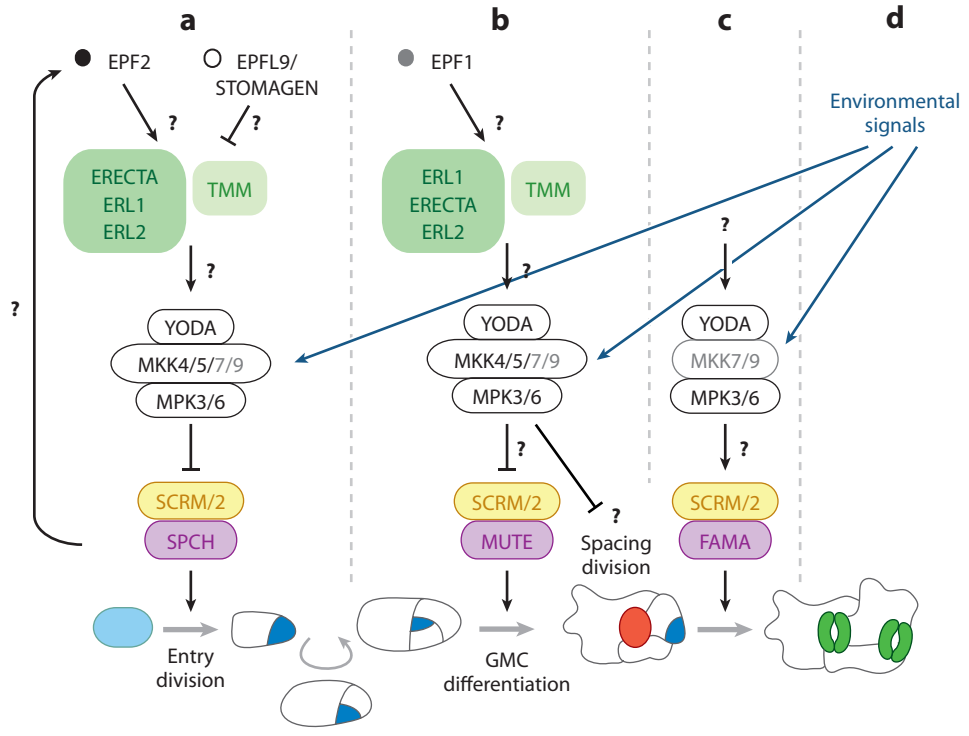


Figure 3

Cell-cell signaling components controlling stomatal patterning and differentiation. Shown is a predicted model of peptide-receptor kinase-mediated signaling pathways and their potential actions on the basic helix-loop-helix (bHLH) heterodimers specifying stomatal cell-state transitions (see **Figure 2**). Cell types and colors are as described in **Figure 1**. Gray text indicates that the in vivo biological functions of these MAPKKs/MAPKs have not yet been demonstrated. (*a*) Entry division is negatively regulated by EPF2, which is perceived primarily by ERECTA LRR-RLKs. TMM likely modulates the signaling outputs. The signal is mediated via a mitogen-activated protein kinase (MAPK) cascade involving YODA, MKK4/5, and MPK3/6 to inhibit SPCH·SCRM/2 heterodimers. It is likely that SPCH·SCRM/2 induces EPF2, thereby forming a negative feedback loop. MKK4/7, when ectopically expressed, can act as MKK4/5. STOMAGEN/EPFL9 is secreted from internal tissues and promotes stomatal development, perhaps via competitive interaction with EPF2 to the same set of receptors. Among the three ERECTA-family members, ERECTA play a major role in this step. (*b*) EPF1 inhibits guard mother cell (GMC) differentiation and orients spacing division primarily via ERL1. TMM likely modulates the signaling outputs. Again, the signal is mediated by the MAPK module, which may, in turn, inhibit MUTE·SCRM/2 heterodimers. (*c*) The MAPK module involving YODA, MKK7/9, and MPK3/6 is likely to promote guard-cell differentiation by acting on the FAMA·SCRM/2 step. Upstream regulators are unknown. (*d*) Environmental signals likely converge into the MAPK module in either step to influence stomatal density and distribution.

receptors and downstream signaling components: ERECTA-family leucine-rich-repeat receptor-like kinases (LRR-RLKs), TMM LRR receptor-like protein (RLP), and components of mitogen-activated protein kinase (MAPK) cascades, including YODA (MAPKKK), MKK4/5 (MAPKK), and MPK3/6 (MAPK) (5, 29, 93, 102) (**Figure 3**). Furthermore, a bioinformatics-based study identified a group of secreted peptides, EPIDERMAL PATTERNING FACTORs (EPFs), as putative signaling ligands coordinating stomatal patterns (39, 40, 44) (**Figure 3*a*,*b***; **Table 1**). Direct connections of ligand-receptors to downstream MAPK cascade are yet to be demonstrated.

In addition, *STOMATAL DENSITY AND DISTRIBUTION1* (*SDD1*), which encodes a subtilisin-type proteinase, was identified as regulator of proper stomatal patterning (4). Currently, several genetic studies indicate *SDD1* acts independently of other signaling components (5, 39, 40, 95), and the identity of its substrates remains unknown.

Leucine-Rich-Repeat Receptors

tmm was the first mutant isolated on the basis of an altered stomatal patterning phenotype (110). Interestingly, the phenotype of *tmm* plants is region (organ) dependent. The cotyledons and leaves of *tmm* produce clustered stomata, whereas hypocotyls and stems are devoid of stomata (30). Thus, *TMM* could act either as a positive or negative regulator of stomatal development. Because *TMM* encodes an LRR-RLP lacking any cytoplasmic effector domain (73), it has been predicted to act as a coreceptor for RLKs. Consistent with this, a recent biochemical study showed that TMM associates with ERECTA-family RLKs in vivo, while it does not associate with itself (61).

The three members of the family, *ERECTA*, *ERL1*, and *ERL2*, exhibit partial redundancy and synergistic interaction to inhibit epidermal cells from adopting stomatal cell fate (93) (**Figure 3*a*,*b***). Detailed analysis of higher-order mutant combinations revealed that *ERECTA* primarily acts at the early steps of stomatal development to suppress asymmetric entry divisions. In contrast, *ERL1* primarily acts to suppress GMC differentiation and orient asymmetric spacing division (93). Consistently, the *ERECTA* promoter is active in the entire protoderm, whereas *ERL1* promoter activity is specific to actively dividing meristemoids and neighboring SLGCs. Stomatal clustering occurs only in the absence of the entire *ERECTA* family. Interactions between the ERECTA family and TMM are dauntingly complex. Although the ERECTA family and TMM act cooperatively in enforcing the one-cell spacing rule in cotyledons and leaves, they act antagonistically in stems where the *erl1* mutation is capable of suppressing the "no-stomata" phenotype of *tmm* (93). Thus, TMM likely modulates ERECTA-family signaling, perhaps by dampening or enhancing ERECTA signal transduction depending on the availability of ligand and/or receptor pools. The diverse activities of their putative ligands, EPFs/EPFLs, support such a hypothesis (see below).

Putative Peptide Ligands

EPF1, the founding member of an eleven *EPF/EPF-LIKE* (*EPFL*) gene family in *Arabidopsis*, was originally identified through a large-scale, function-based screening for novel *Arabidopsis*-secreted peptides with biological activities (39). *EPF/EPFL*-family genes encode cysteine-rich peptides, a large family of secreted peptides that regulates diverse developmental and defense responses (69). *EPF1* is expressed in late-stage stomatal precursors, such as late meristemoids and GMCs, and its overexpression confers arrest of stomatal-lineage cells similar to the *tmm erecta erl2* phenotype (39, 93). The loss-of-function *epf1* mutation confers modest stomatal clustering (39). These findings suggest that EPF1 acts as a cell-cell signal, emitted from late-stomatal precursors to enforce one-cell spacing. The phenotypic resemblance of an *EPF1* overexpressor and *tmm erecta erl2* triple mutant implies that the excessive availability of EPF1 peptide may hyperactivate the ability of ERL1 to repress GC differentiation.

The second member, *EPF2*, limits the asymmetric entry division that initiates the stomatal lineage. The *EPF2* promoter is active in MMCs and early meristemoids, and the loss-of-function *epf2* mutant vastly increases stomatal as well as nonstomatal cell density but does not confer stomatal clustering (40, 44). Conversely, *EPF2* overexpression results in an epidermis solely composed of pavement cells with an apparent lack of stomatal-lineage divisions (40, 44). The phenotype of the *epf2* mutant and *EPF2* overexpression are nearly identical to *SPCH* overexpression and the *spch* mutant, respectively (40, 67, 80). Thus, an exciting scenario is that *SPCH* and *EPF2* constitute

a negative-feedback loop reinforcing lateral inhibition: *SPCH* induces *EPF2* in the MMC, and then EPF2 secreted from SPCH-expressing cells suppresses SPCH activity in the neighboring cells to prevent them from adopting a stomatal-lineage fate (**Figure 3**). In further support of this idea, *EPF2* represses itself (40).

The third member, *EPFL9/STOMAGEN*, was identified on the basis of sequence similarity with EPFs and high *in silico* coexpression coefficiency values with *EPF1*, *TMM*, and *SDD1* (52, 95). Overexpression of *STOMAGEN* confers excessive stomatal clustering resembling the loss-of-function mutations in the *ERECTA* family, *TMM*, and *YODA* MAPK components, whereas reduction in *STOMAGEN* transcripts reduces stomatal density. Thus, unlike *EPF1* and *EPF2*, *STOMAGEN* acts as a positive regulator of stomatal development (52, 95) (**Figure 3*a***). Intriguingly, *STOMAGEN* is expressed in young mesophyll tissues but not in the epidermis (52, 95). STOMAGEN is most likely produced and secreted from mesophyll cells to promote stomatal differentiation in the adjacent epidermal layer. This finding expands our understanding of stomatal development to a three-dimensional view and underscores the importance of cell-layer communication between photosynthetic tissue and the epidermis for proper stomatal patterning.

The fourth member, *EPFL6/CHALLAH* (*CHAL*), was identified as a suppressor of *tmm* phenotype in hypocotyls and stems (1). As described earlier, *tmm* produces no stomata in hypocotyls and stems (29). Loss-of-function *chal* partially resumes stomatal development in these organs of *tmm*. The phenotype of *tmm chal* stems resembles that of *tmm erl1*, implying that the stomataless phenotype of *tmm* may be due to an excessive availability of signaling ligands for ERECTA-family RLKs. Consistent with this hypothesis, *chal* displays complex interactions with *erecta*-family mutants (1). Interestingly, *CHAL* is not expressed in the epidermis but in the internal tissues of hypocotyls and stems (1), further highlighting the importance of inter-cell-layer communication during stomatal patterning. However, the *chal* mutation alone does not confer any stomatal phenotype. The lack of any visible phenotype may reflect the functional redundancies with other *EPFL* genes (85). Alternatively, *CHAL* may not have any normal function in stomatal patterning. Instead, it could act in other developmental processes, and when TMM·ERECTA-family receptor pools are not balanced, loss of *CHAL* may ectopically affect the signaling strength in the epidermis. Further studies should clarify these possibilities.

The additive phenotype of the *epf1 epf2* double mutant indicates that *EPF1* and *EPF2* act independently to negatively regulate stomatal development (40). *STOMAGEN*, by contrast, positively regulates stomatal development (**Figure 3**). It is provocative that, despite their nonredundant roles, these *EPF/EPFL* genes require *TMM* and/or the *ERECTA* family to manifest their overexpression phenotypes. How can different signals elicit different developmental responses using the same set of receptors? Recently, direct association of EPF2 and EPF1 peptides to the ERECTA family was demonstrated *in planta* and in a biosensor platform (61), indicating that ERECTA-family RLKs are the primary receptors for EPFs. In contrast, TMM exhibited limited binding to EPFs, consistent with its predicted role as a signal modulator (61). Interestingly, blocking ERECTA-mediated signaling in vivo by a dominant-negative ERECTA receptor resulted in seedlings resistant to EPF2 application but not to EPF1. Conversely, blocking ERL1-mediated signaling in vivo resulted in seedlings insensitive to EPF1 application but not to EPF2 (61). Taken together, stomatal patterning is regulated by two ligand-receptor pairs, EPF2-ERECTA and EPF1-ERL1, which restrict initial asymmetric entry divisions and orient asymmetric spacing divisions, respectively (61). While the study identified specific ligand-receptor pairs, known redundancies among ERECTA-family RLKs suggest promiscuity and crosstalk between RLKs. It is not know whether ERECTA-family RLKs perceive other EPFL peptides, including STOMAGEN,

which may compete with EPF1/2 for binding. Further biochemical and single cell–based approaches are necessary to address this question.

MAPK Cascades

The signals received by the ERECTA family and TMM are likely mediated via a MAPK cascade containing YODA (MAPKKK), MKK4/5 (MAPKKs), and MPK3/6 (MAPKs) to prevent stomatal differentiation. Constitutively active forms of these kinases confer an epidermis composed solely of pavement cells. Conversely, their loss of function confers high-density stomatal clustering (5, 102). The stomatal-cluster phenotype of *yoda*, *mkk4/5*, and *mpk3/6* are much more severe than their corresponding ligand/receptor mutants (*epf1*, *tmm*, or *erecta erl1 erl2*). This suggests the presence of additional upstream signals (other than EPFs/EPFLs, TMM, and the ERECTA family) that feed into YODA. An alternative scenario is that YODA and downstream MAPKK and MAPKs act as a cell-fate switch, as their on/off status confers extreme phenotypes (no stomata or nearly all stomata). Consistently, the YODA-MKK4/5-MPK3/6 module phosphorylates SPCH protein in vitro, and this phosphorylation is critical for suppression of SPCH activity in vivo (59) (**Figure 3*a***). The mechanism of SPCH downregulation is unknown but may likely involve phosphorylation-dependent degradation, which has been shown for other bHLH proteins in plants (17).

The MPK3/6-mediated phosphorylation of SPCH connects the signaling pathway to a transcription factor that specifies stomatal development. Control of SPCH activity may not be the only point of the MAPK signaling cascade that impinges on transcription-factor control of stomatal differentiation. For instance, even strong, ectopic overexpression of SPCH lacking the MAPK-target domain does not lead to constitutive stomatal differentiation, as seen in the gain-of-function *scrm-D* or loss-of-function *mpk3/6* (47, 59, 102). The phosphorylation protein array has identified MUTE, SCRM, and MYB88 as in vitro targets of MAPKs (82). Recent cell-state-specific expression of various MAPK components revealed a potential role of MKK7/9 in promoting GMC to GC differentiation, as the constitutively active form of MKK7/9 driven by the FAMA promoter conferred a stomatal-cluster phenotype (58). This may represent the mechanism reinforcing stomatal differentiation once its precursor cells commit to becoming GCs (**Figure 3*c***).

MAPK Phosphatases

As described above, stomatal-lineage versus pavement cell-fate specification relies on the activity levels of YODA-MKK4/5-MPK3/6: Higher activity results in less stomatal differentiation. Thus, ensuring their proper activity level is critical for stomatal patterning. MAPK phosphatases inhibit MAPK activity by dephosphorylation, and they counteract MAPK-mediated developmental and environmental processes (13, 60). AP2C3, a putative MAPK phosphatase belonging to the protein phosphatase 2C (PP2C) family, is expressed in the stomatal lineage (98). Excitingly, ectopic overexpression of AP2C3 confers an epidermis composed almost entirely of stomata (98), a phenotype resembling the *mpk3 mpk6* double loss-of-function mutation (102). AP2C3 possesses a kinase interaction motif; it also associates with and inactivates MPK3 and MPK6 *in planta*. Furthermore, the catalytic phosphatase activity and nuclear localization of AP2C3 is required for stomatal-cluster formation (98). Together, these results highlight AP2C3 as a positive regulator of stomatal differentiation via the negative regulation of MPK3/6. Neither *ap2c3* single mutant nor higher-order mutants with related PP2Cs conferred a visible stomatal phenotype. Thus, a role for endogenous AP2C3 and its related phosphatases in stomatal development remains unclear. Both MPKs and PP2Cs have additional roles in GC physiology and stress response (13), which may complicate further analysis to overcome redundancy. Stomatal cell state–specific knockdown of AP2C3, similar to the approach taken by

Lampard et al. (58) may provide informative results.

Intrinsic polarity: the process in which distinct daughter cells are produced from the unequal distribution of cell-fate determinants prior to division

Extrinsic polarity: the process in which distinct daughter cell fates are generated after division in response to environmental cues

CELL-DIVISION POLARITY

Intrinsic Control of Asymmetric Division

During development, a parental cell can impart different developmental fates for its daughter cells through asymmetric distribution of cell-fate determinants (intrinsic) (34), or the daughter cells can establish their cell fate after division through external exposure to different developmental signals (extrinsic) (106). In animals, intrinsic axis polarity is specified, in part, by conserved PAR (partitioning defective) proteins, which exhibit highly polarized expression that directs spindle-apparatus position and the unequal distribution of cell-fate determinants to each daughter cell (34, 50). No homologs to the PAR proteins have been identified in plants, suggesting plant and animal lineages have evolved distinct components for establishing intrinsic polarity during division. Researchers have characterized several plant proteins that directly alter asymmetric divisions or are asymmetrically inherited after division (3, 65, 72, 76, 94, 105). However, only recently have premitotic polarly localized proteins related to plant cell division been identified. These proteins provide initial insight into the variable mechanisms driving intrinsic polarity during cell division in plants.

PAN1: Subsidiary Cell-Division Polarity in *Zea mays*

Although some genes possess conserved functions in stomatal differentiation in monocots and dicots (64, 66), the development of stomatal complexes is different in both classes of flowering plants (64). A stomatal complex in maize consists of a stoma flanked by a pair of specialized subsidiary cells that regulate GC expansion and pore opening (**Figure 4*a***). A GMC is produced through an initial asymmetric division during which the cells flanking the GMC called subsidiary mother cells (SMC) asymmetrically position their nuclei toward the GMC. *PANGLOSS1* (*PAN1*), a Type III LRR-like protein, was identified as a likely receptor for a yet-unknown GMC-produced signal that regulates the polarized cell division of SMCs toward the GMC (14, 28) (**Table 1**). PAN1 and a unique patch of F-actin display clear polar localization in the SMC where they are in contact with the GMC (14). Mutations in *PAN1* cause defects in actin-patch formation and nuclear positioning that result in the abnormal division of ~30% of the subsidiary cells. However, many sister cells from abnormal divisions adopt appropriate cellular fates, indicating that PAN1 is not necessary for cell-fate determination (14). Although several LRR proteins, including TMM and the ERECTA family, are known regulators of stomatal-lineage division and differentiation in *Arabidopsis*, these proteins do not exhibit polar localization (73, 93). Recently, the Rho family of GTPases (Rho-of-plants, ROPs), ROP2 and ROP9, were shown to interact physically with PAN1 and colocalize at the SMC/GMC contact (14, 43). PAN1 and ROPs may work cooperatively as *rop2/9* partial loss of function greatly enhances the *pan1* phenotype (43).

BASL: Meristemoid-Division Polarity in *Arabidopsis*

BREAKING OF ASYMMETRY IN THE STOMATAL LINEAGE (*BASL*) promotes physical asymmetry of MMC and meristemoid divisions in *Arabidopsis* (25) (**Table 1**). Fewer asymmetric divisions take place in *basl*, resulting in equally sized daughter cells that often have an equivalent cell fate. The *BASL* protein displays a dynamic dual-localization pattern at the cell periphery and in the nucleus, which correlates with the developmental fate of the expressing cell (**Figure 4*b***). For example, GFP-BASL localization in restricted regions of the periphery results in pavement-cell differentiation, and nuclear localization is associated with stomatal differentiation. By contrast, simultaneous peripheral (or diffuse) and nuclear localization result in continued asymmetric

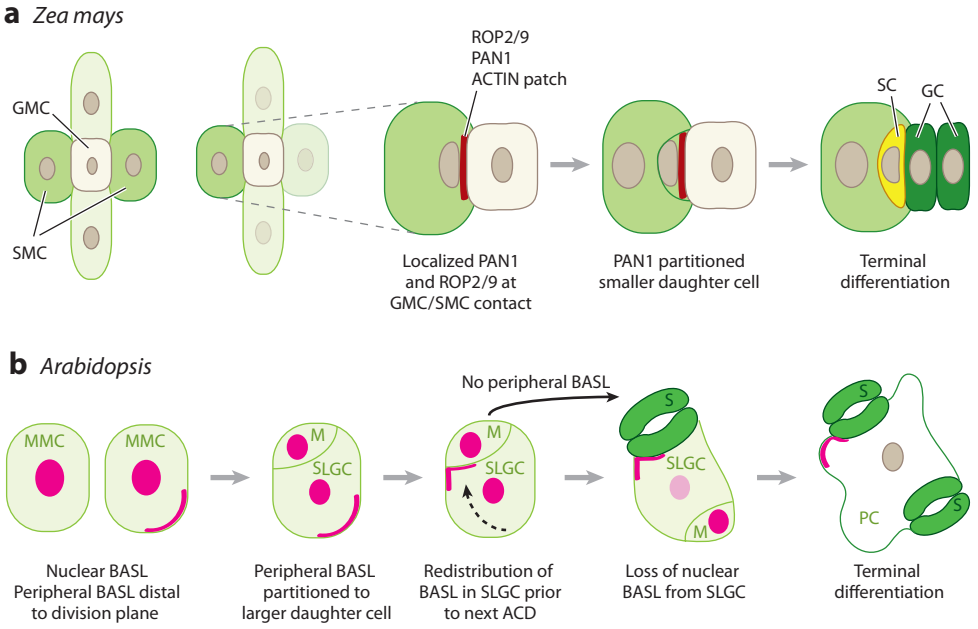


Figure 4

Intrinsic regulators of asymmetric division in maize and *Arabidopsis*. (*a*) In maize, the subsidiary mother cells (SMCs) are adjacent to the guard mother cell (GMC). PAN1 and ROP2/9 (*red*) colocalize with an actin patch at the contact site between the SMC and GMC. Migration of the SMC nucleus occurs after PAN1 localization. The SMC divides asymmetrically toward the GMC to produce a subsidiary cell (SC). The GMC divides symmetrically to produce two guard cells (GC). (*b*) In *Arabidopsis*, nuclear BASL expression (*pink*) and polar localization of BASL to the periphery of the meristemoid mother cell (MMC) occurs prior to the asymmetric division (ACD), producing a meristemoid (M) and a stomatal-lineage ground cell (SLGC). Redistribution of BASL prior to SLGC asymmetric division ensures the new M is distal to the existing stomatal precursor. SLGCs lose nuclear BASL and differentiate into a pavement cell (PC). Loss of peripheral BASL in M causes a cell-state transition to a GMC (not shown), which divides symmetrically to produce two GCs of the mature stomata (S).

division (**Figure 4*b***). Peripheral BASL localization is always distal to the division plane, and disruption of external signaling cues through TMM or EPF1 do not affect the correct positioning of BASL relative to the division (25). An active role of BASL in the nucleus remains to be determined, as the main site of BASL function is at the cell periphery. Ectopic overexpression of BASL causes localized cell expansion in expressing cells, suggesting that the segregation of peripheral BASL to the SLGC may exaggerate size differences of the SLGC and meristemoid after division. The expanded size differences could lead to dilution of cell-fate determinants or signaling molecules in the SLGC, resulting in an alternate developmental path (25).

Recently, POLAR LOCALIZATION DURING ASYMMETRIC DIVISION AND REDISTRIBUTION (POLAR) has been reported as a second polarity protein within stomatal cell lineages in *Arabidopsis* (79) (**Table 1**). POLAR was identified through transcriptome analysis that took advantage of stomatal cell-state transition mutants. Comparison of transcriptomes from seedlings that develop epidermis composed solely of pavement cells (*spch*), meristemoids (*scrm-D mute*), and stomata (*scrm-D*) yielded a set of genes highly associated with meristemoid

states, including *POLAR* (79). POLAR shows unique and intriguing subcellular localization dynamics. It is transiently localized to the cell cortex distal to the division plane in asymmetrically dividing cells. After asymmetric division, POLAR-GFP is upregulated only in the daughter cell that will continue to divide asymmetrically and is rapidly downregulated in differentiating cells (79). Strong peripheral localization of POLAR-GFP is dependent on a functional BASL protein, suggesting that POLAR may be a component in the BASL-mediated pathway that marks the site of rapid cell expansion. The absence of a clear phenotype in loss-of-function *polar* mutant implies that *POLAR* function may be redundant.

The ROP proteins regulate polar cell expansion in plants in different developmental contexts (27, 36, 46), and a Rho-family GTPase is required for asymmetric division in yeast (91). The local expansion of *BASL*-overexpressing cells is reduced in the background expressing a dominant-negative form of ROP2, suggesting that ROPs may act downstream of BASL (25). The inclusion of ROP proteins in both the PAN1 and BASL polarity modules may ultimately reveal a common thread between these distinct mechanisms of asymmetric divisions. However, the general lack of genes that exhibit premitotic polar localization in either maize or *Arabidopsis* precludes a uniform comparison of the two systems. Regardless of distinctions or commonalities between them, understanding the molecular context that regulates the polar localization of PAN1 and, specifically, BASL and POLAR, which can reestablish peripheral expression in dividing SLGCs, will lead to novel and exciting advances in our knowledge of cell polarity in plants.

ENVIRONMENTAL CONTROL OF STOMATAL DEVELOPMENT: LONG-DISTANCE SIGNALS

The sessile nature of plants requires that they be able to adapt their physiology and developmental programs to respond to external surroundings. Alteration of stomatal function or distribution is one of the many flexible processes that allow plants to minimize the impacts of a stressful environment or take advantage of an ideal environment. Plants can rapidly adjust stomatal aperture to moderate CO_2 uptake and water loss in response to transient fluxes in the environment (15, 84). However, long-term or consistent differences in the environment, such as growing in shade versus under sunlight, modify stomatal density in developing leaves (23). Excitingly, the signals that drive stomatal-density changes in developing leaves are derived from mature leaves (23, 55). Thus, stomatal density and patterning are influenced by both short-distance cellular signals (described in Putative Peptide Ligands, above) as well as long-distance systemic signals. Despite advances in our understanding of the genetic regulation of stomatal development over the past decade, questions remain regarding how the activities of stomatal regulators are modulated by long-range environmental signals to determine final stomatal abundance.

Light

Across species, light plays an important role in stomatal development. Dark-grown plants produce significantly fewer stomata compared with light-grown plants, and high light intensity results in an increase in stomatal index (ratio of number of stomata to total number of epidermal cells) (16, 55). Stomatal opening in response to light is mediated primarily by the blue-light receptors, cryptochromes and phototropins (51, 68). Recently, *CRYPTOCHROME1* (*CRY1*) and *CRY2* were also implicated in the light-induced changes in stomatal development (48). Among other developmental alterations, the *cry1 cry2* double mutant under monochromatic blue light produces an epidermis with a lower stomatal index compared with wild type under the same conditions (48). Similarly, stomatal index was lower in a mutant lacking functional phytochrome B

(*phyB*) and phytochrome A (*phyA*) under red- and far-red-light conditions, respectively (8, 16, 48). These data indicate that the mediation of light quality and intensity to promote stomatal development is achieved through the additive function of *CRY1*, *CRY2*, *PHYB*, and *PHYA*.

COP1 (*CONSTITUTIVE PHOTOMORPHOGENESIS 1*), an E3 ubiquitin ligase, is a downstream component in the *CRY* signaling network, and strong *cop1* mutants display large stomatal clusters in both light and dark conditions, similar to those seen in *yoda* mutants, indicating a clear negative role of *COP1* in stomatal development (48, 62) (**Table 1**). The contrasting stomatal phenotypes of the photoreceptor mutants compared with *cop1* are consistent with the role of *CRY1* and *CRY2* in the negative regulation of *COP1* in other photomorphic processes (63, 108). Based on genetic interactions with *TMM* and *YODA*, a model for light-signaling integration into the stomatal pathway proposes that the photoreceptors CRY, PHYA, and PHYB negatively regulate COP1, which acts in parallel with TMM to positively regulate YDA (through unknown mechanisms). The YDA MAPK-signaling module negatively regulates SPCH and MUTE, resulting in alterations in stomatal development under variable light conditions (48, 57, 58) (**Figure 3*d***).

Although *SDD1* is not required for the light response (89), *SDD1* and *SDD1-like* proteins are upregulated in the developing leaves of shaded plants (23), consistent with the production of fewer stomata under shaded conditions. This suggests an alternate pathway, which is not required, but may fine-tune final stomatal abundance in response to light.

Carbon Dioxide

Similar to light intensity, CO_2 plays a role in the regulation of both stomata opening (41, 111) and stomatal development (35). Although not universal, the trend across species is to decrease stomatal density under increasing levels of CO_2; this can be observed both in the laboratory and through geological time (32, 96). *HIGH CARBON DIOXIDE* (*HIC*) modulates changes in stomatal density in response to changing carbon-dioxide levels (35) (**Table 1**). In contrast to wild type, *hic* mutants produce significantly higher numbers of stomata when exposed to elevated CO_2 levels. However, loss of *HIC* function does not disrupt stomatal patterning, as stomata are equally spaced and are not present in clusters. *HIC* encodes a protein similar to the 3-keto acyl CoA synthase involved in the production of cuticular wax polymers (35). Changes in cuticle composition may affect gas/water permeability as well as diffusion gradients of soluble inhibitors, or they may alter the light spectra experience by the leaf and, thus, alter stomatal density via activation of the light-response pathway. This idea is supported by other cuticular wax mutants, which display both increases and decreases in stomatal density compared with wild type (2, 18, 35).

Recently, β-carbonic anhydrase (βCA) genes, which bind CO_2 and catalyze its conversion into HCO_3^- and H^+, were found to affect both stomatal function and development (42) (**Table 1**). *βca2 βca4* plants are insensitive to CO_2-induced stomatal movement. At ambient CO_2 level, mutant plants showed increased stomatal conductance and stomatal density. Although βCA2 and βCA4 are expressed in both mesophyll and GCs, GC expression alone was sufficient to complement the stomatal-density phenotype, suggesting that GC-specific production of bicarbonate is a key step in the production of the long-distance signal altering stomatal density in response to CO_2 (42).

Other Environmental Conditions: Humidity and Temperature

Several other environmental factors, including humidity and temperature, affect the development of stomata, but the genetic basis for the change has not been determined (15, 56). It is well documented that stresses such as low temperature, low humidity, drought, wounding, pathogens, and stress-related molecules and hormones often activate MAPK cascades

in *Arabidopsis* and other species (22, 45, 74, 103). Among such conditions, humidity confers stomatal clustering (56). The characterized role of the YODA-MKK4/5/7/9-MPK3/6 module in stomatal development and the known function of MPK6 in multiple stress responses has prompted the idea that MAPK signaling may be a common mechanism through which environmental signals are integrated into developmental programs (22, 82, 102). Except SPCH, other stomatal regulators have not been identified as direct targets of MPK3 or MPK6 (59). However, through use of functional protein microarrays, MYB88 and MUTE were identified as possible targets of MPK6 and MPK4, respectively (82). Identification of additional MAPKs and substrates for MAPK modules that function at distinct stages of stomatal development will determine the extent to which posttranslational phosphorylation adjusts stomatal development.

SCRM, the core integrator bHLH protein of stomatal differentiation, is identical to ICE1, the key upstream regulator of cold-induced transcriptome and freezing tolerance (20). It has been proposed that cold stress confers posttranslational modification on the ICE1/SCRM protein, which leads to a transcriptional activation of CBF3/DREB1A, one of the key transcription factors that directly induces expression of cold-regulated (*COR*) genes (21). The stability of ICE1 under cold stress is modified posttranslationally by ubiquitination (by HOS1) and SUMOylation (by SIZ1) (24, 70). It is not known whether MAPK-mediated phosphorylation plays a role in ICE1's function as a regulator of cold response. Likewise, whether known modifications of ICE1 (i.e., ubiquitination and SUMOylation) affect stomatal development remains undetermined. It is astonishing that the *scrm-D* gain-of-function mutation is identical to the *ice1-D* gain-of-function mutation, the latter of which confers cold sensitivity (20, 47). This surprising finding indicates that the identical molecular lesion in the KRAAM motif of SCRM/ICE1 confers constitutive active- and dominant-negative effects for stomatal differentiation and cold acclimation, respectively. Thus, upstream regulation of SCRM/ICE function may have opposite effects in development and environmental response.

CONCLUSIONS AND PERSPECTIVES

In the past decade, there have been exciting advancements in our understanding of stomatal development. Key components of stomatal development have been identified in *Arabidopsis*. The discoveries of bHLH proteins specifying stomatal cell-state transitions, cell-cell signaling components enforcing stomatal patterning, and polarity proteins have revealed a core framework of stomatal development. However, it is not known how these components are tied together molecularly to constitute regulatory networks. Large gaps exist; for instance, no biochemical evidence exists demonstrating how EPF binding to ERECTA-family receptors activates MAPK cascades. Likewise, in vivo phosphorylation of stomatal bHLH proteins by MAPKs has not been demonstrated. The molecular and cellular mechanisms of asymmetric localization of BASL, an intrinsic polarity component within stomatal cell lineage, are unknown. Integrated approaches combining emerging disciplines, systems approaches, and traditional developmental genetics may bring a new, dynamic view of stomatal development.

Stomatal density is influenced not only by environmental conditions, but also by endogenous factors, such as phytohormones (49, 88). Understanding how multiple signals molecularly impinge on the core components of stomatal development will be an important next challenge. Knowledge of such molecular intersections will broaden the significance of stomata to whole-plant growth, development, and physiology. Lastly, as genome sequence information of more plant species becomes available, it will also become possible to understand the conservation and uniqueness of the evolution of gene regulatory networks specifying stomatal development.

SUMMARY POINTS

1. Stomata are produced through a characteristic series of divisions controlled via the coordinated activities of transcription factors that can directly regulate core cell-cycle genes.
2. Correct stomatal patterning and initiation requires intercellular communication through the activity of secreted peptide ligands, receptor kinases, and MAPK signaling modules.
3. A receptor-like protein (PAN1) and novel, polarly localized proteins (BASL and POLAR) provide the first examples of premitotic polarity factors in plants.
4. Environmental conditions impact the production of stomata in developing leaves via a long-distance signal initiated in mature leaves. MAPK signaling modules may provide a common integration point among multiple environmental inputs.

FUTURE ISSUES

1. The molecular basis of the establishment of SPCH expression is still an outstanding question. Identification of factors driving SPCH transcriptional expression will provide much needed insight into intriguing questions: What drives SPCH expression, and what underlying program restricts SPCH expression to MMCs to initiate entry division?
2. The current genetic analysis of the organ-specific interactions of the ERECTA family and TMM with their ligands suggests a complex control mechanism. Novel approaches to quantify receptor and ligand interactions and model their dynamics will be necessary to tease apart the complexities of this system to get a true understanding of the delicate balance among receptors and ligands specifying stomatal patterning.
3. Although some players regulating the integration of environmental signaling and stomatal development have been identified, the specifics on the integration point of environmental signaling into the stomatal development pathway remain unknown. Identification of new mutants and additional analysis of known mutants under variable conditions may provide insight into this complex system.
4. Analysis of the conservation among diverse plant groups will allow for a mechanistic understanding of stomatal development and provide a means of assessing the ubiquitous nature of the pathways driving stomatal development. The comparative analysis of these genes in diverse species will provide the reasoning behind future crop-improvement strategies.

DISCLOSURE STATEMENT

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