Mechanisms of Stomatal Development

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Abstract

The main route for CO₂ 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.
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

<table>
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Environmental control genes

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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).

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

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.

Pillitteri • Torii
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, *spcb* loss-of-function mutants produce an epidermis composed solely of interlocking pavement cells (Figure 2a). No known stomatal-lineage-specific markers are expressed in *spcb*, 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 2b). In weak *spcb*-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.

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

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).
a) spch (scrm scrm2)

No MMC transition or stomatal entry divisions
Pavement cell only

b) mute (scrm scrm2/+)

Promotes MMC identity
No GMC transition
Meristemoid arrest after excess division

Ectopic MUTE
Promotes GMC identity

c) Ectopic MUTE

Promotes GMC identity

Ectopic SPCH

Promotes MMC identity
No GMC transition or stomatal entry divisions
Pavement cell only

e) fama (scrm*)

No GC transition
Excessive GMC division
* Not fully penetrant

f) Ectopic FAMA

Promotes GC identity

Excessiveentry division
Excessive stomatal differentiation

Excessive GMC division

Excessive GMC division

h) Increased GMC division

i) myb88 flp

Excessive GMC division

Excessive GMC division

Excessive GMC division

j) SCRM/2

MUTEMUTE

GC transition

CDKB1;1

FLP

MYB88

GC transition

GMC division

596 Pilletteri • Torii
presumably owing to a physical size restriction for further division (80) (Figure 2e). *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 2d). 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, whereas successive loss of *SCRM* and *SCRM2* delays GC transition (75). *FAMA* overexpression causes all epidermal cells to produce identical phenotypes consisting of pavement cells only (Figure 2f). 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 2f). *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: Scream(s)**

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 *spcb* epidermal defect, whereas successive loss of *SCRM* and *SCRM2* produces *fama- and mute-like phenotypes* (Figure 2a,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) *spcb* and *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 *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 produces GC-specific markers or hallmark GC morphology (Figure 2e). 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 2f). *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).

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domain of either SCRM or SCRM2 mimics a MUTE overexpression phenotype (Figure 2g).

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 fbp 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 2b). In fbp 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 2i). The strong phenotypic resemblance and similar expression pattern between fama and fbp 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 2j).

A mechanistic understanding of both FLP/MYB88 and FAMA targets has been clarified through the negative regulation of CYCLOPS 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 fbp myb88 double mutant are dependent on CDKB1;1 and CDKB1;2 function (107) (Figure 2j). 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 fbp myb88 phenotype but not the fama phenotype, and both FLP and MYB88 bind directly to the CYCA2;3 promoter (100) (Figure 2j).

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 to 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. **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).

**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
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.
In addition, STOMATAL DENSITY AND DISTRIBUTION1 (SDD1), which encodes a subtilisin-type proteinase, was identified as a 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 3a,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 coefficient 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 3a). 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 cbal partially resumes stomatal development in these organs of tmm. The phenotype of tmm cbal 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, cbal 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 cbal 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 known 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 3a). 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 3c).

**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
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

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

**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 4a). 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. PAN-GLOSS1 (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 4b). 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
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 4b). 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 (spcb), 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 CO₂ 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, CRYPTOCRHOME1 (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 3d).

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, CO₂ 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 CO₂; 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 CO₂ 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 CO₂ and catalyze its conversion into HCO₃⁻ and H⁺, were found to affect both stomatal function and development (42) (Table 1). βCA2 βCA4 plants are insensitive to CO₂-induced stomatal movement. At ambient CO₂ 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 CO₂ (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.

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LITERATURE CITED


Contents

There Ought to Be an Equation for That

Joseph A. Berry ................................................................. 1

Photorespiration and the Evolution of C$_4$ Photosynthesis

Rowan F. Sage, Tammy L. Sage, and Ferit Kocacinar ........................................ 19

The Evolution of Flavin-Binding Photoreceptors: An Ancient Chromophore Serving Trendy Blue-Light Sensors

Aba Losi and Wolfgang Gärtner ........................................ 49

The Shikimate Pathway and Aromatic Amino Acid Biosynthesis in Plants

Hiroshi Maeda and Natalia Dudareva ........................................ 73

Regulation of Seed Germination and Seedling Growth by Chemical Signals from Burning Vegetation


Iron Uptake, Translocation, and Regulation in Higher Plants

Takanori Kobayashi and Naoko K. Nishizawa ........................................ 131

Plant Nitrogen Assimilation and Use Efficiency

Guohua Xu, Xiaorong Fan, and Anthony J. Miller ........................................ 153

Vacuolar Transporters in Their Physiological Context

Enrico Martinoia, Stefan Meyer, Alexis De Angeli, and Réka Nagy ........................................ 183

Autophagy: Pathways for Self-Eating in Plant Cells

Yimo Liu and Diane C. Bassham ........................................ 215

Plasmodesmata Paradigm Shift: Regulation from Without Versus Within

Tessa M. Burc-b-Smith and Patricia C. Zambryski ........................................ 239

Small Molecules Present Large Opportunities in Plant Biology

Glenn R. Hicks and Natasha V. Raikhel ........................................ 261

Genome-Enabled Insights into Legume Biology

Nevin D. Young and Arvind K. Bharti ........................................ 283
Synthetic Chromosome Platforms in Plants
  Robert T. Gaeta, Rick E. Masonbrink, Lakshminarasimhan Krishnaswamy,
  Changzeng Zhao, and James A. Birchler ........................................ 307

Epigenetic Mechanisms Underlying Genomic Imprinting in Plants
  Claudia Köhler, Philip Wolff, and Charles Spillane .......................... 331

Cytokinin Signaling Networks
  Ildoo Hwang, Jen Sheen, and Bruno Müller ...................................... 353

Growth Control and Cell Wall Signaling in Plants
  Sebastian Wolf, Kian Hématy, and Herman Höfte .............................. 381

Phosphoinositide Signaling
  Wendy F. Boss and Yang Ju Im .................................................. 409

Plant Defense Against Herbivores: Chemical Aspects
  Axel Mitöffer and Wilhelm Boland .............................................. 431

Plant Innate Immunity: Perception of Conserved Microbial Signatures
  Benjamin Schwessinger and Pamela C. Ronald ................................. 451

Early Embryogenesis in Flowering Plants: Setting Up
  the Basic Body Pattern
  Steffen Lau, Daniel Slane, Ole Herud, Jixiang Kong, and Gerd Jürgens .......... 483

Seed Germination and Vigor
  Loïc Rajjou, Manuel Duval, Karine Gallardo, Julie Catusse, Julia Bally,
  Claudette Job, and Dominique Job .............................................. 507

A New Development: Evolving Concepts in Leaf Ontogeny
  Brad T. Townsley and Neelima R. Sinha ...................................... 535

Control of Arabidopsis Root Development
  Jalean J. Petricka, Cara M. Winter, and Philip N. Benfey ...................... 563

Mechanisms of Stomatal Development
  Lynn Jo Pillitteri and Keiko U. Torii ......................................... 591

Plant Stem Cell Niches
  Ernst Aichinger, Noortje Korner, Thomas Friedrich, and Thomas Laux ........... 615

The Effects of Tropospheric Ozone on Net Primary Productivity
  and Implications for Climate Change
  Elizabeth A. Ainsworth, Craig R. Yendrek, Stephen Sitch, William J. Collins,
  and Lisa D. Emberson ............................................................ 637

Quantitative Imaging with Fluorescent Biosensors
  Sakiko Okumoto, Alexander Jones, and Wolf B. Frommer ........................ 663