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Stomatal Development in Arabidopsis

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Stomata consist of two guard cells that function as turgor-operated valves that regulate gas exchange in plants. In Arabidopsis, a dedicated cell lineage is initiated and undergoes a series of cell divisions and cell-state transitions to produce a stoma. A set of basic helix-loop-helix (bHLH) transcription factors regulates the transition and differentiation events through the lineage, while the placement of stomata relative to each other is controlled by intercellular signaling via peptide ligands, transmembrane receptors, and mitogen-activated protein kinase (MAPK) modules. Some genes involved in regulating stomatal differentiation or density are also involved in hormonal and environmental stress responses, which may provide a link between modulation of stomatal development or function in response to changes in the environment. Premitotic polarly-localized proteins provide an added layer of regulation, which can be addressed more thoroughly with the identification of additional proteins in this pathway. Linking the networks that control stomatal development promises to bring advances to our understanding of signal transduction, cell polarity, and cell-fate specification in plants.

INTRODUCTION

Stomatal pores serve as a low-resistance pathway for diffusional movement of gas and water vapor between a plant and the environment. The development of stomata and adjustment of pore aperture are regulated by complex regulatory networks that incorporate environmental cues to optimize photosynthesis capacity and minimize water loss. The accessibility and predictable development of stomata have made them an excellent model for examining intra- and intercellular signaling networks, cell polarity, and cell-type differentiation; processes that are fundamental to both plants and animals. Here, we summarize the developmental events that result in the differentiation of stomata in Arabidopsis and discuss directions for future research. For a list of genes discussed in this chapter, see Table 1.

Stomata Structure and Function

The term “stoma” means mouth in Greek. Just as a mouth contains an opening surrounded by two lips, a stoma consists of two guard cells that surround a pore. Typical of the dicot lineage, guard cells in Arabidopsis are kidney-shaped and capable of rapidly changing internal turgor to alter their shape and pore aperture (Figure 1). Stomatal pores open when guard cells are turgid, allowing mesophyll chloroplasts access to carbon dioxide for photosynthesis. In addition, water vapor release from open stomata promotes water and nutrient uptake through the roots via transpiration (Schroeder et al., 2001; Pillitteri and Torii, 2012).

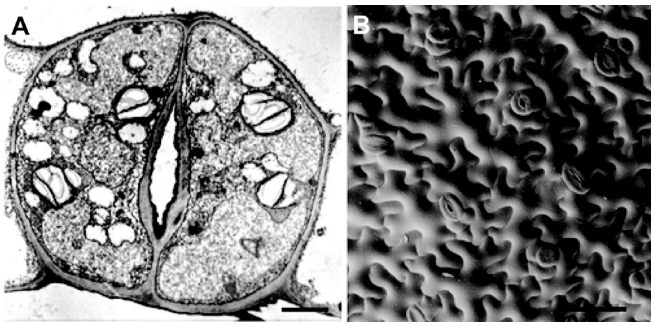

Figure 1. Morphology and Distribution of Arabidopsis Stomata.

(A) Two kidney-shaped guard cells surround a pore.
(B) Cryoscanning electron micrograph of maturing epidermis from a cotyledon. The larger, non-stomatal cells are pavement cells that are shaped like pieces of a jigsaw puzzle. Electron micrographs from Nadeau and Sack, 2002a.

Scale bars = 2 µm (A) and 30 µm (B).

The turgor-driven movements of guard cells are in part driven by changes in K^+ salt and sugar concentration in guard cells. Increased solute concentration results in water uptake from the surrounding apoplast and an overall increase in hydrostatic pressure within the guard cell (Fisher, 1968; Shimazaki et al., 2007). The elevated turgor expands the guard cell, but asymmetrical positioning of cell wall components in guard cells limits expansion in all directions and promotes pore widening (Figure 1) (Franks and

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Farquhar, 2001). The regulation of stomatal movement is coordinately controlled by a large network of signaling pathways that monitor water status, light, CO_2 and various other environmental conditions to optimize plant growth and water use (Schroeder et al., 2001; Shimazaki et al., 2007; Casson and Hetherington, 2010; Tricker et al., 2012). Numerous Arabidopsis mutants have been isolated that are defective in stomatal function (Gray et al., 2000; Assmann, 2003; Miyazawa et al., 2006; Young et al., 2006; Kang et al., 2009a; Clark et al., 2011; Sawinski et al., 2013).

Distribution

In Arabidopsis, stomata are present in the epidermis of all aboveground organs except petals and stamen filaments (Sessions et al., 1997; Geisler et al., 1998; Pillitteri et al., 2008; Robinson et al., 2011). Stomata are distributed nonrandomly across the epidermis and the number and distribution of stomata across the epidermis can vary between organs. For example, there are fewer stomata on the adaxial (upper) epidermis than abaxial (lower) epidermis in Arabidopsis leaves and cotyledons (Serna and Fenoll, 2000a; Geisler et al., 2000; Geisler and Sack, 2002). Altering stomatal distribution impacts CO_2 uptake, evaporation, and internal leaf temperature in much the same way as pore opening and closing (Yoo et al., 2011).

One-cell spacing rule

Despite differences in stomatal density among organs, stomata are always separated from each other by at least one cell (Sachs, 1991; Geisler et al., 2000). A minimum requirement of one intervening cell ensures that guard cells can efficiently exchange ions and water to regulate pore opening and reduce unnecessary evaporation. Current evidence supports the idea that stomatal placement is regulated locally, as there is no evidence for a broad morphogen gradient to promote and organize development across various organs as is commonly reported in animal systems (Schwank and Basler, 2010).

Modified stomata

Arabidopsis and other land plants produce structures such as hydathodes and nectaries, which include modified stomata specifically used for secretion. Hydathodes are found along leaf margins at the end of water-conducting vasculature, where they release excess fluid and ions (guttation) under conditions of high humidity and darkness. Nectaries secrete a carbohydrate-rich solution often used as a reward for pollinators. Nectaries in Arabidopsis are at the base of stamen filaments, although the placement of nectaries varies across species (Figure 2) (Bowman and Smyth, 1999; Baum et al., 2001; Pillitteri et al., 2008; Kram et al., 2009). Modified stomata are generally larger than epidermal stomata and more often found in contact with one another (Figure 2) (Davis and Gunning, 1993). In addition, hydathode and nectaries cannot regulate pore aperture, leaving modified stomata continually open. Therefore, the mechanisms regulating stomatal spacing and opening may be different for modified stomata. However, genes that control the differentiation of these structures are likely the same as those that produce typical epidermal stomata (Pillitteri et al., 2008).

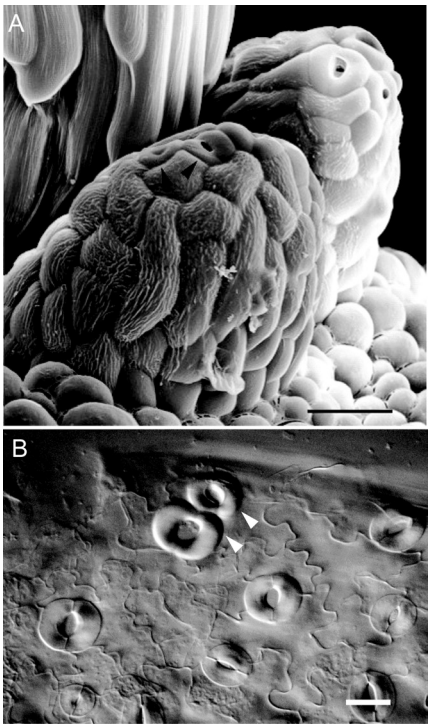

Figure 2. Modified stomata in wild type Arabidopsis.

(A) Cryoscanning electron micrograph showing stomata at the tips of nectaries (Nadeau and Sack, 2002a).
(B) DIC image of a hydathode. Arrowheads indicate modified stomata with large open pore in contact with one another.

Scale bar 30μm (A) and 10μm (B).

OVERVIEW OF STOMATAL DEVELOPMENT

Cell-state transitions

A stoma is produced by a specialized epidermal lineage, which undergoes an organized series of divisions and successive cell-state transitions, where each transitional state is distinct and demonstrates changes in morphology, transcript accumulation and protein localization (Zhao and Sack, 1999; Dong et al., 2009). Through a mechanism that is not well understood, a subset of protodermal cells becomes meristemoid mother cells (MMC) (Figure 3). Through an asymmetric “entry” division, an MMC produces a small triangular cell called a meristemoid and a larger cell called a stomatal-lineage ground cell (SLGC). An SLGC can terminally differentiate into a lobed pavement cell (PC) or alternatively, an SLGC can initiate an asymmetric spacing division to produce a satellite meristemoid, which is always placed distal to an existing stoma or precursor. The

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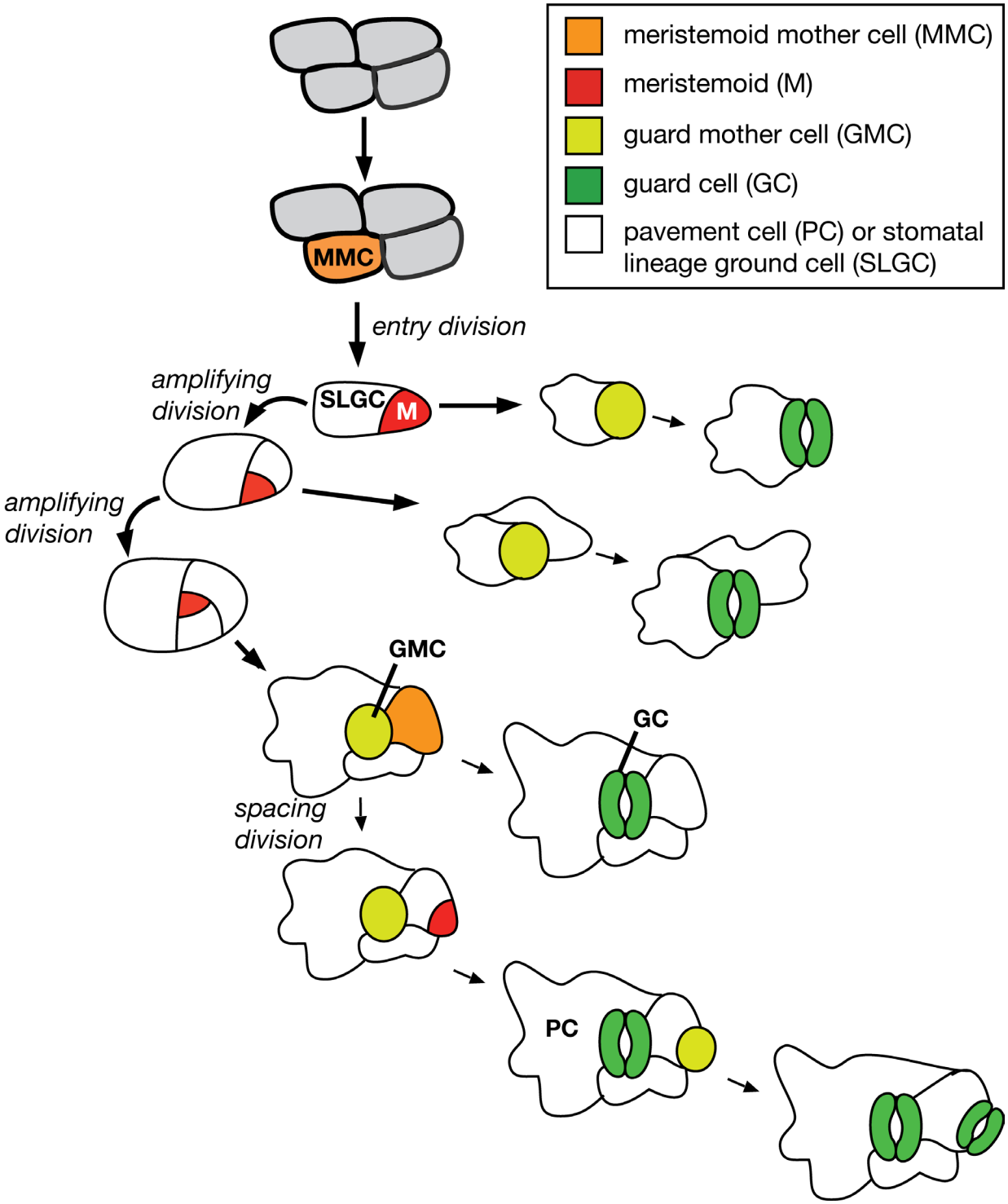

Figure 3. Diagram of stomatal lineage progression in Arabidopsis.

A subset of protodermal cells (*grey*) becomes meristemoid mother cells (MMC, orange). MMCs enter the stomatal lineage through an asymmetric division to create two daughter cells, a meristemoid (M, *red*) and a stomatal-lineage ground cell (SLGC). Meristemoids can undergo a limited number of amplifying divisions, but eventually transition into a guard mother cell (GMC, *yellow*). An SLGC can differentiate into a pavement cell (PC) or attain MMC identity to produce a satellite meristemoid positioned away from an existing stomatal precursor. A GMC divides once symmetrically to produce two equally-sized guard cells (GCs, green).

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orientation of spacing divisions is controlled by a signaling network that provides spatial cues to ensure that stomata are isolated and have at least one non-stomata cell between them (Geisler et al., 2000; Shpak et al., 2005; Hara et al., 2007; Hara et al., 2009; Hunt and Gray, 2009). In the case where two adjacent MMCs produce meristemoids in direct contact, correction occurs later in the lineage when one of the meristemoids divides away from the other or exits the lineage (Sachs, 1991; Geisler et al., 2000; Hara et al., 2007; Hara et al., 2009). All meristemoids (primary or satellite) have stem cell-like character. Meristemoid divisions are called amplifying divisions and can occur up to four times. Each division regenerates the meristemoid and increases, or "amplifies", the total number of SLGCs produced by a single lineage. Cells produced through amplifying divisions greatly increase the total number of cells in the epidermis, and thus provide the main source of both pavement cells and stomata (Geisler et al., 2000; Von Groll et al., 2002). Ultimately, meristemoids transition into a guard mother cell (GMC), which is accompanied by distinct changes in cell shape and cell-division capacity. A GMC divides once symmetrically to yield two cells, which concurrently proceed through a final cell-state transition to form terminally differentiated guard cells (GC). Although lineages at different stages can be observed in proximity to one another across the epidermal surface, overall, stomata initiate and mature in a basipetal (tip to base) direction (Pyke et al., 1991).

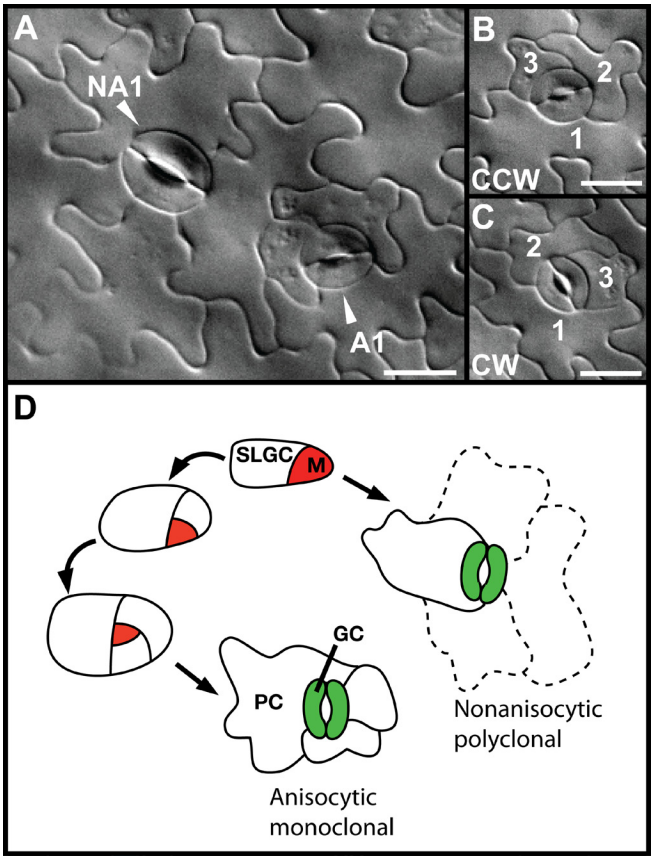

Figure 4. Spacing pattern and stomatal complexes in Arabidopsis.

(A) DIC image of the abaxial side of the leaf of a 20-day old seedling. Stomata are surrounded by non-stomatal cells. Stomatal complexes are anisocytic (A1), others are nonanisocytic (NA1) because they are surrounded by more than three neighbor cells (NA1).
(B,C) Asymmetric division of anisocytic complexes can occur counter clockwise **(B)** or clockwise **(C)**. Cells of sequential divisions are numbered.
(D) Diagram of monoclonal and polyclonal complex formation. Dashed cell outlines indicate cells that were not produced by the meristemoid. Scale bar = 10uM.

Stomatal complexes

A stomatal complex is defined as a stoma plus adjacent epidermal cells. Bordering cells are not always morphologically distinct and are generally referred to as "neighbor cells" which exchange ions and water with a stoma and contribute indirectly to stomatal movement. As mentioned earlier, neighbor cells that are clonally related to the stoma (derived from the meristemoid that produced the stoma) are designated as SLGCs (Figure 3). In many species, a set number and arrangement of neighbor cells surround each stoma and can distinguish different taxa (Zarinkamar, 2007).

Anisocytic complexes

Arabidopsis and other members of the Brassicaceae exhibit anisocytic complexes (aniso meaning unequal) where a stoma is surrounded by three cells of different size that correlate with cell age (smallest cell is the youngest) (Figure 4A-C) (Metcalfe and Chalk, 1950; Pant and Kidwai, 1967). This arrangement results from three consecutive asymmetric divisions of a meristemoid (Berger and Altmann, 2000; Geisler et al., 2000). Because the divisions are organized in an inward spiral, the SLGCs are located to the outside of the meristemoid and completely surround the stoma. An anisocytic complex is considered monoclonal because all five cells (three SLGCs plus two guard cells) originate from the same MMC (Figure 4D). Anisocytic complexes make up 40-50% of all complexes in leaves and cotyledons (Geisler et al., 2000; Serna and Fenoll, 2000). The inward spiral of asymmetric divisions can occur in a clockwise or counterclockwise direction (Figure 4B,C). Modeling the cell division and expansion of epidermal cells in Arabidopsis has provided some mathematical principles that guide cell wall placement during stomatal formation (Robinson et al., 2011; Staff et al., 2012).

Nonanisocytic complexes

Nonanisocytic complexes are composed of a stoma surrounded by 2, 4 or 5 neighbor cells or three cells of roughly equal size (Geisler et al., 2000; Serna and Fenoll, 2000) (Figure 4A). Variability in nonanisocytic complexes results in part from how many times a meristemoid divides. It has been estimated that about 15-30% of all meristemoids in a lineage divide once or not at all before converting to a GMC. The resulting complexes contain at least one non-clonal neighbor cell and are considered poly-

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clonal and nonanisocytic (Larkin et al., 1997; Serna and Fenoll, 2000). Unlike the consistent enforcement of the one-cell spacing rule, the anisocytic pattern is not obligate and permits flexibility during development.

CELL DIVISION AND PROGRESSION THROUGH THE STOMATAL LINEAGE

Basic helix-loop-helix (bHLH) proteins

Basic helix-loop-helix (bHLH) transcription factors are an evolutionarily ancient group that are known to specify cellular identity in both plants and animals (Rhodes and Konieczny, 1989; Weintraub et al., 1991; Ghysen et al., 1993; Modolell and Campuzano, 1998; Zhao et al., 2012). The bHLH domain consists of two functionally distinct regions, the basic region and HLH region, which are involved in DNA binding and protein dimerization, respectively. Arabidopsis codes for 158 bHLH proteins that are characterized into 26 subfamilies (Pires and Dolan, 2010). Five bHLH genes representing two distinct subfamilies act as positive regulators of stomatal-lineage initiation and progression (Ohashi-Ito and Bergmann, 2006; MacAlister et al., 2007; Pillitteri et al., 2007b; Kanaoka et al., 2008). The genes *SPEECHLESS* (*SPCH*: At5g53210), *MUTE* (At3g06120), and *FAMA* (At3g24140) are members of subfamily Ia and share conserved motifs outside the bHLH region (Pires and Dolan, 2010). These three genes act sequentially to promote cellular transition during stomatal development (Ohashi-Ito and Bergmann, 2006; MacAlister et al., 2007; Pillitteri et al., 2007). Despite the similarities among SPCH, MUTE and FAMA, they are unable to functionally replace one another during stomatal development due to distinct features of each protein (MacAlister et al., 2007; MacAlister and Bergmann, 2011). Two additional bHLH proteins from subfamily IIIb, SCREAM (SCRM: At3g26744) and SCRM2 (At1g12860), act redundantly to coordinate the activities of SPCH, MUTE and FAMA through heterodimerization (Kanaoka et al., 2008; Pires and Dolan, 2010).

Although the molecular mechanism driving the transition from PDC to MMC is not well understood, it requires the activity of SPCH. SPCH is expressed in MMCs and is restricted to the meristemoid after asymmetric division. *spch* mutants fail to initiate stomatal lineages and produce an epidermis composed solely of interlocking pavement cells (Figure 5A, B). *spch* mutants do not express any known stomatal lineage markers, indicating that *SPCH* initiates the lineage and doesn't maintain lineage identity after initiation (MacAlister et al., 2007; Pillitteri et al., 2007b). Consistent with this, *SPCH* overexpression results in excessive entry division and an epidermis of small highly-divided cells. *SPCH* also plays a role in prolonging meristemoid identity, as meristemoids divide significantly fewer times in weak *spch* mutants compared to wild type (MacAlister et al., 2007; Robinson et al., 2011). Inhibition of SPCH function predominantly affects MMCs and meristemoids and their ability to divide asymmetrically (MacAlister et al., 2007; Pillitteri et al., 2007b; Robinson et al., 2011), therefore, *SPCH* may participate in a fundamental pathway which promotes asymmetric division in the stomatal lineage.

In contrast to role of SPCH, the bHLH protein MUTE is required to terminate asymmetric division. *mute* meristemoids undergo excessive amplifying divisions and fail to transition to a GMC or GC. Meristemoids of *mute* divide significantly more than wild type (4-6 divisions) and simply arrest due to spatial limitations. (Figure 5C)(Pillitteri et al., 2007b). The *MUTE* promoter is not active in all meristemoids, but is specifically activated in late-stage meristemoids that will undergo GMC transition. Unrestricted *MUTE* expression in the protoderm using a ubiquitous promoter results in an epidermis composed solely of stomata (Pillitteri et al., 2007b; Pillitteri et al., 2008) indicating that cells outside the stomatal lineage can respond to MUTE activity. Because the number of meristemoid divisions is not predicable, it is unclear how meristemoids control the timing of *MUTE* expression and hence the end of meristemoid division. Interruption of cell-cell signaling can cause an extension or reduction of meristemoid division, consistent with the fact that epidermal cell number (including stomata) is a plastic trait that is monitored and adjusted based on internal and external cues (Pillitteri et al., 2007b; Zhang et al., 2008; Bhave et al., 2009)

The final transition from GMC to GC requires *FAMA*. This transition is divided into two distinguishable events; symmetric cell division of the GMC and GC transition. *FAMA* restricts GMC cell division and promotes GC transition (Ohashi-Ito and Bergmann, 2006). The GMCs in *FAMA* loss-of-function mutants undergo more than one symmetric division, resulting in the production of short columns of elongated cells (Figure 5D), which have no hallmarks of guard cell morphology or expression of guard cell-specific reporter genes (Ohashi-Ito and Bergmann, 2006). Ectopic expression of *FAMA* results in the production of cells that express guard cell specific markers in the epidermis and the underlying mesophyll layer (Ohashi-Ito and Bergmann, 2006). This further confirms that asymmetric division of the stomatal lineage is not required for stomatal differentiation.

SPCH, MUTE, and FAMA act non-redundantly to positively regulate specific transitions at defined points of the stomatal lineage. Two additional bHLH proteins, SCRM and SCRM2, act broadly throughout the stomatal lineage to promote cellular transitions. *SCRM* was identified as a gain-of-function mutation (*scrm-D*), which produces a stomata-only phenotype that mimics a *MUTE* overexpression phenotype (Figure 5E). The phenotypes of *SCRM* and *SCRM2* single and double null mutants resemble the phenotypes of *spch*, *mute,* and *fama* mutants. For example, a *scrm* single mutant produces occasional *fama*-like columns, while the *scrm scrm2* double knock-out phenocopies a *spch* mutant. Based on gene expression, mutant phenotypes, and protein interaction assays, SCRM and SCRM2 heterodimerize with SPCH, MUTE and FAMA to promote stomatal-lineage transitions (Chinnusamy et al., 2003; Kanaoka et al., 2008) (Figure 5E,F). Kanaoka et al., (2008) suggested that the dominant *scrm-D* mutation stabilizes SCRM/SPCH heterodimerization and enhances entry into the stomatal lineage. SCRM, known as ICE1, is also involved in chilling and freezing tolerance in Arabidopsis (Chinnusamy et al., 2003). Both environmental and developmental factors regulate stomatal development, which implicates SCRM as a mechanism for integration of environmental signals directly into the stomatal differentiation pathway (Hetherington and Woodward, 2003; Dong and Bergmann, 2010).

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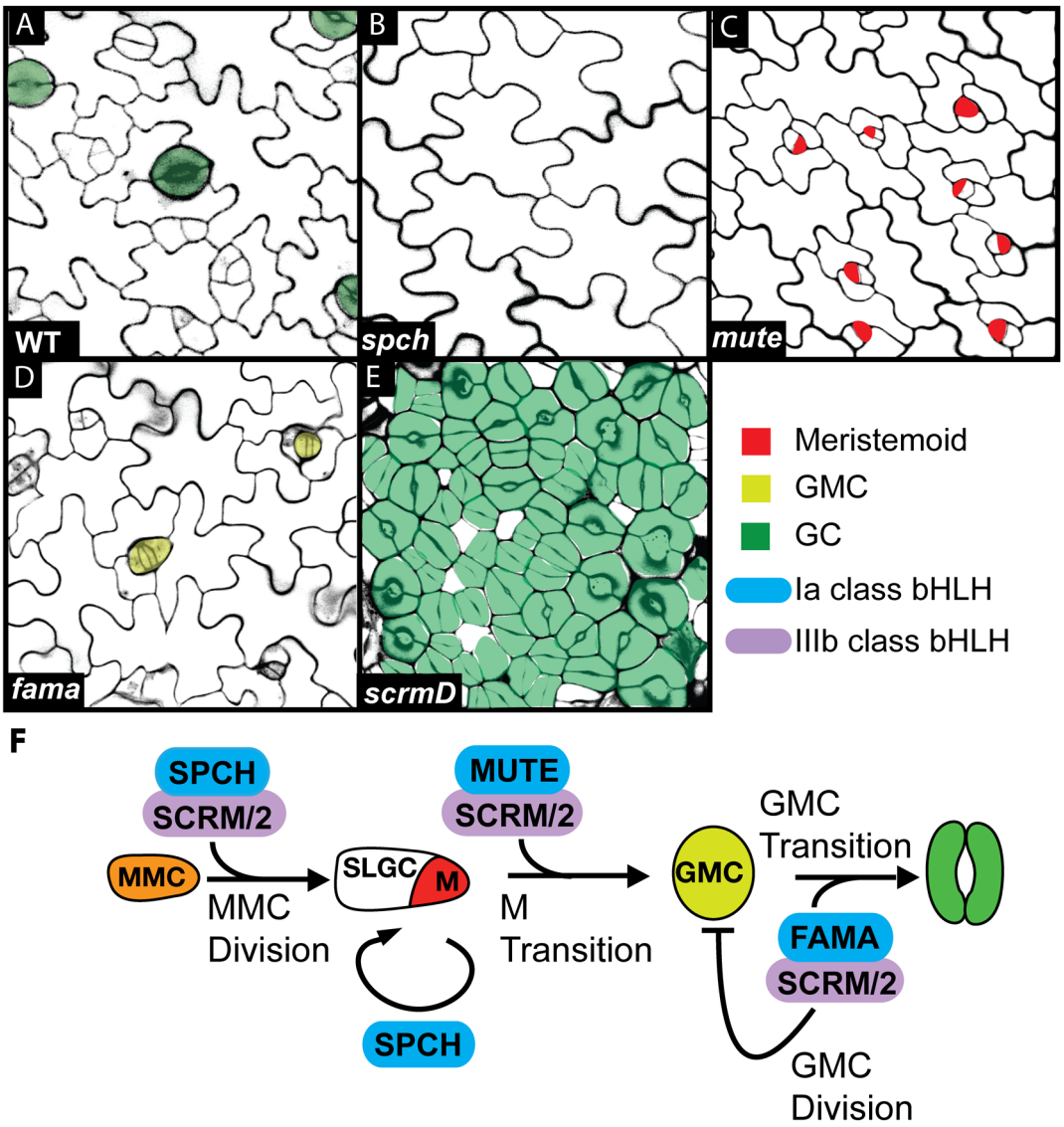

Figure 5. Phenotypes of bHLH transcription factor mutants.

(A-E) Confocal images of the abaxial leaf epidermis of 14-day old seedlings. Specific cell types in each panel are false colored according to Figure 3.
(A) WT
(B) *spch,* pavement cell only.
(C) *mute*, lineage progression arrests at meristemoid stage.
(D) *fama*, GMCs divide more multiple times and no stomata are produced.
(E) *scrm-D*, stomata only.
(F) Diagram of the actions of the stomatal bHLH proteins. SPCH and SCRM/2 direct MMC transition, MUTE and SCRM/2 direct the GMC transition and FAMA and SCRM/2 promote the GC transition.

MYB domain proteins

MYB transcription factors are characterized by a highly conserved DNA-binding domain (MYB domain) consisting of up to four imperfect sequence repeats. These proteins are categorized into subgroups based on the number of repeats present in the MYB domain. MYB-family proteins are involved in a variety of plant-specific processes including the regulation of cell-fate; two proteins of the R2R3 subgroup have roles in stomatal development (Kirik et al., 2005; Baumann et al., 2007; Kang et al., 2009b; Dubos et al., 2010). *FOUR LIPS (FLP*: At1g14350) and *MYB88* (At2g02820) act redundantly to restrict GMC cell division and promote the final stomatal transition from GMC to GCs (Yang and Sack, 1995; Lai et al., 2005; Xie et al., 2010). In weak *flp*

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mutants, GMC division occurs, but GC differentiation is delayed. Therefore, the daughter cells of the first GMC division often divide again, resulting in two adjacent stomata (i.e. four lips) oriented in parallel relative to each other (Figure 6). A *myb88* single mutant has no phenotypic defects; however, a *flp myb88* double mutant more closely resembles *fama*. A notable difference is that a *flp myb88* mutant does produce cells with GC identity, whereas the production of cells with GC identity is completely blocked in a *fama* mutant (Lai et al., 2005; Ohashi-Ito and Bergmann, 2008).

MYB and bHLH proteins are known to interact in several developmental contexts (Zimmermann et al., 2004; Bernhardt et al., 2005; Lai et al., 2005). Despite the strong phenotypic similarities between *fama* and *flp myb88,* neither FLP or MYB88 have recognized sites necessary for bHLH/MYB binding and no physical interaction between these proteins has been established (Ohashi-Ito and Bergmann, 2006) suggesting that FAMA and FLP/MYB88 act in parallel to restrict GMC division. A common downstream target of FLP/MYB88 and FAMA is *CYCLIN DEPENDENT KINASE B1;1* (*CDKB1;1*: At3g54180). Both FLP and FAMA bind directly to the *CDKB1;1* promoter (Xie et al., 2010; Hachez et al., 2011) and the additional GMC divisions characteristic of the *flp myb88* double mutant are dependent on CDKB1;1 and CDKB1;2 (At2g38620) function (Xie et al., 2010). A dominant negative form of CDKB1;1 restricts GMC division (Boudolf et al., 2004) most likely through the combined interference with both CDKB1;1 and CDKB1;2 (Xie et al., 2010). Additionally, FLP and MYB88 can both bind the promoter of *CYCLINA2;3* (*CYCA2;3*: At1g15570) (Vanneste et al., 2011). CDKB1;1 functionally interacts with CYCA2;3 (Boudolf et al., 2009) and the *CYCA2* gene family has been shown to promote GMC divisions (Vanneste et al., 2011). A combinatorial mutant of *CYCA* genes produces significantly more single GCs than WT, highlighting that the transition from GMC to GCs occurs as two distinct processes and in the absence of cell division, differentiation can still proceed.

Additional proteins

Several genes with diverse functions have been identified that produce a range of stomatal defects, however their direct placement or connection in the gene network driving stomatal development remains to be determined. A mutation in the microRNA, *miR824* (At4g24415), which targets the MADS-box transcription factor *AGAMOUS-LIKE16* (AGL16: At3g57230) mRNA for deg-

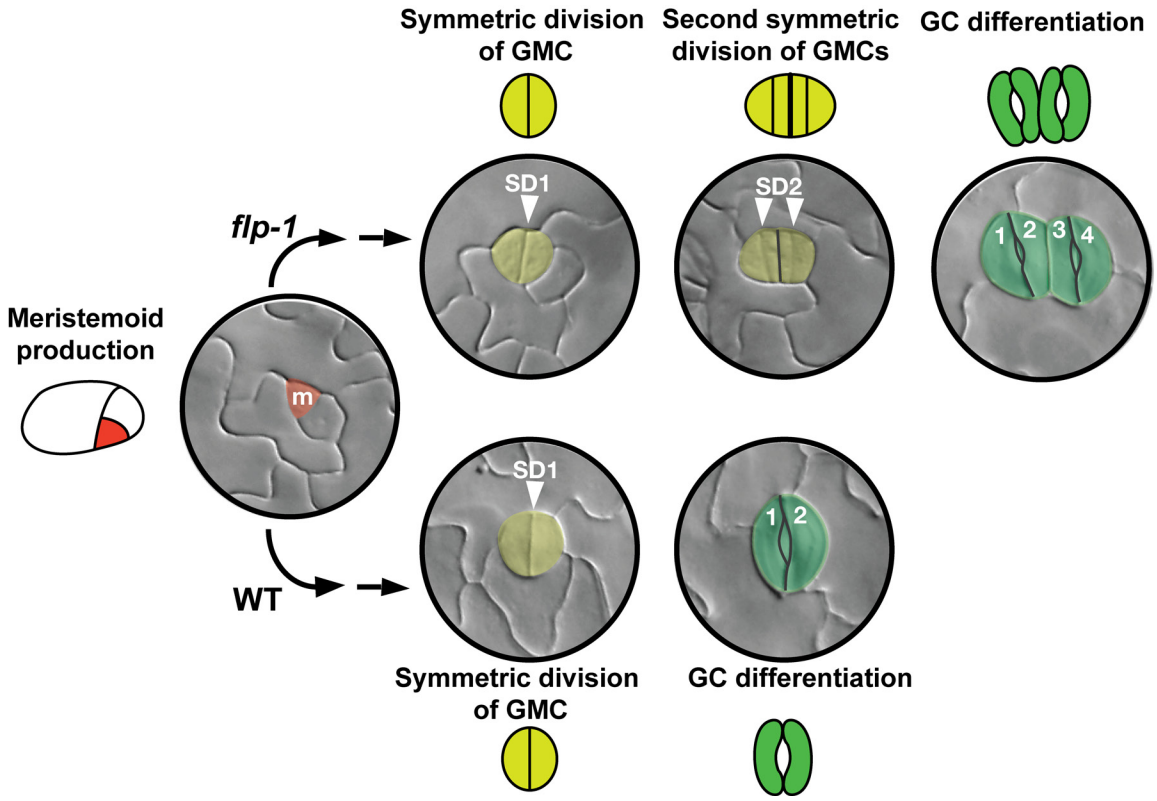

Figure 6. Representative images of the stages of stomata production in WT and the *flp-1* mutant.

DIC images of abaxial leaf epidermis of WT and *flp-1* mutants. Images do not capture a single cell through time. Meristemoids are produced similarly in WT and *flp-1.* Two symmetric divisions (SD1 and SD2) of the GMC in *flp-1* compared to one symmetric division in WT results in a pair of stomata or "four lips" (numbered). Arrowheads indicate division plane. Colors and abbreviations for cells types are as in Figure 3.

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radation causes increased production of satellite meristemoids (Kutter et al., 2007). Recently, a loss-of-function mutation in the meristemoid-enriched HD-ZIP IV gene, *HOMEODOMAIN GLABROUS2* (*HDG2*: At1g05230), was found to delay stomatal differentiation, specifically the meristemoid–to- GMC transition. HDG2 likely functions as a transcriptional activator and consistent with the delay in stomatal differentiation, HDG2 can activate expression of *MUTE in planta* (Peterson et al., 2013). Mutations in genes involved in cell wall production or cytokinesis have highlighted the importance of restricting the movement of cell-fate determinants and regulatory proteins for maintenance of cell identity. Loss of callose deposition at plasmodesmata due to a mutation in *GLUCAN SYNTHASE-LIKE 8* (*GSL8*: At2g36850) resulted in an increase in the number of entry divisions and small stomatal clusters (Thiele et al., 2008; Chen et al., 2009; Guseman et al., 2010). *STOMATAL CYTOKINESIS-DEFECTIVE1* (*SCD1*: At1g49040) encodes an unknown protein with a DENN domain known to interact Rab-family GTPases (Falbel et al., 2003; Marat et al., 2011). Loss of SCD1 function disrupts the final division of the GMC resulting in binucleate guard cells with a false pore structure. *scd-1* guard cells display typical guard cell markers, indicating the phenotype is the result of cytokinesis, not guard cell specification. *CYTOKINESIS DEFECTIVE1* (*CYD1*: uncloned) produces similar phenotypes to those described for *scd1* (Yang et al., 1999). Mutations in *GIGAS1* (*GIG1*: At3g57860), a plant specific inhibitor of APC/C complex, results in the production of cells with characteristics of both guard cells and pavement, suggesting it may have a role in stomatal cell-fate determination (Iwata et al., 2011).

STOMATAL PRODUCTION IN HYPOCOTYLS

The hypocotyl is the embryonic stem that bridges the cotyledons and seedling root. It is evident that the some of the mechanisms driving the development of stomata in this embryonic organ are different than in leaves. The hypocotyl epidermis is composed of two clearly patterned cell files, protruding and non-protruding (Kono et al., 2007). Hypocotyl stomata are restricted in a position-dependent manner to non-protruding files that overlay two cortical cell files, which preferentially places stomata over intercellular spaces (Berger et al, 1998). The pattern of stomata development in the hypocotyl mimics that of root hair development, with hairs produced only in specific cell files. Consistently, genes that regulate the production of root hairs also control the production of stomata in the hypocotyl. Three proteins, TRANSPARENT TESTA GLABRA (TTG: At5g24520, WD-repeat containing protein), WEREWOLF (WER: At5g14750, MYB-related protein), and GLABRA2 (GL2: At1g79840, leucine zipper protein) inhibit the production of stomata in protruding cell files thereby restricting lineage initiation to non-protruding files. These proteins were originally implicated in root-hair specification (Galway et al., 1994; Di Cristina et al., 1996; Lee and Schiefelbein, 1999); however *ttg*, *wer*, or *gl2* mutants produce stomata in both non-protruding and protruding cell files, thus increasing the overall number of stomata produced (Hung et al, 1998; Serna, 2005). Another root-hair specification gene, *CAPRICE* (*CPC*: At2g46410), promotes stomatal cell fate in non-protruding cell files, further highlighting regulatory similarities between the root and hypocotyl (Serna, 2008; Wada et al., 2002). The cell cycle regulators, *CYCLIND4;1* (*CYCD4;1*: At5g65420) and *CYCLIND4;2* (*CYCD4;2*: At5g10440), have been implicated as downstream targets of the these proteins and a loss-of-function mutation in either *CYCD* gene results in a reduction in the total number of cells in non-protruding cell files compared to wild type (Kono et al., 2007). Although the SPCH, MUTE and FAMA proteins have been shown to promote stomatal development across all organs, the root-hair specification genes and *CYCD* genes have not been implicated in stomatal development in non-embryonic tissue, indicating that some developmental regulation may differ between embryonic and post-embryonic development (Berger et al., 1998; Serna, 2008).

GENERATION OF SPACING PATTERN

The synchronized development of a tissue requires communication among cell types. During epidermal development, the one-cell spacing rule is regularly enforced to ensure that two stomata are not produced in direct contact with one another. Separation of stomata promotes the efficient regulation of stomatal opening and closure, which requires movement of water and ions from neighboring cells. The identification of several patterning mutants revealed a core group of signaling molecules which include, a proteinase, a leucine-rich repeat receptor like kinases (LRR-RLKs), an LRR receptor-like protein, MAP kinase components, and a family of small cysteine-rich secreted peptides.

Peptide ligands: Cysteine-rich secreted proteins

All eukaryotes use small secreted peptides as intercellular signals (Dohlman and Thorner, 2001; Gao and Guo, 2012; Karamboulas and Ailles, 2012). The *EPIDERMAL PATTERNING FACTOR* (*EPF*) and *EPIDERMAL PATTERNING FACTOR-LIKE* (*EPFL*) genes encode a family of secreted cysteine-rich peptides with six or eight conserved carboxy-terminal cysteines that form disulfide bridges. These cysteine linkages expose a variable loop domain believed to be the major source of the biochemical and functional diversity among family members (Ohki et al., 2011) (Figure 7). The EPF protein family (EPFf) is made up of 11 members (EPF1, EPF2 and EPFL1-9) that participate in diverse developmental processes (Hara et al., 2007; Hara et al., 2009; Hunt and Gray, 2009; Rowe and Bergmann, 2010; Rychel et al., 2010; Torii, 2012). STOMAGEN (EPFL9: At4g12970) has been experimentally shown to be post-translationally modified and it is likely that the other members are similarly processed (Kondo et al., 2009; Ohki et al., 2011).

Four EPFf members affect stomatal development, *EPF1* (At2g20875), *EPF2* (At1g34245), *CHALLAH* (*CHAL/EPFL6*: At2g30370), and *STOMAGEN*/EPFL9; however, only *EPF1* and *EPF2* are specifically expressed in the stomatal lineage. EPF2 is produced in *SPCH*-expressing protodermal cells (MMCs) early in the lineage, whereas EPF1 is produced in late-stage meristemoids, GMCs and young guard cells (Hara et al., 2007; Hara et al., 2009; Hunt and Gray, 2009). Loss of *EPF2* function results in excessive entry divisions, similar to a *SPCH* overexpression phenotype. In contrast, ectopic expression of *EPF2* (or external application of bioactive EPF2) inhibits entry divisions, resulting in

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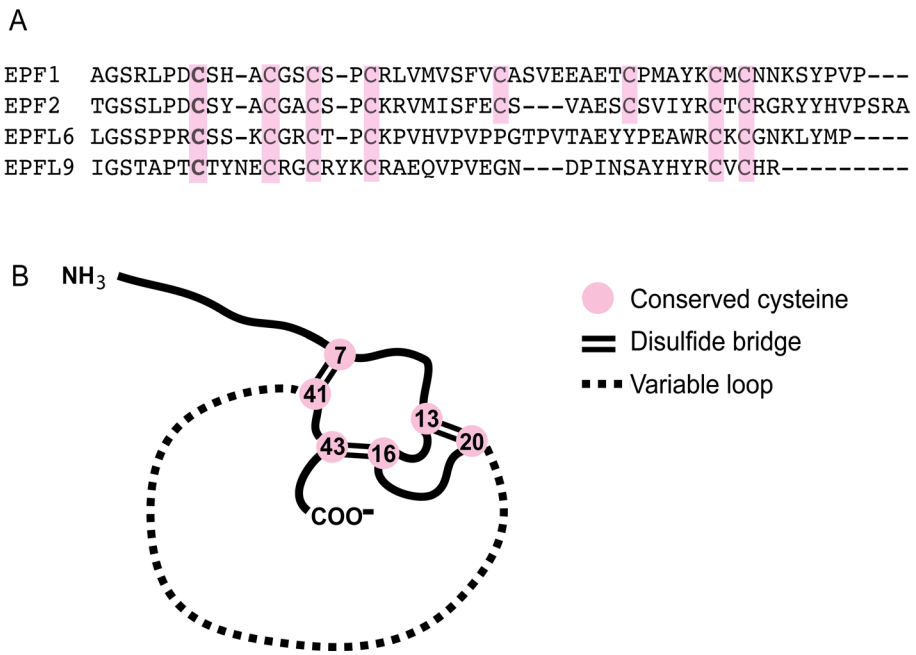

Figure 7. Primary sequence alignment of stomatal EPFf proteins and experimentally determined structure of EPFL9 (STOMAGEN).

(A) Sequence alignment generated using CLUSTALW. Conserved cysteines are highlighted in pink.
(B) A diagram of the structure of STOMAGEN with conserved cysteines (pink) and intramolecular disulfide bonds (double black bars) indicated. Residues of the exposed variable loop structure are shown as a dashed line. EPFL9 structure modeled after Kondo et al., 2010.

an epidermis composed of pavement cells only. The inverse relationship between *epf2* and *spch* phenotypes supports a model in which *SPCH*-expressing MMCs secrete EPF2 to inhibit neighboring cells from entering the stomatal lineage. This strategy reduces the occurrence of adjacent meristemoids (Figure 8).

Whereas EPF2 inhibits adjacent cells from entering the linage, EPF1 functions as an orientation signal to provide positional information about the location of stomata and stomatal precursors (meristemoids, GMCs and GCs) relative to one another. A null *epf1* mutation produces occasional adjacent stomata that are paired in a non-parallel orientation, indicating a defect in new stomata placement relative to established stomata. Overexpression of *EPF1* (or application of bioactive EPF1) represses meristemoid to GMC transition, resulting in transdifferentiation of meristemoids into pavement cells. Together, EPF1 and EPF2 communicate positional information to promote and enforce the one-cell spacing rule. However, their functions are not interchangeable and each peptide is perceived differently (Hara et al., 2009; Lee et al., 2012).

EPF1 and EPF2 function within the epidermis, whereas STOMAGEN and CHAL influence stomatal development through intertissue signaling, specifically between the epidermis and internal tissue of the leaf and stem, respectively. *STOMAGEN* is expressed in the underlying photosynthetic mesophyll tissue in leaves, where it likely acts as a signal to coordinate stomatal differentiation with photosynthetic capacity (Kondo et al., 2009; Sugano et al., 2010). In contrast to the inhibitory role of EPF1 and EPF2, STOMAGEN promotes the production of stomata. *STOMAGEN* overexpression (or application of bioactive STOMAGEN) increases stomatal density and stomatal cluster formation (Kondo et al., 2009; Sugano et al., 2010). Conversely, fewer stomata are produced in RNAi knockdown lines. Based on co-application studies, STOMAGEN and EPF2 peptides compete to influence stomatal differentiation. Simultaneous application of EPF2 and STOMAGEN reduces the efficacy of STOMAGEN resulting in an almost normal epidermis. The competing nature of the EPFf peptides provides an excellent system to fine-tune stomatal production during development and in response to changing stimuli (Ohki et al., 2011).

The final *EPFf* member implicated in stomatal development, *CHAL*, is expressed in the internal tissues of stems and hypocotyls, but not leaves (Abrash and Bergmann, 2010). Currently, it is unclear if CHAL has an endogenous role in stomata production; however, in the absence of a receptor component, loss of *CHAL* function alters stomata density in the stem (Abrash and Bergmann, 2010). Specifically, *CHAL* was identified in a screen to identify suppressors of another stomatal patterning mutant called *too many mouths* (*TMM*: At1g80080) and only presents a phenotype in the presence of a *tmm* mutation. TMM is one of the receptors for the EPFf, discussed further in the following section. A *chal* mutation independent of a *tmm* mutation confers no phenotype (Abrash and Bergmann, 2010).

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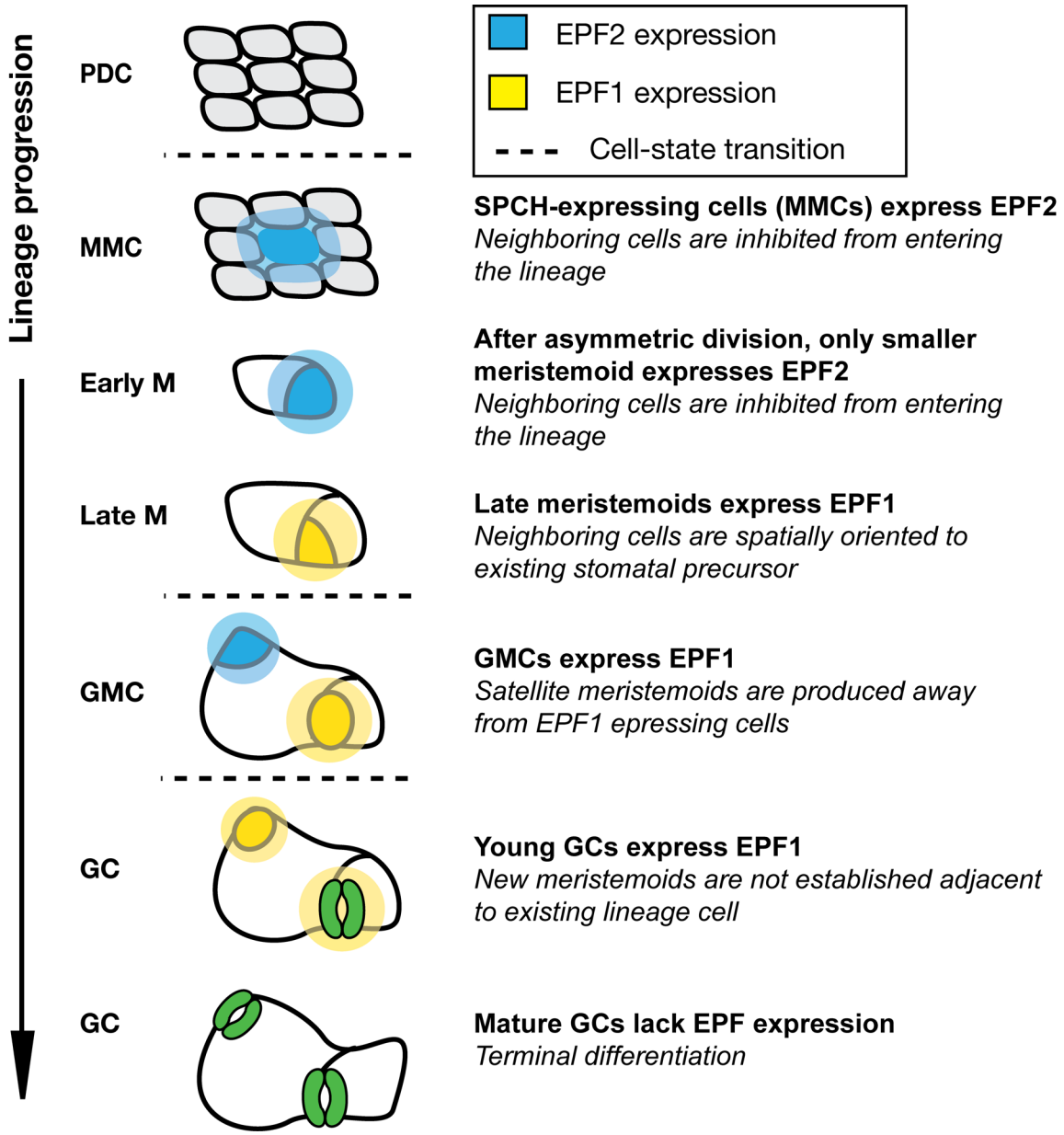

Figure 8. Model for EPF1 and EPF2 action during stomatal development.

Protodermal cells that express *SPCH*, secrete EPF2 (blue) and inhibit neighboring cells from entering the stomatal lineage. The EPF2-expressing cell becomes a meristemoid mother cell (MMC) and initiates asymmetric entry division. Late-stage meristemoids, GMCs and young GCs express EPF1, which spatially orients neighbor cells to existing stomatal precursors.

Leucine–Rich Repeat Receptors

EPFf peptides are recognized by at least four receptors that contain extracellular leucine-rich repeat (LRR) domains. Three are LRR-receptor-like kinases (LRR-RLKs), ERECTA (ER: At2g26330), ERECTA-LIKE1 (ERL1: At5g62230) and ERL2 (At5g07180), and the fourth is a LRR receptor-like protein (LRR-RLP) called TOO MANY MOUTHS (TMM). The ERECTA-family (ERf) possess a canonical LRR extracellular domain with 20 continuous repeats and a cytoplasmic serine-threonine kinase domain (Torii et al., 1996; Shpak et al., 2003), whereas TMM contains only 10 LRRs and lacks a cytoplasmic kinase domain (Nadeau and Sack, 2002b). EPF1, EPF2, and STOMAGEN require TMM and/or the ERf to manifest their overexpression phenotypes indicating their

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importance in perception of the EPFf. TMM functions exclusively in the stomatal lineage, although the ERf has roles in many aspects of development outside of stomatal development (Torii et al., 1996; Pillitteri et al., 2007a; Hord et al., 2008).

The three members of the ERf RLKs are partially redundant and produce an excessive stomatal clustering phenotype only in the absence of all three family members, though more subtle phenotypes are observed in single or double ERf mutants (Shpak et al., 2003; Shpak et al., 2005). Overall, the role of these receptors is to inhibit stomatal production. ER primarily suppresses entry divisions and ERL1 inhibits GMC differentiation and promotes the correct orientation of satellite meristemoids. The phenotype of *tmm* is organ specific as leaves of *tmm* mutants exhibit stomatal clusters, while *tmm* stems and hypocotyls produce no stomata (Geisler et al., 2000). In leaves, the ERf and TMM work cooperatively to inhibit stomata production; however, loss of *ERL1* function suppresses the "no stomata" stem phenotype of *tmm* mutants, suggesting they work antagonistically in the stem. The apparent paradox of TMM function in different organs can be resolved by incorporating an explanation that includes both receptors and ligands. The resumption of stomata production in *erl1 tmm* stems is phenocopied by a *chal tmm* double mutant. Based on this, a model has been proposed that CHAL signaling through ERL1 is overactive and represses stomatal development in stems in the absence of TMM. When CHAL is removed, stomata production resumes due to reduced signaling from ERL1. Because *CHAL* is not expressed in leaves, the "stomataless" phenotype of *tmm* mutants is only exposed in stems and hypocotyls.

Biochemical studies using a biosensor chip have established ER-EPF2 and ERL1-EPF1 as the predominant ligand-receptor pairs, where ER-EPF2 regulates entry divisions and ERL1-EPF1 regulates spacing divisions (Lee et al., 2012). A dominant-negative ER receptor, which blocks ER-mediated signaling, results in seedlings resistant to EPF2 application but not to EPF1. Similarly, blocking ERL1-mediated signaling only effects sensitivity to EPF1, but not EPF2. TMM has no kinase domain (Geisler et al., 2000), does not efficiently bind the EPF peptides and preferentially forms heterodimers with ERL1 (Lee et al., 2012). This strongly suggests that TMM does not function via direct signal transduction. Currently, the model predicts that TMM prevents the formation of functional ERL1 heterodimers in stomatal precursors to prevent them from inhibiting their own differentiation.

MAPK Kinase signaling

Mitogen-activated protein kinase (MAPK) signaling is universal among eukaryotes and mediates a variety of environmental and developmental responses. Although direct connections have not been established, downstream signaling from ERf and TMM are likely transduced through a MAPK module, consisting of the MAPKKK YODA (YDA: At1g63700), MKK4/5 (At1g51660/At3g21220), and MPK3/6 (At3g45640/At2g43790) (Bergmann et al., 2004; Lukowitz et al., 2004; Wang et al., 2007; Umbrasaite et al., 2010). These MAPKs are broadly expressed and have pleiotropic effects on development, but uniformly work to promote pavement cell differentiation and inhibit the initiation of stomata. Constitutively active forms of YDA and MKK4/5 kinases produce an epidermis composed solely of pavement cells, whereas severe stomatal clustering phenotypes are observed in null mutants. Because the clustering phenotype of MAPK mutants exceeds those observed when the ligands (EPF1, EPF2) or receptors (TMM, ERf) are absent, additional upstream signals may feed into this pathway. A role for MKK7/9 (At1g18350/At1g73500) in promoting GMC transition was suggested based on cell-specific expression of MAPK components in stomatal-lineage cell types. When constitutively active forms of MKK7/9 are expressed specifically in GMCs and GCs, a stomatal-cluster phenotype is observed (Lampard et al., 2009).

Constitutive activation of many of the stomatal MAPKs phenocopies *spch* loss-of-function, which implicates SPCH as a downstream target of the MAPK signaling cascade that inhibits stomatal production. SPCH contains a MAPK target domain (MPKTD) that is phosphorylated by MKP3/6 *in vitro* and is required for suppression of SPCH activity *in vivo* (Lampard et al., 2008). Deletion of the MPKTD from SPCH results in higher activity (hence more stomata), consistent with the inhibitory role of MAPKs on stomatal development. Among the stomatal bHLH proteins, a MPKTD is found only in SPCH. Although MUTE, SCRM and MYB88 have been implicated as targets of phosphorylation (Popescu et al., 2009), no evidence has been reported. Indirect support for kinase regulation of SCRM has been suggested based on mutations near regulatory phosphorylation sites (Chinnusamy et al., 2003; Kanaoka et al., 2008).

MAPK phosphatases

Phosphorylation of MAPK modules increases their activity while dephosphorylation converts them to an inactive state (Canagarajah et al., 1997). The modification of YDA, MKK4/5, and MPK3/6 activities through the action of phosphatases represents a regulation point to adjust the initiation of stomata during development. The Arabidopsis protein phosphatase 2C (*AP2C3*: At2g40180) is expressed specifically in the stomatal lineage from meristemoid onwards and results in an epidermis composed solely of stomata when ectopically expressed (Umbrasaite et al., 2010). This phenotype is similar to *yda* or *mpk3/6* loss-of-function mutants and implicates AP2C3 as a suppressor of MAPK activity in epidermal cells. AP2C3 colocalization with MAPKs in the nucleus can effectively inactivate MPK3/6 *in vitro*. This predicts that AP2C3 deactivates MAPK3/6 specifically in the stomatal lineage, providing a mechanism to tightly adjust the signaling pathway controlling stomatal production (Figure 9). Further work needs to be initiated to investigate the endogenous or redundant role of AP2C3, because loss-of-function mutants have no detectable phenotype (Umbrasaite et al., 2010).

Proteinase

STOMATAL DENSITY AND DISTRIBUTION1 (SDD1: At1g04110), a subtilisin-type proteinase, was identified as regulator of proper stomatal patterning. SDD1 is a negative regulator of stomata development, where loss of SDD1 function results in properly spaced but higher density stomata (Berger and Altmann, 2000; Von Groll et al., 2002). Genetic studies indicate that SDD1 acts independently of other signaling components in the stomatal

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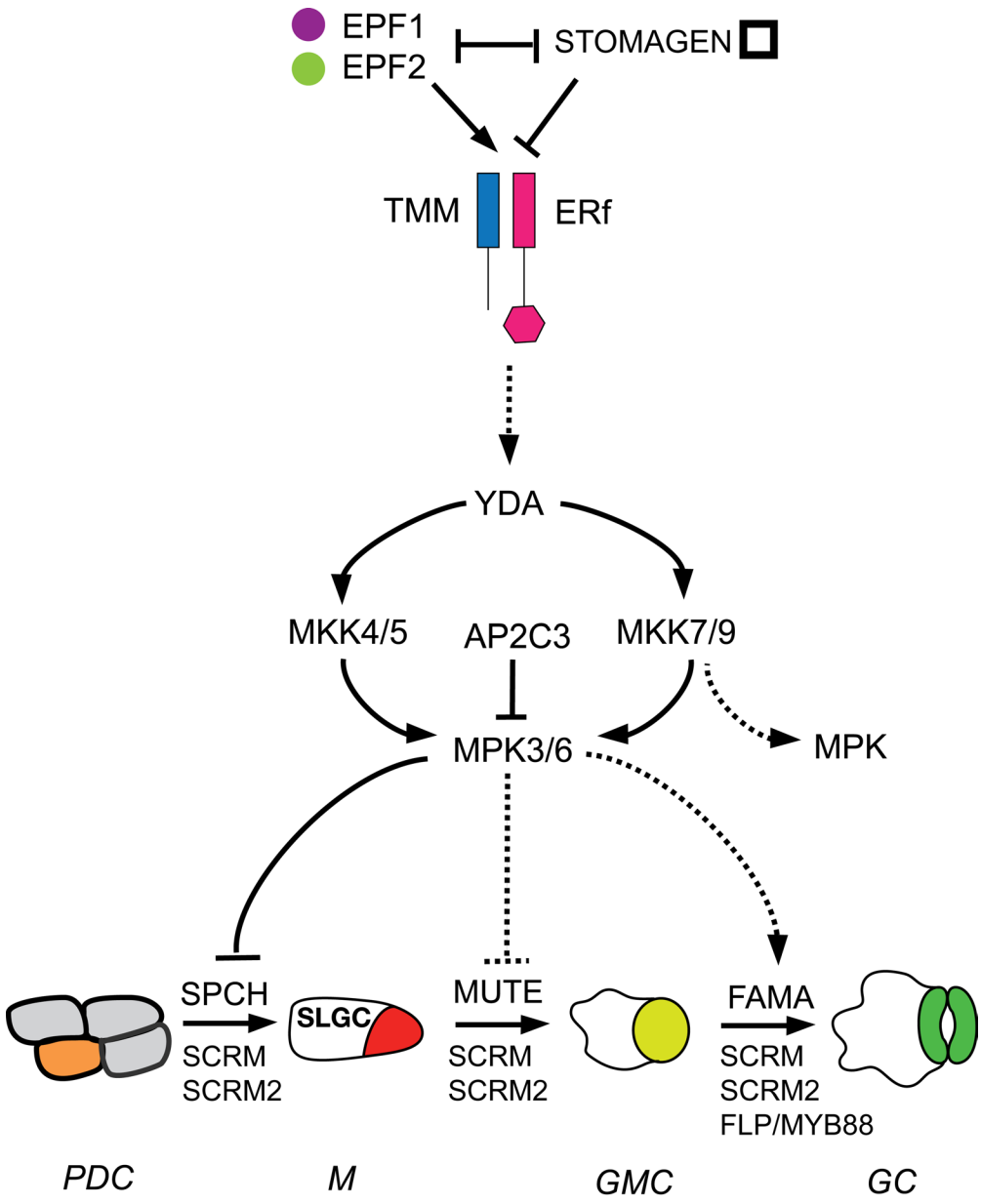

Figure 9. Diagram of the signal transduction pathway regulating stomatal development.

Stages of stomatal development are given at the bottom of the figure. Cell color and cell-type abbreviations are as described in Figure 3. EPF1, EPF2 and STOMAGEN activate or inhibit signaling via TMM and the ERf, which transduce the signal to the YDA MAPK cascade. MPK3/6 inactivates SPCH through phosphorylation, reducing the number of entry divisions. Experimental evidence supporting inhibition or activation are shown with solid lines. Dashed lines indicate predicted interactions.

pathway (Bergmann et al., 2004; Hara et al., 2007; Hara et al., 2009; Sugano et al., 2010). Currently, the identity of SDD1 substrates and hence its molecular role in stomatal development remains unknown. Regardless, due to its clear impact on stomatal density, it has been investigated as a potential means of modifying water use efficiency in plants (Yoo et al., 2011; Hamanishi et al., 2012).

CELL POLARITY AND DIVISION ASYMMETRY

More than a decade of research has provided ample knowledge regarding the positive and negative regulators that direct cell fate and cell self-renewal (Bergmann and Sack, 2007; Lau and Bergmann, 2012; Pillitteri and Torii, 2012). However, our understanding of the molecular pathways that execute oriented asymmetric

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divisions within the stomatal lineage are just emerging due to the recent identification of polarity proteins in both Arabidopsis and maize (Cartwright et al., 2009; Dong et al., 2009; Pillitteri et al., 2011; Facette and Smith, 2012).

Researchers often consider two distinct models to describe asymmetric cell division, which rely on "intrinsic" or "extrinsic" regulation (Gonczy, 2008; Knoblich, 2008; Abrash and Bergmann, 2009; Petricka et al., 2009; Abrash and Bergmann, 2010; de Smet and Beeckman, 2011; Inaba and Yamashita, 2012). "Intrinsic" regulation implies an innate polarization process in the mother cell that determines the unequal cell fates of daughter cells, whereas in "extrinsic" regulation, the daughter cells differentiate based on their distinct microenvironments. Asymmetric divisions in the stomatal lineage involve both intrinsic and extrinsic mechanisms to guide self-renewal, fate determination and stomatal distribution (Abrash and Bergmann, 2009; Petricka et al., 2009; Pillitteri and Torii, 2012; Torii, 2012) (Figure 10). In contrast, the asymmetric divisions in the monocot maize may more heavily rely on extrinsic regulation from neighboring cells to generate polarity and cell fate (Facette and Smith, 2012) (Figure 10).

Intrinsic polarity proteins, BASL and POLAR

The "intrinsic" model for asymmetric division in animal systems (flies and nematodes) utilizes conserved PAR (partitioning defective) protein complexes, which asymmetrically localize to induce "symmetry breaking" of the mother cell and direct differential segregation of the cell fate determinants into two daughter cells (Gonczy, 2008). No homologues of PAR proteins have been identified in plants; however, two novel polarity proteins, BREAKING OF ASYMMETRY IN THE STOMATAL LINEAGE (BASL: At5g60880) (Dong et al., 2009) and POLAR LOCALIZATION DURING ASYMMETRIC DIVISION AND REDISTRIBUTION (POLAR: At4g31805) (Pillitteri et al., 2011), mimic the behaviors of the PAR proteins and likely provide a plant-specific solution to the challenge of asymmetric division. Both BASL and POLAR are unknown proteins, with no predicted functional domains.

BASL and POLAR show largely overlapping localization at the cell cortex during stomatal asymmetric divisions (Figure 10A) (Dong et al., 2009; Pillitteri et al., 2011). Mutations in *BASL* have clear defects in the physical asymmetry of stomatal lineage divisions, whereas *polar* mutants have no discernable defects, suggesting genetic redundancy (Pillitteri et al., 2011). In addition to the cortex, BASL is also localized to the nucleus, though nuclear localization is dispensable for its function and is likely used as a sequestration mechanism (Dong et al., 2009). BASL is required for POLAR localization, thus POLAR likely functions downstream of BASL, although whether they physically interact is not known (Pillitteri et al., 2011). Before visual signs of division, BASL and POLAR accumulate at a restricted region on the plasma membrane of the mother cell and are always distal to the prospective division plane. Although the mechanism of how the BASL-POLAR polarity module directs cellular asymmetry is not well understood, ectopic over-expression of *BASL* in hypocotyl cells induces confined cell expansion at the site of BASL localization. This prompted a novel hypothesis that pre-mitotic BASL polarity may induce regional cortical extension to establish physical asymmetry of the mother cell (Dong et al., 2009; Menke and Scheres, 2009). It is not clear whether POLAR is required for the BASL-induced cell expansion. One potential downstream effector is the small GTPase, RHO OF PLANTS2 (ROP2, At1g20090), because BASL-induced cell outgrowth is greatly reduced in plants with compromised ROP2 function (Dong et al., 2009). Identification of genetic and physical partners of BASL and POLAR will provide insight into this plant-specific polarity mechanism based on directional cell expansion.

Polarization of BASL and POLAR appears to be an innate property of the stomatal lineage cells and the initial orientation of the proteins is random relative to leaf axis and shape. It is not understood how BASL-POLAR polarity is initiated, but SPCH activity is likely a required upstream event, because elevated *SPCH* expression precedes the appearance of BASL polarity (Robinson et al., 2011) and the meristemoids in weak *spch* mutants have less potential for prolonged asymmetric division (MacAlister and Bergmann, 2011).

BASL-POLAR polarization is reiteratively initiated in meristemoids during each amplifying division, which produces the inward division spiral of anisocytic complexes (Figure 3; Figure 10A). Computational modeling has proposed an intrinsic "polarity switching" mechanism that relies on cell aging. This process reiteratively places BASL distant from new cell walls and ensures the inward division pattern (Robinson et al., 2011). The model precisely predicted the localization and expression pattern of fluorescent protein-labeled SPCH (the decision maker) and BASL (the division operator) in developing leaves.

Extrinsic cue-mediated protein, PAN1

Stomatal complexes in monocots such as maize are composed of a stoma (two dumbbell-shaped GCs) flanked by a pair of subsidiary cells (SCs) that regulate GC expansion and pore opening (Figure 10B). Formation of SCs requires asymmetric division and provides an excellent system to study extrinsic cue-mediated cell division. Formation of a GMC (produced by an unknown mechanism) causes the flanking cells, called subsidiary mother cells (SMCs), to position their nuclei and divide toward the GMC, resulting in one SC adjacent to each GC. Two LRR receptor-like kinases, PANGLOSS1 (PAN1) and PAN2, are strong polarity markers that play an important role in orientating the asymmetric division of SMCs (Cartwright et al., 2009; Zhang et al., 2012).

Both PAN1 and PAN2 proteins are expressed in SMCs and localize to the plasma membrane at the SMC/GMC contact site. As putative receptor-like kinases, PAN proteins likely perceive an unknown signal from GMCs (Cartwright et al., 2009; Zhang et al., 2012). Loss-of-function *pan1* or *pan2* results in an increased number of abnormal non-polarized SMC divisions. Protein interaction studies show that PAN2 functions as a homodimer and that PAN1 and PAN2 do not physically interact (Zhang et al., 2012). Polarization of PAN1 requires the presence of PAN2, suggesting PAN2 is an upstream factor in polarity formation. Neither PAN1 nor PAN2 are catalytically active kinases, which suggests that they act as co-receptors with a third receptor-like kinase in the SMC, similar to TMM and the ERf in Arabidopsis (Cartwright et al., 2009; Zhang et al., 2012). Alternatively, PAN1 and PAN2 may serve as scaffolding proteins to recruit cytosolic protein kinases to the plasma membrane for signal transduction.

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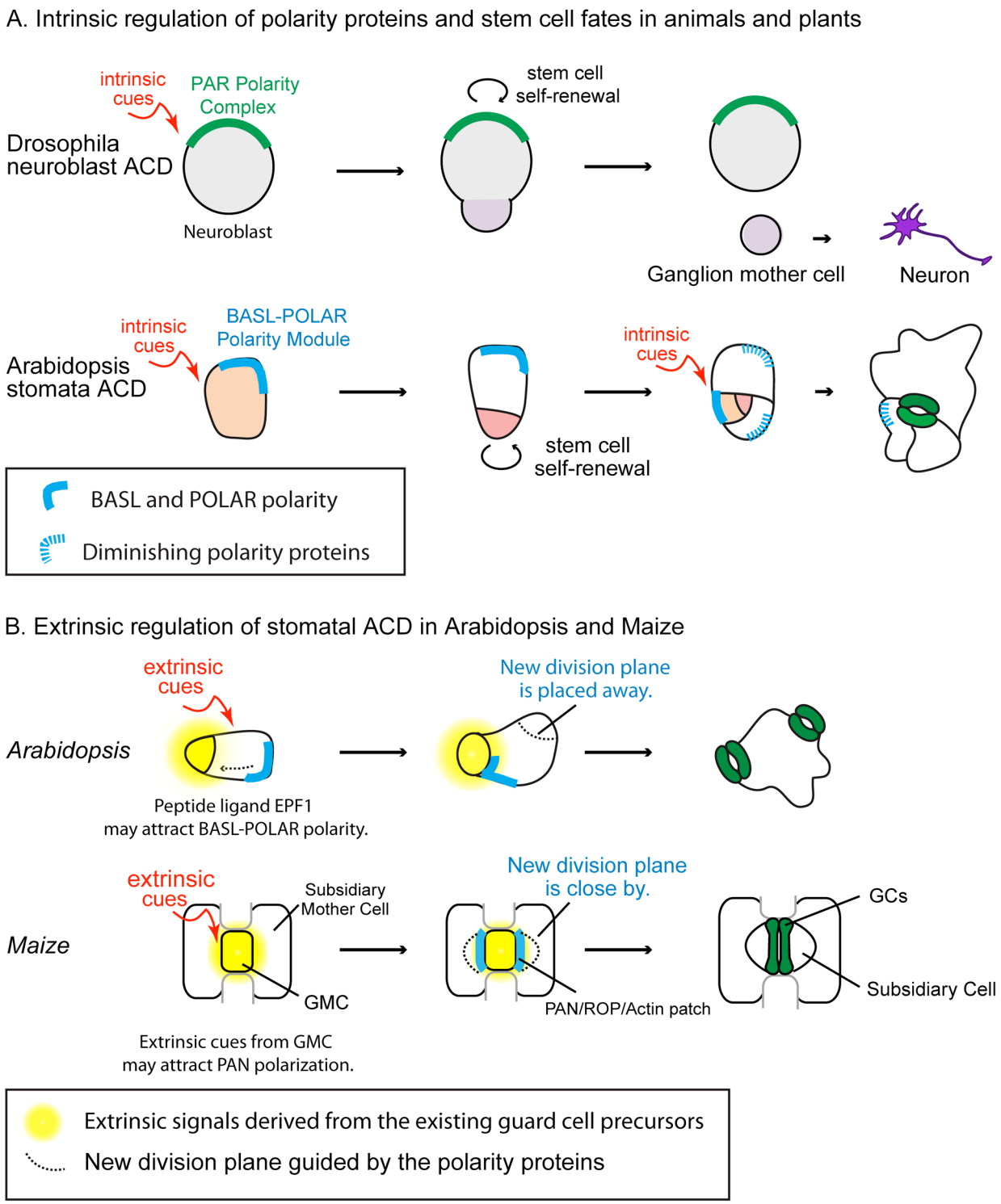

Figure 10. Intrinsic and extrinsic regulation of cell polarity during asymmetric division.

(A) Differential association of intrinsic polarity proteins in animals (PAR proteins) and plants (BASL-POLAR). The BASL-POLAR module reorients after each meristemoid division.
(B) Extrinsic signals guide the polarity orientation in Arabidopsis and maize. An extrinsic cue from the meristemoid or GMC attracts the polarity proteins. The nature of the extrinsic cue for either system has not been experimentally determined.

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Polar localization of PAN proteins is the earliest event identified in SMC asymmetric division, which precedes the polar accumulation of a series of additional cellular components, including small GTPases (ROPs) and F-actin (Humphries et al., 2011; Zhang et al., 2012) (Figure 10B). In the absence of PAN1, ROP2 and ROP9 (Type I ROPs) are not enriched at the SMC/GMC contact site compared with wild type (Humphries et al., 2011). Compromised activity of ROP2 and ROP9 in maize results in defective SMC division polarity, but PAN1 polarization is not disturbed. Considering the potential involvement of ROPs in BASL function (previous section), it is attractive to speculate a general role of ROP signaling in translating polarity cues into cellular changes, such as reorganization of cytoskeletal components. Indeed, one important step in SMC polarization is the formation of a dense patch of F-actin, which likely aids in nuclear migration or anchoring in division. The F-actin patch is diminished in *pan1* and *rop2/9* mutants, indicating it is downstream of these components (Cartwright et al., 2009; Humphries et al., 2011). Although ROP proteins may bridge polarity signaling (both BASL and PANs) with cytoskeletal reorganization and cellular polarization, it should be noted that the nucleus migrates away from BASL, but toward PANs, signifying that distinct molecular machineries are involved.

Polarity proteins and cell fate

Besides the defect in the physical asymmetry of stomatal lineage divisions, *basl* mutants also fail to specify two distinct daughter cells, because both daughter cells can express MUTE and thus form stomata (Dong et al., 2009). The pole where BASL and POLAR are polarized relative to the new division plane resembles the position of the PAR proteins in premitotic neuroblasts. However, after the division, the PAR complex is always associated with the self-renewing stem cell and the other cell differentiates into a neuron. In contrast, BASL and POLAR are always inherited into the daughter cell (SLGC) that withdraws from self-renewal and often differentiates into a pavement cell (Figure 10A). This might reflect a novel difference in the mechanism regulating how intrinsic polarity proteins control cell-fate determination in plants. Instead of distributing cell-fate determinants to the other side of the cell, as PAR polarity does in neuroblasts, the regional cell expansion induced by the BASL may result in diluted stem cell determining factors (e.g. SPCH) or other signaling molecules in the SLGC, which leads each cell to distinct developmental paths.

Even though extrinsic cue-induced PAN polarity induces SMC polarization, loss of PAN1 function does not interfere with cell-fate determination (Wright et al., 2009). For instance, in *pan1* and *pan2* mutants, some SMC divisions are not asymmetric, but SC fate is not affected (Cartwright et al., 2009). This is a key difference between the PAN and BASL polarity pathways, in that *BASL* loss-of-function influences cell fate. From this, it is predicted that the maize system represents an extrinsic niche-regulated process, in that the fate of the SC is determined by the adjacent microenvironment (GMC). It is possible that the unknown extrinsic signal from GMC that triggers SMC polarization may also determine SMC differentiation or that a FAMA-like bHLH transcription factor, which has conserved function in monocots and dicots (Liu et al., 2009), exist for SC differentiation as well.

Extrinsic signaling spatially overrides internal polarity

In budding yeast, cell polarity is generated by an intrinsic "symmetry-breaking" mechanism, but the polarity direction can be spatially biased by extrinsic cues (Johnson et al., 2011). This phenomenon is similar to what occurs during an SLGC spacing division (Figure 10B). The first asymmetric division always results in BASL in the SLGC, distal to the newly formed meristemoid. If an SLGC divides again to produce a satellite meristemoid (Figure 3), the placement of the new cell wall must be reoriented (Figure 10B), so that the secondary meristemoid is produced away from the existing meristemoid (one-cell spacing rule). BASL leads this reorientation process by switching its polarity orientation from distal to proximal of the existent meristemoid (Dong et al., 2009). The pathway or molecules that spatially determine polarization of BASL are not known, but EPF1 and TMM are potential candidates. EPF1 and TMM function to enforce the orientation of the spacing divisions by providing spatial cues about the location of existing stomatal precursors. In *epf1* and *tmm* mutants, the formation of BASL intrinsic polarity is intact, but its reorientation during the spacing division is compromised (Dong et al., 2009). This observation suggests that EPF1 might be the extrinsic cue from the meristemoid that attracts BASL, and TMM may mediate the signal from EPF1 to BASL (Figure 10B). However, neither TMM nor the ERf are polarly distributed in the SLGC membrane. The molecular mechanisms for how EPF1-TMM spatially controls BASL re-positioning and the orientation division polarity remain to be elucidated.

An alternative to the "symmetry-breaking" model is the "autocrine" signaling model (Facette and Smith, 2012), which also explains how EPF ligands and TMM-ERf receptors differentiate daughter cell fates and division polarity. The autocrine model predicts that the existent meristemoid produces EPF1 and perceives it through its own TMM-ERf receptors to trigger the release of an unknown polarizing protein or molecule into the SLGC. An unidentified receptor in the SLGC responds to the polarizing cue and orientates the SLGC division away from the EPF1-producing cell. Identification of additional signaling molecules and receptors is needed to substantiate this model.

ENVIRONMENTAL AND HORMONAL INFLUENCES

Plants are sessile organisms that produce many cell types, tissues and organs post-embryonically, including stomata. Due to this, plants need a high level of flexibility in developmental and physiological programs to adapt to an ever-changing environment. Although much is known about the regulation of guard cell function (Kim et al., 2010), relatively little is known about how environmental and hormonal changes feed into the modulation of stomatal number. Recently, pivotal players have been identified in the light, carbon dioxide and brassinosteroid signaling pathways that are important in stomatal development (Figure 11).

Light

Plants grown in light produce more stomata than those grown in dark (Schoch, 1980) and high light intensity results in a higher stomatal index (SI, the ratio of the number of stomata to the total

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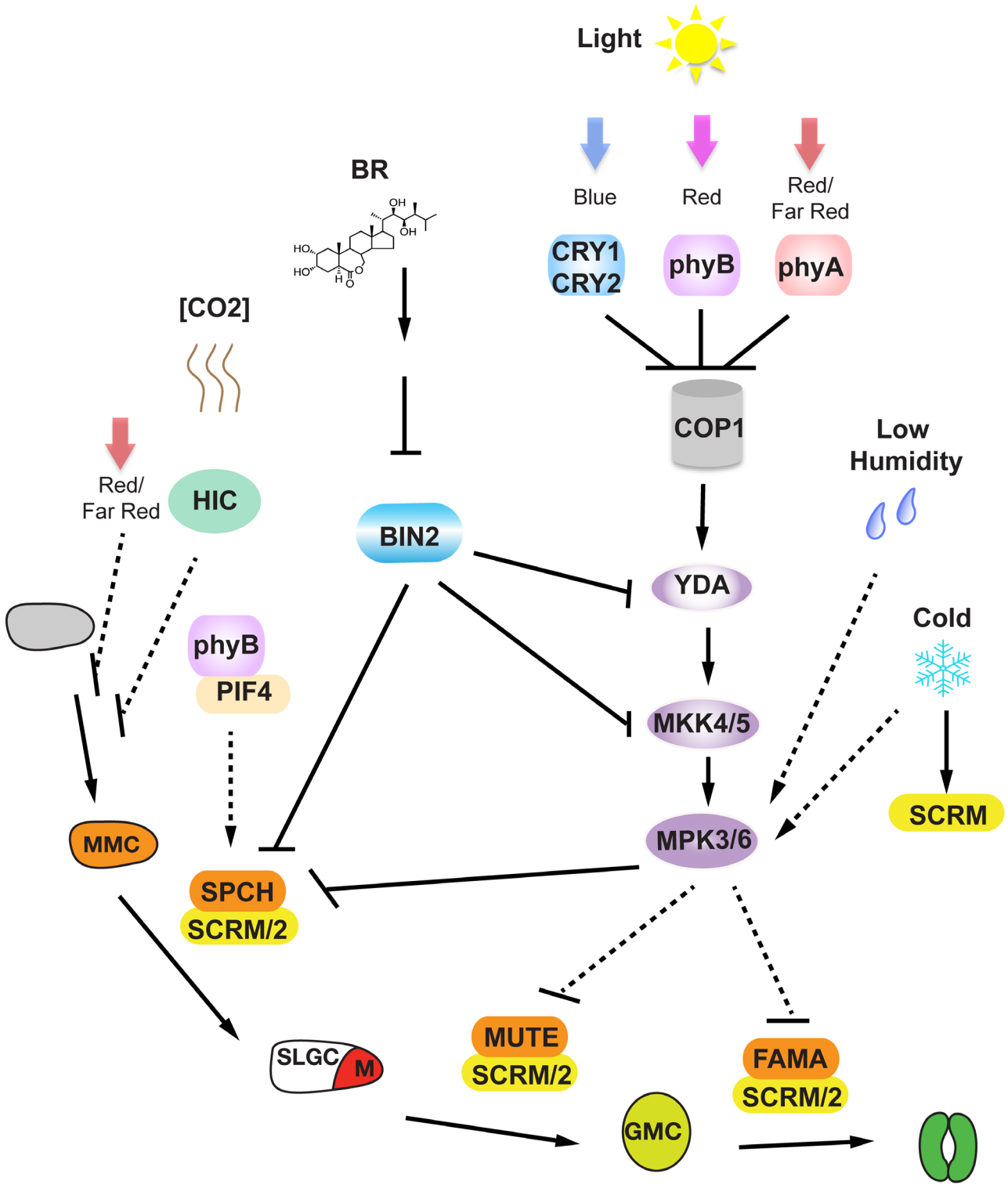

Figure 11. Summary of the hormonal and environmental signaling involved in stomatal development.

Stages of stomatal development from protodermal cell to GC are given at the bottom of the figure. Colors and abbreviations for cells types are as in Figure 3. Arrows indicate positive regulation and blunt lines indicate negative regulation. Experimentally confirmed genetic interactions are represented by solid lines and speculated genetic interactions are indicated by dashed lines.

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number of epidermal cells) without dramatic changes in patterning. Different wavelengths of light have contrasting effects on stomatal density; for example, high intensity red light increases SI, whereas far-red treatment decreases it (Boccalandro et al., 2009; Casson et al., 2009). The red light photoreceptor, phytochrome B (phyB: At2g18790), plays a dominant role in red light-induced increase in SI (Casson et al., 2009). In the absence of *phyB*, plants fail to produce more stomata under high intensity red light. Mutations in *PHYTOCHROME-INTERACTING FACTOR 4* (*PIF4*: At2g43010) display a similar defect. PIF4 is a bHLH transcription factor, which is transported to the nucleus upon binding to phyB. PIF4 is speculated to interact with the stomatal bHLH transcription module to alter stomatal production. The binding of phyB to PIF4 might influence the interaction strength of PIF4 with other bHLHs (Casson et al., 2009) (Figure 11). In addition to phyB, the blue-light receptors, CRYPTOCHROME1 (CRY1: At4g08920) and CRY2 (At1g04400), and the red/far-red photoreceptor, phytochrome A (phyA: At1g09570), all act to promote stomata production under high light intensity (Kang et al., 2009a). The link between light signaling and stomatal development is through the E3 ubiquitin-protein ligase, CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1: At2g32950), a well-known repressor of light-mediated development. COP1 is downstream of the CRY signaling and a strong *cop1* mutant displays a striking overproduction and clustered stomatal phenotype in both light and dark conditions. This phenotype strongly resembles a *yda* mutant, indicating a clear negative role of COP1 in stomatal development (Yang et al., 2001; Liu et al., 2008). Genetic analysis placed COP1 upstream of YDA and in parallel with TMM in the regulation of YDA activity. How TMM and COP1 regulate YDA is still unknown, but it is established that the YDA MAPK module negatively regulates SPCH (Lampard et al., 2008), so that stomata number is altered under variable light conditions. A detailed time-course tracing the stomatal-lineage divisions in another ubiquitin-protein ligase mutant, *CONSTITUTIVE PHOTOMORPHOGENIC 10* (*COP10*: At3g13550) revealed a severely clustered-stomata phenotype due to extended stomatal-lineage proliferation and defects in spacing division (Delgado et al., 2012). Although alternate pathways have not been established, evidence suggests that the light-mediated pathway may be fine-tuned by parallel pathways, which incorporate SDD1 (Schluter et al., 2003; Coupe et al., 2006).

CO_2

Stomata are the major route for CO_2 uptake to use during photosynthesis and CO_2 regulates both stomata opening and development (Gray et al., 2000; Hashimoto et al., 2006; Young et al., 2006). The majority of plant species investigated (~75%) produce fewer stomata when CO_2 levels increase. This has been observed both in the geological record and under laboratory conditions (Woodward, 1987; Woodward, 1995; Lawson et al., 2002; Driscoll et al., 2006).

Arabidopsis *HIGH CARBON DIOXIDE* (*HIC*: At2g46720) plays a role in modulating SI when CO_2 levels vary. Unlike wild type plants, *hic* mutants produce significantly more stomata than wild type under elevated CO_2 concentrations (Gray et al., 2000). *HIC* encodes a protein similar to 3-keto acyl CoA synthase, which synthesizes long-chain fatty acids in cuticular wax. *HIC* is expressed specifically in guard cells, but how cuticular changes in GCs regulates stomatal development is still unknown. One hypothesis is that the diffusion rate or gradient of GC signaling molecules is altered in *hic* mutants. Alternatively, cuticular wax or an intermediate could be a signaling compound in stomatal development. It is likely that cuticle permeability to water or CO_2 plays a role in the *hic* mutant phenotype (Gray et al., 2000). This is suggested by the fact that several other mutants defective in cuticular wax production display increased or decreased SI (Chen et al., 2003; Aharoni et al., 2004).

The CO_2-binding proteins, β-carbonic anhydrases (βCA1: At3g01500 and βCA4: At1g70410), function during early CO_2 signaling and control stomatal movement (Hu et al., 2010). The double mutant, *ca1 ca4,* is not only impaired in CO_2-induced stomatal closure, but also displays increased stomatal density. Both enzymes are highly expressed in guard cells and mesophyll cells, however, only guard cell-specific expression is required to rescue the stomatal density phenotype of the double mutant. As stomatal development is defined at the early stages of the lineage, the ability to change stomatal density by altering processes at the GC stage suggests a non-cell-autonomous signal produced via βCA1 and βCA4 from differentiated cells to non-differentiated cells (Hu et al., 2010).

Long-range signals have been suspected in the regulation of stomatal development at the whole plant level. A cuvette system can independently manipulate the environment surrounding a single old leaf of a plant and an emerging young leaf to investigate long-distance signaling on stomatal density (Lake et al., 2002). In Arabidopsis, when mature leaves are exposed to elevated CO_2, developing leaves in ambient CO_2 show reduced stomatal density. Therefore, a long-range signal must transduce the information from mature leaves to emerging leaves. Given the complexity of the transduction pathway regulating stomatal development, the elusive long-distance signal is likely to be a combination of signals that converge at multiple points in the pathway. Investigation of how impaired stomatal activity in mature leaves modulates stomatal development of young leaves would provide additional levels of long-distance communication to investigate.

Brassinosteroids

Brassinosteroids (BRs) are phytohormones that are produced broadly and regulate a wide range of developmental and physiological processes (Kim and Wang, 2010). A series of discoveries implicating a core regulator in BR signaling, BRASSINOSTEROID INSENSITIVE2 (BIN2: At4g18710), in the regulation of several stomatal regulatory components has provided an excellent example of how specific developmental processes can be fine-tuned by a broadly generated signaling molecule (Gudesblat et al., 2012b; Fuentes et al., 2012; Kim et al., 2012; Khan et al., 2013).

BRs inhibit stomatal formation in cotyledons.

BIN2 is a glycogen synthase kinase 3 (GSK3)-like kinase and a negative regulator of the nuclear transcription factor BRASSINAZOLE RESISTANT1 (BZR1: At1g75080) (He et al., 2002). Under high BR levels, BIN2 is inactivated by the upstream phosphatase BRI SUPPRESSOR1 (BSU1: At1g03445) (Mora-Garcia,

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2004). Dephosphorylation of BIN2 releases inhibition on BZR1 and BR responses are triggered (Li and Nam, 2002). The epidermal phenotype of the quadruple loss-of-function mutant of BSU1-related phosphatases (*bsu-q*) was the first evidence suggesting BR signaling controlled stomatal development (Kim et al., 2012). The cotyledon of *bsu-q* resembles that of *yda*, which produces an epidermis entirely covered by stomata. Consistent with fact that BSU inhibits BIN2, overexpression of *BIN2* induces a similar stomatal overproduction phenotype as *bsu-q*. The direct connection between BR signaling and stomatal development is through the negative regulation of YDA kinase activity via BIN2 phosphorylation. Collectively, BRs negatively regulate stomatal development by inhibiting BIN2 kinase-mediated inhibition of YDA (Kim et al., 2012). MKK4/5, the downstream effector of YDA, are also targeted and inhibited by BIN2 phosphorylation (Khan et al., 2013).

BRs promote stomatal formation in hypocotyls.

Brassinosteroids have been directly implicated in promoting stomatal development in hypocotyls (Gudesblat et al., 2012b; Fuentes et al., 2012). In addition to YDA, BIN2 can phosphorylate and inhibit another key stomatal regulator, the bHLH protein SPCH (Gudesblat et al., 2012b). As YDA and SPCH have antagonistic function during stomatal development, this implies conflicting roles for BR signaling, in that BR signaling may inhibit BIN2 kinase, an inhibitor of SPCH, to promote SPCH activity and thus stomata production. Indeed, external application of excessive BRs increases the number of stomata in the hypocotyl (Gudesblat et al., 2012b). Several hypotheses have been proposed to address the two apparently conflicting functions of BR in stomatal development (Casson and Hetherington, 2012; Gudesblat et al., 2012a; Serna, 2013). Possibly, different experimental conditions and alleles of the mutants employed in these two studies produced varied phenotypes that may have lead to discrepant interpretation (Gudesblat et al., 2012a). Another possibility is that tissue-specific regulation may play a role. The interaction between BIN2 and YDA were mainly examined in the cotyledon epidermis (Kim et al., 2012), whereas the study on BIN2 and SPCH focused in the hypocotyl epidermis (Gudesblat et al., 2012b). As discussed earlier, the receptor-like protein TMM titers the stem/hypocotyl-specific ligand CHAL and reduces the signaling through the ERf (Abrash and Bergmann, 2010). Therefore, in hypocotyls, TMM confers a negative effect on downstream MAPK activities. At the whole plant level, higher levels of MAPK activity is expected in the cotyledons, but lower levels in the hypocotyls. Based on this, the differential effects of BRs in cotyledons and in hypocotyls can then be resolved. In cotyledons, BIN2 may target high levels of YDA activity for inhibition, but in hypocotyls, where MAPK activities are low and SPCH activities are high, BIN2 may target SPCH for inhibition (Serna, 2013). This interesting hypothesis awaits confirmation by measuring the MAPK activity in different tissues.

Other environmental influences

A direct connection between low-temperature tolerance and stomatal development is based on the identification the *scrm-D/ice1-D* mutant (Chinnusamy et al., 2003; Kanaoka et al., 2008). In addition to the prominent role of SCRM in stomatal development, *scrm-D* mutants show impaired cold-induced gene expression and impaired freezing tolerance (Chinnusamy et al., 2003). *scrm-D* is a gain-of-function mutant, which is proposed to stabilize the interaction with SPCH to promote stomatal production (Kanaoka et al., 2008), although the regulatory mechanisms for SCRM function in the two pathways may be different. For example, an ICE1 protein with an S403A amino acid substitution is substantially more resistant to cold-induced proteasomal degradation and confers more freezing tolerance than ICE1(WT). However, either ICE1(WT) or ICE1(S403A) rescues the growth and stomatal phenotypes of a *scrm scrm2* double mutant and both isoforms appear to function similarly during stomatal development (Miura et al., 2011). SUMOylation is also a source of regulation on ICE1 during cold treatment, but has not been investigated in the stomata system (Miura et al., 2007).

While several environmental (CO_2, humidity, temperature) and hormonal factors (ethylene and abscisic acid (ABA)) are known to effect stomatal density or SI, very little is known about the underlying molecular or genetic basis for the changes (Serna and Fenoll, 1997; Gray et al., 2000; Casson and Hetherington, 2010). The known role of MPK6 in multiple stress responses suggests that MAPK signaling may be a common mechanism through which environmental cues modify developmental programs (Colcombet and Hirt, 2008; Popescu et al., 2009). In addition, fluctuations in ABA levels due to altered transpiration rate under variable humidity and CO_2 conditions has been suggested as a mechanism for inhibiting stomatal development. Although the specific pathway through ABA is not established, non-cell-autonomous regulation from the stomatal lineage cells is important for ABA-mediated pavement cell expansion (Lake and Woodward, 2008; Tanaka et al., 2013), highlighting the importance of coordinated cell expansion and growth during leaf development.

EVOLUTIONARY PERSPECTIVE

It is likely that stomata only arose once during plant evolution, coincident with the mass expansion of plants in terrestrial environments approximately 400 million years ago. Although the basic core structure of a stoma (two guard cells surrounding an intervening pore) is invariant, the divisions of the stomatal lineage are divergent among different plant lineages. Stomata likely originated in the bryophyte lineage (Ligrone et al., 2012). Functional conservation of MUTE and FAMA orthologs between the bryophyte and angiosperm lineages suggests these proteins have an ancient role in specification of stomata (Peterson et al., 2010; MacAlister and Bergmann, 2011). With some functional conservation among components, the question remains as to the sources of the diverse stomatal patterns seen today.

Analysis of the genes that regulate stomatal development and spacing has been examined most closely in the angiosperm lineages, monocots (rice (*Oryza sativa*) and maize (*Zea mays*)) and dicots (Arabidopsis). Both lineages have dedicated cell lines that produce stomata; however the development of stomata complexes and spacing pattern formation are not entirely comparable. For instance, monocots produce dumbbell-shaped guard cells that are always associated with a subsidiary cell and arranged

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in linear files, as opposed to a more dispersed development in dicots. A key difference between the two lineages is the production of stem-cell like populations that undergo several amplifying divisions. Arabidopsis undergoes amplifying divisions that produce the intervening pavement cells required for GC function. In contrast, no self-renewing cells are produced in the stomatal lineage of monocots reflecting the difference in their leaf development (Liu et al., 2009). Based on cross-complementation results, MUTE and FAMA function is generally conserved between the two lineages (Liu et al., 2009), although SPCH function has diverged somewhat. In addition, LRR-RLKs are involved in the development of the stomatal complex in monocots (Cartwright et al., 2009). However, they are not direct orthologs of the receptors used in Arabidopsis and do not play a similar role. Overall, it appears that the formation of stomatal complexes in different plant lineages have maintained the function of master regulators of cell-type differentiation like the bHLH proteins, but have also recruited novel genes to regulate unique aspects of patterning that are specific to separate lineages. Phylogenetic comparison of genes important for stomatal development and spacing and speculation about the alterations of the regulatory mechanisms in Arabidopsis have been proposed for other species (Peterson et al., 2010).

Table 1. Stomatal development and patterning genes

Gene Name	AGI	Symbol	Gene Description	References
Differentiation Genes				
SPEECHLESS	At5g53210	*SPCH*	bHLH protein	MacAlister et al., 2007; Pillitteri et al, 2007
MUTE	At3g06120	*MUTE*	bHLH protein	Pillitteri et al., 2007; MacAlister et al., 2007
FAMA	At3g24140	*FAMA*	bHLH protein	Ohashi-Ito and Bergmann 2007
SCREAM/ICE1	At3g26744	*SCRM*	bHLH protein	Kanaoka et al., 2008
SCREAM2	At1g12860	*SCRM2*	bHLH protein	Kanaoka et al., 2008
FOUR LIPS	At1g14350	*FLP*	R2R3 MYB protein	Lai et al., 2005; Xie et al., 2010
MYB88	At2g02820	*MYB88*	R2R3 MYB protein	Lai et al., 2005; Xie et al., 2010
HOMEODOMAIN GLABROUS2	At1g05230	*HDG2*	HD-ZIP IV protein	Peterson et al, 2013
Spacing and Patterning Genes				
YODA	At1g63700	*YDA*	MAPKKK	Bergmann et al., 2004; Lampard et al., 2008
MKK4/MKK5	At1g51660 At3g21220	*MKK4* *MKK5*	MAPKK	Wang et al., 2007
MKK7/MKK9	At1g18350 At1g73500	*MKK7* *MKK9*	MAPKK	Lampard et al., 2009
MPK3/MPK6	At3g45640 At2g43790	*MPK3* *MPK7*	MAPK	Wang et al., 2007
ARABIDOPSIS PROTEIN PHOPHATASE 2C	At2g40180	*AP2C3*	Phosphatase	Umbrasaite et al., 2010
ERECTA	At2g26330	*ER*	Leucine-rich repeat receptor kinase	Shpak et al, 2005
ERECTA-LIKE1	At5g62230	*ERL1*	Leucine-rich repeat receptor kinase	Shpak et al, 2005
ERECTA-LIKE2	At5g07180	*ERL2*	Leucine-rich repeat receptor kinase	Shpak et al, 2005
TOO MANY MOUTHS	At1g80080	*TMM*	Leucine-rich repeat receptor protein	Yang and Sack, 1995; Geisler et al., 2000; Bhave et al., 2009
STOMATAL DENSITY AND DISTRIBUTION1	At1g04110	*SDD1*	Subtilisin-like protease	Berger et al., 2000; Von Groll et al., 2002

(continued)

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Table 1. (continued)

Spacing and Patterning Genes (continued)				
EPIDERMAL PATTERNING FACTOR1	At2g20875	*EPF1*	Cysteine-rich secreted peptide	Hara et al., 2007
EPIDERMAL PATTERNING FACTOR2	At1g34245	*EPF2*	Cysteine-rich secreted peptide	Hara et al., 2009; Hunt and Gray 2009
STOMAGEN/EPF-LIKE9	At4g12970	*EPFL9*	Cysteine-rich secreted peptide	Sugano et al., 2010
CHALLAH/EPF-LIKE6	At2g30370	*CHAL* *EPFL6*	Cysteine-rich secreted peptide	Abrash and Bergmann, 2010
Polarity and Division Asymmetry Genes				
BREAKING OF ASYMMETRY IN THE STOMATAL LINEAGE	At5g60880	*BASL*	No homology	Dong et al., 2009
PANGLOSS1 *PANGLOSS2*	N/A	*PAN1* *PAN2*	Leucine-rich repeat receptor protein	Gallagher and Smith, 2000; Cartwright et al., 2009
POLAR LOCALIZATION DURING ASYMMETRIC DIVISION AND REDISTRIBUTION	AT4g31805	*POLAR*	No homology	Pillitteri et al., 2011
Cell Division Genes				
CYCLIN DEPENDENT KINASE B1;1	At3g54180	*CDKB1;1*	Cyclin-dependent kinase	Boudolf et al., 2004
CYCLIN D4;2	At5g10440	*CYCD4;2*	Cyclin	Kono et al., 2007
CYCLIN A2 gene family	At5g25380 At5g11300 At1g15570 At1g80370	*CYCA2;1* *CYCA2;2* *CYCA2;3* *CYCA2;4*	Cyclin	Vanneste et al., 2011
CYTOKINESIS DEFECTIVE 1	unknown	*CYD1*	Not cloned	Yang et al., 1999
STOMATAL CYTOKINESIS DEFECTIVE 1	At1g49040	*SCD1*	DENN-WD40 protein	Falbel et al., 2003
GLUCAN SYNTHASE-LIKE 8	At2g36850	*GSL8*	Callose synthase	Guseman et al., 2009; Chen et al., 2009
Hormone and Environmental Signaling Genes				
CRYPTOCHROME	At4g08920 At1g04400	CRY1 CRY2	Blue-light photoreceptor	Kang et al., 2009; Boccalandro et al., 2009; Casson et al., 2009
PHYTOCHROME	At1g09570 At2g18790	PHYA PHYB	Red/far-red photoreceptor	Kang et al., 2009
PYTOCHROME-INTERACTING FACTOR 4	At2g43010	PIF4	bHLH protein	Casson et al., 2009
CONSTITUTIVE PHOTOMORPHOGENIC 1	At2g32950	*COP1*	Ubiquitin Ligase	Kang et al., 2009
CONSTITUTIVE PHOTOMORPHOGENIC 10	At3g13550	*COP10*	Ubiquitin Ligase	Delgado et al., 2012
HIGH CARBON DIOXIDE	At2g46720	*HIC*	3-keto acyl coenzyme A synthase	Gray et al., 2000
BRI SUPPRESSOR1	At1g03445	*BSU1*	Phosphatase	Kim et al., 2012
BRASSINOSTEROID INSENSTIVIE 2	At4g18710	*BIN2*	GSK3-like Kinase	Kim et al., 2012
Hypocotyl Stomatal Genes				
TRANSPARENT TESTA GLABRA	At5g24520	*TTG*	WD-repeat protein	Galway et al, 1994
WEREWOLF	At5g14750	*WER*	MYB-related protein	Lee et al., 1999
GLABRA2	At1g79840	*GL2*	Leucine zipper protein	Di Cristina et al, 1996

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FUTURE DIRECTIONS

Arabidopsis stomatal development has been used as an exemplary system for the analysis of cell fate, cell polarity, and intercellular communication. However, several outstanding questions still remain.

1. Identification of the molecular mechanism that influences the transcriptional activation of *SPCH* will provide needed insight into the outstanding question: What restricts *SPCH* expression to MMCs to initiate entry division? On the other hand, prolonged activity of SPCH needs to be precisely inhibited to restrict stomatal overproduction. Elucidating how SPCH activity is post-transcriptionally regulated in a spatiotemporal manner promises to provide insight into the systematic regulation of tissue homeostasis in plants.
2. How do stomatal lineage cells monitor aging? Investigation of the processes which monitor cell age or division capacity will be critical for a better understanding of the activation of *MUTE* expression and relocalization of the intrinsic polarity proteins, BASL and POLAR.
3. Is the BASL-POLAR intrinsic polarity module fundamental in the regulation of plant asymmetric division? Identification of the molecular components and understanding the mechanisms for BASL and POLAR localization and function will clarify whether intrinsic polarity is widely used to regulate asymmetric division in different developmental contexts in plants (e.g. embryo, shoot and root development).
4. How does environmental and developmental signaling, through the core MAPK pathway, impinge on the stomata pathway? Investigation of the subcellular targeting of individual components in the MAPK cascade is vital to understand how specificity is conferred in interconnected signaling networks at the subcellular level.

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APPENDIX: TERMINOLOGY

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

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

Stomatal lineage: The cell lineage that initiates the production of a stoma. Lineages are produced in a mosaic pattern across the leaf surface in Arabidopsis.

Stomatal Complex: The stoma and all adjacent neighbor cells.

Anisocytic complex: A stomatal complex common to the Brassicaceae in which the stoma is surrounded by three neighbor cells, each of sequentially smaller size.

Monoclonal vs. polyclonal: Stomatal complexes in which all cells, guard cells and non-stomatal cells, derive from a single precursor cell are considered to be monoclonal.

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

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

Neighbor cell: General term to describe an epidermal cell in contact with a guard cell or precursor.

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

Stomatal lineage ground cell (SLGC): The larger cell produced clonally through MMC or meristemoid asymmetric division.

Meristemoid: A small, often triangular cell that can reiteratively divide asymmetrically. Some meristemoids do not divide and directly transition to a guard mother cell.

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

Satellite meristemoid: A type of meristemoid that forms from a neighbor cell. A satellite meristemoid is located away from the pre-existing stoma/precursor. This is a major mechanism ensuring a minimal distance between stomata in Arabidopsis.

Pavement cell: Generic, relatively large, jigsaw puzzle-shaped epidermal cells.

Subsidiary cell (SC): A cell located next to a guard cell that is morphologically distinct from other epidermal cells. Two subsidiary cells flank two guard cells to regulate stomatal opening and closing.

Subsidiary mother cell (SMC): The precursor of subsidiary cell. SMC is recruited by the contacting developing guard mother cell (GMC) and divides asymmetrically to produce a SC next to a GMC.

Symmetry breaking: A term popularly used to describe the early events/processes in asymmetric cell division that lead to different cell fates. Symmetry breaking typically involves protein asymmetric localization (polarization), cytoskeletal re-organization and unequal organelle distribution.

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