

Breaking the silence: three bHLH proteins direct cell-fate decisions during stomatal development

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Summary

Stomata are microscopic pores on the surface of land plants used for gas and water vapor exchange. A pair of highly specialized guard cells surround the pore and adjust pore size. Studies in *Arabidopsis* have revealed that cell–cell communication is essential to coordinate the asymmetric cell divisions required for proper stomatal patterning. Initial research in this area identified signaling molecules that negatively regulate stomatal differentiation. However, genes promoting cell-fate transition leading to mature guard cells remained elusive. Now, three closely related basic helix–loop–helix (bHLH) proteins, *SPEECHLESS*, *MUTE* and *FAMA* have been identified as positive regulators that direct three consecutive cell-fate decisions during stomatal development. The identification of these genes opens a new direction to investigate the evolution of stomatal development and the conserved functions of bHLH proteins in cell type differentiation adopted by plants and animals. *BioEssays* 29:861–870, 2007. © 2007 Wiley Periodicals, Inc.

Introduction

Stomata are plant epidermal structures that consist of two specialized epidermal cells called guard cells, which surround a microscopic pore. The size of the pore opening dictates the amount of gas and water vapor exchange that can take place between the plant and the atmosphere. Stomatal development requires a series of asymmetric divisions followed by a single symmetric division to produce the paired guard cells⁽¹⁾ (Fig. 1). During stomatal development in *Arabidopsis thaliana*, the stomatal lineage is initiated by a subset of protodermal cells called meristemoid mother cells (MMC). The MMC undergoes an asymmetric “entry” division that produces a small triangular cell called a meristemoid, and a larger sister cell called a

stomatal-lineage ground cell (SLGC), which often becomes a cuticularized epidermal pavement cell. The meristemoid is self-renewing and can repeat multiple asymmetric amplifying divisions that regenerate the meristemoid as well as additional SLGCs. Meristemoids ultimately lose their self-renewing character and differentiate into a round guard mother cell (GMC). New meristemoids are always oriented away from an existing GMC or stomata, which ensures stomata develop at least one-cell apart from one another. The GMC undergoes a final symmetric division to produce two highly specialized guard cells, which work in concert to control the size of the pore. The mature guard cells are terminally differentiated and do not divide further.

This process has been studied in *Arabidopsis* over the past decade and factors that inhibit stomatal development have been identified^(2–5) (Table 1). These genes are involved in the regulation of proper spacing and density of stomata, and their loss of function leads to the formation of aggregated stomata. More recent studies have identified three master switch genes that positively direct three steps of cell-fate transition through the stomatal lineage, from protoderm to differentiated guard cells^(6–8) (Table 1). We discuss the genetic and molecular functions of the master switch genes *SPEECHLESS* (*SPCH*), *MUTE* and *FAMA* in *Arabidopsis* stomatal development and their utility as tools to investigate stomatal patterning diversity, stomatal evolution and the conservation of developmental programs between plants and animals.

Stomatal patterning requires cell–cell communication

Stomata (from the Greek work ‘mouth’) are separated from one another by at least one non-stomatal cell because proper opening and closing of stomata requires water and ions supplied from surrounding cells.⁽⁹⁾ In addition to genetic control, placement of stomata across the epidermal surface is also affected by numerous extrinsic factors through both local and long-distance signaling.⁽¹⁰⁾ Conserved cell-surface receptors and intracellular signaling molecules have been implicated in mediating the perception and response to these signals. Mutations in these signaling molecules result in increased stomatal density and mis-orientation of asymmetric divisions that result in adjacent stomata (clustering). The

Department of Biology, University of Washington, Seattle, WA.

Funding agencies: Our research on stomatal development has been supported by the grant from NSF (IOB-0520548), DOE (DE-FG02-03ER15448), and the CREST award from Japan Science and Technology Agency to K.U.T. L.J.P. was supported by a CSREES USDA postdoctoral fellowship.

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DOI 10.1002/bies.20625

Published online in Wiley InterScience (www.interscience.wiley.com).

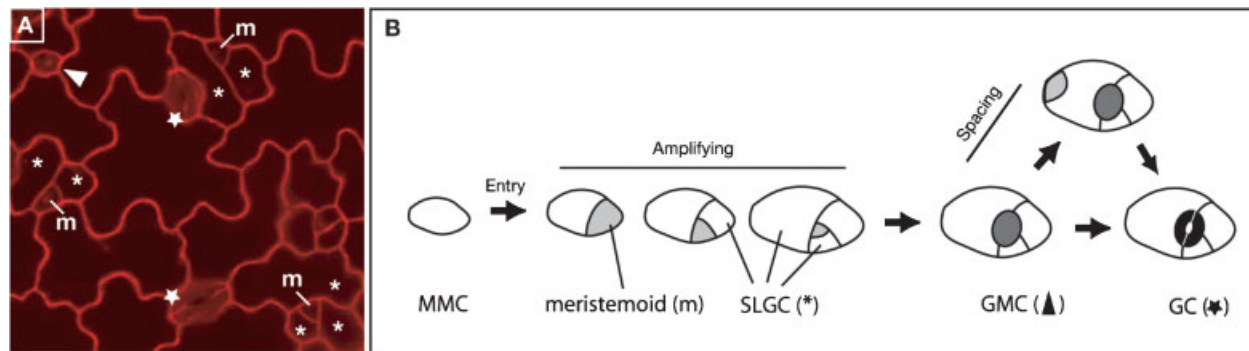


Figure 1. Cell types of the stomatal lineage. **A:** Confocal image of the abaxial epidermis of a wild-type cotyledon. Cell walls are stained with propidium iodide. Cell types are labeled as described in panel B. **B:** Stages of divisions during stomatal development. A protodermal cell (not shown) is converted into a meristemoid mother cell (MMC) through an undefined mechanism. The MMC undergoes an asymmetric entry division to create a meristemoid (light grey). Meristemoids are self-renewing and can reiterate one to three rounds of amplifying divisions before differentiating into a guard mother cell (GMC, dark grey). The stomatal lineage ground cells (SLGCs) produced from amplifying divisions can initiate an entry division that is always oriented away from an existing GMC or stomata (spacing division). GMCs divide symmetrically to produce two guard cells (GC, black), which eventually form the mature stomata.

current model suggests that *STOMATAL DENSITY AND DISTRIBUTION 1 (SDD1)*, a subtilisin-like serine protease, acts to process a yet unidentified ligand.⁽⁵⁾ The SDD-processed ligand is perceived by *TOO MANY MOUTHS*

through potential interaction with the three *ERECTA* family proteins (*ERECTA*, *ERECTA-LIKE 1* and *ERL2*). *TMM* and the *ERECTA* family are a plasma-membrane localized leucine-rich repeat receptor-like protein (LRR-RLP) and

Table 1. Genes involved in *Arabidopsis* stomatal development in rosette leaves*

Gene name	Symbol	Molecular homology	Mutant Phenotype	Overexpression/Constitutively Active Phenotype
Patterning genes				
<i>STOMATAL DENSITY AND DISTRIBUTION 1</i>	<i>SDD1</i>	Subtilisin-like protease	Increased SI, small clusters	Represses stomatal divisions Arrested meristemoids and GMCs
<i>TOO MANY MOUTHS</i>	<i>TMM</i>	Leucine-rich repeat receptor-like protein	Increased SI, clusters	ND
<i>ERECTA</i> -family	<i>ER.ERL1, ERL2</i>	Leucine-rich repeat receptor-like kinase	Greatly increased SI, large clusters	<i>ERECTA</i> - no phenotype ⁺ <i>ERL1</i> and <i>ERL2</i> -ND
<i>YODA</i>	<i>YDA</i>	Mitogen-activated protein kinase kinase	Greatly increased SI, large clusters	No stomata, pavement cell only
<i>MAPKK4/MAPKK5</i>	<i>MKK4/MKK5</i>	Mitogen-activated protein kinase kinase	Entire epidermis converted to stomata	No stomata, pavement cell only
<i>MAPK3/MAPK6</i>	<i>MPK3/MPK6</i>	Mitogen-activated protein kinase	Entire epidermis converted to stomata	No stomata, pavement cell only
Differentiation genes				
<i>SPEECHLESS</i>	<i>SPCH</i>	bHLH protein	No initiation of asymmetric cell division in the epidermis—no stomata	Excessive epidermal divisions, no extra stomata
<i>MUTE</i>	<i>MUTE</i>	bHLH protein	Initiation and reiteration of asymmetric cell division in the epidermis—no stomata	Entire epidermis converted to stomata
<i>FAMA</i>	<i>FAMA</i>	bHLH protein	Reiterative divisions of the GMC—no stomata	Entire epidermis converted to single guard cells
<i>FOUR LIPS</i>	<i>FLP</i>	R2R3 MYB protein	Reiterative divisions of the GMC, small clusters	ND
<i>MYB88</i>	<i>MYB88</i>	R2R3 MYB protein	None, enhances <i>flp</i> phenotype	ND

**TMM*, *ER*-family and *YODA* have varying phenotypes in different organs. SI, stomatal index (number of stomata/total cell number); ⁺, E.D. Shpak and K.U. Torii, unpublished; ND, no data.

LRR-receptor-like kinases (RLK), respectively.^(3,11,12) The identification of several mutants has implicated a mitogen-activated protein kinase cascade in transduction of the receptor signal to the ultimate gene targets. Mutations in the MAP kinase kinase kinase (MAPKKK) gene, *YODA* (*YDA*), cause the formation of large stomatal clusters.⁽¹³⁾ Recently, two MAP kinase kinase genes and two MAP kinase genes, *MKK4/MKK5* and *MPK3/MPK6*, respectively, were shown to act downstream of *YDA* in this signaling pathway.⁽¹⁴⁾ The culmination of these studies revealed that cell–cell signals were required for proper orientation of divisions, density of stomata, and control of entry and amplifying divisions (Fig. 2). However, they are not intrinsically required for differentiating cell types within the stomatal lineage. Now, three genes have been identified that lift the “black box” in our understanding of how stomata are produced.

Stomatal differentiation requires the consecutive action of three bHLH proteins

SPEECHLESS (*SPCH*), *MUTE* and *FAMA* encode closely related bHLH proteins required for stomatal development. A mutation in any one gene results in aerial organs that lack stomata. The gene names reflect their phenotypes: *SPCH* and

MUTE have ‘no-mouths’ and *FAMA* is the goddess of rumor with ‘fake mouths’. Expression analysis and loss- and gain-of-function phenotypes indicate that these genes make up a three-step transcriptional cascade where each gene has a distinct role in key transitional states of the stomatal lineage:^(6–8) (1) the transition from MMC to meristemoid by *SPCH*, (2) meristemoid to GMC by *MUTE*, and (3) GMC to guard cell by *FAMA* (Fig. 3A).

SPCH is the first gene from this trio to be expressed. *SPCH* was identified through a genetic screen to isolate genes necessary to promote stomatal fate.⁽⁶⁾ The strong *spch-1* mutant is completely devoid of stomatal cell lineages, meaning the epidermis comprises jig-saw-puzzle-shaped pavement cells with no stomata (Fig. 3C). Therefore, *SPCH* is required for the first transition to initiate the asymmetric entry division of the stomatal lineage. The weak *spch-2* allele has a reduced number of entry divisions and stomata compared to wild type. Close examination of *spch-2* revealed that fewer SLGCs were associated with mature stomata suggesting that *SPCH* may have an additional role in promoting amplifying divisions. Consistent with this, *SPCH* promoter activity is detected early, broadly in the protoderm but also later in a stomatal-lineage-specific pattern.^(6,8) *SPCH* is not only necessary but also

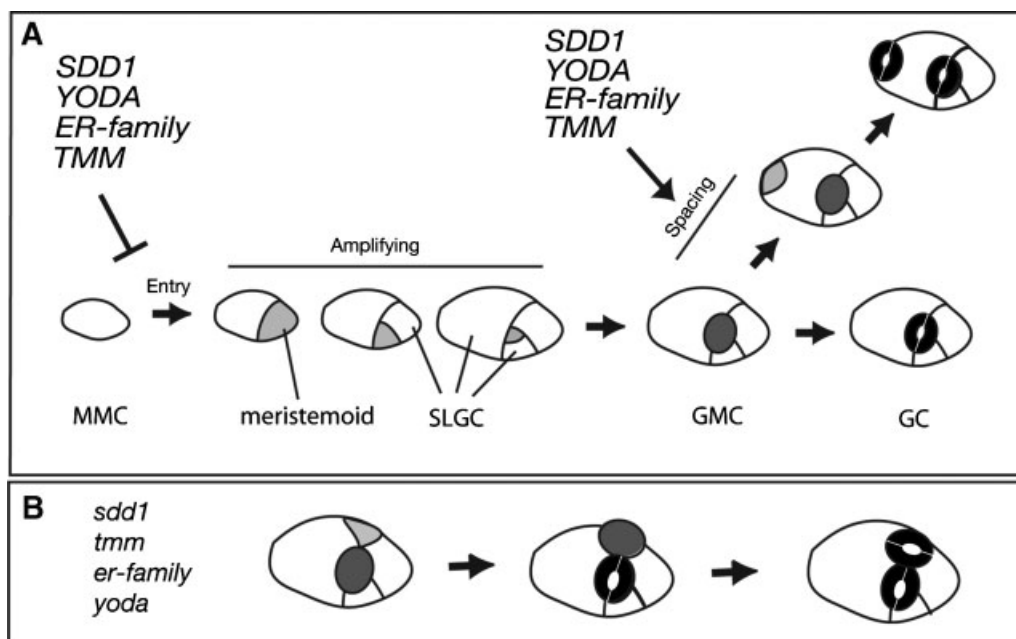


Figure 2. Genetic control of stomatal lineage cell divisions. **A:** Diagram of the stages of divisions during stomatal development and the points of action of several signaling components implicated in mediating the perception and response to external signals to inhibit stomata production via control of cell lineage divisions. *SDD1* (subtilisin protease), *YODA* (MAPKKK), *TMM* (LRR-RLP) and the *ER* family (LRR-LRKs) act as negative regulators of stomatal development through limiting the number entry divisions. They also act to inhibit stomatal cluster formation through orienting spacing divisions. **B:** Example of signaling component mutants showing misorientation of spacing divisions and the production of a stomatal cluster. The role of *TMM* in entry division inhibition is organ specific. MMC, meristemoid mother cell; SLGC, stomatal lineage ground cell; GMC, guard mother cell; GC, guard cell.

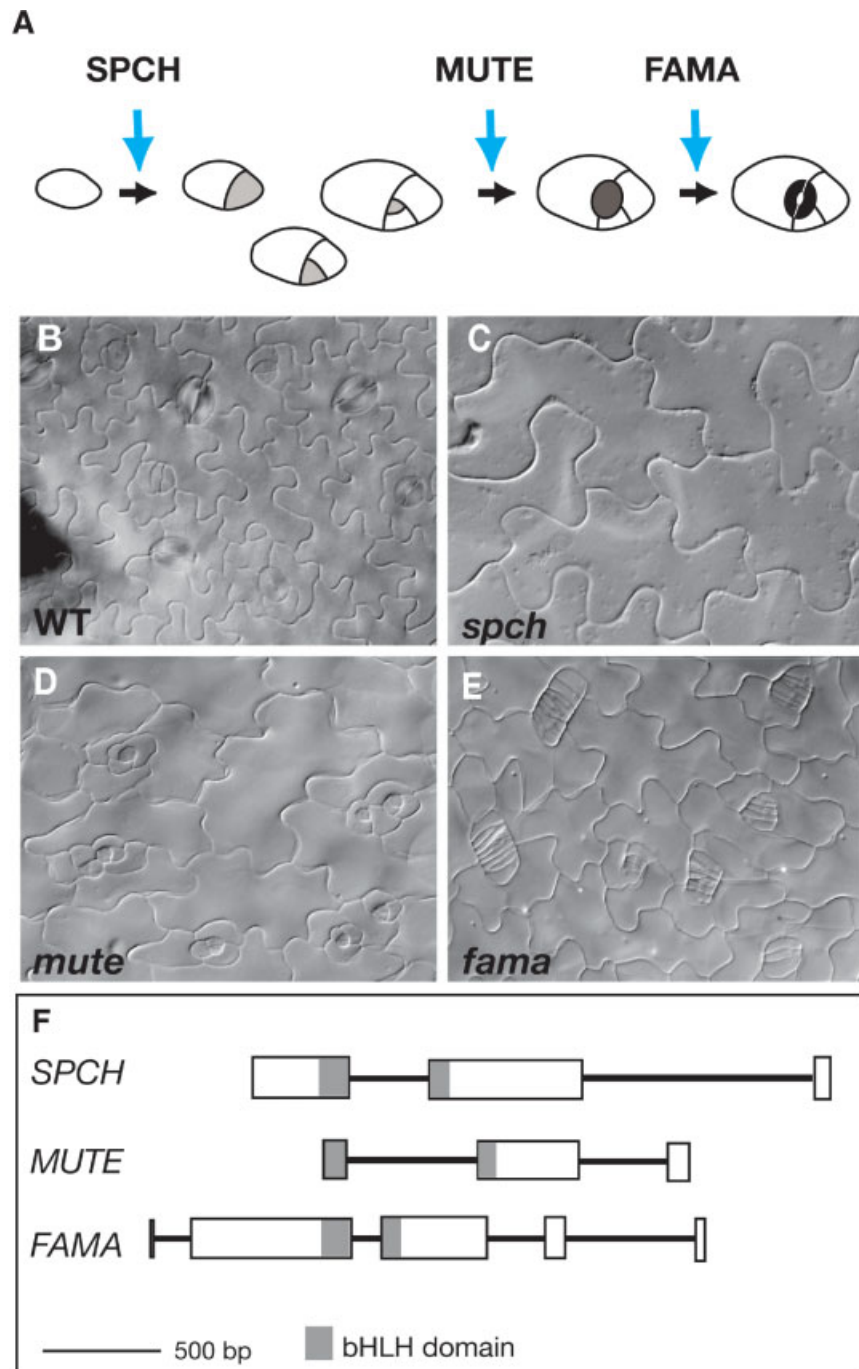


Figure 3. bHLH protein mutant phenotypes. **A:** Site of stomatal bHLH protein action: the three bHLH proteins work in a three-step transcriptional cascade starting at the transition from MMC to meristemoid by *SPCH*, meristemoid to GMC by *MUTE*, and GMC to guard cell by *FAMA*. Blue arrows indicate points of gene action. **B–E:** Abaxial leaf epidermal surface of wild type (**B**), *spch* (**C**), *mute* (**D**), and *fama* (**E**). **F:** Schematic diagram of *SPCH*, *MUTE* and *FAMA*. Exons are indicated as boxes, introns as lines. The bHLH domain of each gene is indicated as a grey box.

sufficient to create the stomatal lineage, as ectopic inducible expression of *SPCH* produced excess cell divisions in cells, which do not normally divide (i.e. differentiated pavement cells).^(6,8) In addition, these highly divided cells expressed the stomatal lineage markers *TMM::TMM-GFP* and *ERL1::GUS*.⁽⁸⁾ *SPCH* activity is required for the expression of both *MUTE* and *FAMA*, and *SPCH* function may be specific to stomatal development, as asymmetric divisions in other organ types, such as the root or embryo, were not affected by *SPCH* loss-of-function.⁽⁶⁾

MUTE activity drives the second transition: the transition from meristemoid to GMC. *MUTE* was identified through a sensitized genetic screen for genes required for stomatal development.⁽⁸⁾ The epidermis of *mute* plants produces no stomata, but unlike *spch*, *mute* plants are able to initiate the first entry division in the stomatal lineage and produce a meristemoid. Typically, this meristemoid completes an excessive number of amplifying divisions, but fails to transition to a GMC. Therefore, the *mute* epidermis is characterized by inwardly spiraled SLGCs with a triangular, aborted meristemoid at the center (Fig. 3D). *MUTE* promoter activity and *MUTE* protein is only observed in a subset of meristemoids, presumably those that have undergone a sufficient number of amplifying asymmetric divisions and are 'ready' to transition. The fact that *mute* meristemoids are surrounded by an excess number of SLGCs suggests that *MUTE* is also involved in limiting the number of amplifying divisions, most likely as a secondary consequence of the failure of the self-renewing meristemoid to differentiate. The mechanism through which a plant monitors the number of cell divisions and triggers *MUTE* is not understood, however, the genetic study by Pillitteri et al.⁽⁸⁾ suggested that the cell signaling receptor *ERL1* may be involved in regulating the number of amplifying asymmetric division. In any event, the timing of *MUTE* expression is critical for proper stomatal spacing and function. This is strikingly demonstrated by the ectopic overexpression of *MUTE* in the epidermis, which converts the entire epidermis to stomata.^(6,8) The weaker ectopic overexpression of *MUTE* conferred hybrid pavement/guard cells that possess chloroplasts and form a symmetric division with a faux pore.⁽⁸⁾ This peculiar phenotype indicates that a moderate level of forced *MUTE* expression can cause epidermal cells to adopt both pavement- and guard cell developmental programs.

FAMA acts at the third transition and was identified through a genome-wide search for genes that are expressed at higher levels in *yda* mutant plants compared to wild type.⁽¹³⁾ The strong *fama-1* mutant produces abnormal 'caterpillar-like' rows of GMCs (tumors) on the epidermal surface (Fig. 3E) and its expression is restricted to GMCs and newly divided stomata. Therefore, *FAMA* regulates the critical differentiation event between GMC and guard cell and, in addition, halts cell division at the final stage of the stomatal lineage to ensure a single symmetric division.⁽⁷⁾ Its role in guard cell differentiation

was demonstrated by overexpression analysis, which led to the formation of unpaired guard cells even in non-epidermal tissues, indicating that *FAMA* is sufficient to specify guard cell fate. In addition, *fama* tumors do not express the mature guard cell marker *KAT1::GUS*, suggesting that they do not progress past GMC, further supporting a role for *FAMA* in final differentiation. Negative regulation of the cyclin-dependent kinase, *CDKB1;1*, by *FAMA* was suggested as a possible mechanism for halting cell divisions in guard cells.⁽⁷⁾

SPCH, *MUTE* and *FAMA* are the three most-closely related bHLHs of Arabidopsis subfamily 3.⁽¹⁵⁾ This subgroup shares high amino-acid conservation in addition to similar intron distribution and position (Fig. 3F). For bHLH proteins, the basic region is involved in DNA binding and the HLH region functions in dimerization. Dimer formation is required for DNA binding and subsequent transcriptional activation. Although they are closely related, the function of each gene is specific as complementation experiments indicated that they can not functionally substitute for one another.⁽⁶⁾ Compared to either *SPCH* or *FAMA*, a notable structural feature of *MUTE* is that the N-terminal region is missing. For *MUTE*, the presumed translational start site begins three amino acids into the basic region. Interestingly, induced expression of *FAMA* variants containing only the HLH and C-terminal domain or a mutated DNA-binding domain produced a phenotype similar to that described for *MUTE* overexpression (i.e. all stomata). These variants were not affected in dimerization properties. Ohashi-Ito and Bergmann⁽⁷⁾ postulated that the *FAMA* variants may act in a dominant negative manner by sequestering other bHLHs in non-functional heterodimers or, alternatively, acquired neomorphic activity within the stomatal differentiation pathway. To test whether the neomorphic phenotype of the *FAMA* variants is an artifact of overexpression or mimicry of *MUTE* requires further investigation into the DNA-binding properties of the stomatal bHLHs.

The strong *fama-1* phenotype is similar to that described for a double mutant knockout of *FOUR LIPS (FLP)* and *MYB88* (R2R3 MYB-type transcription factors).^(4,16) In addition, weak *fama-2* mutants are similar to *flp-1* single mutants that produce 3- to 4-celled stomata but no tumors. In root epidermal patterning, bHLH and R2R3 MYB proteins interact to produce a complex that controls cell-fate specification.⁽¹⁷⁾ Genetic interaction studies did not suggest that *FAMA* and *MYB88/FLP* form heterodimers. In addition, *FAMA* failed to interact with *FLP* or *MYB88* in yeast or in plants. Together, Ohashi-Ito and Bergmann⁽⁷⁾ concluded that *FAMA* and *FLP/MYB88* do not form an active complex similar to that in root epidermal cell-fate specification.

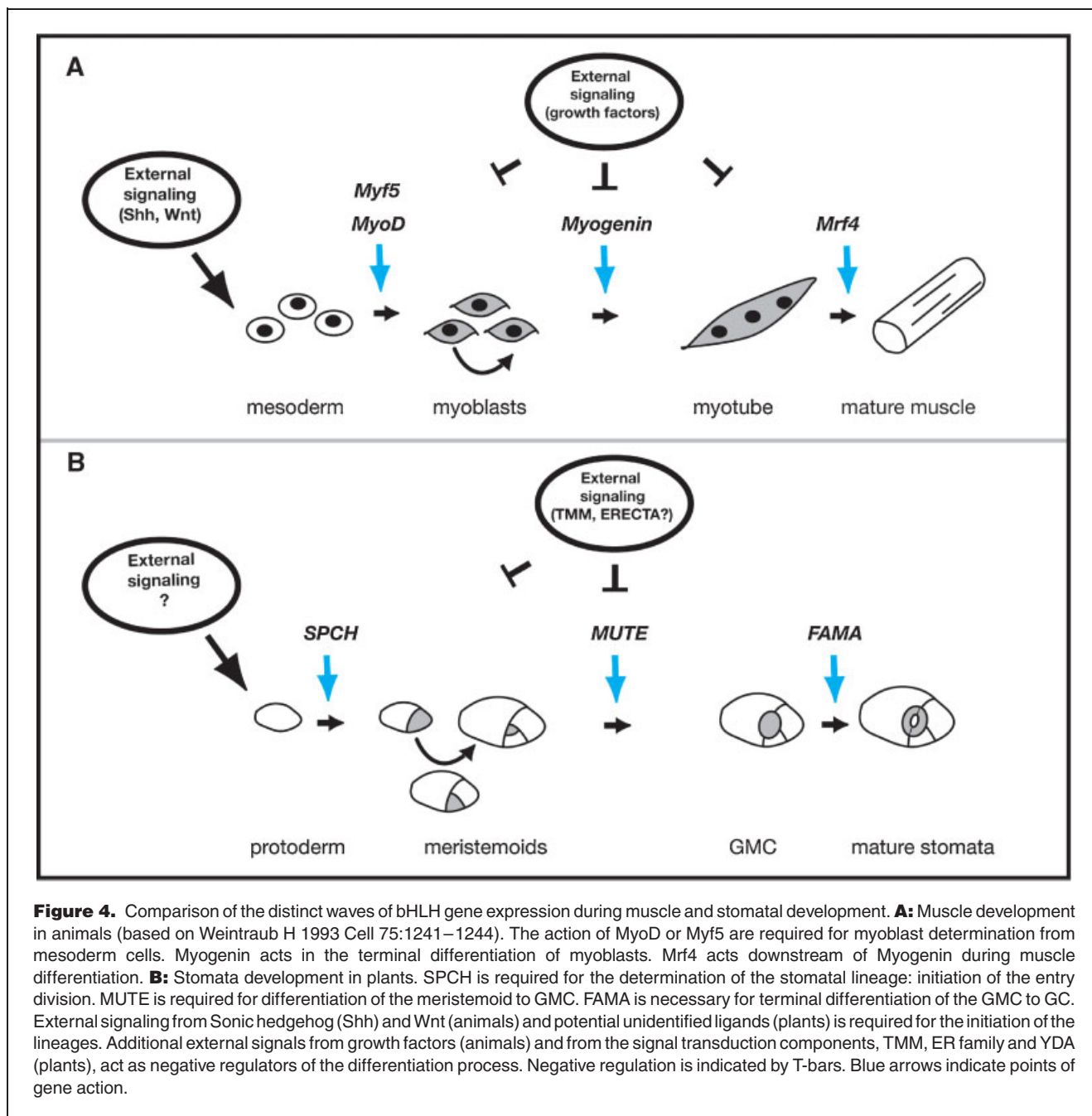
Possible *FAMA* dimerization partners may include the bHLH071 and bHLH093, which were identified from a yeast two-hybrid screen.⁽⁷⁾ Both bHLH071 and bHLH093 belong to the same subgroup as *SPCH*, *MUTE* and *FAMA*.⁽¹⁵⁾ While loss-of-function mutations in bHLH071 and bHLH093 did not

produce a noticeable phenotype, overexpression of either gene produced a mild *fama* phenotype.⁽⁷⁾ It is possible that these genes have redundant roles in suppressing FAMA function. Whether they enhance the phenotype of *fama* or other stomatal bHLH mutations remains to be addressed.

Drawing parallels between plants and animals

The last common ancestor of plants and animals was likely unicellular and existed around 1.6 billion years ago.⁽¹⁸⁾

Therefore, plants and animals independently developed mechanisms for cell communication and pattern formation even though the molecules that carry out these processes are often unrelated.⁽¹⁹⁾ In contrast, the discovery that three closely related bHLH proteins control stomatal lineage cell-fate decisions is strikingly parallel to the consecutive use of closely related bHLH proteins during myogenesis (Fig. 4). The myogenic bHLH proteins, including MyoD, Myf5, myogenin and MRF4^(20–23) act in a consecutive manner to direct cell-fate



decisions during successive steps in myoblast differentiation: MyoD and Myf5 specifying myoblast precursor state, and myogenin and MRF4 directing terminal differentiation of muscles.⁽²⁴⁾ The MyoD family proteins are considered 'master regulators' of skeletal myogenesis, as their forced expression is capable of converting nearly all cell types into skeletal muscle fibers.^(25,26) Similarly, overexpression of SPCH, MUTE and FAMA induces ectopic stomatal-lineage cells. Both stomatal and MyoD family bHLHs are expressed in a spatially restricted manner.

The myogenic bHLH proteins induce muscle-specific transcription via binding of their basic domain to the canonical DNA E-box consensus (CANNTG) of downstream target genes and regulating their expression. Comparatively, SPCH, MUTE and FAMA all contain the conserved H-E-R amino acid residues in the basic domain characteristic of proteins that bind the E-box.⁽²⁷⁾ Therefore, SPCH, MUTE and FAMA may act as transcription factors that bind directly to the regulatory elements (promoters) of target genes to direct cell-fate transition. Mutating these critical residues in the FAMA basic domain resulted in a protein that could not rescue the *fama-1* mutant.⁽⁷⁾

For the MyoD protein, homodimers are transcriptionally inactive, however, heterodimerization with ubiquitously expressed bHLHs (E proteins) results in a complex with high DNA-binding affinity, which is pivotal for controlling downstream targets.^(28,29) Therefore, in addition to conserved transcriptional molecules, stomatal development may also share similar regulatory mechanisms with myogenesis. This idea stems from the identification of the two ubiquitously expressed bHLHs that specifically bind to FAMA.⁽⁷⁾ Multiple mutant analyses with these two bHLHs will provide some insight as to whether this type of regulatory conservation exists between these two developmental programs.

In animals, non-DNA-binding HLH proteins lacking the basic region, such as human Id protein, act as transcriptional inhibitors.⁽³⁰⁾ They negatively regulate bHLH transcription factors via direct protein–protein interaction that results in non-functional heterodimers. The Arabidopsis genome contains at least 27 proteins that are categorized as non-DNA-binding HLHs.⁽¹⁵⁾ Among them, KIDARI inhibits photomorphogenesis by direct association with the bHLH protein HFR1, a known downstream component of light signal transduction.⁽³¹⁾ It will be fascinating to address whether the activity of SPCH, MUTE and FAMA may also be regulated by such non-DNA-binding HLHs.

The activity of bHLH transcription factors has also been shown in several systems to be regulated by phosphorylation/dephosphorylation circuits, which can influence the choice and affinity for dimerization partners. Specifically during myogenesis, the MAP kinase p38 phosphorylates the MyoD-binding partner, E47.⁽³²⁾ This phosphorylation event is required for the formation of a functional MyoD/E47 hetero-

dimer. Furthermore, DNA-binding affinity of bHLHs is also regulated by phosphorylation. Growth factors, such as FGF (fibroblast growth factor), promote cell proliferation and inhibit terminal differentiation of muscles by inactivating the myogenic bHLHs.⁽³³⁾ This inhibition is mediated by protein kinase C, which phosphorylates the conserved site in the DNA-binding domain of myogenic bHLH and prevents its association to target DNA sequence.⁽³⁴⁾ During stomatal development, a MAP kinase cascade, including YDA, MKK4/MKK5 and MPK3/MPK6, negatively regulates asymmetric division and stomatal formation, and their loss-of-function mutations resulting in large clusters of stomata.^(13,14) Conversely, constitutively active forms of YDA and MPKs result in a phenotype resembling *spch-1*, consisting of an epidermis of pavement cells only.^(13,14) YDA and the ERECTA family of receptor-like kinases promote cell proliferation and organ growth while inhibiting entry divisions to limit stomatal formation.^(2,3) Therefore, the parallel between myogenesis and stomatal development may extend to how these master regulatory bHLHs are regulated by extrinsic cell–cell signals (Fig. 4). It remains to be seen whether the phosphorylation circuit works directly or indirectly to regulate stomatal bHLH activity.

Although not directly paralleled in myogenesis, regulation of the stomatal bHLHs may involve a C-terminal regulatory region. MUTE, SPCH and FAMA contain a C-terminal domain weakly similar to an ACT domain, which serves as a regulatory module in a diverse group of proteins. The ACT domain has been most studied in amino acid metabolic enzymes where it is involved in allosteric regulation through the binding of specific small molecule ligands.^(35,36) This domain is involved in protein–protein interactions and recently shown to be important for dimerization of several plant bHLH proteins.⁽³⁷⁾ Dimerization and function of MUTE, SPCH or FAMA with specific partners may require the ACT domain. Evidence for this comes from the loss-of-function *spch-1* mutant, which results in a 7-amino acid C-terminal truncation of the wild-type protein, yet is indistinguishable from the complete T-DNA knock-out allele.⁽⁶⁾ The truncation removes part of the ACT domain, highlighting the importance of this domain in SPCH function.

Epidermal diversity: SPCH, MUTE and FAMA action

The paralogous relationship among SPCH, MUTE and FAMA strongly suggests that they are derived from a genomic duplication event. It appears that both expression and functional changes have occurred within the stomatal bHLHs given that their expression patterns do not significantly overlap and that they cannot functionally substitute for one another.⁽⁶⁾ One can hypothesize that the variation of stomatal patterning among land plants could in part be a result of the diversification of the stomatal bHLHs through the course of evolution.

Numerous models relating to SPCH, MUTE or FAMA action can be postulated to explain stomatal pattern variation. For instance, the simplest model predicts that expression of *MUTE* immediately after the initial entry division without additional amplifying divisions would produce a modified pattern to that described for *Arabidopsis*. Indeed, many monocots, including the *Liliaceae*, have linear rows of elongated epidermal cells. Stomata develop at the basal end of these cells after a single asymmetric division with no subsidiary cells present.⁽³⁸⁾ For *Liliaceae*, spacing of stomata could be ensured by a mechanism that allowed the meristemoid to be produced only at the basal end of cells leaving each stomata at least one cell away from the next (Fig. 5). Additional models could be postulated to explain stomatal complexes with subsidiary cells totaling >4, as is seen in the *Araceae*.⁽³⁸⁾ In these cases, a delay in *MUTE* activity could predict this phenotype as is observed in the *mute* mutant itself. Previous work has determined that stomatal patterning and the division series required for a functional stomatal complex in the model monocot *Zea mays* varies from *Arabidopsis*.⁽³⁹⁾ The large amount of genetic and molecular tools available for maize will allow for testing of some of these models.

Finally, beyond examining the evolutionary diversity of stomatal patterning, the key stomatal regulators are useful tools to investigate how stomata initially developed in early land plants. The earliest land plants include the liverworts, hornworts and mosses, collectively called the Bryophytes. The gametophyte of these plants do not produce stomata; however, the sporophyte of some mosses and hornworts do produce responsive stomata. Further, the fern *Selaginella*

appears to produce stomata without extensive asymmetric division of a precursor cell.⁽⁴⁰⁾ Perhaps, prototypical function of stomatal bHLHs may be to trigger initial entry asymmetric divisions and then immediately specify GMC differentiation. Similar to the extensive work done piecing together the evolution of flowering using floral master regulatory genes such as *LEAFY* (*LFY*), these ‘primitive’ plants provide an excellent comparative system to investigate the stomatal bHLHs and their importance in land plant evolution. The true challenge may lie in identifying true homologous stomatal bHLH genes in other plant systems. Unlike *LFY*, which is generally found as a unique single copy transcription factor, the bHLH gene family has undergone a large radiation in plants,⁽¹⁵⁾ containing hundreds of members. However, based on homology, convincing stomatal bHLH homologues have been identified in rice (K.U.T., unpublished data) and as more basal plant genomes are sequenced, homology searches will likely reveal others. Identifying whether the stomatal bHLHs have alternate roles in astomatous gametophyte plants in the Bryophytes, as was discovered for *LFY* in the non-flowering moss *Physcomitrella patens*,⁽⁴¹⁾ is an area of exciting future research.

Conclusions

Stomatal development shares a common theme with other developmental programs in which cells must communicate and coordinate to adopt different cell fates. Many aspects of the emerging model for stomatal development remain to be tested. These include how the cell–cell signaling pathway specifying patterning integrates with the cell differentiation

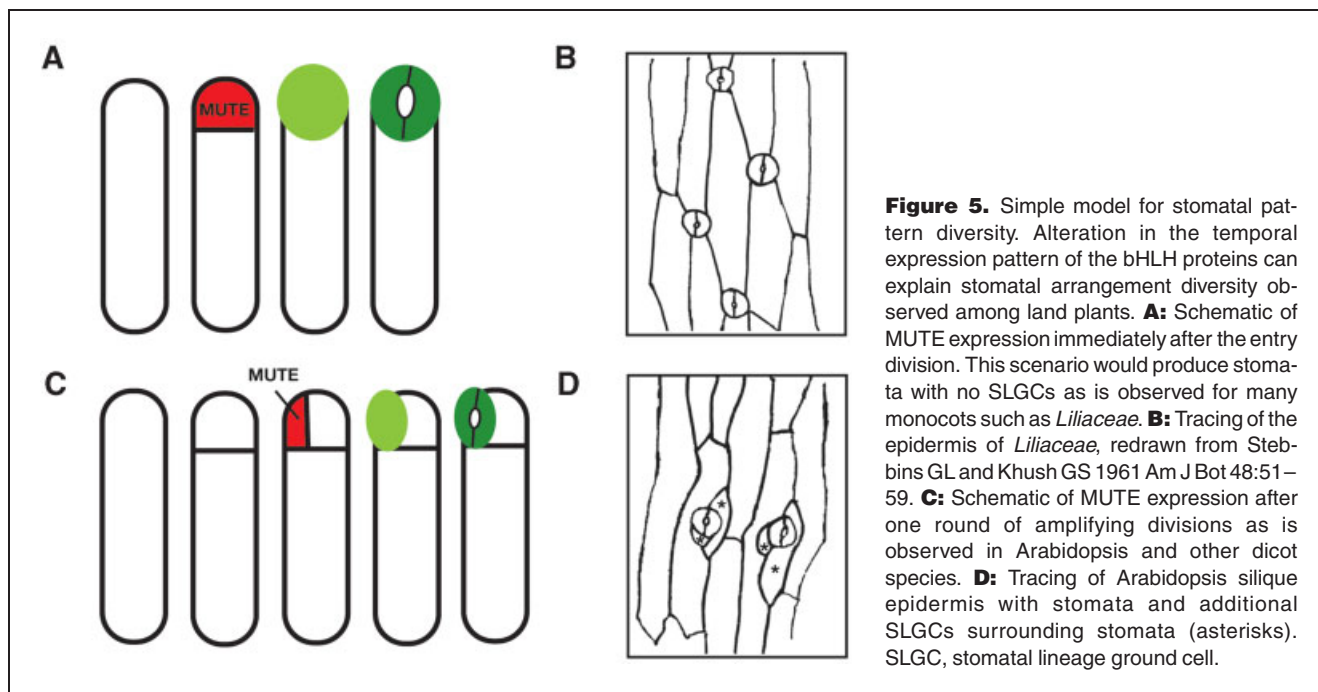


Figure 5. Simple model for stomatal pattern diversity. Alteration in the temporal expression pattern of the bHLH proteins can explain stomatal arrangement diversity observed among land plants. **A:** Schematic of *MUTE* expression immediately after the entry division. This scenario would produce stomata with no SLGCs as is observed for many monocots such as *Liliaceae*. **B:** Tracing of the epidermis of *Liliaceae*, redrawn from Stebbins GL and Khush GS 1961 Am J Bot 48:51–59. **C:** Schematic of *MUTE* expression after one round of amplifying divisions as is observed in *Arabidopsis* and other dicot species. **D:** Tracing of *Arabidopsis siliqua* epidermis with stomata and additional SLGCs surrounding stomata (asterisks). SLGC, stomatal lineage ground cell.

pathway. What is the mechanism by which a subset of cells are “chosen” from a seemingly identical pool of protodermal cells that uniformly express SPCH? Similarly, what mechanism determines if MUTE is activated in meristemoids after one division or three? How or when were the stomatal bHLHs recruited for stomata production? The identification of additional players, including up and downstream factors and potential binding partners, and the molecular examination of basal land plants will aid in our understanding. Because the second and third transitions of the stomatal lineage can be interpreted as cell-cycle exit, one potential gene group to investigate as downstream targets are core cell-cycle genes as was suggested by the negative regulation of CDKB1;1 by FAMA. Though many details remain to be addressed, numerous parallels have emerged between the molecules and regulatory mechanisms used by plant and animal developmental programs, even though each group independently recruited bHLHs for these processes through evolution. This makes the isolation of the three stomatal bHLHs a valuable tool for plant and animal comparative analysis.

Acknowledgments

We thank Dr. Masa Kanaoka for the confocal image used in Fig. 1.

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