DOI 10.1002/bies.200800231 **Review article**

Cell fate transitions during stomatal development

*Laura Serna**

Facultad de Ciencias del Medio Ambiente, Universidad de Castilla-La Mancha. Real Fábrica de Armas, Avda. Carlos III, Toledo, Spain

Stomata, the most influential components in gas exchange with the atmosphere, represent a revealing system for studying cell fate determination. Studies in Arabidopsis thaliana have demonstrated that many of the components, functioning in a signaling cascade, guide numerous cell fate transitions that occur during stomatal development. The signaling cascade is initiated at the cell surface through the activation of the membrane receptors TOO MANY MOUTHS (TMM) and/or ERECTA (ER) family members by the secretory peptide EPIDERMAL PATTERNING FACTOR1 (EPF1) and/or a substrate processed proteolytically by the subtilase STOMATAL DENSITY AND DISTRIBUTION1 (SDD1) and transduced through cytoplasmic MAP kinases (YODA (YDA), MKK4/MKK5, and MPK3/MPK6) towards the nucleus. In the nucleus, these MAP kinases regulate the activity of the basic helix-loop-helix (bHLH) proteins SPEECHLESS (SPCH), MUTE, and FAMA, which act in concert with the bHLH-Leu zipper protein SCREAM (SCRM) (and/or its closely related paralog, SCREAM2). This article reviews current insights into the role of this signaling cascade during stomatal development.

Keywords: epidermis; stomata

Introduction

Gas exchange between the plant and the atmosphere is regulated through the ''stoma'', which is a Greek word meaning mouth or opening. Stomata are microscopic pores found in the epidermal tissue of most aerial organs of all terrestrial plants. Each stomatal pore is bounded by two guard cells. The pore opening (and gas exchange) depends on changes in the turgor of the guard cells, which is regulated by the flow of water and ions between the guard cells and the adjacent epidermal cells.[1] Gain in turgor of the guard cells triggers stomatal opening, whereas loss of turgor induces its closure. Gas exchange depends not only on the opening and closing of the stomata but also on their density and spatial distribution within the epidermal tissue.

***Correspondence to:** L. Serna, Facultad de Ciencias del Medio Ambiente, Universidad de Castilla-La Mancha. Real Fábrica de Armas, Avda. Carlos III, s/n, 45071 Toledo, Spain.
E-mail: laura.serna@uclm.es

In *A. thaliana*, the first sign of stomatal development is an asymmetric cell division from a protodermal cell called the meristemoid mother cell[2,3] (Fig. 1). This cell division produces a small triangular cell, the meristemoid, and a larger cell. Meristemoids can divide asymmetrically, in an inward spiral, up to three times and always yield a larger cell and a smaller meristemoid that maintains its stem cell activity. The meristemoid, after these asymmetric cell divisions, loses its stem cell character and adopts a rounded shape giving rise to the guard mother cell. The guard mother cell undergoes a symmetric cell division that produces the paired guard cells. The paired guard cells and their adjacent epidermal cells make up the primary stomatal complex. The larger cells that result from the asymmetric divisions and that come into contact with the stoma (or its precursor) can either enter into the stomatal pathway or become pavement cells. When they enter into the stomatal pathway, they copy the developmental program of the meristemoid mother cell, giving rise to secondary complexes. Higher complex order can also be formed.

Wild-type stomata almost never develop directly next to each other, as would be expected if they were randomly distributed, which indicates that there must be an underlying patterning mechanism (Fig. 2). The inward-spiraling nature of the reiterative asymmetric divisions that the meristemoids undergo tends to result in the stomata being surrounded by a full complement of clonally related nonstomatal cells, which helps in preventing the development of stomatal clusters[4,5] (Fig. 1). However, it has been demonstrated that the orientation of the cell division plane in cells entering the stomatal pathway that contact a stoma or its precursor (spacing divisions) places the new meristemoid (satellite meristemoid) away from the pre-existing stoma, ensuring a minimal distance of one cell between stomata neighbors[6] (Fig. 1). It seems that a combination of several strategies ensures that guard cells never make direct contact with their neighbors, guaranteeing their function.[2,3]

During the last few years, the understanding of the stomatal development and patterning in a molecular context has been advanced by the cloning of a large number of relevant genes (Table 1). These include those encoding for extracellular molecules,[4,7] cell membrane components[8–10]

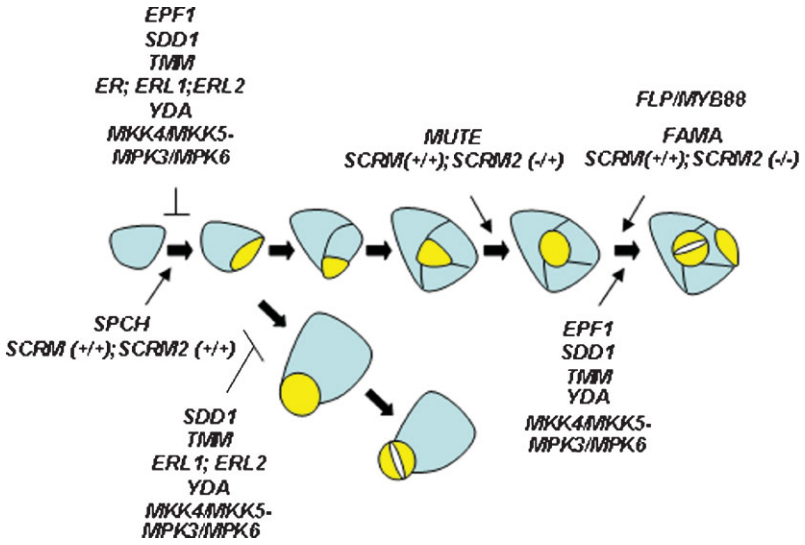

Figure 1. Genetic control of the progressive steps of stomatal development. *SDD1*, *EPF1*, *TMM*, members of the *ER* family receptor-like kinases (*ER*, *ERL1*, and *ERL2*), *YDA* and *MKK4/MKK5-MPK3/MPK6* repress the entry into the stomatal pathway. In contrast, *SPCH* starts stomatal development by inducing the first asymmetric division, giving rise to the first meristemoid. Two or three divisions after the formation of the first meristemoid, *MUTE* drives the last asymmetric cell division producing the guard mother cell. The *FLP/MYB88* and *FAMA* then drive the symmetric division that gives rise to the two guard cells. Both *TMM* and *SDD1*, among other genes, also maintain the stem cell character of the meristemoids and orient the cell division plane of meristemoid mother cells that contact stomata so that the new meristemoids form away from the pre-existing stomata. *SCRM* and *SCRM2*, in a dosage-dependent manner, specify the actions of *SPCH*, *MUTE*, and *FAMA*. Adapted from.[16–18,36]

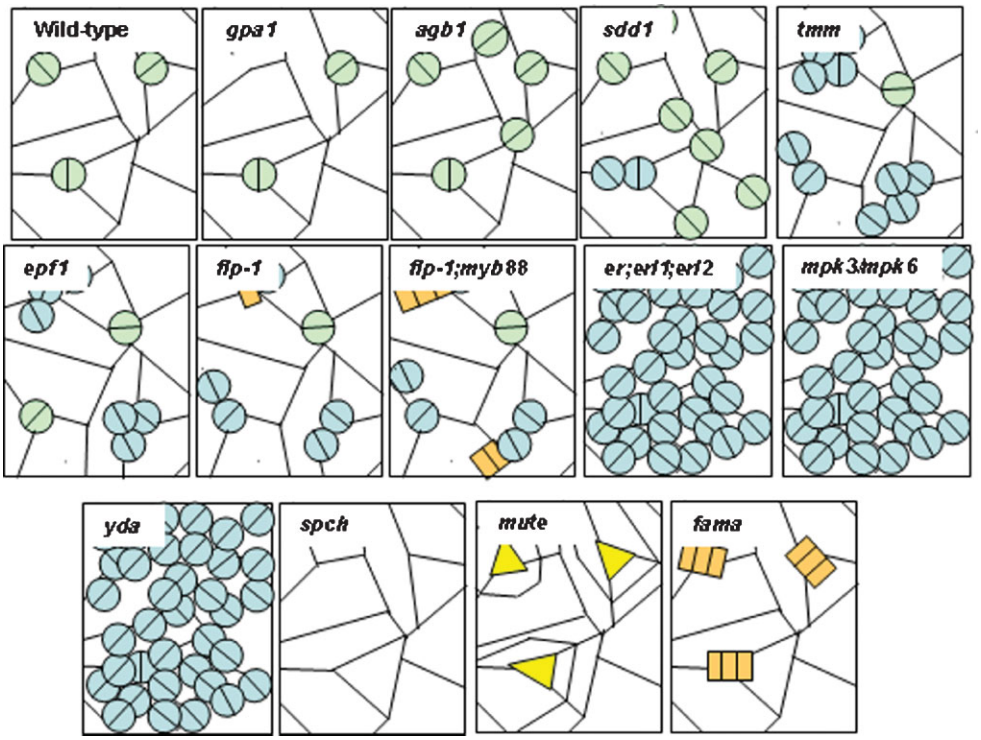

Figure 2. Stomatal phenotype of wild-type and mutant plants. Wild-type stomata are spaced by intervening cells. The *gpa* mutant exhibits a reduced stomatal index. In contrast, the *agb1* mutant exhibits an increased stomatal index. In *sdd1*, *tmm*, *epf1*, *flp-1*, *flp-1 myb88*, *er erl1erl2*, loss of function of *MPK3/MPK6* (or *MKK4/MKK5*) and *yda*, stomata develop adjacent to one another. The *spch*, *mute*, and *fama* mutants fail to develop mature stomata. The *scrm*, *scrm scrm2/+*, and *scrm scrm2* mutants phenocopy *fama*, *mute*, and *spch*, respectively. *AGL16* does not regulate stomatal pattern (not shown). Adapted from.[16,36]

Table 1. Genes regulating stomatal development in Arabidopsis.

Gene name	Mutant phenotype	Proposed wild-type function	Protein encoded
STOMATAL DENSITY AND DISTRIBUTON 1 (*SDD1*)	Increased stomatal index, some stomatal clusters	Represses stomatal initiation, promotes stem cell fate maintenance of the Ms, orients spacing divisions, reduces the number of higher order complexes	Subtilisin-related protease
EPIDERMAL PATTERNING FACTOR1 (*EPF1*)	Stomatal clusters	Orients spacing divisions, represses stomatal initiation	Secretory peptide
ERECTA (*ER*) *family*	Almost all epidermal cells are GCs	Represses stomatal initiation, promotes stem cell fate maintenance of the Ms	Leucine-rich repeat-receptor-like kinases
TOO MANY MOUTHS (*TMM*)	Stomatal clusters	Represses stomatal initiation, promotes stem cell fate maintenance of the Ms, orients spacing divisions, reduces the number of higher order complexes	Leucine-rich repeat-receptor-like protein
YODA (*YDA*)	Almost all epidermal cells are GCs	Represses stomatal initiation, promotes stem cell fate maintenance of the Ms, orients spacing divisions	Mitogen-activated protein kinase kinase kinase
MITOGEN-ACTIVATED PROTEIN KINASE KINASE (*MKK4/MKK5*)	Almost all epidermal cells are GCs	Represses stomatal initiation, promotes stem cell fate maintenance of the Ms, orients spacing divisions	Mitogen-activated protein kinase kinase
MITOGEN-ACTIVATED PROTEIN KINASE (*MPK3/MPK6*)	Almost all epidermal cells are GCs	Represses stomatal initiation, promotes stem cell fate maintenance of the Ms, orients spacing divisions	Mitogen-activated protein kinase
SPEECHLESS (*SPCH*)	Only jigsaw-puzzle-shaped pavement cells	Drives the first asymmetric cell division	bHLH protein
MUTE	Ms aborted after excessive asymmetric cell divisions	Represses stem cell fate of Ms and promotes GMC formation	bHLH protein
FAMA	Clusters of GMCs or immature GCs	Drives the transition from the GMC to the GCs	bHLH protein
SCREAM (*SCRM*)	Clusters of GMCs or immature GCs	Specifies the actions of SPCH, MUTE and FAMA	bHLH-leucine zipper protein
SCREAM2 (*SCRM2*)	None; *scrm scrm2* phenocopies *spch; scrm scrm2/+* phenocopies *mute*	Specifies the actions of SPCH, MUTE, and FAMA	bHLH-leucine zipper protein
FOUR LIPS (*FLP*)	Stomatal clusters with arrested GCs	Drives the transition from the GMC to the GCs	R2R3 MYB protein
MYB88	None; enhances *flp* phenotype	Drives the transition from the GMC to the GCs	R2R3 MYB protein
AGAMOUS-LIKE16 (*AGL16*)	Reduced higher-order stomatal complexes	Promotes the formation of higher order complexes	MADS box protein
G PROTEIN α*-SUBUNIT* (*GPA1*)	Reduced stomatal index	Promotes the formation of higher order complexes and reduces the formation of primary complexes	Heterotrimeric G protein
G PROTEIN β*-SUBUNIT* (*AGB1*)	Increased stomatal index	Reduces the formation of higher order complexes and promotes the formation of primary complexes	Heterotrimeric G protein

M, meristemoid; GMC, guard mother cell; GC, guard cells. Phenotypes of leaves and/or cotyledons.

and cytoplasmic[11,12] and nuclear factors.[13–18] Here, I review current insights into the role played by these genes and the interplay among them.

The switch from protodermal to meristemoid mother cell identity

Entry into the stomatal pathway is under the control of several genes, which also control additional steps in the stomatal pathway (see the next sections). Analysis of stomatal development through serial imprints has shown that mutations in the *TOO MANY MOUTHS (TMM)* or *STOMATAL DENSITY AND DISTRIBUTION (SDD1)* genes, encoding for a plasma membrane-anchored leucine-rich repeat-receptor-like protein (LRR-RLP) and a subtilisin-related extracellular protease, respectively,[4,8] result in an increased number of protodermal cells entering the stomatal pathway.[4,6,19] This indicates that these genes negatively regulate stomatal pathway initiation.[4,6,19] Studies of epidermal patterning in fixed samples have shown an inverse correlation between the stomatal density and the level

of expression of *EPIDERMAL PATTERNING FACTOR1* (*EPF1*), with plants that exhibit higher expression levels producing no stomata and being infertile as a consequence of the absence of stomata.[7] This indicates that *EPF1*, which encodes a secretory peptide, also represses stomatal initiation.[7] This finding is supported by the *epf1* mutant, which exhibits an increased stomatal density with some stomatal clusters.[7] Analysis of epidermal patterning in fixed plants also revealed that members of the *ERECTA* family [*ERECTA* (*ER*), *ERECTA LIKE1* (*ERL1*), and *ERL2*], which control several aspects of plant growth and also its responses to biotic and abiotic signals,[20,21] also repress the entry into the stomatal pathway, although in a redundant manner.[9,22] The three members of the *ER* family encode for members of the membrane leucine-rich repeat-receptor-like kinases (LRR-RLK).[21,23] Cells in plants with a permanently active version of the mitogen-activated protein kinase kinase kinase (MAPKKK) YODA (YDA) do not initiate stomatal development, which indicates that stomatal initiation requires a reduction in or suppression of YDA activity.[11] In agreement with this finding, the number of meristemoid mother cells is increased in the *yda* mutant.[11] *YDA* also controls cell fate determination during plant embryonic development with most *yda* mutants dying at the embryo stage and those that survive remaining very small and fuzzy.[24] Similar to *yda*, loss of function of *MITOGEN-ACTIVATED PROTEIN KINASE4 (MKK4)/MKK5* or of their downstream *MPK3/MPK6* increases the number of cells entering the stomatal pathway, and activation of MKK4/MKK5-MPK3/MPK6 switches off stomatal development initiation.[12] MKK4 and MKK5 are also upstream kinases for MPK3 and MPK6 in responses to multiple stress stimuli.[25–27]

Mutations in these loci promote the entry into the stomatal pathway (Fig. 1), but they produce different stomatal phenotypes in mature organs (Fig. 2). Mutations in *TMM* or *EPF1* lead to many single stomata being replaced by stomatal clusters.[7,28] Mutations in *SDD1* mainly increase the stomatal index ([stomatal density/(stomatal density + epidermal cell density)] $\times$ 100, where epidermal cells include guard cells as well as others that constitute the epidermis) but also produce some stomatal clusters.[4] In both *yda* and *er erl1 erl2* almost all epidermal cells are guard cells.[11,21] Similarly, silencing of *MPK3* and *MPK6* or *MKK4* and *MKK5* genes results in the entire epidermal tissue being composed of stomata.[12]

In addition to these negative regulators of stomatal pathway initiation, positive ones have also been identified. Mutations in the *SPEECHLESS* (*SPCH*) gene, which encodes a member of the basic helix-loop-helix (bHLH) family of transcriptional factors, produce an epidermal tissue consisting of jigsaw-puzzle-shaped pavement cells[16] (Fig. 2). This suggests that *SPCH* drives the first asymmetric cell division that initiates stomatal development[16] (Fig. 1). Supporting such as role, the overexpression of *SPCH* increases the number of cells entering the stomatal pathway.[16,17] The *INDUCER OF CBF EXPRESSION1* (*ICE* also named *SCREAM*) and *SCREAM2* (*SCRM2*) genes also redundantly initiate stomatal lineage with *scrm scrm2* double loss-of-function mutants phenocopying the *spch* epidermal phenotype[18] (Figs. 1 and 2). In agreement with this positive role in stomatal development, gain-of-function mutations in either *SCRM* or *SCRM2* increase the number of stomata, producing stomata in direct contact.[18] *SCRM* and *SCRM2* also encode nuclear, closely related bHLH-leucine zipper proteins.[18,29]

G protein signaling can be transmitted through the activated Gα, the G$\beta\gamma$ dimer, and/or the heterotrimeric complex. *G protein α-subunit* (*GPA1*) and *G protein β-subunit* (*AGB1*) encode the α-subunit and β-subunit, respectively, of the G protein.[30,31] *GPA1* positively regulates stomatal formation, with *gpa1* loss-of-function mutant exhibiting a reduced stomatal index (Fig. 2), whereas plants overexpressing the constitutively active form of *GPA1* show an increased stomatal index compared with that of wild-type plants.[10] In agreement with the positive role of *GPA1* in stomatal formation, plants with an elevated steady-state pool of activated GPA1 (*rgs1*) exhibit an increased stomatal index compared with that of both wild-type and *gpa1* plants.[10] In contrast with the *gpa1* mutant, plants with an inactive form of the G protein β-subunit (*agb1*) exhibit an increased stomatal index (Fig. 2), which suggests that G$\beta\gamma$ dimer represses stomatal formation.[10] In accord with this view, overexpression of the *AGB1* subunit reduces the stomatal index.[10] These results can be also attributable to the lack of GPA1. However, because the stomatal index in *agb1* is higher than that in *rgs1* or in plants overexpressing *GPA1*, the increased stomatal index in *agb1* most probably has not resulted from the positive role of the active form of GPA1.[10] Thus, the antagonistic action between GPA1 and AGB1 in stomatal initiation indicates that both Gα and G$\beta\gamma$ can transduce signals to downstream effectors. This is supported by the stomatal density in the double *gpa1 agb1* mutant, which exhibits the mean value of the single *gpa1* and *agb1* mutants.[10]

Driving the number of asymmetric cell divisions

Many of the genes that control the switch from protodermal to meristemoid mother cell identity (*SDD1*, *TMM*, members of the *ER*-family, *YDA*, *MKK4/MKK5*, *MPK3/MPK6*, *SPCH*, *SCRM*, and *SCRM2*) also regulate the number of asymmetric cell divisions that occur before stoma formation. Analysis of serial imprints and of fixed specimens has shown that guard mother cell identity is prematurely assumed, after the first or second asymmetric cell division, in both *tmm* and *sdd1* mutants.[4,6,32] In the *yda* mutant, almost all epidermal cells are guard cells.[11] Because asymmetric divisions contribute

to the formation of pavement cells, we infer that in the *yda* mutant, guard mother cell identity is also prematurely assumed. The same argument can be applied to the *er erl1 erl2* triple mutant or to plants silencing *MPK3* and *MPK6* or *MKK4* and *MKK5*, whose epidermis consists basically of paired guard cells.[9] In addition, *erl1*, *erl2* and *erl1erl2* mutants seem to exhibit a reduction in the number of larger cells that surround the stoma (or its precursor), which arise from the asymmetric divisions.[9] This suggests that both *ERL1* and *ERL2* maintain the stem cell character of the meristemoids, preventing guard mother cell formation.[9,22] Either direct or indirect observations indicate that the affected genes in these mutants maintain the stem cell character of the meristemoids in wild-type plants (Fig. 1).

SPCH may also play roles other than that related with the entry into the stomatal pathway. However, the fact that cells in the *spch* mutant do not enter into the stomatal pathway limits further characterization of hypothetical *SPCH* functions. Interestingly, a missense mutation affecting the carboxy terminus of SPCH reduces but does not repress stomata formation.[16] The analysis of static pictures from the pedicel epidermis showed that *SPCH* mutation reduces the number of sister cells of the meristemoids that surround every stoma. This led to the proposal that *SPCH*, in addition to promoting the first asymmetric cell division during stomatal development, also maintains the stem cell activity of the meristemoids.[16] Whether *SPCH* also plays this role in the leaf and/or cotyledon remains to be established.

Normal stomatal development requires a delicate balance between the maintenance of meristemoid cell activity and its differentiation into guard mother cell. The genes described here maintain the stem cell character of the meristemoids. However, how do meristemoids lose their stem cell properties to differentiate into guard mother cells? The loss-of-function *mute* mutant does not develop paired guard cells but forms meristemoids that abort after excessive asymmetric cell divisions (Fig. 2), indicating that *MUTE* negatively regulates stem cell activity and promotes guard mother cell formation[16,17] (Fig. 1). *MUTE* encodes a bHLH protein,[17] and it is also required for the production of the hydathode pore.[33] Both *SCRM* and *SCRM2* are also required to drive the transition from meristemoid to guard mother cell (Fig. 1), with *scrm scrm2/+* mutant exhibiting an identical phenotype to *mute*[18] (Fig. 2). The reduced stomatal index of the *gpa1* loss-of-function mutant, together with the increased index of plants overexpressing the constitutively active form of *GPA1*, suggests that *GPA1* represses the stem cell fate of the meristemoids.[10] *AGB1* modulates this process in the opposite direction to that of *GPA1*.[10] As previously discussed, both Gα and G$\beta\gamma$ probably transduce signals to downstream effectors regulating the stem cell fate of the meristemoids.

Stopping cell divisions: From the guard mother cell to the guard cells

A group of four genes controls the last and symmetric cell division in the stomatal pathway that gives rise to the two guard cells from the guard mother cell (Fig. 1). Two of them, *FOUR LIPS* (*FLP*) and *MYB88*, encode R2R3 MYB regulators with very similar sequences and structures.[13] Mutations in *FLP* gene (severe *flp* alleles) induce stomatal clusters with arrested guard cells[13,28] (Fig. 2) and, although mutations in *MYB88* induce no apparent stomatal phenotype,[13] plants homozygous for mutations in both genes show more and larger clusters than *flp* alone, with some developmentally arrested guard cells.[13] This suggests that they play redundant functions during stomatal development. The complementation of the *flp* phenotype by expression of a genomic *MYB88* construct supports such a conclusion.[13]

A combination of techniques, including monitoring the cell division pattern through time and the determination of cell identity by cytological (wall thickenings) and molecular (*KAT* gene expression) markers, has led to the proposal that in the *flp* mutant the daughter cells of the guard mother cells divide symmetrically one or more times before assuming guard cell identity.[13] This indicates that the *FLP* gene drives the transition from the guard mother cell to the guard cells. The presence of arrested guard cells in both *flp myb88* and severe *flp* alleles suggests that *FLP* may also drive guard cell differentiation. Alternatively, the role for these genes in guard cell differentiation might be only indirect due to cell packing constraints or the dilution of intrinsic positive regulators of guard cell differentiation.[13] If this is true, *flp* and *flp myb88* should show full guard cell differentiation in single stomata. The presence of fully differentiated guard cells placed in the border line of the clusters would support the hypothesis that packing limits guard cell differentiation and lead to discarding the hypothesis that dilution of the intrinsic regulators prevents guard cell differentiation.

Plants lacking detectable *FAMA* expression form clusters of guard mother cells or immature guard cells instead of single and mature stomata[14] (Fig. 2). We therefore conclude that the *FAMA* gene, which encodes a protein belonging to the bHLH family of transcriptional factors, also controls the symmetrical cell division of the guard mother cell and guard cell differentiation.[14] The loss-of-function mutation in *SCRM* also induces the formation of groups of guard mother cells or immature guard cells instead of single and fully differentiated stomata.[18] This indicates that *SCRM* plays a role similar to that of *FAMA* and *FLP/MYB88*[18] (Fig. 1).

Higher order complexes and spacing divisions

Primary stomatal complexes can give rise to higher order complexes derived from satellite meristemoids.[2] The MADS box gene *AGAMOUS-LIKE16* (*AGL16*) promotes the formation of higher order stomatal complexes and is subject to miRNA regulation.[15] The number of higher order stomatal complexes increases in plants expressing *miRNA824*-resistant *AGL16* mRNA and decreases in *agl16-1* mutant or *miR824*-overexpressing plants.[15] This result and the fact that both AGL16 and miR824 locate to stomatal complexes, but have no overlapping pattern, suggest that in wild-type stomatal development the down-regulation of *AGL16* by *miR824* reduces the number of higher order stomatal complexes.[15] The reduced number of higher order complexes in *gpa1* indicates that *GPA1* promotes the formation of higher order complexes. *GPA1* also reduces the formation of primary complexes.[10] Like that for the stomatal index, *AGB1* counteracts the action of *GPA1*.[10] In contrast to *AGL16* and *GPA1* function, both *TMM* and *SDD1* reduce the number of higher order complexes, with *tmm* and *sdd1* mutants developing an increased number of higher order stomatal complexes.[4,6]

When cells in contact with a stoma (or its precursor cell) enter into the stomatal pathway, their division planes are orientated, so that the new meristemoids are placed away from the pre-existing stomata (or stomatal precursors)[3] (Fig. 1). Analysis of serial imprints has shown that, in both *tmm* and *sdd1* mutants, the orientation of the cell division plane of these meristemoid mother cells is random, which produces meristemoids adjacent to the pre-existing stomata.[4,6] Studies of epidermal patterning in fixed plants have also shown that mutations in *YDA*, *EPF1*, *MKK4/MKK5* or *MPK3/MPK6* also trigger the formation of ectopic meristemoids.[7,11,12] Therefore, *TMM*, *SDD1*, *YDA*, *EPF1*, and *MKK4/MKK5- MPK3/MPK6* orient the cell division plane of meristemoid mother cells, preventing the development of the meristemoids in contact with the stomata or their precursors. Because this is the main mechanism that prevents stomatal cluster formation,[6] these genes play a relevant role in stomatal pattern formation. Although no additional genes have been shown to be involved in this process, it does not exclude the possibility that genes such as the *ER* family, for example, can play a role in such a process.

A model for stomatal development

Membrane receptors transmit information from the cell surface to the interior cells. Interestingly, TMM lacks a cytoplasmic domain.[8] Based on well-characterized systems as prediction models, it has been proposed that TMM, which locates to the plasma membrane, physically interacts with a partner that would provide the cytoplasmic domain required to transmit the signal across the membrane.[8,19] The molecular nature of the members of the ER-family and their domains of promoter induction, which overlap with that of *TMM* in stomatal lineage cells, suggest that they may be the proposed partner(s)[8,21,23,34] (Fig. 3). The activated receptor complexes signal to the MKK4/MKK5-MPK3/MPK6 module *via* YDA.[11,12] The activation of this signaling cascade negatively regulates SPCH activity, repressing stomatal initiation. Because primary complexes can give rise to higher order complexes when their cells resulting from the asymmetric divisions copy the meristemoid mother cell program,[2,3] the induction of SPCH activity should start in the precursor cell of the primary complex and should progress through the precursor cells of secondary (and higher order) complexes. This signaling cascade also maintains the stem cell character of the meristemoids by repressing MUTE activity, and guides the cell division orientation of cells entering the stomatal pathway in contact with stomata (or their precursors). Both mechanisms contribute to the stomatal pattern, with the second one being the main mechanism preventing stomatal cluster formation.[2,3] This cascade, therefore, controls not only stomatal initiation but also its distribution in the epidermal layer. However, how are these receptors activated? A possibility is that either an SDD1-processed molecule or the EPF1 peptide, which are expressed in stomatal lineage cells,[7,35] diffuse from the stomatal lineage cell and are recognized by the extracellular domain of the hypothetical heterodimer(s).[4,7] Both extracellular molecules seem to be involved in at least the entry into the stomatal pathway and placing meristemoids away from existing stomata.[4,7] However, the differences in the stomatal phenotypes of plants with mutations in the different genes that have a role in this signaling cascade indicate that the reality is probably more complex than this simple model.

Genetic analyses support the relationship between these factors. For example, the finding that constitutive *SDD1* expression reduces the number of stomata in the epidermis of wild-type plants (and of *sdd1* and *flp1* mutants), but is unable to suppress the *tmm-1* stomatal phenotype, suggests that *TMM* acts downstream of *SDD1*.[35] The stomatal pattern of the *epf1 tmm* double mutant, which is similar to that of *tmm* single mutant, supports that *TMM* also functions downstream of *EPF1*.[7] This is in agreement with the fact that the stomatal pattern of *35S::EPF1* plants is dependent on the *TMM* gene (and ER-family genes).[7] Interestingly, the effect of *epf1 sdd1* on the stomatal pattern is additive, indicating that *EPF1* and *SDD1* function independently, and arguing against the theory that SDD1 cleaves and activates the EPF1 peptide.[7] This is supported by the fact that the *35S::EPF1* stomatal pattern does not depend on *SDD1*: the number of stomata in *35S::EPF1* wild-type plants is similar to those in *35S::EPF1*

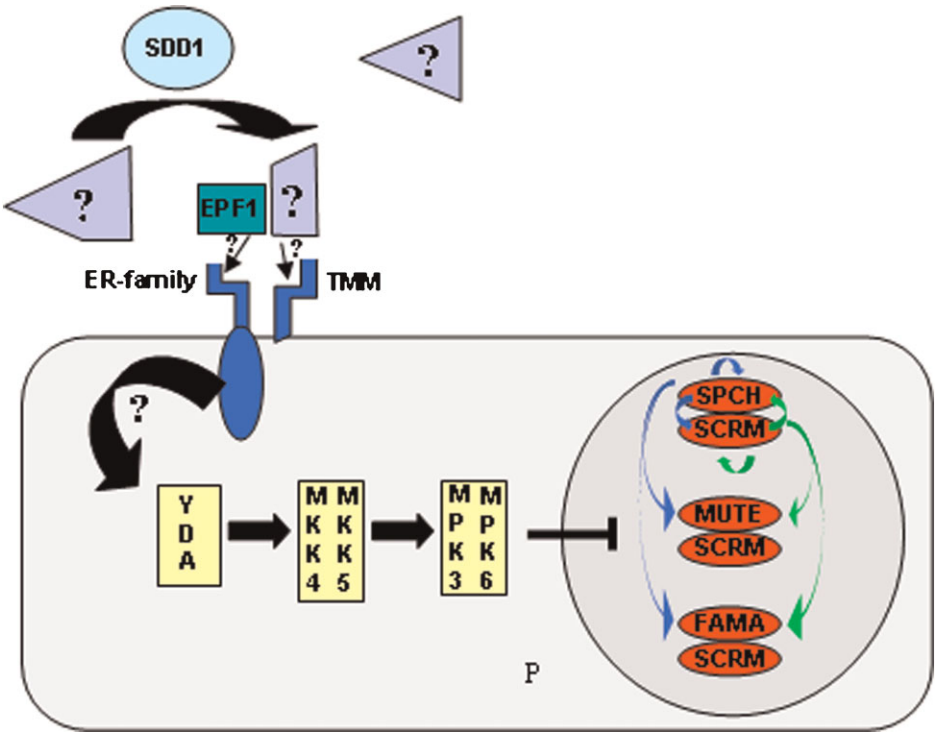

Figure 3. Model for stomatal development. At the plasma membrane, the leucine-rich repeat receptor-like protein (LRR-RLP) TMM, which lacks a cytoplasmic domain, physically interacts with members of the ER family of LRR-receptor-like kinases (LRKs). Either the EPF1 peptide or a molecule processed by the SDD1 subtilase is recognized by the extracellular domain of the hypothetical heterodimer. The activated TMM-ER complex signals to the MKK4/MKK5-MPK3/MPK6 module *via* YDA. Such activation negatively regulates the activities of the bHLH heterodimers (SPCH-SCRM/SCRM2 and/or MUTE-SCRM/SCRM2) triggering a normal stomatal development. In addition, SPCH positively autoregulates its own transcription, and it is also required to maintain the wild-type expression levels of *FAMA* and *MUTE*. SCRM/SCRM2 is also required to maintain its/their expression and also to activate both *MUTE* and *FAMA* expression. *SPCH* and *SCRM/SCRM2* regulate each other's expression?, unknown interactions or components.

sdd1.[7] The epidermal patterning phenotype of *tmm*, *yda*, and *er erl1 erl2* is epistatic to *35S::EPF1*, which suggests that *EPF1* acts upstream of *TMM*, *YDA*, and *ER* family genes.[7] The fact that the constitutive activation of YDA can dominantly suppress the defects of *tmm* suggests that YDA acts downstream of TMM.[11] The gain-of-function Nt-MEK2, the tobacco homolog of MKK4 and MKK5, rescues the *yda* stomata clustered phenotype, suggesting that YDA plays a role upstream of MKK4/MKK5-MPK3/MPK6.[12] In agreement with this finding, MPK3 and MPK6 are activated in constitutively active YDA plants.[12] The genetic interactions among *TMM* and members of the *ER* family seem to be more complex. The stomatal phenotypes arising from combinations of mutants in the *TMM* gene with single and multiple mutants in the *ER* family support the hypothesis that the affected proteins are part of one physical complex, but in several organs they can also be explained by *TMM* acting independently of the *ER* family, *e.g.*, in stems.[8,9]

Genetic analysis also supports the fact that these signaling components act upstream of *SPCH*, *MUTE*, and *FAMA*.[36] Moreover, an elegant set of experiments has shown that MPK3 and MPK6 phosphorylate to SPCH *in vitro* and modulate its activity *in vivo*[37] In addition, all the phosphorylation sites are contained within the 93-amino acid MAPK target domain of SPCH.[37] *SPCH*, *MUTE*, and *FAMA* exhibit a sequential expression pattern, with *SPCH* being expressed in all protodermal cells,[16,17] *MUTE* mainly in meristemoids[16,17] and *FAMA* in guard mother cells and developing guard cells.[14] The proteins encoded by these genes exert their actions through dimer formation with both SCRM and SCRM2, which are broadly expressed in the epidermis.[18] Although *SPCH* and *SCRM/SCRM2* are broadly expressed in the epidermal tissue, their expression patterns are not completely overlapping. *SPCH* is expressed in the entire protoderm,[16,17] and was the first of the regulatory genes to be transcribed. Kanaoka et al.[36] have proposed that *SPCH* just confers the competency to enter into the stomatal pathway and that *SCRM* and *SCRM2* are required to initiate stomatal development. In addition, these bHLH factors autoregulate their own expression and activate that of one

another (Fig. 3). Such interactions might serve to reinforce the decision to differentiate and confer stability to the phenotype. Strikingly, the mechanism regulating stomatal development seems to be similar to that in animal systems:[18,38–42] skeletal muscle differentiation depends on the interaction of several broadly expressed bHLH proteins with bHLH proteins exhibiting more restricted expression patterns.

It is largely known that some MYB proteins regulate their activity by physically interacting with proteins belonging to the bHLH family.[43] However, *FLP/MYB88* and *FAMA*, in spite of having overlapping expression patterns and playing similar roles,[13,14] do not contain the amino acid signatures required for the interaction between members of these two families.[44] Consistently, FLP and MYB88 fail to physically interact with FAMA.[14] It also seems that neither protein is required for the transcriptional activation of the other.[14] It is therefore likely that *FAMA* and *FLP/MYB88* control the transition from the guard mother cell to the guard cell independently.

Some link between G proteins and the signaling cascade has been found. Certainly, *GPA1* positively regulates *SPCH* and *MUTE* expression. *AGB1* plays a reverse role.[10] The transcriptional regulation of *ER*, *YDA*, *TMM*, and *FAMA* is not regulated by these genes.[10]

Conclusion

Evidence is accumulating that helps to resolve the central question on how stomata are specified and patterned. Perhaps the main conclusion emerging from the study of stomatal development is that many genes regulate several developmental stages. How the discrimination between the genes that must be activated in each developmental state functions is at present unknown. The study of stomatal development has unraveled not only stomatal lineage-specific processes but also processes that are common to many other cells in the regulation of proliferation *versus* differentiation or the orientation of the cell division plane. Comparison of the signaling network reviewed here with other networks regulating these common processes in other cell types will help to deepen our knowledge of these processes. Most of the results to date concern the signaling cascade from SDD1/EPF1 to the bHLH proteins involved in stomatal development, and further work will be necessary to extend it. Future challenges include, for example, unraveling the targets of the bHLH heterodimers.

Acknowledgments: This work was supported by grants from both the Communities Council of Castilla-La Mancha (grant, PCI08-0041-1136) and the Ministry of Science and Innovation of Spain (grant, BIO2008-02149).

References

1. **Taiz, L. and Zeiger, E.,** Plant Physiology, 4th edition. Sunderland, Sinauer Associates, Inc., 2006.
2. **Serna, L. and Fenoll, C.,** Stomatal development in *Arabidopsis*: how to make a functional pattern. *Trends Plant Sci* 2000. **5**: 458–561.
3. **Bergmann, D. C. and Sack, F. D.,** Stomatal Development. *Annu Rev Plant Biol* 2007. **58**: 163–181.
4. **Berger, D. and Altmann, T.,** A subtilisin-like protease involved in the regulation of stomatal density and distribution in *Arabidopsis thaliana*. *Genes Dev* 2000. **14**: 1119–1131.
5. **Serna, L., Torres-Contreras, J. and Fenoll, C.,** Clonal analysis of stomatal development and pattening in *Arabidopsis* leaves. *Dev Biol* 2002. **241**: 24–33.
6. **Geisler, M., Nadeau, J. and Sack, F. D.,** Oriented asymmetric divisions that generate the stomatal spacing pattern in *Arabidopsis* are disrupted by the *too many mouths* mutation. *Plant Cell* 2000. **12**: 2075–2086.
7. **Hara, K., Kajita, R., Torii, K. U., Bergmann, D. C. and Kakimoto, T.,** The secretory peptide gene *EPF1* enforces the stomatal one-cell-spacing rule. *Genes Dev* 2007. **21**: 1720–1725.
8. **Nadeau, J. A. and Sack, F. D.,** Control of stomatal distribution on the *Arabidopsis* leaf surface. *Science* 2002. **296**: 1697–1700.
9. **Shpak, E. D., McAbee, J. M., Pillitteri, L. J. and Torii, K. U.,** Stomatal patterning and differentiation by synergistic interactions of receptor kinases. *Science* 2005. **309**: 290–293.
10. **Zhang, L., Hu, G., Cheng, Y. and Huang, J.,** Heterotrimeric G protein α and β subunits antagonistically modulate stomatal density in *Arabidopsis thaliana*. *Dev Biol* 2008. **324**: 68–75.
11. **Bergmann, D. C., Lukowitz, W. and Somerville, C. R.,** Stomatal development and pattern controlled by a MAPKK kinase. *Science* 2004. **304**: 1494–1497.
12. **Wang, H., Ngwenyama, N., Liu, Y., Walker, J. C. and Zhang, S.,** Stomatal development and patterning are regulated by environmentally responsive mitogen-activated protein kinases in *Arabidopsis*. *Plant Cell* 2007. **19**: 63–73.
13. **Lai, L. B., Nadeau, J. A., Lucas, J., Lee, E.-K., Nakagawa, T.,** *et al.* The *Arabidopsis* R2R3 MYB proteins FOUR LIPS and MYB88 restrict divisions late in the stomatal lineage. *Plant Cell* 2005. **17**: 2754–2767.
14. **Ohashi-Ito, K. and Bergmann, D. C.,** Arabidopsis FAMA controls the final proliferation/differentiation switch during stomatal development *Plant Cell* 2006. **18**: 2493–2505.
15. **Kutter, C., Schöb, H., Stadler, M., Meins, F., Jr and Si-Ammour, A.,** MicroRNA-mediated regulation of stomatal development in *Arabidopsis*. *Plant Cell* 2007. **19**: 2417–2429.
16. **MacAlister, C. A., Ohashi-Ito, K. and Bergmann, D. C.,** Transcription factor control of asymmetric cell divisions that establish the stomatal lineage. *Nature* 2007. **445**: 537–540.
17. **Pillitteri, L. J., Sloan, D. B., Bogenschutz, N. L. and Torii, K. U.,** Termination of asymmetric cell division and differentiation of stomata. *Nature* 2007. **445**: 501–505.
18. **Kanaoka, M. M., Pillitteri, L. J., Fujii, H., Yoshida, Y., Bogenschutz, N. L.,** *et al. SCREAM/ICE1* and *SCREAM2* specify three cell-state transitional steps leading to Arabidopsis stomatal differentiation. *Plant Cell* 2008. **20**: 1775–1785.
19. **Serna, L. and Fenoll, C.,** Reinforcing the idea of signalling in the stomatal pathway. *Trends Genet* 2002. **18**: 597–600.
20. **Lease, K. A., Lau, N. Y., Schuster, R. A., Torii, K. U. and Walker, J. C.,** Receptor serine/threonine protein kinases in signalling: analysis of the erecta receptor-like kinase of *Arabidopsis thaliana*. *New Phytol* 2001. **151**: 133–143.
21. **Shpak, E. D., Berthiaume, C. T., Hill, E. J. and Torii, K. U.,** Synergistic interaction of three ERECTA-family receptor-like kinases controls *Arabidopsis* organ growth and flower development by promoting cell proliferation. *Development* 2004. **131**: 1491–1501.
22. **Ingram, G. C.,** Plant development: spacing out stomatal pores. *Curr Biol* 2005. **15**: R663–R665.
23. **Torii, K. U., Mitsukawa, N., Oosumi, T., Matsuura, Y., Yokoyama, R.,** *et al.* The Arabidopsis *ERECTA* gene encodes a putative receptor protein kinase with extracellular leucine-rich repeats. *Plant Cell* 1996. **8**: 735–746.

24. **Lukowitz, W., Roeder, A., Parmenter, D. and Somerville, C.,** A MAPKK kinase gene regulates extra-embryonic cell fate in Arabidopsis. *Cell* 2004. **116**: 109–119.
25. **Yang, K. Y., Liu, Y. and Zhang, S.,** Activation of a mitogen-activated protein kinase pathway is involved in disease resistance in tobacco. *Proc Natl Acad Sci USA* 2001. **98**: 741–746.
26. **Asai, T., Tena, G., Plotnikova, J., Willmann, M. R., Chiu, W. L.,** *et al.* MAP kinase signalling cascade in *Arabidopsis* innate immunity. *Nature* 2002. **415**: 977–983.
27. **Ren, D., Yang, H. and Zhang, S.,** Cell death mediated by MAPK is associated with hydrogen peroxide production in *Arabidopsis*. *J Biol Chem* 2002. **277**: 559–565.
28. **Yang, M. and Sack, F. D.,** The *too many mouths* and *four lips* mutations affect stomatal production in *Arabidopsis*. *Plant Cell* 1995. **7**: 2227–2239.
29. **Chinnusamy, V., Ohta, M., Kanrar, S., Lee, B. H., Hong, X.,** *et al.* ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. *Genes Dev* 2003. **17**: 1043–1054.
30. **Offermanns, S.,** G-proteins as transducers in transmembrane signalling. *Prog Biophys Mol Biol* 2003. **83**: 101–130.
31. **Jones, A. M. and Assmann, S. M.,** Plants: the latest model system for G-protein research. *EMBO Rep* 2004. **5**: 572–578.
32. **Bhave, N. S., Veley, K. M., Nadeau, J. A., Lucas, J. R., Bhave, S. L.,** *et al.* TOO MANY MOUTHS promotes cell fate progression in stomatal development of *Arabidopsis* stems. *Planta* 2009. **229**: 357–367.
33. **Pillitteri, L. J., Bogenschutz, N. L. and Torii, K. U.,** The bHLH protein, MUTE, controls differentiation of stomata and the hydathode pore in *Arabidopsis*. *Plant Cell Physiol* 2008. **49**: 934–943.
34. **Shpak, E. D., Lakeman, M. B. and Torii, K. U.,** Dominant-negative receptor uncovers redundancy in the *Arabidopsis* ERECTA leucine-rich repeat receptor–like kinase signaling pathway that regulates organ shape. *Plant Cell* 2003. **15**: 1095–1110.
35. **von Groll, U., Berger, D. and Altmann, T.,** The subtilisin-like serine protease SDD1 mediates cell-to-cell signaling during *Arabidopsis* stomatal development. *Plant Cell* 2002. **14**: 1527–1539.
36. **Serna, L.,** bHLH proteins know when to make a stoma. *Trends Plant Sci* 2007. **12**: 483–485.
37. **Lampard, G. R., MacAlister, C. A. and Bergmann, D. C.,** *Arabidopsis* stomatal initiation is controlled by MAPK-mediated regulation of the bHLH SPEECHLESS. *Science* 2008. **322**: 1113–1116.
38. **Murre, C., McCaw, P. S. and Baltimore, D.,** A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. *Cell* 1898. **56**: 777–783.
39. **Sun, X. H. and Baltimore, D.,** An inhibitory domain of E12 transcription factor prevents DNA binding in E12 homodimers but not in E12 heterodimers. *Cell* 1991. **64**: 459–470.
40. **Massari, M. E. and Murre, C.,** Helix-loop-helix proteins: Regulators of transcription in eucaryotic organisms. *Mol Cell Biol* 2000. **20**: 429–440.
41. **Gray, J. E.,** Plant development: three steps for stomata. *Curr Biol* 2007. **17**: R213–R215.
42. **Serna, L.,** Emerging parallels between stomatal and muscle cell lineages. *Plant Physiol* 2009. **149**: 1625–1631.
43. **Serna, L. and Martin, C.,** Trichomes: different regulatory networks lead to convergent structures. *Trends Plant Sci* 2006. **11**: 274–280.
44. **Barton, M. K.,** Making holes in leaves: promoting cell state transitions in stomatal development. *Plant Cell* 2007. **19**: 1140–1143.