# Stomatal Development and Perspectives toward Agricultural Improvement

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Stomata are small pores on the surface of land plants that facilitate gas exchange—acquiring $CO_2$ from surrounding atmosphere and releasing water vapor. In adverse conditions, such as drought, stomata close to minimize water loss. The activities of stomata are vital for plant growth and survival. In the last two decades, key players for stomatal development have been discovered thanks to the model plant *Arabidopsis thaliana*. Our knowledge about the formation of stomata and their response to environmental changes are accumulating. In this review, we summarize the genetic and molecular mechanisms of stomatal development, with specific emphasis on recent findings and potential applications toward enhancing the sustainability of agriculture.

Stomata are small pores found on the epidermis of the aerial part of plants, which have an important role in efficient photosynthesis and water use via controlling gas exchange and transpiration. This activity balances carbon and water level in the atmosphere, thus playing a critical role in maintaining our ecosystem and greatly contributing to crop production. To facilitate gas exchange while avoiding desiccation, plants alter stomatal apertures in response to environmental stresses, and in the long run adjust the number of stomata (Hetherington and Woodward 2003; Shimazaki et al. 2007; Hu et al. 2010; McKown and Bergmann 2018). As the world population continues to grow, meeting the demand for food production has become a pressing issue for food security and sustainability of our society (life sustainability) (Godfray et al. 2010; Tilman et al. 2011; Long et al. 2015). Stomatal function is highly relevant for photosynthetic productivity and plant water use efficiency to improve productivity to meet such demands (Lawson and Vialet-Chabrand 2018).

In the dicotyledonous plant *Arabidopsis*, stomata are formed via a series of cell divisions and cell fate determination processes (Qi and Torii 2018; Zoulias et al. 2018). The initial state is undifferentiated epidermal cells, known as protodermal cells, that enter the stomatal lineage by adopting a meristemoid mother cell (MMC) identity. The MMC divides asymmetrically to generate two distinct cell types, a meristemoid and its larger sister cell, called stomatal-lineage ground cell (SLGC). The meristemoid follows either destination: differentiates into a guard mother cell (GMC) or progress several additional rounds of amplifying asymmetric divisions to generate more SLGCs while renewing itself. On the other hand, the SLGCs undergo spacing division to create a satellite meristemoid or al-

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ternatively differentiate into a jigsaw puzzle–shaped pavement cell. Finally, the GMC divides symmetrically into paired guard cells (GCs) to form stomata (Fig. 1A). These paired GCs are crucial for functioning as a turgor-driven valve in response to internal and external stimuli (Shimazaki et al. 2007).

Cereal crops, such as rice, wheat, and corn produce grains and are our major source of food (Godfray et al. 2010; Long et al. 2015). In these monocotyledonous grass species, a stomatal complex is composed of characteristic dumbbell-shaped, paired GCs, each accompanying a subsidiary cell (Hepworth et al. 2018; McKown and Bergmann 2018). In the grass model plant *Brachypodium distachyon*, stomatal development starts from the establishment of a stomatal file (Fig. 1B). A single asymmetric cell division within the stomatal cell file generates a GMC. While the GMC is maturing, the cell file adjacent to the stomatal cell file starts to acquire a subsidiary mother cell (SMC) identity. Before the GMC divides symmetrically to form a stoma, SMC divides asymmetrically. A daughter cell adjacent to the GMC retains the SMC identity and eventually differentiates into a subsidiary cell comprising a functional stomatal complex unique in grass species. The thickness and the composition of GC walls as well as the specialized function of subsidiary cells are likely to contribute to the highly adaptive nature of grass plants to dry conditions (Franks and Farquhar 2007; Raissig et al. 2017). However, besides the subsidiary cell formation and arrangement of stomata, the core

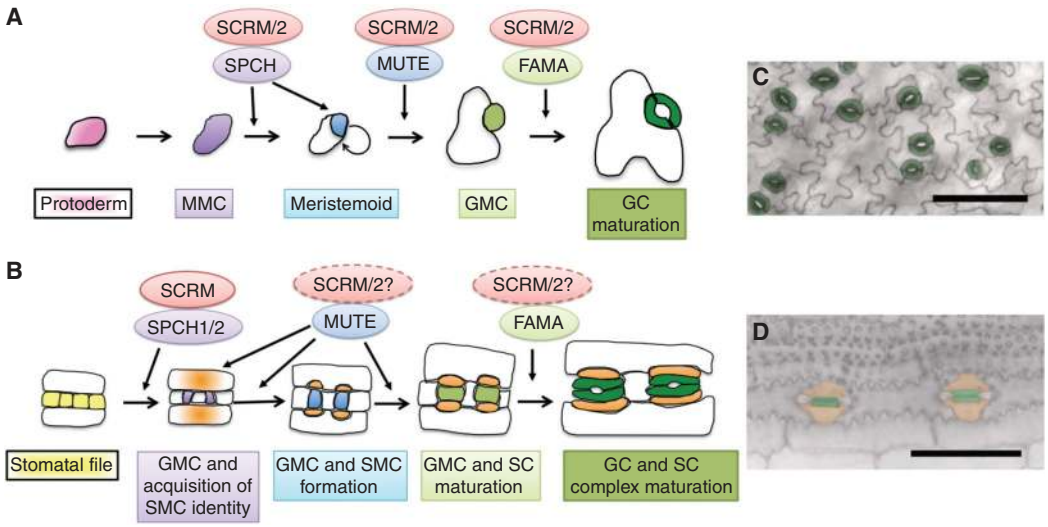


**Figure 1.** Schematic diagrams of stomatal development in dicot and monocot plants. (*A*) Stomatal development in dicots. Protodermal cell (pink) acquires the MMC identity (purple) and divides asymmetrically to generate a meristemoid (blue) and SLGC (white). The meristemoid differentiates into GMC (light green), which divides symmetrically to generate the paired guard cells (green). In each differentiation state, master bHLH transcription factors, SPCH, MUTE, and FAMA govern transition of cell state together with partner bHLH proteins SCRM/2 in *Arabidopsis*. (*B*) Stomatal development in monocot grass species. Establishment of stomatal file (yellow) occurs first, and subsequently asymmetric cell division follows to generate GMC (purple then blue). The cell files adjacent to the stomatal file acquire SMC identity and asymmetrically divides to form SMC (orange). GMC and SMC undergo maturation (light green and orange) to form a functional stomatal complex composed of GCs and SCs (green and orange). In each differentiation state, master bHLH transcription factors, SPCH1/2, MUTE, and FAMA, play pivotal roles in rice, maize, and *Brachypodium* (Liu et al. 2009; Raissig et al. 2017). SCRM protein has been shown to play a role in an establishment of GMC in *Brachypodium* (Raissig et al. 2016). It is not known whether SCRM (dashed, red oval) also functions as partner protein together with MUTE or FAMA. Examples of stomata in the (*C*) *Arabidopsis* and (*D*) rice, with stomata false colored in green and subsidiary cells in orange. Scale bars, 100 μm.

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anatomical feature of stomata seems to be conserved across the species (for instance, *Arabidopsis* and *Brachypodium*).

The molecular identifications of genes regulating stomatal development now enable us to explore whether manipulation of stomatal numbers and density could also improve plant productivity and drought tolerance. In this review, we focus on the molecular basis of stomatal development and discuss the potential application of these findings toward improved water use efficiency and/or productivity of agriculturally relevant species.

## Transcriptional Control of Stomatal Development

Extensive molecular-genetic studies in stomatal development using the model plant *Arabidopsis* have identified key players of stomata formation. At the molecular level, a series of cell-state transitions from the protodermal cell into a GC is controlled by sequential actions of three basic helix-loop-helix (bHLH) transcription factors SPCHLESS (SPCH), MUTE, and FAMA, each forming a heterodimer with the partner bHLH proteins, SCRM1/ICE1, and SCRM2 (Fig. 1A) (Chinnusamy et al. 2003; Ohashi-Ito and Bergmann 2006; MacAlister et al. 2007; Pillitteri et al. 2007; Kanaoka et al. 2008). SPCH specifies the initiation of stomatal cell lineages: an acquisition of the MMC identity and a subsequent entry of asymmetric cell division. SPCH also drives amplifying asymmetric divisions (MacAlister et al. 2007). The loss-of-function mutant *spch* fails to form any stomatal precursor cells, and results in the epidermal solely composed of pavement cells (MacAlister et al. 2007). The chromatin immunoprecipitation analysis of SPCH-binding targets predicted that the expression of about 400 of the *Arabidopsis* genes can be directly regulated by the SPCH protein (Lau et al. 2014). These include genes that are involved in stomatal development, such as *SCRM/ICE1*, *SCRM2*, *TOO MANY MOUTHS* (*TMM*), and *ERECTA LIKE 2* (*ERL2*) (Geisler et al. 2000; Shpak et al. 2005; Kanaoka et al. 2008).

MUTE, on the other hand, terminates asymmetric divisions of a meristemoid and promotes its differentiation into a GMC. The loss-of-function mutant of *MUTE* produces no stomata, but meristemoids keep dividing to form a rosette-like cluster of unequally divided cells (Pillitteri et al. 2007). A recent, genome-wide study of MUTE targets revealed that MUTE switches the initial stomatal precursor state to the later state by inhibiting the early patterning gene, *EPIDERMAL PATTERNING FACTOR2* (*EPF2*), while inheriting the shared targets between SPCH and MUTE, including *SCRM/ICE1, SCRM2, TMM, ERL2*, and so on (Han et al. 2018). Furthermore, MUTE coordinates the expression of cell-cycle regulators, including cyclin-dependent kinase gene *CDKB1;1* and both A-type and D-type cyclins, as well as the negative regulators of these cell-cycle genes, *FAMA* and *FOUR LIPS* (*FLP*), to ensure the single symmetric division of GMC to form a functional stoma with paired GCs (Han et al. 2018; Weimer et al. 2018).

The final step of stomatal differentiation is regulated by FAMA, which restricts the GMC division and promotes GC differentiation (Ohashi-Ito and Bergmann 2006). In addition, the interplay of FAMA, FLP, and MYB88 (a paralogous protein of FLP), together with their physical interacting partner protein RETINOBLASTOMA RELATED (RBR), is required for GC fate maintenance (Lee et al. 2014; Matos et al. 2014). Loss of *FAMA* function progressively continues GMC divisions and fails to differentiate the GCs, thus producing caterpillar-like cell tumors (Ohashi-Ito and Bergmann 2006). SCREAM/ICE1 and SCRM2, the partner bHLHs for SPCH, MUTE, and FAMA, are expressed in all stomatal-linage cells, and consistently, their progressive loss-of-function recapitulates *fama*, *mute*, and *spch* loss-of-function phenotypes (Kanaoka et al. 2008). In contrast, the dominant form of *SCRM* (*scrm-D*) causes the epidermis solely composed of stomata because of its insensitivity to an inhibitory cell signaling (Kanaoka et al. 2008; Horst et al. 2015).

Importantly, these bHLH genes are conserved across the land plants, from moss, ferns, to flowering plants, including monocots (Liu et al. 2009; Peterson et al. 2010; Ran et al. 2013; Raissig et al. 2016; Chater et al. 2017; Qu

et al. 2017). In rice, maize, and *Brachypodium*, there are two copies of *SPCH* and a single copy of *MUTE* and *FAMA* in the gnome (Qu et al. 2017). In rice, all of these bHLH proteins, OsSPCH1/2, OsMUTE, and OsFAMA possess a crucial role in stomatal development (Liu et al. 2009). For instance, loss-of-function mutants of *OsSPCH2* (*osspch2-1*) exhibit reduced stomatal density, whereas loss-of-function mutants of *OsFAMA* (*osfama-1*) produce immature GCs. The overexpression of *OsMUTE* in *Arabidopsis* phenocopies the *AtMUTE* overexpression (Liu et al. 2009). From these observations, it has been proposed that (1) OsSPCH is required for establishing the stomatal cell file; (2) OsMUTE regulates the transition from stomatal file to GMC; and (3) OsFAMA regulates the maturation of GCs and stomatal complexes. In *Brachypodium*, BdSPCH1 and BdSPCH2 redundantly drive stomatal development because loss-of-function of both *BdSPCH1/2* (*bdspch1 bdspch2*) confers a stomata-less phenotype resembling the *Arabidopsis spch* mutant (Fig. 1B) (Raissig et al. 2016). Interestingly, it has been shown that BdMUTE controls subsidiary cell differentiation in a non-cell-autonomous fashion: the movement of BdMUTE from the stomatal precursor cell to the neighboring cell file triggers the differentiation of subsidiary cells, specifying the eventual formation of grass-specific, subsidiary-GC complexes (Fig. 1B) (Raissig et al. 2017). It will be interesting to investigate the molecular characteristics of BdMUTE that enable cell-to-cell trafficking, and how the stomatal bHLH regulatory networks evolved in dicot and monocot plants.

## Ligands and Receptors that Control Stomatal Patterning

Proper stomatal density, spacing, and patterning are critical for optimizing the stomatal function. Normally, stomata maintain at least one non-stomatal cell type in between, and this is defined as the "one-cell spacing rule" (Dow et al. 2014; Dow and Bergmann 2014). Characterizing the molecular makeup of this one-cell spacing rule has highlighted the roles of peptide hormones and receptor kinase signaling cascades in stomatal patterning (Torii et al. 1996; Geisler et al. 2000; Nadeau and Sack 2002; Shpak et al. 2004, 2005; Hara et al. 2007, 2009; Hunt et al. 2010; Sugano et al. 2010). During leaf development in *Arabidopsis*, three plant peptide hormones belonging to the EPIDERMAL PATTERNING FACTOR (EPF) family, EPF1, EPF2, and EPFL9/STOMAGEN play a pivotal role (Rychel et al. 2010). Among them, EPF1 and EPF2 act as negative regulators of stomatal development (Hara et al. 2007, 2009; Hunt and Gray 2009). However, the findings that single mutants of *epf1* and *epf2* exhibit distinct phenotypes, and the lack of phenotypic rescue when EPF1 and EPF2 were reciprocally expressed into their respective mutant backgrounds through promoter swapping suggest that the functions of EPF1 and EPF2 are not identical (Hara et al. 2009). *EPF2* expression is detected in stomatal precursor cells, MMCs, and meristemoids earlier than the onset of *EPF1* expression in late meristemoids and GMCs (Hara et al. 2007, 2009).

In contrast to EPF1 and EPF2, EPFL9/STOMAGEN acts as a positive regulator of stomatal development (Hunt et al. 2010; Kondo et al. 2010; Sugano et al. 2010; Ohki et al. 2011; Lee et al. 2015). Interestingly, *EPFL9/STOMAGEN* is expressed in immature mesophyll cells, but not in the epidermis. EPFL9/STOMAGEN competes for receptor binding with EPF1 and EPF2 and, thus, blocking the downstream signal transduction (Ohki et al. 2011; Lee et al. 2012, 2015; Lin et al. 2017). In fact, if the STOMAGEN signal is high, epidermal cells form clustered stomata, whereas when the EPF1 and EPF2 signal is high, epidermal cells form less or no stomata on the leaf surface, suggesting that the intricate balance of EPF1, 2, and EPFL9/STOMAGEN fine tunes proper stomatal patterning (Hara et al. 2007, 2009; Hunt and Gray 2009; Ohki et al. 2011; Lee et al. 2012, 2015; Lin et al. 2017).

EPF proteins are processed to become a mature active form. Two subtilisin-like serine protease, STOMATAL DENSITY AND DISTRIBUTION1 (SDD1) and $CO_2$ RESPONSE SECRETED PROTEASE (CRSP) are known to influence stomatal development (Berger and Altmann 2000; Von Groll et al. 2002; Engineer

et al. 2014). Like *EPF1*, *SDD1* is expressed in meristemoid and GMC. The loss-of-function mutant of *SDD1* (*sdd1-1*) shows higher stomatal density than the wild type, thus suggesting its possible role in maturation of EPF peptides. However, so far there is no evidence showing that this protease cleaves EPF peptides to form a mature ligand. In contrast, CRSP has been shown to cleave EPF2 propeptides (Engineer et al. 2014). In the elevated $CO_2$ condition (500 ppm), wild-type *Arabidopsis* plants exhibit a reduced stomatal index (i.e., stomata number per the number of epidermis cells; Engineer et al. 2014). Expressions of *EPF2* and CRSP are induced by high $CO_2$ treatment. Loss-of-function mutants of either of these genes confer a higher stomatal index than the wild type under high $CO_2$ conditions. Moreover, EPF2, but not EPF1 or STOMAGEN, can be cleaved by CRSP to form mature peptides in vitro. Together, these data suggest that CRSP specifically cleaves EPF2 to produce mature, active peptides, thereby inhibiting stomatal development under high $CO_2$ conditions in *Arabidopsis* (Engineer et al. 2014).

Genetic, structural, and biochemical evidence show that these peptide hormones directly bind to a receptor complex composed of leucine-rich repeat receptor kinases (LRR-RKs), ERECTA (ER), ER-LIKE 1 (ERL1), ERL2, and LRR receptor protein, TOO MANY MOUTHS (TMM) (Hara et al. 2007, 2009; Sugano et al. 2010; Ohki et al. 2011; Lee et al. 2015). The ligand binding triggers association of ER-family/TMM receptor complex with SOMATIC EMBRYOGENESIS RECEPTOR KINASEs (SERKs) to transmit the cellular signal (Fig. 2; Shpak et al. 2005; Lee et al. 2012, 2015; Meng et al. 2015; Lin et al. 2017; Qi et al. 2017). ER, ERL1, and ERL2 exhibit partial redundancy; excessive overproduction and clustering of stomata occurs in the absence of all three *ER*-family genes (Shpak et al. 2005). The structural analysis of ERL1 and TMM1 complex as well as binding assays in vitro and in planta indicate that ERs and TMM form a constitutive receptor complex, creating a binding pocket for EPF1 and EPF2 (Shpak et al. 2005; Lee et al. 2012, 2015; Meng et al. 2015; Lin et al. 2017; Qi et al. 2017). Moreover, that EPFL9/STOMAGEN competes with EPF1 and EPF2 for this binding pocket (Ohki et al. 2011; Lee et al. 2015).

The *tmm* loss-of-function mutant produces clustered stomata in the leaf but displays no stomata in hypocotyls (Geisler et al. 1998). The rather peculiar, organ-specific *tmm* effects can be explained by the binding preference of additional EPF ligands to the ER-TMM complex. A subclade of *EPF* family members, namely, *EPFL6/CHALA*, *EPFL5/CLL1*, and *EPFL4/CLL2* are expressed in the hypocotyl and/or stem endodermis (Abrash and Bergmann 2010; Abrash et al. 2011; Uchida et al. 2012). Indeed, EPFL4/5/6 can bind to ER in the absence of TMM (Lin et al. 2017). Moreover, the loss-of-function triple mutant of *EPFL4/5/6* induced severe stomatal clusters in *tmm* hypocotyl, thus phenocopying *er erl1 erl2 tmm* quadruple mutant hypocotyl epidermis (Shpak et al. 2005; Abrash et al. 2011). Normally, EPFL4/6 expressions are restricted in the endodermis and they function as a ligand for the phloem-expressed population of ER to promote inflorescence elongation (Uchida et al. 2012). These pieces of evidence, collectively, emphasize the role of TMM as a "buffer" for EPF peptide signaling. TMM is specifically expressed in the epidermis (Nadeau and Sack 2002; Shpak et al. 2005) and guides the epidermal-expressed ER-family receptors to "correctly" perceive positional cues (EPF1, 2, and EPFL9/STOMAGEN) for stomatal development. In the absence of TMM, however, internally expressed EPFL4/6, which do not normally play a role in stomatal development, activate the ER-family receptors in the epidermis to suppress stomatal differentiation in the stems and hypocotyls. Consistent with this scenario, the ectopic *EPFL4/6* overexpression represses the stomatal development in cotyledons (Abrash et al. 2011; Uchida et al. 2012).

The third receptor component, SERKs, function as coreceptors for a broader range of receptor-kinase signaling including immunity, brassinosteroid hormone signaling, organ abscission, and root development (Ma et al. 2016). In stomatal development, SERK1, SERK2, SERK3 (also known as BRASSINOSTEROID INSENSITIVE1-ASSOCIATED KINASE [BAK1]), and SERK4 play a role in EPF peptide

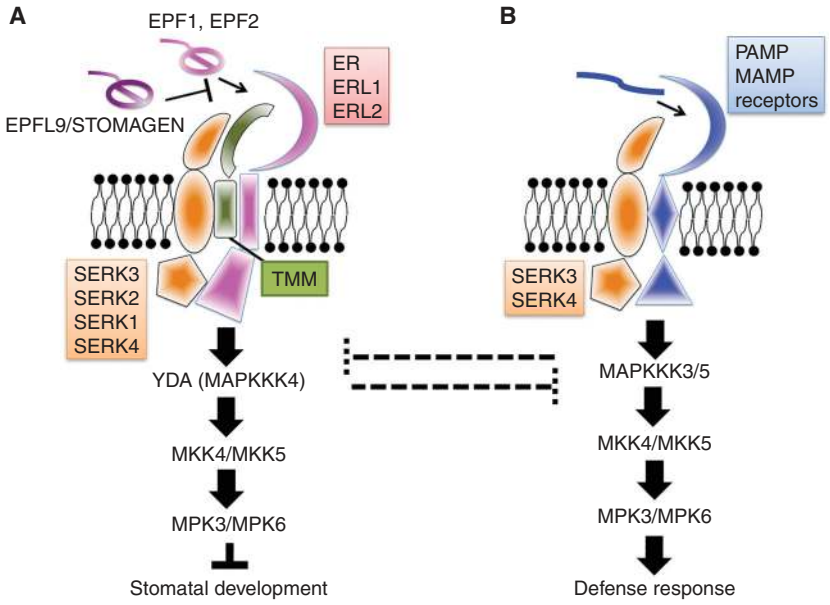


**Figure 2.** A model for the leucine-rich repeat receptor kinase (LRR-RK) complexes, which mediate stomatal development and defense responses. (*A*) In the ERECTA (ER) pathway, endogenous ligands, EPIDERMAL PATTERNING FACTORs (EPF1), and (EPF2), bind the ER-TOO MANY MOUTHS (TMM)-SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) complex to transduce downstream signaling cascades composed of the YODA (YDA)-MKK4/5-MPK3/6 module. EPFL9/STOMAGEN antagonistically inhibits EPF1 and/or EPF2 binding to receptor complex. (*B*) In the pattern-triggered immunity, external ligands, pathogen-associated molecular patterns (PAMPs/MAMPs) such as flagellin and an elongation factor bind the PAMP/MAMP receptor-SERK complex to transduce downstream signaling cascades composed of mitogen-activated protein kinase kinase kinase (MAPKKK)3/5-MKK4/5-MPK3/6 module. It has been proposed that YDA-MKK4/5-MPK3/6 and MAPKKK3/5-MKK4/5-MPK3/6 modules antagonistically interact to regulate precise signal output (Sun et al. 2018).

perception along with ERs and TMM (Meng et al. 2015). The *serk1-1 serk2-1 bak1-4* triple mutant showed a clustered stomata phenotype resembling *er erl1 erl2* triple mutants. Interestingly, different alleles of *BAK1*, *bak1-4*, and *bak1-5*, can discriminate immune- and BR-signaling pathways through specific phosphorylation codes of partner LRR-RKs: *bak1-5* is impaired in immunity response but not in BR signaling (Schwessinger et al. 2011; Perraki et al. 2018). The higher order *serk* mutants of *serk1-1 serk2-1 bak1-5 serk4-1* exhibited severe stomatal clustering while retaining nearly normal BR response (Meng et al. 2015), thus refuting the hypothesis that stomatal phenotypes of *serks* are a result of impaired BR signaling. Indeed, biochemical analysis demonstrated that BAK1 interacts with ER or ERL1 in planta. Moreover, coimmunoprecipitation experiments using protoplasts showed that SERKs form a ligand-induced receptor complex with ER/ERLs (Meng et al. 2015).

Orthologous genes of *EPF*s and *TMM/ER/SERK* receptors can be found in grass genomes (Rychel et al. 2010; Takata et al. 2013; Aan den Toorn et al. 2015; Lee et al. 2015; Hepworth et al. 2018). Several lines of evidence imply that this signaling module is conserved in grass species. For instance, overexpression of *EPF1* or *EPF2* orthologs in barley and rice or disruption of the rice *EPFL9/STOMAGEN* ortholog cause decreased stomatal production (Hughes et al. 2017; Yin et al. 2017; Caine et al. 2019). Another piece of evidence is that the *OsEPF1* expression under the *AtEPF2* promoter partially rescued the *epf2* mutant phenotype in *Arabidopsis*

(Caine et al. 2019). Therefore, it is worth investigating whether those orthologous genes indeed have similar function in cell–cell signaling of stomatal development in grass spices.

## Cellular Signaling: Connecting Extrinsic Signals and Intrinsic Polarity

Genetic evidence revealed sequential phosphorylation steps comprised of a MAP kinase cascade at the downstream of EPF and TMM/ER/SERK ligand-receptor complex. This cascade starts from MAPKKK gene encoding YDA followed by MAPKK genes MKK4/5 and MAPKs MPK3/6 (Fig. 2A; Bergmann 2004; Wang et al. 2007). Complete loss of function of any of these genes causes exaggerated stomatal clusters. In contrast, if their constitutive active forms are expressed, stomata formation is strongly inhibited (Bergmann 2004; Wang et al. 2007; Lampard et al. 2009, 2014). In addition, MAP KINASE PHOSPHATASE1 (MKP1), which is expressed and functions at an early stage of stomatal development, is shown to control MPK3 and MPK6 phosphorylation status downstream of YDA (Tamnanloo et al. 2018). Loss of function of *MKP1* causes hyperactivation of stomatal MAPK in *Arabidopsis* and produces less stomata with clusters of small cells on leaf epidermis. These observations highlight that not only the phosphorylation of MPK3 and MPK6 but also the dephosphrylation by MKP1 is critical for proper formation and distribution of stomata.

One of the prominent downstream targets of MAP kinase cascade in stomatal development is the SPCH protein. SPCH is phosphorylated at Ser193, Ser211, Thr214, Ser219, and Ser255 positions (first to fifth positions) of its amino acid sequence by MPK3 and MPK6 in vitro and these sites seem to be critical for SPCH regulation (Lampard et al. 2008). The sequence containing these amino acid residues was defined as MAPK target domain (MPKTD). The MPKTD is unique to the SPCH protein among the three master bHLHs. SPCH phosphorylation confers complex effects on its protein function. In brief, phosphorylation of Ser193 contributes to SPCH activity while phosphorylation of other residues leads to inactivity via probably change in protein stability or affinity to functional partners such as SCRM (Lampard et al. 2008). Later studies demonstrated that SPCH can be subjected to phosphorylation by multiple, different classes of protein kinases, including glycogen synthase kinase (GSK), BRASSINOSTEROID INSENSITIVE2 (BIN2) (Gudesblat et al. 2012) and cyclin-dependent kinase A1 (CDKA;1) (Yang et al. 2014). These findings further emphasize SPCH protein activity/stability as a key regulatory point by which environmental and physiological signals intersect.

During the asymmetric amplifying division, only one daughter cell, a meristemoid, retains high SPCH expression whereas its sister cell, SLGC, eventually loses SPCH expression. BREAKING OF ASYMMETRY IN THE STOMATAL LINEAGE (BASL) is involved in controlling SPCH activity during the asymmetric cell division via recruiting the MAP kinase cascade module (Dong et al. 2009; Zhang et al. 2015, 2016). In a *basl* loss-of-function mutant, the SPCH protein level is maintained in both meristemoids and SLGCs. BASL is phosphorylated by MPK3/6, and this phosphorylation is essential for correct BASL localization and its function (Zhang et al. 2015, 2016). BASL directly associates with YDA, and thus acts as a scaffold protein to polarly localize the MAPK complex to a cell cortex in SLGCs. This polar localization is necessary for negative control of SPCH protein levels in SLGCs (Zhang et al. 2015). It remains unclear, however, how polarly tethered MAPK activities in SLGC can downregulate SPCH protein in the nucleus.

Recent findings revealed that POLAR proteins (Pillitteri et al. 2011) and family members function as scaffold proteins to regulate the polarity of the GSK3/SHAGGY-like kinase, BIN2, and its close homologs during stomatal asymmetric cell divisions (Houbaert et al. 2018). BIN2 phosphorylates YDA, MKK4/5, and SPCH proteins to suppress activity of these proteins (Gudesblat et al. 2012; Kim et al. 2012; Khan et al. 2013). Therefore, it has been a mystery how BIN2 both positively and negatively regulates asymmetric cell division: specifically, phosphorylation of the MAP kinase module and SPCH proteins, respectively. Here, the study

found that POLAR proteins function as a scaffold to polarly confine BIN2 to the cell cortex together with BASL, thus enabling the attenuation of the MAP kinase module, which colocalized with the BIN2-POLAR-BASL module at the cell cortex before asymmetric cell division. This regulation alleviates inhibition of SPCH by BIN2 and results in accumulation of SPCH proteins in the nucleus to drive the asymmetric cell division (Houbaert et al. 2018). Interestingly, phosphorylation of POLAR by BIN2 increases its stability, suggesting the involvement of BIN2 in POLAR protein turnover (Houbaert et al. 2018). This finding further implies that BIN2 and POLAR fine tune each other during stomatal development

All residues in MPKTD of the SPCH protein, except for the fifth residue of the phosphorylation sites, are conserved in one of two copies of *SPCH* genes in rice and maize (Lampard et al. 2008). Likewise, *YDA* orthologs can be found in grass species, and a hypomorphic allele of *BdYDA1* gene *bdyda1-1* shows defects in GMC specification and SC formation in which one spacing rule is broken; thus the stomata and subsidiary cells form a clustered complex (Abrash et al. 2018). These findings imply that the core MAP kinase module in the stomatal developmental pathway is also conserved in grass species.

## Stomatal Development and Environmental Factors

Environmental signals or external signals such as light, $CO_2$, osmotic stress, and water alter stomatal development (for review, see Qi and Torii 2018). High temperature and pathogen infections also influence stomatal development, and their molecular intersections with the stomatal development pathways have been reported recently. In *Arabidopsis*, transferring a plant from 22°C ambient temperature to higher 28°C results in an elongation of the hypocotyl and petioles to form an open rosette structure with improved cooling capacity (Quint et al. 2016). Interestingly, this treatment also induces reduction of stomatal density and index (Crawford et al. 2012; Lau et al. 2018). The heat stress signaling directly feeds into the core component of stomatal development, *SPCH* (Lau et al. 2018). *SPCH* promoter activity and expression decreased upon higher temperature conditions, implying the regulation at the transcriptional level. Indeed, a core transcription factor of high-temperature signaling, PHYTOCHROME-INTERACTING FACTOR 4 (PIF4), directly binds to the *SPCH* promoter to repress its expression at high temperatures (Lau et al. 2018). *PIF* family genes are also known to negatively regulate light signaling, and *PIF4* promotes stomatal development under high light conditions (Casson et al. 2009). However, among the *PIF* genes, only *PIF4* is involved in a high-temperature response (Koini et al. 2009; Leivar and Quail 2011). The high-temperature-mediated inhibition of stomatal development is impaired in *pif4* mutants regardless of photoperiods (i.e., short days or long days) (Lau et al. 2018). In turn, SPCH protein can directly bind and repress the expression of the *PIF4* gene, suggesting that PIF4 and SPCH form a negative feedback loop (Lau et al. 2018). This loop may contribute to the control of stomatal development in fluctuating temperature in nature.

It is well known that microbial infections trigger stomata closure; this may not be surprising because stomatal pores serve as bacterial entry gates (Melotto et al. 2006, 2017). Whether plants also reduce a number of the entry gates, stomata, as a long-term solution to pathogen infection, is a matter of debate. To countervail plant defense systems, pathogens have elaborate strategies to interfere with the host immune system (He et al. 2006; Torres et al. 2006; Hann and Rathjen 2007). For instance, gram-negative bacteria *Pseudomonas syringae* inject effector proteins through their type III secretion system to promote infection by blocking the plant innate immune system (Alfano and Collmer 2004; Abramovitch et al. 2006; Grant et al. 2006; Varden et al. 2017). Intriguingly, inducible overexpression of two effector proteins of *P. syringae* pathovar tomato strain, DC3000 (Pst DC3000), AvrPto and AvrPtoB, produces a severe clustered stomata phenotype in *Arabidopsis* (Meng et al. 2015). Together with the previous observations that SERK3/BAK1 is a target of AvrPto and AvrPtoB (Shan et al. 2008; Cheng et al. 2011; Meng et al. 2015), the finding led to

unravel the role of SERKs in stomatal development as a coreceptor with the ER-TMM complex (Fig. 2). *ER* affects resistance to bacterial wilt pathogen, *Ralstonia solanacearum* (Godiard et al. 2003). More recently, higher-order loss-of-function mutants of *tmm er erl1 erl2* and *er bak1* are shown to be more susceptible to necrotrophic fungus *Plectosphaerella cucumerina* BMM (PcBMM; Jordá et al. 2016; Sopeña-Torres et al. 2018). These findings imply a potential involvement of ER/TMM receptor signaling in defense signaling or, alternatively, the importance of enforcing the one-cell spacing rule to reduce bacterial infection.

The above study proposed that the ER/TMM/SERK receptor complex transduces defense signaling via a downstream YDA-MKK4/MKK5-MPK3/6 module (Jordá et al. 2016; Sopeña-Torres et al. 2018). In contrast, other studies have shown that the MKKK3/MKKK5-MKK4/MKK5-MPK3/6 module plays critical roles in the defense response triggered by a variety of elicitors, including chitin (Yamada et al. 2016), flg22, elf18, nlp20, and pep23 (Sun et al. 2018). How can these two distinct MAP kinase cascades operate in plants to control immunity and stomatal development? A recent report highlights a possible, antagonistic interaction between stomatal YDA-MKK4/MKK5-MPK3/6 and pathogen mediated PAMP (pathogen-associated molecular patterns)-triggered immunity MKKK3/MKKK5-MKK4/MKK5-MPK3/6 modules (Fig. 2; Sun et al. 2018). Here, Agrobacterium-mediated transient overexpression of MAPKKK3 or MAPKKK5 together with YDA and MKK5 strongly inhibits YDA and MKK5 interaction in *Nicotiana benthamiana*. A similar result was obtained by *YDA* overexpression—the interaction between MAPKKK3 or MAPKKK5 and MKK5 are strongly inhibited. Although stomatal phenotypes were not examined in depth in this study, the observed antagonism emphasizes the intricate regulation of these two signaling pathways. In nature, plants are constantly challenged by multiple pathogens. Hence, it remains an important question how signaling in stomatal development and immunity is coordinated to facilitate optimal signal output.

## Impact of Stomatal Development on Gas Exchange, Plant Photosynthetic Capacity, and Water-Use Efficiency

Stomata facilitate gas and water exchange by opening or closing via GC expansion or shrinkage (Shimazaki et al. 2007). This activity can be calculated as stomatal conductance (gs; Gerosa et al. 2012). By developing the prediction methods to calculate stomatal conductance under different environmental conditions using *Arabidopsis* as a model, Dow et al. (2014a,b) showed that number, size, and distribution of stomata (stomatal trait) affect gs. Here, plants with higher number of stomata tend to have higher gs, although severe stomatal clustering impaired normal stomatal function and reduced its maximum gs (Dow et al. 2014a,b), therefore emphasizing the importance of the one-cell spacing rule.

In grass species, alternating stomatal density affects the fitness of plants under challenging conditions (Hughes et al. 2017; Caine et al. 2019). For example, overexpression of barley *Hordeum vulgare EPF1* (*HvEPF1OE*) causes reduction in stomatal density, stomatal index, and stomatal length, all of which affect gs (Dow et al. 2014a,b; Hughes et al. 2017). Consequently, the overexpression lines have a lower gs and a lower $CO_2$ assimilation rate, but higher intrinsic water use efficiency (the value of assimilation divided by gs) compared to the wild type in a well-watered condition (60% soil water content; Hughes et al. 2017). There is no difference in the photosynthetic capacity (by measuring the light-adapted quantum yield of PSII) between the wild-type and *HvEPF1OE* lines in a well-watered condition. Strikingly, the *HvEPF1OE* plants showed significant enhancement in rates of photosynthesis in a water-withheld condition. Consistently, these lines maintained relatively higher water content after being challenged in a water-withheld condition and were much less susceptible to wilting compared to the wild type (Hughes et al. 2017). Importantly, overexpression of *HvEPF1* showed no drastic impact on grain yield neither in well-watered nor water-restricted (25% soil water content) conditions, but improved its water use efficiency in the water-restricted condition and displayed drought tolerance (Hughes et al. 2017).

The study in barley indicates that mild-to-moderate overexpression of *EPF1* could be a means for increasing water-use efficiency of crop plants without significant yield penalty. Similar results were obtained by the overexpression of *OsEPF1* in rice (Caine et al. 2019). Moreover, the study revealed that maintaining a suitable number of stomata is important for survival under high temperature and drought conditions: only the *OsEPF1* overexpression line survived when subjected to a combined condition of high growth temperature (40°C) and severe drought stress treatments (Caine et al. 2019). Altogether, these data encourage the alteration of stomatal development as a potential means for better performance and yield under the challenged conditions in crops.

## Perspectives: Synthetic Chemistry Approaches with Agricultural Application in Mind

Cultivation of genetically modified (GM) or "bioengineered" plants have a great potential as a solution to food shortage by vesting tolerance to multiple stresses (Godfray et al. 2010). However, in many countries, growing GM plants is restricted by polices implemented to prevent the disturbance of the natural environment (Kuzma 2016). Some plant species are still difficult to genetically engineer because of their resistance toward *Agrobacterium* infection, low regeneration capacity, and/or lack of complete genome information. Small chemical compounds or synthetic peptides could be an alternative solution for manipulating specific signaling pathways of plant development (Zhao et al. 2007; De Rybel et al. 2009; Park et al. 2015; Nemhauser and Torii 2016; Hirakawa et al. 2017a,b; Kinoshita et al. 2018; Uchida et al. 2018; Uraguchi et al. 2018).

Thus far, four small compounds, Bikinin, Bubblin, CL1, and CL2, have been reported to modulate stomatal development (Kim et al. 2012; Sakai et al. 2017; Ziadi et al. 2017). Bikinin was identified from a commercially available DIVERSet compound library (ChemBridge) (De Rybel et al. 2009). This compound has a highly specific inhibitory activity for the seven *Arabidopsis* GSK3-like kinases including BIN2 (De Rybel et al. 2009). GSK3-like kinases regulate brassinosteroid signaling and they also appear to be involved in stomatal development (Li and Nam 2002; Kim et al. 2009, 2012; Rozhon et al. 2010; Gudesblat et al. 2012; Houbaert et al. 2018). Indeed, treatment of Bikinin reduces the number of stomata in a leaf epidermis (Kim et al. 2012). Bubblin is a small compound found from a screening of the Library of Active Compounds on *Arabidopsis* (Zhao et al. 2007; Sakai et al. 2017). This compound affects asymmetric cell division of MMCs presumably by alternating the correct localization of cell polarity factors including BASL, and, consequently, induces clustered stomata formation in *Arabidopsis* (Sakai et al. 2017). The CL1 and CL2 were found from a specially curated small-compound chemical library (Ziadi et al. 2017). Originally these compounds have an effect not only on the enhancement of stomatal development but also on plant growth where seedlings showed stunted root growth with pale green cotyledons. To minimize the cell toxicity of the chemicals, Ziadi et al. (2017) derivatized the original compound and successfully found that one derivative named ZA144 confers stomatal developmental activity with no growth defect. At present, physiological targets of Bubblin and ZA144 or their modes of action remain unknown. Elucidating the mechanisms of how these molecules control stomatal development may provide new insights into stomatal development biology and opportunities to precisely manipulate stomatal development on other species.

Exogenous manipulation of stomatal development can be achieved via peptide application, albeit high costs of peptide synthesis precludes large-scale agricultural applications. Very recently, small dodecapeptides CLE9/10 were found to restrict stomatal development via the HAESA-LIKE1 (HSL1) receptor in parallel to EPF2 (Qian et al. 2018). CLE family peptides can be synthesized and applied to test, for instance, how reduced stomatal density could improve water use efficiency in the field. The EPF-family peptides share conserved cysteine residues that make disulfide bonds form a stem region (Ohki et al. 2011). A variable loop region

determines the antagonistic activity between the EPF1, 2, and EPFL9/STOMAGEN (Ohki et al. 2011). Swapping the variable loops between EPF2 and STOMAGEN revealed that the loop region, but not the disulfide bond-formed scaffold, determines specificity (Ohki et al. 2011). Based on this information, we can predict that cyclic EPF peptides can mimic functional counterparts. Because cyclic peptides are often more stable in vivo than the linear counterparts (Lambert et al. 2001), cyclic EPF peptides, if their three-dimensional structures and bioactivities can be retained, could be a powerful way to control stomatal development in the field. There has been a great advance in the cyclization technique of peptide in the last decades—it is becoming easier and faster (Bashiruddin and Suga 2015; Rohrbacher et al. 2015; Bode 2017). In the future, it might become feasible to synthesize cyclic peptides or even more complex polypeptides with high efficiency to be commensurate with the costs (Fig. 3). Altogether, we envisage that such technologies will not only open a new era in basic plant biology research but also a postgreen revolution era in agriculture.

## CONCLUSION

In the past decade, our knowledge of stomatal development has accumulated. Cereal crops, such as rice, wheat, and maize, account for the majority of the world's food supply and livestock feed (Godfray et al. 2010; Long et al. 2015), thus these species could be a primary target for genetic manipulation for better yield. Because key regulators of stomatal development, originally identified from *Arabidopsis*, are conserved in grass species, manipulating their expression levels and patterns could be a route to modify plant performance under challenging conditions. Because of the rapid advancement in sequencing technology and genome-editing techniques such as the CRISPR-Cas9 system (Jinek et al. 2012; Feng et al. 2013; Mao et al. 2013; Miao et al. 2013; Tsutsui and Higashiyama 2017), we predict that the core stomatal development

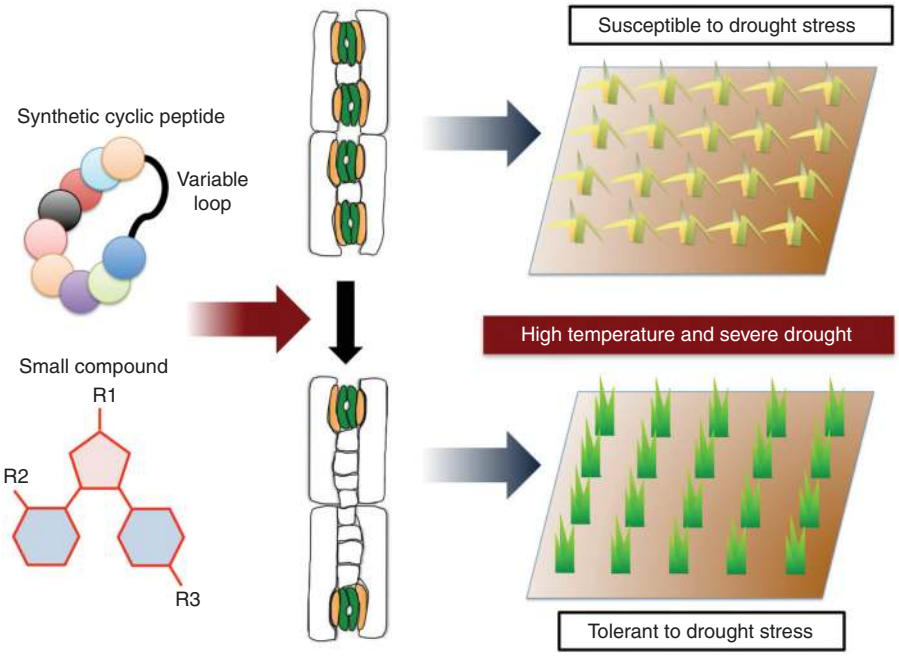


**Figure 3.** A strategy for manipulation of stomatal development by using synthetic peptides or small compounds. It has been shown that a reduced number of stomata in grass species show tolerance to dry and/or high temperate conditions, implying the potential usage of synthetic chemicals or compounds for better crops.

pathways of other species will soon be revealed. However, many fundamental questions still remain even in the widely studied model plant *Arabidopsis*. For instance, how plants integrate external and internal signals to facilitate proper stomatal development and patterning via complex ligand and receptor pairs followed by their downstream signaling cascade; what kind of factors or mechanisms determine the entering of stomatal development in protodermal cells before a *SPCH* expression. Therefore, it is necessary to continue basic studies to fully understand the molecular mechanisms that control precise stomatal development and patterning. At the same time, to sustain our food supply, it is important to accelerate the development of tools or techniques manipulating plant development of major crops to prepare for climate change, which is expected to deteriorate in the near future (IPCC 2018).

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