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Stomatal patterning and development in grasses

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Grass stomata provide an exemplary model of how form can improve functionality and promote the success of a plant family. The four-celled grass stomata are composed of dumbbell-shaped guard cells, each flanked by a single parallel subsidiary cell–arguably the most derived and fastest stomatal morphotype. The grasses' breathing pores develop in a strictly linear gradient within a stereotypically patterned epidermis, making it a highly accessible and spatiotemporally predictable developmental study system. Here, we highlight our current understanding of how vein-associated establishment of stomatal identity, tightly regulated asymmetric and symmetric cell division programs and extraordinary morphogenetic processes orchestrate the development of these uniquely shaped graminoid stomata. The innovative geometry and cellular composition of grass stomata have been repeatedly linked to rapid stomatal opening and closing kinetics, thus contributing to the grasses' water-use-efficient photosynthesis. Therefore, besides revealing fundamental aspects of plant development and plant cell biology, the dissection of the developmental processes forming grass stomata can also highlight strategies to engineer stomatal morphology for improved plant-atmosphere gas exchange.

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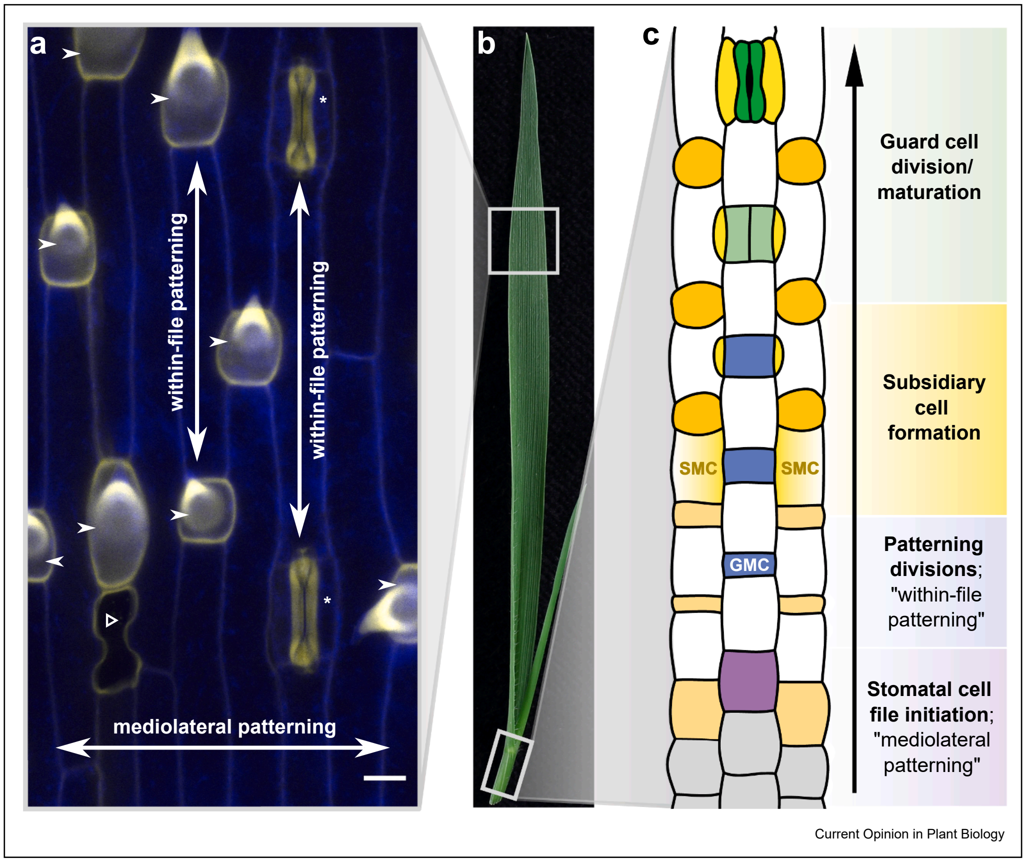
Introduction

Plant stomata are epidermal complexes on leaves and stems that connect the photosynthetic plant tissues with the atmosphere by regulating plant-atmosphere gas exchange. Stomata can open and close to enable carbon dioxide (CO_2) uptake required for photosynthesis and regulate evapotranspiration of water, which facilitates water and nutrient transport from root to shoot and controls leaf temperature. Stomatal complexes appear in diverse shapes and cellular compositions across the plant kingdom [1,2]. In *Arabidopsis thaliana*, stomata consist of two kidney-shaped guard cells (GCs) surrounding the central pore [3]. In contrast, grasses (Poaceae), form a highly derived stomatal morphotype with two dumbbell-shaped GCs flanked by two subsidiary cells (SCs; Figure 1). In the last decade, the developmental processes shaping these so-called graminoid stomata gained increasing attention [3,4], mainly because the graminoid morphology facilitates faster stomatal opening and closing kinetics [5–9]. This contributes to the grasses' water-use efficiency and their domination of many different habitats and ecosystems, including human agriculture [10–13]. In addition, grass stomata present an extraordinary developmental study system; they form in a linear, spatiotemporally stereotyped developmental gradient, divide symmetrically and asymmetrically in only two (perpendicular) orientations, recruit an additional cell type (i.e. SCs) and form a novel, dumbbell-shaped GC morphology. Genes with a described function in grass stomatal development are summarized in Table 1.

Distinct developmental modules coordinate stomatal initiation between and within tissues

Grass leaf epidermal development occurs, as in many monocots, in semi-clonal linear cell files, in which cell file identity is determined close to the leaf base during symmetric protodermal cell divisions (Figure 1b and c; [14,27,54]). The abaxial leaf epidermis of *Brachypodium distachyon*, for example, consists of stomatal and hair cell files where the specialized cells (i.e. GCs and hair cells) are usually interspersed by one larger cell (Figure 1a–c). Stomatal cell files only produce future GCs and interstomatal cells, which either differentiate into pavement cells or are reprogrammed to adopt subsidiary mother cell (SMC) identity to then form SCs when two or more adjacent stomatal cell files are initiated. Mutants of the bHLH transcription factors *SPEECHLESS1* (*SPCH1*) and *SPCH2* or *INDUCER OF CBF EXPRESSION 1* (*ICE1*/*SCRM*) in *B. distachyon* and rice fail to initiate the stomatal lineage [14,16,15]. In maize, *ZmICEb*, one of three *AtICE1*/*AtSCRM* homologues, affects all stages of stomatal development, with the mutant still forming ~30 % normal stomata but displaying a range of

Figure 1



Stomatal developmental stages in the grass leaf. (a) Confocal image of the mature abaxial leaf epidermis of the model grass *Brachypodium distachyon*; cell outlines in blue (UV-autofluorescence) and lignification in yellow (basic fuchsin-staining). Stomatal complexes (asterisk) with dumbbell-shaped guard cells (in yellow) and lateral subsidiary cells, prickle hair cells (arrowhead), silica cells (triangle) and pavement cells can be seen. Horizontal arrow indicates mediolateral patterning across cell file identities and vertical arrows indicate within-file patterning of the hair cell files (left) and stomatal files (right). Scale bar: 10 µm. **(b)** *Brachypodium distachyon* seedling with a mature first leaf and an emerging second leaf. Top box highlights the mature zone of the first leaf; bottom box indicates leaf developmental zone of the second, not yet unrolled leaf enveloped by the sheath of the older leaf. **(c)** Schematic of grass stomatal development. Protodermal stem cells (gray) divide symmetrically and identity programs mediolaterally pattern the leaf epidermis and establish distinct cell file fates (stomatal lineage precursor: purple; hair cell lineage precursor: orange). Independent of identity, all cell files undergo a transverse, asymmetric pattering division. In stomatal files, the stomatal lineage precursor (purple) divides asymmetrically to form a guard mother cell (GMC; blue) and an interstomatal cell. Subsidiary mother cells (SMCs) are established and polarized (yellow gradient), which undergo a longitudinal asymmetric division to form subsidiary cells (SCs; yellow). The GMC then symmetrically divides in a longitudinal manner to form two guard cell (GC) precursors (light green), which then form a central pore and mature into dumbbell-shaped GCs (green).

phenotypes from aborted guard mother cells (GMCs) to GC and SC division defects [17].

Interestingly, in fully penetrant non-initiating mutants like *bdice1*, the rows that should form stomata form hair cells instead [14]. Conversely, overexpressing *BdSPCH2* reprograms hair cell precursors and forms ectopic GC pairs [14]. Similarly in maize, mutants of *SQUAMOSA-PROMOTER BINDING PROTEIN-LIKEs* (*ZmSPL10/14/26*) cause a lack of hair cells but form ectopic stomatal complexes instead [27]. These opposing phenotypes indicate two different, antagonistic cell identity modules that pattern stomatal versus hair cell file identities early in the leaf epidermis (Figures 1a and 2).

In addition to mediolateral coordination of file identity (Figure 1a–c), stomatal cell files in grasses are always formed next to underlying veins, suggesting a developmental module that coordinates vein development and stomatal identity [3]. SCARECROW (SCR) and SHORTROOT (SHR) are two GRAS-domain transcription factors well known for their diversified roles in vascular patterning in flowering plants [55]. Mutations in *SCR* and *SHR* in rice, maize and *Setaria viridis* show

Table 1

Overview of genes involved in grass stomatal development.

Gene name	Gene id	Species	Function	Reference
BdSPCH1	Bradi1g38650	*B. distachyon*	Establishment of stomatal lineage	[14]
OsSPCH1	Os06g33450	*O. sativa*	Establishment of stomatal lineage	[15]
BdSPCH2	Bradi3g09670	*B. distachyon*	Establishment of stomatal lineage	[14]
OsSPCH2	Os02g15760	*O. sativa*	Establishment of stomatal lineage	[16,15]
BdICE1	Bradi4g17460	*B. distachyon*	Establishment of stomatal lineage	[14]
OsICE1	Os11g32100	*O. sativa*	Establishment of stomatal lineage & differentiation of stomatal complex	[15]
ZmICEa	Zm00001d042263	*Z. mays*	Not yet known	[17,18]
ZmICEb; ZmDSD1	Zm00001d049294	*Z. mays*	Differentiation of stomatal complex	[17,18]
ZmICEc	Zm00001d007382	*Z. mays*	Not yet known	[17,18]
BdSCRM2	Bradi2g59497	*B. distachyon*	Differentiation of stomatal complex	[14]
OsICE2; OsSCRM2	Os01g70310	*O. sativa*	Establishment of stomatal lineage & differentiation of stomatal complex	[15]
OsCYCA2;1	Os012g31810	*O. sativa*	Role during patterning division making GMCs	[19]
BdYDA1	Bradi5g18180	*B. distachyon*	Maintenance of fate asymmetry	[20,21]
HvYDA1	HORVU2Hr1G096350; HORVU.MOREX.r2.2HG0155920; HORVU.MOREX.r3.2HG0188270	*H. vulgare*	Maintenance of fate asymmetry	[22]
HvBRX-Solo	HORVU.MOREX.r2.2HG0144720; HORVU.MOREX.r3.2HG0175040	*H. vulgare*	Maintenance of fate asymmetry	[22]
OsSCR1	Os11g03110	*O. sativa*	Promotion of stomatal identity	[15,23,24]
SvSCR1	Sevir.7G316501	*S. viridis*	Promotion of stomatal identity	[25]
ZmSCR1	GRMZM2G131516	*Z. mays*	Promotion of adaxial stomatal identity	[25]
ZmSCR1h	GRMZM2G015080	*Z. mays*	Promotion of adaxial stomatal identity	[25]
OsSCR2	Os12g02870	*O. sativa*	Promotion of stomatal identity	[15,23]
SvSCR2	Sevir.8G008100	*S. viridis*	Promotion of stomatal identity	[25]
OsSHR1	Os07g39820	*O. sativa*	Promotion of stomatal identity	[15]
ZmSHR1	GRMZM2G132794	*Z. mays*	Promotion of stomatal identity	[26]
OsSHR2	Os03g31880	*O. sativa*	Promotion of stomatal identity	[15]
ZmSPL10	Zm00001d015451	*Z. mays*	Promotion of hair cell identity/Inhibition of stomatal identity	[27]
ZmSPL14	Zm00001d036692	*Z. mays*	Promotion of hair cell identity/Inhibition of stomatal identity	[27]
ZmSPL26	Zm00001d053756	*Z. mays*	Promotion of hair cell identity/Inhibition of stomatal identity	[27]
BdSTOMAGEN-1	Bd2g58540	*B. distachyon*	Promotion of stomatal identity	[28]
OsEPFL9-1; OsSTOMAGEN	OsIR64_00032g010800; LOC_01g6859	*O. sativa*	Promotion of stomatal identity	[29,30,31,32]
TaSTOMAGEN-1	TraesCS3A02G419900	*T. aestivum*	Promotion of stomatal identity	[28]
BdSTOMAGEN-2	Bd3g40846	*B. distachyon*	Promotion of stomatal identity	[28]
OsEPFL9-2; OsEPFL10	LOC_08g4136	*O. sativa*	Promotion of stomatal identity	[30,31]
TaSTOMAGEN-2	TraesCS7A02G255900	*T. aestivum*	Promotion of stomatal identity	[28]
OsEPF1	Os04g54490	*O. sativa*	Inhibition of stomatal identity	[30]
SbEPF1	Sobic006G233600; SbiTx43006G248600	*S. bicolor*	Inhibition of stomatal identity	[33]
TaEPF1	TraesCS2A02G526100	*T. aestivum*	Inhibition of stomatal identity	[28,34]
BdEPF2-1	Bd5g12220	*B. distachyon*	Inhibition of stomatal identity	[28]
BdEPF2-2	Bd5g23357	*B. distachyon*	Inhibition of stomatal identity	[28]
OsEPF2	Os04g38470	*O. sativa*	Inhibition of stomatal identity	[30]
TaEPF2	TraesCS2A02G343000	*T. aestivum*	Inhibition of stomatal identity	[28,34]
BdMUTE	Bradi1g18400, BdiBd21-3.1G0240400	*B. distachyon*	GMC division plane orientation & SC recruitment	[7,35]
OsMUTE	Os05g51820	*O. sativa*	Maintenance of GMC identity & SC recruitment	[15]
ZmMUTE	GRMZM2G417164	*Z. mays*	Maintenance of GMC identity & SC recruitment	[36]
ZmBRK1	GRMZM5G842058	*Z. mays*	Polarization of SMC & pavement cell morphogenesis (SCAR/WAVE)	[37–39]
ZmBRK3	GRMZM5G88636	*Z. mays*	Polarization of SMC & pavement cell morphogenesis (SCAR/WAVE)	[38,39]

(*continued on next page*)

Table 1 (*continued*)

Gene name	Gene id	Species	Function	Reference
OsLPL2	Os03g05020	*O. sativa*	Polarization of SMC & pavement cell morphogenesis (SCAR/WAVE)	[40]
OsLPL3	Os08g43130	*O. sativa*	Polarization of SMC & pavement cell morphogenesis (SCAR/WAVE)	[40]
BdPAN1	BdiBd21-3.3G0526300; Bradi3g39910	*B. distachyon*	Polarization of SMC	[41]
ZmPAN1	Zm00001d031437	*Z. mays*	Polarization of SMC	[42,43]
ZmPAN2	Zm00001d007862	*Z. mays*	Polarization of SMC & SC morphogenesis	[44,43]
ZmWPRA1	Zm00001d023629 (V4.0); Zm00001eb408590 (V5.0)	*Z. mays*	Polarization of SMC	[45]
ZmWPRA2	Zm00001d041088 (V4.0); Zm00001eb133280 (V5.0)	*Z. mays*	Polarization of SMC	[45]
ZmWPRB1	Zm00001d0475516 (V4.0); Zm00001eb395070 (V5.0)	*Z. mays*	Polarization of SMC	[45]
ZmWPRB2	Zm00001d007164 (V4.0); Zm00001eb111490 (V5.0)	*Z. mays*	Polarization of SMC	[45]
BdPOLAR	BdiBd21-3.3G0715200; Bradi3g54060	*B. distachyon*	Polarization of SMC	[41]
ZmROP2	Zm00001d053899	*Z. mays*	Polarization of SMC	[46]
ZmROP9	Zm00001d015036	*Z. mays*	Polarization of SMC	[46]
ZmMLKS2	Zm00001d052955	*Z. mays*	SMC nuclear migration	[47,48]
ZmOPAQUE1; ZmDCD2	Zm00001d052110 (V4.0); Zm00001eb193160 (V5.0)	*Z. mays*	Regulation of SMC phragmoplast orientation	[49]
ZmTAN1	Zm00001d038060	*Z. mays*	Regulation of SMC division plane orientation	[50,51]
OsFLP	Os07g43420	*O. sativa*	GMC division plane orientation	[15]
ZmNOD	GRMZM2G027821	*Z. mays*	GMC division & differentiation of stomata	[52]
BdFAMA	Bradi2g22810	*B. distachyon*	GC differentiation & SC recruitment	[53]
OsFAMA	Os05g50900	*O. sativa*	Differentiation of stomatal complex	[16,15]

pleiotropic effects affecting both roots and leaves [15,23,25,26,56,57]. In rice and *S. viridis*, *scr* and *shr* mutants affect stomatal formation indicating that these proteins are involved in the initiation of stomatal files, fitting with *SCR* expression in early stomatal stages or *SHR* expression in the vasculature during stomatal initiation [15,26,23,25,24]. Interestingly, *SCR* mutants in maize show a much less severe phenotype where stomatal formation is only affected on the adaxial side of the leaf [23]. This deviation from the phenotype observed in rice, along with the expression of *ZmSCR* in the inner leaf and not the epidermis, suggests species-specific diversification of the SCR-SHR module during leaf development [56,23].

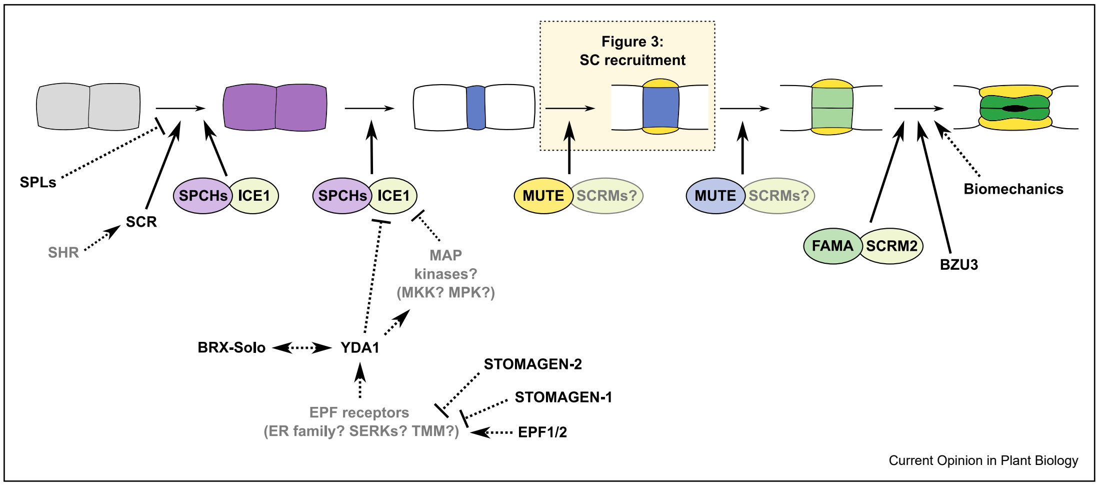
A conserved cell signalling module regulates stomatal file patterning

In *Arabidopsis*, stomatal density and patterning are controlled by regulating division potential and division orientation in stomatal lineage ground cells (SLGCs) and meristemoids. Grasses, however, have neither SLGCs nor meristemoids. Here, density can be regulated through the number of adjacent stomatal files that are initiated (usually between one and three) and the number of stomata per row. How the number of adjacent stomatal files is determined is unknown. How many stomata per single stomatal cell file are formed, though, is likely controlled by the number of SPCH-driven symmetric precursor divisions (Figures 1c and 2).

The within-file pattern of specialized epidermal cells (i.e. hair cells or stomata) interspersed with at least one large pavement cell depends on asymmetric, transverse "patterning divisions" (Figure 1a–c). Maintenance of the post-division fate asymmetry involves players of the conserved stomatal signaling cascade (Figure 2). In *Arabidopsis*, ligand peptides (EPIDERMAL PATTERNING FACTORs, EPFs) are recognized by leucine-rich repeat receptor-like kinases (LRR-RLKs) of the ERECTA family and its co-receptors TOO MANY MOUTHS (TMM) and members of the SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE (SERK) family (reviewed in Refs. [4,58]). They induce MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) phospho-signalling cascades of YODA (YDA), MKK4/5/7/9 and MPK3/6, ultimately leading to the phosphorylation and degradation of SPCH and, thus, inhibition of stomatal formation (reviewed in Refs. [4,58]).

In grasses, the mesophyll-derived STOMAGEN-1 (also known as EPFL9 or EPFL9-1) and its paralog EPFL10 (EPFL9-2, STOMAGEN-2) promote stomatal initiation

Figure 2



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Genetic regulation of grass stomatal development. Schematic depicting the stomatal stages as described in Figure 1. Key stomatal transcription factors with their heterodimerization partners are indicated in colored ellipses. Gray-font factors indicate putative pathway components inferred from studies in *Arabidopsis*; black-font factors indicate experimentally supported roles during grass stomatal development (referenced in main text). Arrow shape indicates effect of a protein on another component of the pathways (T shape: inhibition; arrowhead: promotion). Dashed lines suggest putative interactions that require experimental confirmation. SC = Subsidiary cell.

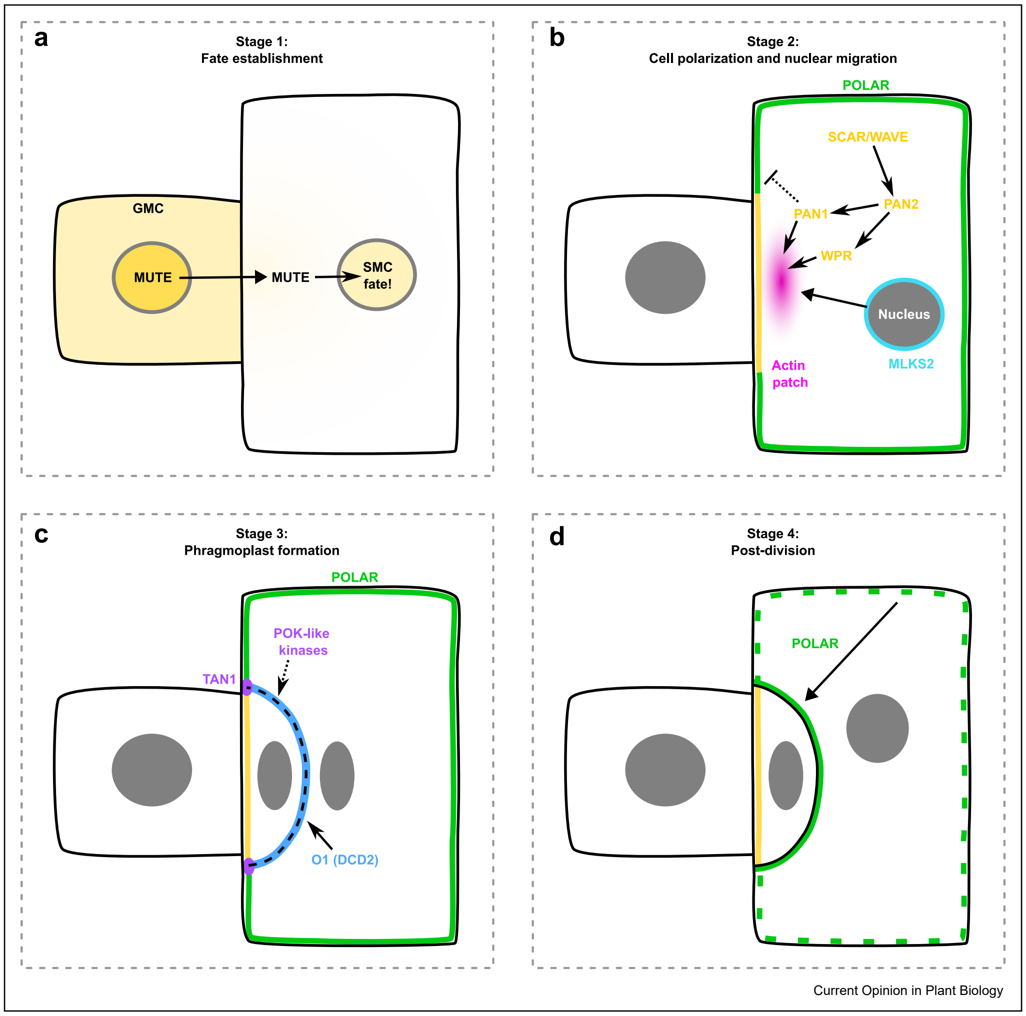
[28–32,59]. In contrast, grass EPF1 and EPF2 decrease leaf stomatal density, yet it remains unclear whether this occurs during early symmetric divisions or later after GMC establishment [28,30,31,33,34,60]. In *B. distachyon* and barley, *yda1* mutants show within-file clusters of stomata, hair cells and silica or cork cells, suggesting the involvement of YDA1 in repressing stomatal and hair cell fate in the larger daughter cell after the transverse patterning division [20,22]. Indeed, BdYDA1 is polarized towards the base of the larger daughter cell after division, suggesting an asymmetric, post-mitotic fate inhibition of specialized epidermal cell fate (i.e. hair or stomata) in the large, basal daughter cells [21]. Accordingly, a mutation in the putative stomatal polarity gene *HvBREVIS RADIX-Solo* (*HvBRX-Solo*) phenocopies the barley *yda1* mutant, hinting at a link between cell polarity and MAPK signalling much like in *A. thaliana* [22]. To date, the receptors of the EPF signals, the additional components of the MAPK phosphorelay and its protein target(s) have not been experimentally identified in the context of grass stomatal development (Figure 2).

Establishment, polarization and division of the subsidiary cell lineage

After the transverse patterning divisions that produce a small GMC and a large interstomatal pavement cell, the established GMC recruits lateral, parallel (=paracytic) SCs. First, the GMC-neighboring cell acquires SMC fate. Second, SMCs strongly polarize, causing nuclear migration toward the GMC/SMC interface. Third, a longitudinal asymmetric division forms a single SC, which then differentiates and is functionally integrated into the stomatal complex (Figure 3).

The master regulator of SC development is the bHLH transcription factor MUTE with grass *mute* mutants lacking SCs [7,15,36]. *MUTE* is expressed in GMCs and the protein then moves into neighboring cell files to initiate SMC identity (Figures 2 and 3; [7,36,35]). If this mobility is impaired, the spatiotemporal coordination of GMC and SMC divisions is severely disturbed [35]. In addition to its role in SC formation, MUTE also ensures correct GMC divisions. Mutants in rice and maize completely abort stomatal complex formation, because all GMCs divide transversally rather than longitudinally leading to failed pore formation and arrest of GC differentiation [15,36]. Even though *mute* mutants in *B. distachyon* form 75 % functional, yet two-celled stomata that lack SCs, 25 % of complexes also abort and show skewed, oblique, or even transverse division [35]. Low levels of functional BdMUTE in GMCs are sufficient to rescue the division plane misorientation but not the recruitment of SCs, indicating a GMC-autonomous role of MUTE in GMC division plane orientation [35]. This dual role of grass MUTE likely causes the GMC division orientation defect and stomatal abortion phenotype in rice and maize [7,15,36,35]. We speculate that the much shorter division zone in the small grass *B. distachyon* compared to rice and maize causes significant overlap between *BdMUTE* and *BdFAMA* expression, which regulates GC differentiation [53]. Therefore, in *bdmute*, *BdFAMA* might activate some of the genetic programs required for accurate GMC division. Similarly, ectopic

Figure 3



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Subsidiary cell development. The four developmental stages of subsidiary cell (SC) formation are shown. **(a)** Stage 1: MUTE is expressed in guard mother cells (GMCs) and moves into lateral cell files to induce subsidiary mother cell (SMC) fate. **(b)** Stage 2: A proximal polarity domain (yellow) at the SMC/GMC interface is formed by a polarization protein cascade (yellow), which leads to the formation of a localized actin patch (pink). The proximal domain protein PAN1 also polarizes the distal domain protein POLAR (green). The nucleus (gray), guided by MLKS2 (light blue) and the proximal polarity domain (yellow), moves towards the future division site. **(c)** Stage 3: POLAR is involved in cortical division sites formation, which are established above and below the GMC and marked by TAN1 (purple). During the division, the phragmoplast forms under the guidance of O1 (blue) and, putatively, POK-like kinases. **(d)** Stage 4: Once the SC division is complete, the previously distal POLAR domain (green) relocalizes to the newly formed SC wall.

expression of *BdFAMA* under the *BdMUTE* promoter can partially rescue SC recruitment in the *bdmute* background, suggesting functional redundancy of these two genes in each other's absence [53].

SMC divisions are highly asymmetric and, therefore, require an interplay of proximal and distal polarity factors that ensure nuclear migration towards the GMC/SMC interface and the establishment of the cortical division site above and below the GMC (Figure 3b). A suite of polarity factors localize specifically to the GMC/SMC interface. Initially, BRICK family (BRK) proteins that encode for SCAR/WAVE components polarly localize at the GMC/SMC interface (Figure 3b; [3,37–39]). The initial (GMC-derived?) signal that triggers this polarization remains elusive. BRK1 then polarizes the receptor-like kinase PANGLOSS2 (PAN2) and subsequently PAN1 [39,42,44]. The proximal polarity domain seems to be established independently of SMC identity [41] and precedes and contributes to the proximal actin patch formation [39,61,43]. PAN2 but not PAN1 is required to polarize WEB1/PMI2-Related (WPR) proteins, which directly interact with actin and are, thus, likely involved in actin patch formation [45]. The SMC nucleus then migrates towards the future division site near the GMC/SMC interface, which is guided by the proximal domain (Figure 3b; [41]). The nuclear membrane protein MAIZE LINC KASH SINE-LIKE2 connects the SMC nucleus to the cytoskeleton to guide nuclear migration and to stabilize the nuclear position at the division site, which is required for correct division plane orientation [47]. In *B. distachyon* SMCs, a transient, distal polarity domain is formed by BdPOLAR, which is polarized by the proximal domain (i.e. BdPAN1) and guides SMC division plane orientation, potentially through repelling cortical division site formation [41].

In maize, the phragmoplast is then formed at the SMC division plane and guided by the myosin OPAQUE1/DISCORDIA2 (O1) and putatively PHRAGMOPLAST ORIENTING KINESIN (POK)-like kinesins towards the cortical division sites (Figure 3c, [49]). Additionally, recent findings have shown that O1 and intact actin filaments seem to be necessary for the accumulation of the micotubule-binding protein TANGLED1 (TAN1) to the cortical SMC division site to reorganize microtubule orientation and thereby division plane orientation [50,51]. In *B. distachyon*, BdPOLAR quickly re-localizes to the newly formed SC after the division (Figure 3d, [41]), a relocalization not seen in the *Arabidopsis* stomatal lineage.

Guard cell morphogenesis and stomatal maturation

After the recruitment of SCs, a symmetric, longitudinal GMC division forms the GC pair (Figure 1c). Upon GMC division, a pore is formed, the cytoskeleton is reorganized and the cell wall is anisotropically modified to establish the unique dumbbell shape of grass GCs [62–64]. In addition, and unlike in related Poales like Flagellariaceae, large symplastic connections are formed between the apices of grass GCs [62,65,66]. Finite element modelling approaches indicate that extensive cell wall thickening at the central rods and symplastic GC connections are required for efficient stomatal opening and equalizing pressurization between GCs, respectively [8,66]. These biomechanical modelling approaches also confirm the extensive experimental data suggesting that reciprocal pressurization of SCs and GCs is required for the superior stomatal kinetics [5,7,8,67].

GC differentiation and pore formation in grasses require the transcription factors FAMA and SCRM2, which might act as heterodimerization partners [14,16,53]. Their respective mutants in *B. distachyon* abort stomatal development after the GMC division, fail to form a pore and do not initiate dumbbell-shaped GC morphogenesis [14,53]. While this is reminiscent of FAMA's role in *Arabidopsis*, grass FAMA lacks the LxCxE motif for interaction with the cell cycle inhibitor RETINOBLASTOMA-RELATED (RBR) and seems to have no role in division suppression [53]. A key role in GC morphogenesis might be assigned to grass SCRM2, which has apparently subfunctionalized in *B. distachyon* to control GC morphogenesis and differentiation [14]. Strikingly, grass *SCRM2* homologs are absent in sedges (Cyperaceae), which are Poales but lack grass-like dumbbell GCs. This indicates a role of grass *SCRM2* in generating dumbbell-shaped GCs [68].

Apart from this initial transcription factor module, only a few factors controlling GC morphogenesis and maturation are known. For example, the maize epimerase *BIZUI3* (*BZU3*) seems to be involved in the morphogenesis of stomatal complexes [69]. BZU3 catalyzes the conversion of UDP-Galactose to UDP-Glucose, a compound potentially required for stomatal wall formation, and of UDP-GalNAc to UDP-GlcNAc, a donor for protein N-glycosylation in plants. Indeed, glycoproteomics of the *bzu3* mutant reveal changes in the N-glycosylation status of proteins like PAN2 [69], which is required for SC morphogenesis [43]. It was further shown that SC formation is non-cell-autonomously required for normal GC morphogenesis in *B. distachyon*, as GCs that do not recruit SCs fail to form a dumbbell shape [35]. This could indicate a role of the shared GC-SC apoplast, where PAN2 primarily resides from pre-division throughout stomatal maturation, in stomatal complex morphogenesis [35,43].

Finally, changing cell wall properties and the size of leaf hair cells by mutating a hair cell-specific peroxidase (*BdPOX*) in *B. distachyon* indirectly causes extensive GC elongation [70]. This effect might be due to a biomechanical feedback between hair cell elongation and stomatal elongation to balance and coordinate epidermal cell sizes [70]. These data suggest the coordination of stomatal and hair cell development at late stages of differentiation for these cells in addition to coordinated development during mediolateral patterning of the leaf epidermis.

Outlook

The graminoid stomatal morphology in grasses is arguably the most derived stomatal form in the plant kingdom. Even though the number and arrangement of SCs is variable and diverse in land plants [1], most land plant families form kidney-shaped GCs, whereas dumbbell GCs are exclusively found in Poaceae and some species of closely related Poales like the Cyperaceae [68]. Graminoid morphology was experimentally linked to fast stomatal kinetics, providing an advantage in harsh climatic conditions [5,6,71]. While the physiology of grass stomata within and across species is a focal point of research, much remains to be investigated regarding their development. For example, the molecular pathways that accurately place the stomatal rows next to veins, differentiate and functionally integrate

SCs or guide the unique GC morphogenesis remain enigmatic. While progress regarding cell wall maturation and composition has been made [64,72,73], the biochemical and biomechanical details of stomatal cell walls in grasses are poorly understood. Furthermore, even though some genetic players of the stomatal signalling pathways have been shown to be required for within-file patterning, it remains elusive which receptors sense EPF ligands, how the signal is transmitted beyond YDA and which transcription factors are phosphorylated and degraded. Furthermore, there is strong indication that intrinsic cell polarity is regulated by different players in the grass stomatal lineage and during epidermal patterning compared to *Arabidopsis* [22,39,41]. Finally, even though many of the developmental modules seem to be functionally conserved among domesticated cereal crops (i.e. rice, maize, barley) and wild model grasses such as *B. distachyon*, important differences like distinct effects of the *mute* mutation on GC development and differential expansion of gene family numbers (i.e. SPCH, ICE1) will have to be addressed in the future. Comparative (single-cell) transcriptomics and forward and reverse genetics will further improve our understanding of how grasses form the fastest, morphologically most derived stomatal complexes in the plant kingdom and how these developmental modules can be harnessed to understand how the graminoid stomatal form improves function.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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