

Gymnosperm and fern multifunctional bHLH transcription factors control stomatal development

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Abstract

Stomatal development is driven by a conserved family of basic Helix-loop-helix (bHLH) transcription factors. In the angiosperm model *Arabidopsis thaliana*, *SPEECHLESS* (*SPCH*), *MUTE* and *FAMA* show pronounced yet incompletely separated stage-specific functions. How the functional specialization observed within angiosperms evolved has remained unclear. Here, we perform the first functional analysis of stomatal regulators from the fern *Ceratopteris richardii* and the gymnosperm *Picea abies* (Norway spruce). We show that both species possess a single *SPCH/MUTE*-like gene (*CrSM* and *PaSM*) together with a single *FAMA*-like gene (*CrFAMA* and *PaFAMA*). Cross-species complementation in *Arabidopsis* showed that all the four bHLHs displayed broad developmental capacities and promoted asymmetric stomatal-lineage entry divisions, a function associated with angiosperm *SPCH*. Moreover, *PaSM* promoted pore-like structure formation in the *fama* background, indicating a capacity extending into a developmental transition controlled by angiosperm *FAMA*. To examine the functions of *PaSM* and *PaFAMA* in the native context, we generated transgenic spruce lines expressing dominant-negative variants of these genes. Perturbation of either bHLH was associated with fewer epidermal cells between adjacent stomata, whereas the two constructs produced partially distinct effects on mature stomatal-complex morphology. Together, our findings show that the fern and gymnosperm stomatal bHLHs examined here possess broad and overlapping developmental capacities. These results support a model in which the stage-specific functions of angiosperm stomatal regulators evolved through the progressive refinement and partitioning of broader, partially overlapping developmental capacities.

Introduction

Stomata are microscopic epidermal pores that mediate CO₂ uptake for photosynthesis while limiting transpirational water loss, and their acquisition was likely a key innovation enabling the colonization of land by plants¹. In most land plants, the basic stomatal structure is conserved and consists of a pair of guard cells (GCs) surrounding a stomatal pore^{2,3}. In some lineages, however, this core structure is accompanied by additional specialized cells termed subsidiary cells (SCs)⁴. For example, grasses develop SCs that facilitate GC movements and contribute to the distinctive morphology and function of grass stomatal complexes⁵. Despite such morphological variation, the core regulatory machinery underlying stomatal development appears to be widely conserved.

Stomatal development is controlled by bHLH transcription factors of the subgroup 1a (bHLH1a). In angiosperm model plant, *Arabidopsis thaliana*, three genes encoding bHLH transcription factors *SPCH*, *MUTE*, and *FAMA* act sequentially, together with the broadly expressed bHLH partners ICE1/SCRM and SCRM2, to control successive stomatal-lineage transitions⁶⁻⁹. *SPCH* initiates the stomatal lineage by driving asymmetric cell divisions (ACDs), *MUTE* promotes guard mother cell (GMC) fate, and *FAMA* establishes GC identity. Orthologues of these genes are broadly conserved across angiosperms and generally retain similar core functions¹⁰⁻¹², although in some lineages, they have acquired additional lineage-specific roles^{5,11-16}. Although *SPCH*, *MUTE* and *FAMA* have distinct stage-defining functions, their activities are not completely temporally separated. Recent work showed that *SPCH* persists into GMCs and young GCs¹⁷, where the relative levels and activities of *SPCH* and *FAMA* regulate GC division and expansion¹⁸.

How this pronounced, yet partially overlapping, stage-biased specialization evolved has remained unresolved. Phylogenetic analyses have yielded alternative reconstructions of bHLH1a gene-family evolution. In one scenario, the angiosperm stomatal bHLH module arose through duplication and subsequent diversification of an ancestral bHLH1a gene¹⁹. However, other reconstructions cannot exclude the possibility that early land plants already possessed multiple bHLH1a genes and that the smaller gene content within some extant lineages resulted from secondary gene loss²⁰. These alternative gene-family histories do not, by themselves, reveal the ancestral functional states of the encoded proteins or the extent to which developmental functions are partitioned among coexisting paralogs outside angiosperms. Domain-swapping and mutational analyses of *Arabidopsis* stomatal bHLHs have shown how sequence divergence could generate the specialized activities of *SPCH*, *MUTE*, and *FAMA*²¹. Although these findings provide a mechanistic model for progressive functional divergence following gene duplication, they do not establish whether this was the historical route by which stomatal bHLH specialization evolved.

Functional studies in bryophytes have nevertheless provided insight into the developmental capacities of early-diverging land-plant bHLH1a. In the moss *Physcomitrium patens*, *PpSMF1* partially complements *Arabidopsis mute* and *fama*, whereas native loss of *PpSMF1* abolishes stomatal formation in the moss sporophyte^{3,22}. In the astomatous liverwort *Marchantia polymorpha*, its sole bHLH1a gene functions in non-stomatal developmental contexts but retains the capacity to partially support stomatal development in *Arabidopsis*^{23,24}. Together, these findings demonstrate that extant single-copy bryophyte bHLH1a proteins can possess broad developmental capacities. However, neither they distinguish between alternative histories of bHLH1a gene duplication and loss nor determine whether, under a scenario of ancestral gene multiplicity, coexisting paralogs were already functionally specialized. Thus, irrespective of gene-family history, how the pronounced yet incomplete stage-biased specialization of angiosperm stomatal bHLHs evolved has not been resolved.

Available genomes from several fern and gymnosperm species encode, or are predicted to encode, one *SPCH/MUTE*-like gene together with one *FAMA*-like gene^{19,20}. This two-gene complement provides an

opportunity to test whether coexisting paralogs retain broadly overlapping developmental capacities or exhibit substantial functional partitioning. Comparative functional characterisation of these genes can therefore provide evidence about the degree of functional specialization among stomatal bHLHs outside angiosperms.

Here, we compare the regulatory capacities of *SPCH/MUTE*- and *FAMA*-like transcription factors from the gymnosperm *Picea abies* and the fern *Ceratopteris richardii* through cross-species complementation in *Arabidopsis*. In parallel, we relate *PaSM* and *PaFAMA* transcript abundance to developmental stage-enriched spruce needle regions and examine the effects of fusing *PaSM* and *PaFAMA* to a transcriptional repression domain in transgenic spruce. Together, these approaches enable functional comparison of evolutionary divergent bHLHs in *Arabidopsis* context while independently providing native-context evidence for *PaSM*- and *PaFAMA*-associated functions in gymnosperm stomatal development.

To our knowledge, this study provides the first comparative functional analysis of stomatal bHLH transcription factors from a fern and a gymnosperm. All four fern and gymnosperm transcription factors promoted asymmetric stomatal-lineage entry divisions in *Arabidopsis*, and *PaSM* additionally promoted stomatal pore formation, revealing developmental capacities that span transitions primarily controlled by distinct regulators in angiosperms. Native-context analyses further provided initial evidence for partially distinct *PaSM*- and *PaFAMA*-associated activities in spruce. Together, these findings uncover previously unrecognised broad and overlapping developmental capacities among fern and gymnosperm stomatal bHLHs, with evidence for partial functional differentiation between the two paralogs in spruce.

Results

Norway spruce stomatal development shows conserved and distinct features

We first investigated stomatal development in Norway spruce needles. Stomatal development proceeded from the base toward the tip of the needle and was restricted to specific cell files (Fig. 1a, Supplementary Fig. 1a,b). Spruce needles were rhombic in cross-section, with up to three stomatal cell files present on each of the four faces (Supplementary Fig. 1b). We sometimes observed stomatal files shifting between adjacent epidermal cell files, suggesting some flexibility in the spatial patterning of stomatal development. To analyse early stages of stomatal development, we sampled young needles located near the vegetative meristem of spruce seedlings. Because stomatal development was largely complete in needles longer than 10 mm, we focused on needles shorter than 10 mm (Supplementary Fig. 1a). In spruce stomatal files, protodermal cells underwent divisions that appeared morphologically symmetric (Fig. 1a,b). The resulting daughter cells subsequently diverged in morphology: one elongated into a rectangular cell, whereas the other became rounded, consistent with distinct developmental trajectories (Fig. 1a,c). This divergence was already apparent at the earliest post-division stages that we could resolve, suggesting that daughter-cell fate asymmetry is established before, during, or shortly after division. By reconstructing stomata-forming cell files from mature stomata toward younger developmental stages, we inferred that the elongated rectangular cell was most likely the GMC, as it later underwent a symmetric division, whereas the rounded daughter cell became a non-stomatal cell. Consistent with previous observations in pines²⁵, these results suggest that spruce protodermal cells undergo an equal division whose daughter cells differentiate into a GMC and a polar SC, respectively. We therefore refer to the non-stomatal daughter cell in spruce as the polar SC (Fig. 1a,c). During GMC differentiation, cells in the adjacent non-stomatal row differentiated into lateral subsidiary mother cells (SMCs) (Fig. 1a,c). Subsequently, the GMC and lateral SMCs underwent symmetric and asymmetric divisions, respectively, producing a pair of GCs and two SCs flanking the GCs (Fig. 1a,d). After these divisions, the GCs began to sink into the hypodermal layer (Fig. 1a,e,f). In the mature stomatal complex, the GCs were fully

sunken within the hypodermal layer, as reported in other conifers²⁵. Together, these observations indicate that spruce stomatal development includes conserved features such as the establishment of cell fate asymmetry, differentiation and symmetric division of the GMC, and GC formation. At the same time, it also shows distinctive characteristics, including the recruitment of multiple SCs and the displacement of GCs from the epidermis into a subepidermal position.

Norway spruce genome contains two genes encoding bHLH1a members in SM and FAMA subclades

To identify bHLH1a coding genes in spruce, we performed a phylogenetic analysis and constructed a corresponding phylogenetic tree containing a set of species including bryophytes, a lycophyte, ferns, gymnosperms and angiosperms (Fig. 2a). The phylogenetic tree resolved five subclades. The single *Marchantia polymorpha* bHLH1a gene is placed outside these subclades, near the root defined by the two *Arabidopsis* bHLH3c outgroup genes. One of the subclades consists only fern sequences. *SPCH/MUTE* (SM) and *FAMA* subclades form sister groups. The *FAMA* subclade included genes from all the analysed species except *Marchantia polymorpha*, whereas *SM* subclade contains only ferns, gymnosperms and angiosperms. Gymnosperms have also subclade with conserved number of homologs closely related to angiosperm subclade including *TEC1/6* and *MYC67/70* homologs. We identified that *Picea abies* genome assembly contains a single *SPCH/MUTE*-like gene (*PaSM*; MA_120602g0010) and a *FAMA*-like gene (*PaFAMA*; MA_57244g0010) (Fig. 2a). We next compared the domain structures of *Arabidopsis thaliana* and *Picea abies* *SPCH*, *MUTE*, and *FAMA* orthologues (Fig. 2b). Both *PaSM* and *PaFAMA* contain the conserved bHLH1a and C-terminal domains characterizing the subgroup 1a transcription factors^{26,27}. Additionally, *PaSM* has a truncated MITOGEN-ACTIVATED PROTEIN KINASE target domain (MAPKTD), a region important for the regulation of AtSPCH protein stability^{21,28}. Because AtSPCH variants lacking MAPKTD phosphorylation sites can promote GMC differentiation, a capacity normally associated with *MUTE*, the truncated MAPKTD in *PaSM* raised the possibility that *PaSM* might possess both *SPCH*- and *MUTE*-like activities. However, we noticed that *PaSM* lacks a *MUTE* unique domain, which function has not been defined. *PaFAMA* contains putative *FAMA*-specific domains as well as the LxCxE motif, which is essential for the interaction with RETINOBLASTOMA-RELATED (RBR) protein in *Arabidopsis*²⁹. Based on phylogenetic analysis and domain structures, we hypothesized that *PaSM* may functionally resemble both *SPCH* and *MUTE* or alternatively either *SPCH* or *MUTE*, whereas *PaFAMA* is likely to retain *FAMA*-like functions.

***PaSM* and *PaFAMA* transcripts peak in needle regions enriched for different stages of stomatal development**

Our phylogenetic analyses identified *PaSM* and *PaFAMA* as candidate regulators of stomatal development in spruce. To examine whether their expression patterns vary among needle regions enriched with different phases of stomatal development, we collected basal and distal regions from needles of different size classes to generate four developmental stages enriched for successive stages of stomatal development. We then analysed *PaSM* and *PaFAMA* transcript abundance in these regions by quantitative real-time PCR (qRT-PCR). Based on confocal microscopy-based imaging, developmental stage 1, 2, 3 were enriched predominantly for ACDs, GMC differentiation/division, and maturing stomata, respectively (Fig. 3a–c). Stage 4, collected from the distal region of longer needles, contained more mature stomata and was used to represent the latest developmental stage. We nevertheless observed some morphological variation among needles of similar size, suggesting that needle length alone does not fully reflect stomatal developmental progression.

We next quantified *PaSM* and *PaFAMA* transcript levels across stages 1–4. *PaSM* expression was highest in the ACD-enriched stage and decreased markedly in the GMC differentiation/division-enriched stage. *PaSM*

transcripts were not detected in the stages enriched for maturing and mature stomata, as no Ct values were obtained (Fig. 3d). These results indicate that *PaSM* is associated primarily with early stomatal lineage stages and was most abundant in regions enriched for ACDs.

By contrast, *PaFAMA* expression showed a broader developmental distribution. Its transcript levels were highest in the GMC differentiation/division-enriched stage. However, *PaFAMA* transcripts were also clearly detectable in the ACD-enriched stage and remained detectable in the later stages enriched with maturing and more mature GCs stages (Fig. 3d). Although expression in the later stages showed some variation among samples, these data indicate that *PaFAMA* expression persists well beyond the GMC-enriched stage. This broader pattern suggests that *PaFAMA* is associated most strongly with GMC differentiation and division, while transcripts remained detectable in regions enriched for both earlier and later stages of stomatal development.

Together, these results indicate that *PaSM* and *PaFAMA* transcript abundance is associated with distinct peaks, yet partially overlapping, stage-enriched regions of developing spruce needles. Considered alongside their conserved sequence features (Fig. 2b), these expression patterns prompted us to test whether *PaSM* possesses *SPCH*- and/or *MUTE*-like regulatory capacities and whether *PaFAMA* retains *FAMA*-like activity.

PaSM* and *PaFAMA* can promote early stomatal-lineage transitions in *Arabidopsis

To assess the developmental capacities of *PaSM* and *PaFAMA* in *Arabidopsis*, we introduced *PaSM* and *PaFAMA* with triple yellow fluorescence protein (Venus) fusion in the *Arabidopsis spch-3*, *mute-1*, and *fama-1* mutants under the control of the respective *AtSPCH*, *AtMUTE*, or *AtFAMA* promoters and assessed their ability to restore stomata production and to rescue seedling lethality. This approach allowed us to test whether these proteins could support stomatal-lineage transitions controlled by *AtSPCH*, *AtMUTE* or *AtFAMA* in *Arabidopsis*.

Expression of *pAtSPCH:PaSM* in *spch-3*, which normally produces an epidermis composed only of pavement cells and display seedling lethality⁶, resulted in frequent cell divisions, production of irregularly shaped stomata and occasional single GCs (Fig. 4a). Moreover, *pAtSPCH:PaSM* was sufficient to rescue seedling lethality and recover seed production. However, we noticed that these lines displayed cracks between epidermal cells and occasionally partial detachment of the epidermis from inner layers (Supplementary Fig. 2a), which prevented accurate quantification of stomatal number in mature cotyledons collected from 10-day-old seedlings. To mitigate this issue, we analysed younger cotyledons from 5-day-old seedlings, which exhibited fewer cracks, and confirmed that *pAtSPCH:PaSM* expression restored some extent of stomata production in the absence of *AtSPCH* (Fig. 4b). These results indicate that *PaSM* supported entry into the stomatal lineage, although it does not fully restore the wild-type epidermal phenotype.

Next, we tested whether *PaFAMA* could support stomatal-lineage entry when expressed under the *AtSPCH* promoter in the *spch* mutant. *pAtSPCH:PaFAMA* expression was sufficient to drive formation of stomata and stomatal lineage cells in the cotyledons of the *spch-3*, albeit infrequently (Fig. 4a,b). The seedling-lethal phenotype of *spch-3* was occasionally rescued; however, these plant lines failed to produce seeds. We observed substantial variability in stomatal numbers both across the cotyledons and between individual seedlings. To obtain more accurate view of this phenotype, we therefore counted the total number of stomata per cotyledon. Similar to *pAtSPCH:PaSM/spch*, *pAtSPCH:PaFAMA/spch* seedlings also showed an epidermal peeling-off phenotype (Supplementary Fig. 2a). In most cases, however, this phenotype was less severe than that observed in *pAtSPCH:PaSM/spch*, enabling us to quantify stomatal numbers in 10-day-old seedlings. Our analysis showed that three independent lines with *pAtSPCH:PaFAMA* consistently induced stomata production in the *spch-3* (Fig. 4b), indicating that *PaFAMA* can support stomatal lineage entry.

Because the unique function of AtSPCH among group 1a bHLHs is to promote ACDs, we next examined whether PaSM and PaFAMA can drive ACDs in the absence of AtSPCH. We observed abaxial epidermis of young cotyledons in the *pAtSPCH:PaSM/spch-3* and *pAtSPCH:PaFAMA/spch-3* lines and found that some of the divisions were indeed morphologically asymmetric (Supplementary Fig. 2b). These findings indicate that both PaSM and PaFAMA can support stomatal-lineage entry and induce occasional morphologically asymmetric divisions in the *spch* background. The presence of stomata in these lines suggests that at least some morphologically asymmetric divisions may have been accompanied by fate asymmetry. These two genes show different ability to rescue seedling lethality, suggesting a functional and/or structural difference in the stomatal complexes produced within these lines.

We then introduced *PaSM* and *PaFAMA* into *mute-1* mutant. While both *pAtMUTE:PaSM/mute-1* and *pAtMUTE:PaFAMA/mute-1* lines showed wild-type like stomata, they still retained some arrested meristemoids similar to *mute* mutant⁷. Additionally, we occasionally observed small cells with unknown identity (Fig. 4c). In *pAtMUTE:PaFAMA/mute-1*, we frequently found single GCs, which were rarely observed in *pAtMUTE:PaSM/mute-1* (Supplementary Fig. 2c). We compared stomatal density (SD) between the wild type, *mute-1*, and *mute-1* complemented by *pAtMUTE:PaSM* or *pAtMUTE:PaFAMA*. Two independent lines of both *pAtMUTE:PaSM/mute-1* and *pAtMUTE:PaFAMA/mute-1* showed significantly increased SD compared to the *mute* mutant, although SD remained lower than that of the wild type (Fig. 4d). We defined relative proportions of stomatal cell types and found that *pAtMUTE:PaSM/mute-1* showed higher relative number of stomata, whereas *pAtMUTE:PaFAMA/mute-1* displayed higher relative numbers of meristemoids and single GCs (Supplementary Fig. 2c). Both transgenes partially rescued the defects in seedling viability and seed production observed in *mute*. These results indicate that both PaSM and PaFAMA can partially support AtMUTE function in *Arabidopsis*. However, PaSM promoted production of a higher proportion of stomata relative to meristemoids than PaFAMA did, consistent with a greater capacity to promote progression through MUTE-dependent developmental steps, including stages of GMC differentiation and symmetric division.

To further test whether PaSM act as a multifunctional transcription factor spanning both SPCH- and MUTE-associated steps, we introduced *PaSM* into *spch-3 mute-1* double mutant under the control of *AtSPCH* promoter. Notably, the *AtMUTE* promoter is active in a subset of the broader stomatal-lineage domain covered by the *AtSPCH* promoter^{6,30}. The resulting plants showed similar phenotype as *pAtSPCH:PaSM/spch-3* lines, which showed stomata with aberrant morphology and cracks between epidermal cells (Supplementary Fig. 2a). We noticed that *pAtSPCH:PaSM/spch-3 mute-1* displayed fewer number of stomata per cotyledon than *pAtSPCH:PaSM/spch-3* (Fig. 4b). However, stomata formation in the absence of both *AtSPCH* and *AtMUTE* indicates that PaSM has ability to partially support both stomatal-lineage entry and subsequent MUTE-dependent progression to stomata formation when expressed in *Arabidopsis*. The broad developmental capacity of PaSM raises the possibility that overlapping SPCH- and MUTE-like activities can coexist within a single SPCH/MUTE-like transcription factor.

PaSM* promotes pore-like structures whereas *PaFAMA* partially restores GC differentiation in *Arabidopsis

We explored whether PaSM and PaFAMA can drive the last cell fate transition in the *Arabidopsis* stomatal lineage and support the function of AtFAMA. Expression of *PaSM* in *fama-1* under the control of the *AtFAMA* promoter produced viable seedlings with a partially rescued aspects of the *fama* phenotype, although the plants later failed to produce seeds. We found clusters of small tumour-like structures resembling caterpillar-like clusters of cells present in the *fama*⁸; however, their division orientation was altered compared to typical *fama* tumours. We also occasionally found stomata-like structures; they were composed of GC-like cells with additional divisions, consistent with incomplete maintenance of terminal GC differentiation (Fig. 4e). We

classified both *fama* tumour-like and stomata-like cells as “stomatal tumours” and quantified the proportion exhibiting pore-like characteristics. In *fama* mutant, tumours did not exhibit pore-like characteristics, whereas 15–20% of stomatal tumours in the *pAtFAMA:PaSM/fama-1* lines formed pore-like structures (Fig. 4f). These findings indicate that PaSM can promote the appearance of pore-like structures within a subset of *fama* tumours but does not restore stable terminal GC differentiation in *Arabidopsis*.

In the *pAtFAMA:PaFAMA/fama-1* lines, we observed both normal stomata and “abnormal stomata”, which was characterised by additional cell divisions within GCs (Fig. 4e). We first quantified the Stomatal Index (SI) including only normal stomata; one of the three independent lines exhibited SI similar to that of the wild type. When both normal stomata and “abnormal stomata” were included, two out of the three lines displayed SI values that were not significantly different from wild type (Fig. 4f). “Abnormal stomata” showed diverse structures; most abundant defects resembled the stomata-in-stomata phenotype²⁹. Additional variants included: (i) parallel divisions along the stomatal pore axis within GCs, (ii) both parallel and vertical divisions in each GC within the same pair of GCs, and (iii) vertical divisions followed by extra divisions of the smaller daughter cell, producing structures reminiscent of *fama*-like tumours (Supplementary Fig. 2d,e). These data indicate that PaFAMA can partially substitute for AtFAMA in *Arabidopsis* by supporting stomatal pore formation and guard cell differentiation. However, the frequent additional divisions indicate incomplete enforcement of terminal cell-cycle arrest and are consistent with PaFAMA retaining residual capacity to promote cell divisions at late stomatal-lineage stages.

C.fern SM and FAMA display overlapping stomatal regulatory capacities in *Arabidopsis*

Our phylogenetic analysis showed that both SM and FAMA orthologs are also present in ferns (Fig. 2a,b). To place the gymnosperm findings in a broader evolutionary context, we investigated two bHLH1a transcription factors from the fern *Ceratopteris richardii*, CrSM and CrFAMA, and asked whether they exhibit overlapping or specialized developmental capacities. We tested their activities through cross-species complementation in *Arabidopsis* stomatal mutants.

Expression of *CrSM* or *CrFAMA* under the *AtSPCH* promoter in the *spch-3* background restored seedling viability and seed production, and induced stomatal-lineage cell formation and occasional ACDs. This indicates that both *CrSM* and *CrFAMA* can support entry into the stomatal-lineage in *Arabidopsis*. However, their effects differed: *CrSM* frequently produced excessive stomata or stomatal clusters, whereas *CrFAMA* primarily generated abnormally differentiated cells and only infrequently produced stomata. (Fig. 5a,b, Supplementary Fig. 3a,b).

Both *CrSM* and *CrFAMA* also partially complemented *mute-1* when expressed under the *AtMUTE* promoter and restored seedling viability and seed production, indicating that they can support MUTE-associated developmental transitions in *Arabidopsis*. *CrSM* lines produced paired stomata and Venus-tagged *CrSM* was detected in young GCs, where the endogenous *AtMUTE* is typically not expressed. The persistence of *CrSM* protein into this later developmental stage may lead to an additional round of symmetric division in young GCs (Supplementary Fig. 3d,e). In *CrSM* lines, SD was comparable to or higher than wild type (Fig. 5b), whereas *CrFAMA* resulted in a weaker rescue with mixed stomatal-lineage phenotypes (Fig. 5c,d, Supplementary Fig. 3f).

When expressed under the *AtFAMA* promoter, *CrFAMA*, but not *CrSM*, partially complemented *fama-1*. *CrFAMA* produced both normal and “abnormal stomata”, with phenotypes similar to those observed in *pAtFAMA-PaFAMA/fama-1* lines, whereas *CrSM* failed to restore stomatal differentiation and instead enhanced further cell proliferation within *fama*-like tumours (Fig. 5e,f, Supplementary Fig. 3g–j).

Together, these results suggest that *C.fern* SM and FAMA show overlapping activities during early and middle stomatal development, and possess overlapping capacities during early and intermediate stages of stomatal development, whereas only the CrFAMA display the capacity to support FAMA-associated terminal GC differentiation when expressed in *Arabidopsis*.

Perturbation of PaSM and PaFAMA produces overlapping and distinct effects on spruce stomatal development

Although our cross-species complementation analyses revealed the developmental capacities of stomatal bHLHs in *Arabidopsis*, their native roles and the extent of functional overlap remain unclear. To investigate the function of PaSM and PaFAMA in the native context, we generated transgenic spruce lines constitutively expressing chimeric repressor domain SRDX³¹ fused with PaSM and PaFAMA under the control of 35S promoter, hereafter referred to as *PaSM-SRDX* and *PaFAMA-SRDX*. Transgene expression was confirmed by qRT-PCR using proembryogenic mass samples (Supplementary Fig. 4a). Transgenic lines from both constructs germinated and produced viable seedlings resembling the control lines (Fig.6 a, Supplementary Fig. 4b).

We analysed mature needles collected from approximately three-month-old transgenic and control plants. All the lines formed stomata, suggesting either functional redundancy with other putative regulators of stomata production or reduced efficiency of SRDX system in spruce. To assess whether perturbation of PaSM and PaFAMA affected the cell division behavior in stomatal files, we quantified the number of cells between adjacent stomata. In both *PaSM-SRDX* and *PaFAMA-SRDX* lines, two out of four independent transgenic lines showed a significant reduction in the number of cells locating between stomata compared with the antibiotic resistance control line (Fig.6 b). The reduced number of intervening cells may reflect changes in cell proliferation or patterning within stomatal files. However, analysis of mature needles cannot distinguish whether there are changes within stomatal-lineage initiation events, division of intervening cells.

To determine whether perturbation of PaSM and PaFAMA was associated with changes in mature stomatal-complexes, we performed detailed characterisation of stomatal complex morphology in control, *PaSM-SRDX* and *PaFAMA-SRDX* lines. Strikingly, two out of four transgenic lines with *PaSM-SRDX* showed distinct differences in GC and stomatal complex morphology with a larger pore length/GC length ratio compared with antibiotic resistance control line (Fig.6 c). This indicates that *PaSM-SRDX* expression can alter stomatal pore geometry relative to GC length. In addition, the GC length/width ratio was consistently increased in the all four *PaFAMA-SRDX* lines compared to the antibiotic resistance control line (Fig.6 d), suggesting that *PaFAMA* contributes to the regulation of GC shape, possibly by promoting lateral expansion.

Because GC shape is strongly influenced by cell wall properties, we next examined whether this morphological phenotype was associated with altered pattern of pectin epitopes in the stomatal complex. We analysed labelling with LM19 and LM20 antibodies, which preferentially recognize homogalacturonan (HG) epitopes associated with lower and higher degrees of methyl esterification, respectively. In *PaFAMA-SRDX* lines, LM20 labelling was significantly reduced, whereas LM19 labelling was significantly increased at the SC wall adjacent to GCs in two out of four independent lines. This reciprocal labelling patterns suggest that there is a relative shift in detectable HG epitopes towards lower methyl-esterification within this cell wall domain.

Thus, PaFAMA perturbation is associated with altered HG epitope pattern at the GC–SC interface. However, because GC shape was altered in all the four *PaFAMA-SRDX* lines whereas significant changes in LM19 and LM20 labelling were detected in only two lines, altered HG epitope patterning alone cannot fully explain the GC shape phenotype. Notably, *PaSM-SRDX* lines did not show altered GC shape or HG epitope patterning,

distinguishing the effects of the two constructs on mature stomatal complexes (Fig. 6e,f, Supplementary Fig. 4d).

In addition to changes in mature stomatal morphology, we examined whether the GMC division pattern was affected. In both *PaSM-SRDX* and *PaFAMA-SRDX* lines, we occasionally observed cells containing oblique or perpendicular division walls within stomatal files (Supplementary Fig. 4c). These cells were more rounded than surrounding non-stomatal epidermal cells, suggesting that they may represent GMC-like cells with abnormal division orientation. The rarity of this phenotype was consistent with the narrow developmental window during which GMC divisions occur and because of this, we were not able to quantify the phenotype reliably. Nevertheless, this observation suggests that PaSM and PaFAMA activity may contribute to the proper cell division plane orientation within GMCs.

Taken together, perturbation of PaSM and PaFAMA produced partially overlapping and distinct effects on spruce stomatal development. Both *PaSM-SRDX* and *PaFAMA-SRDX* were associated with fewer epidermal cells separating adjacent stomata, indicating altered cell division patterns of stomatal cell files. The effects on mature stomatal complexes were distinct; *PaFAMA-SRDX* altered GC shape (4/4 lines) and was associated (2/4 lines) with altered HG epitope patterning at SC wall adjacent to GC, whereas *PaSM-SRDX* altered stomatal pore geometry relative to GC length (2/4 lines) without detectable changes in GC shape or HG labelling. Together with their developmental expression patterns, these results support the involvement of both PaSM and PaFAMA in spruce stomatal development while indicating partially distinct effects on stomatal-complex formation.

Discussion

While the morphology and ontogeny of conifer stomatal complex were described decades ago²⁵, their underlying molecular mechanisms have remained poorly understood. Bioinformatic analyses have revealed that gymnosperm genomes encode a single SPCH/MUTE-like gene together with a FAMA-like gene, in contrast to angiosperms, which carry three distinct genes identifiable as *SPCH*, *MUTE*, and *FAMA*^{19,20}. However, the functions of these genes have not been experimentally examined in gymnosperms.

Here, we functionally characterised SPCH/MUTE- and FAMA-like bHLH1a transcription factors from Norway spruce and *C. fern*. In *Arabidopsis*, all four transcription factors displayed developmental capacities extending beyond the stage-restricted activities of their angiosperm counterparts. Native-context analyses in spruce further supported roles for both PaSM and PaFAMA in stomatal development while revealing partially distinct effects on stomatal-complex formation. Together, these findings suggest that stomatal bHLHs in the fern and gymnosperm species studied here show broader and more overlapping functions than those of the corresponding angiosperm regulators.

Previous work showed that AtMUTE has only a limited ability to compensate for loss of AtSPCH function³², consistent with functional divergence between SPCH and MUTE. In contrast, PaSM partially complemented both *spch* and *mute* mutants and supported stomata formation in the *spch mute* double-mutant background. These findings suggest PaSM possesses broad developmental capacities spanning from stomatal-lineage entry to the subsequent MUTE-associated progression. The excessive number of cell divisions and aberrant stomatal structures observed in the *pAtSPCH:PaSM/spch-3* lines resembled phenotypes produced by an AtSPCH variant with disrupted MAPK-dependent negative regulation²⁸. This is consistent with the presence of only a partial MAPKTD in PaSM and raises the possibility that PaSM is regulated differently from AtSPCH with respect to protein stability. At the same time, some high-stringency MAPK target sites implicated in SPCH regulation are present in PaSM²⁸, suggesting that elements of this regulatory mechanism may predate the full

elaboration of the angiosperm *SPCH* module. Notably, *PaSM* did not rescue the *fama* mutant, however, *PaSM* supported pore formation within tumour-like stomatal structures in the *fama* background, a phenotype not reported for *AtSPCH/fama* complementation lines³. Thus, it remains possible that developmental capacity of *PaSM* in *Arabidopsis* extends beyond that associated with *AtSPCH*.

PaFAMA, by contrast, partially rescued the *fama* and *mute* mutant. This pattern is broadly consistent with previous reports showing that *AtFAMA* and *BdFAMA* can rescue both *mute* and *fama* mutants¹², and with studies demonstrating that the *Marchantia* ortholog *MpSETA* and the moss ortholog *PpSMF1* can partially rescue *Arabidopsis* *mute* and *fama* mutants^{3,23}. Unexpectedly, *PaFAMA* also partially supported stomatal-lineage entry in the *spch* background, a capacity not reported for *AtFAMA*, *MpSETA*, or *PpSMF1*^{3,12,23}. This suggests that besides supporting a conserved late-lineage role similar to *AtFAMA*, *PaFAMA* may also possess a broader developmental capacity than previously recognised for *FAMA*-like transcription factors. Together, these results show that both *PaSM* and *PaFAMA* can act across multiple stomatal-lineage transitions in *Arabidopsis*, although their functional emphases differ: *PaSM* can more effectively support early and intermediate transitions, whereas *PaFAMA* is more effective in promotion of terminal GC differentiation.

Comparison of the gymnosperm and fern transcription factors places the broad developmental capacities observed for *PaSM* and *PaFAMA* in a wider evolutionary context. Cross-species complementation assays showed that the two bHLH1a transcription factors from *C.fern* also function across multiple stages of the *Arabidopsis* stomatal lineage. *CrSM* partially complemented both *spch* and *mute* mutants, indicating that, like *PaSM*, it can support stomatal-lineage entry and subsequent *MUTE*-associated progression. Whereas *PaSM* contains a truncated *MAPKTD*, *CrSM* lacks this domain entirely (Fig. 2b). The *pAtSPCH:CrSM/spch* lines formed stomatal clusters resembling those produced by constitutively active *AtSPCH* variants lacking the *MAPKTD*²⁸. This phenotypic similarity raises the possibility that the absence of a *MAPKTD* contributes to altered regulation of *CrSM* activity in *Arabidopsis*. Both *PaSM* and *CrSM* also retain an N-terminal region that is absent from *AtMUTE*, which cannot substitute effectively for *AtSPCH*⁶. Although this correlation does not establish a causal role, it raises the possibility that the N-terminal region contributes to *SPCH*-like activity. Unlike *PaSM*, *CrSM* failed to rescue *fama* and instead enhanced cell proliferation within *fama* tumours. This resembles the phenotype caused by ectopic expression of *CYCD5;1* under the control of *AtMUTE* promoter in *fama-1* mutants³³. Because *AtFAMA* normally contributes to *CYCD5;1* repression, one possible interpretation is that *CrSM* cannot impose the *FAMA*-dependent cell-cycle arrest required for terminal GC differentiation. Alternatively, *CrSM* may retain an active capacity to promote the expression of cell-cycle regulators. Also *CrFAMA* displayed a broad activity profile: it partially complemented *spch*, *mute*, and *fama* mutants, thus demonstrating capacities spanning early, intermediate, and terminal stages of stomatal development. Together, these findings show that broad and overlapping developmental capacities are not restricted to the Norway spruce bHLHs. Both fern bHLHs functioned across more than one stomatal-lineage transition in *Arabidopsis*, but their functional emphases remained distinct: *CrSM* predominantly supported early and intermediate development.

The additional divisions in GCs observed in *pAtFAMA:PaFAMA/fama-1* and *pAtFAMA:CrFAMA/fama-1* could reflect incomplete maintenance of the terminal cell-cycle arrest normally imposed by *AtFAMA*. However, an alternative interpretation is that *PaFAMA* and *CrFAMA* possess division-promoting activity. Recent work showed that *SPCH* persists at low levels into late GMCs and young GCs and that elevated *SPCH* expression in the *FAMA* domain induces ectopic symmetric divisions without reverting cells to an early stomatal-lineage identity¹⁸. The resemblance of this phenotype to the additional divisions induced by *PaFAMA* and *CrFAMA*, together with their capacities to support earlier stomatal-lineage transitions, raises the possibility that these

FAMA-like transcription factors possess an SPCH-like capacity to promote cell division when expressed during late stomatal development.

The transgenic spruce lines provided native-context evidence that both PaSM and PaFAMA are associated with stomatal development. Dominant-negative perturbation of either gene was associated, in subsets of independent lines, with fewer epidermal cells separating adjacent stomata. This phenotype raises the possibility that both genes alter cell proliferation or patterning within stomatal files. *PaSM-SRDX* and *PaFAMA-SRDX* also resulted in distinct effects on mature stomatal complexes. *PaSM-SRDX* altered pore geometry relative to GC length in two independent lines, whereas *PaFAMA-SRDX* reproducibly altered GC shape and was associated in two lines with altered HG epitope patterning at the GC–SC interface. These differences suggest that perturbation of PaSM and PaFAMA affects later stages of stomatal-complex formation in partially distinct ways.

These phenotypes were broadly consistent with the developmental transcript profiles of the two genes. *PaSM* transcript abundance was highest in needle regions enriched for ACDs and GMC differentiation and was low or undetectable in regions dominated by later stages of stomatal maturation. One possible explanation for the stomatal pore phenotype is that *PaSM-SRDX* perturbs an earlier developmental event with downstream consequences for pore formation. In *Arabidopsis*, OCTOPUS-LIKE-associated polarity established during the transition from ACDs to GMC differentiation has been proposed to contribute to specification of the future ventral, pore-forming domain³⁴. If a related polarity mechanism operates in spruce, its perturbation could indirectly alter pore geometry in mature stomata within *PaSM-SRDX* lines. *PaFAMA*, in contrast, was detected across a broader range of stage-enriched needle regions, extending from regions enriched for early stomatal development to those dominated by mature stomata. This broader transcript distribution is consistent with the effects of *PaFAMA-SRDX* on GC shape and cell wall epitope patterning in mature stomatal complexes. The pattern of *PaFAMA* transcript abundance observed here differs from the predominantly late-stage expression reported for *AtFAMA* and *BdFAMA*^{8,12,35}, raising the possibility that PaFAMA has a broader developmental deployment in spruce. A broader expression pattern could also reflect presence and putative functions outside stomatal lineage and development. *Arabidopsis* FAMA participates in myrosin cell differentiation and contributes to stomatal immune responses through interactions with F-BOX STRESS-INDUCED proteins^{35,36}. Myrosin cells are specialized defence-related cells involved in the myrosinase–glucosinolate system, which protects plants against herbivores. This raises the possibility that PaFAMA may also have acquired a lineage-specific role in a defence-related cell type, such as resin ducts, which store and deliver resin to seal wounds and protect tissues against insects and pathogens³⁷. Whether PaFAMA is expressed or functions during resin-duct development remains to be tested.

pAtSPCH-driven expression of *PaSM*, *PaFAMA*, *CrSM*, and *CrFAMA* in the *spch* background resulted in a loss of epidermal integrity. Cryo-scanning electron microscopy (Cryo-EM) images of *pAtSPCH:PaSM/spch* revealed defects resembling those observed in the *Arabidopsis bodyguard (bdg)* mutant³⁸ (Supplementary Fig. 2a). *BDG* has been proposed to contribute to the polymerization of carboxylic esters in either the cuticular layer of the cell wall or the cuticle proper^{38,39}. This phenotypic similarity therefore raises the possibility that epidermal rupture in these transgenic lines involves defects in cuticle formation or in the organisation of the cuticle–outer wall interface. However, defects in cuticle biosynthesis alone are unlikely to account for the loss of epidermal cells and exposure of underlying mesophyll tissue. We therefore propose that expression of *SM/FAMA* homologues in the *AtSPCH* domain disrupts the coordination between stomatal lineage differentiation with outer wall and cuticle assembly, thereby compromising epidermal integrity.

Beyond the comparative functional analyses, our anatomical observations provide additional context for stomatal development in Norway spruce. Like many gymnosperms and some tropical species, Norway spruce forms sunken stomata^{25,40,41}. We observed that the GMC expands not only longitudinally and transversely but also along the adaxial–abaxial axis of the needle (Fig. 1f), consistent with previous observations in pines²⁵. Unlike in pine, however, Norway spruce stomata are not fully sunken immediately after GMC division, and the substomatal chamber remains only partially enclosed at this stage. These observations suggest that key developmental features—including asymmetric fate acquisition, GMC differentiation, and GC formation—are shared between Norway spruce and previously described conifers, while species-specific differences may exist in the timing of symmetric division and stomatal sinking. Such differences may reflect environmental influences or as-yet-unidentified regulatory mechanisms. As we could not find the un-sunken stomata formation in our spruce transgenic lines, its underlying mechanisms remain to be studied in future.

Several limitations of this study should be acknowledged. First, our expression analyses lack cellular-resolution spatial information. *In situ* hybridization or reporter-based approaches will be necessary to define the spatiotemporal expression domains of *PaSM* and *PaFAMA* more precisely. Second, although the SRDX-fusion lines provide native-context evidence supporting roles for *PaSM* and *PaFAMA* in spruce stomatal development, definitive functional characterisation in spruce will require loss-of-function approaches such as CRISPR-based mutagenesis or complementation under native regulatory sequences, since 1) we cannot remove repressed target by this technology so we can only see effects of removed upregulation, 2) our SRDX-fusion lines showed milder phenotype than *AtFAMA-EAR* which mimicked *fama* mutant phenotype in *Arabidopsis*⁸. The latter could be hypothetically caused by inefficient recruitment of chromatin remodelling components or histone acetylation modification enzyme by SRDX in spruce. An alternative explanation could be related to the promoter strength; since the promoter used in *Arabidopsis* line was inducible, it can potentially drive stronger expression than constitutive promoter used in spruce in this study. CRISPR lines remain technically challenging in gymnosperms but will be essential for establishing the precise developmental functions of these genes in their native context.

Taken together, our findings show that the fern and gymnosperm stomatal bHLH1a examined here possess broad and partially overlapping developmental capacities. *PaSM* and *CrSM* each supported both ACDs and GMC differentiation in *Arabidopsis*, indicating that SPCH- and MUTE-like activities can coexist within a single SPCH/MUTE-like transcription factor. *PaSM* additionally promoted pore-like structure formation in the *fama* background, suggesting that its developmental capacity may extend beyond the canonical SPCH/MUTE-associated transitions defined in *Arabidopsis*. *PaFAMA* and *CrFAMA* also acted across multiple stomatal-lineage stages. Although their strongest activities were associated with intermediate and terminal differentiation, both displayed capacities extending into earlier developmental transitions. Native-context analyses in spruce further supported roles for both *PaSM* and *PaFAMA* in stomatal development while indicating partially distinct contributions to stomatal-complex formation.

Rather than functioning as fully discrete stage-specific modules, the fern and gymnosperm bHLHs examined here may therefore operate across overlapping developmental windows with different functional emphases. This pattern is consistent with a model in which the pronounced functional specialization of stomatal bHLHs in angiosperms evolved through the progressive refinement of a more broadly acting regulatory toolkit. Our results further suggest that this specialization involved more than changes in spatiotemporal expression alone. Differences in protein stability, phosphoregulation, cofactor interactions, target-gene regulation, and other protein-intrinsic properties may also have contributed to the emergence of distinct SPCH-, MUTE-, and FAMA-associated activities.

Our data support a model in which stage-specific stomatal bHLH regulators emerged through the gradual differentiation of broadly and partially overlapping developmental capacities.

Materials and Methods

Phylogenetic analysis

The liverwort *Marchantia polymorpha*⁴², moss *Physcomitrium patens*⁴³, lycophyte *Selaginella moellendorffii*⁴⁴, ferns *Alsophila spinulosa*⁴⁵ and *Ceratopteris richardii*⁴⁶, gymnosperms *Ginkgo biloba*⁴⁷, *Picea abies*⁴⁸, *Pinus lambertiana*⁴⁹, *Pseudotsuga menziesii*⁵⁰ and *Sequoiadendron giganteum*⁵¹ and angiosperms *Arabidopsis thaliana*⁵², *Amborella trichopoda*⁵³ and *Oryza sativa*⁵⁴ were selected for phylogenetic analyses. Orthofinder⁵⁵ (version 3.1.3) was run using amino acid sequences from the selected species obtained from Phytozome⁵⁶, Fernbase (<https://fernbases.org/>), TreeGenes⁵⁷, PlantGenIE⁵⁸ and Ginkgo Database⁵⁹ (Supplementary file 1). The orthogroup containing AtSPCH, AtMUTE and AtFAMA was selected as the bHLH1a orthogroup. The *Arabidopsis* bHLH3c genes AT5G57150 and AT4G29930 were included as outgroups.

Amino acid sequences were aligned using MAFFT (version 7.505)⁶⁰ with the L-INS-i option. A phylogenetic tree was constructed with RAXML⁶¹ version 8.2.12. Based on the preliminary alignment and phylogenetic tree, the sequences looking potentially partial or otherwise misannotated gene models were manually re-annotated using FGENESH⁶². The TBLASTN⁶³ was used to identify unannotated genes and manual annotation was carried out with FGENESH+. The curated data (Supplementary file 2) was aligned with MAFFT with L-INS-i option. The resulting sequence alignment (Supplementary file 3) was used to construct a phylogenetic tree with RAXML using PROTGAMMAUTO option to define the best-fit protein substitution model, which for this data was JTT and -f a for tree estimation and bootstrapping with the 1000 bootstraps (Supplementary file 4). Tree was illustrated with FigTree (version 1.4.4) (<https://github.com/rambaut/figtree/>).

The amino acid sequences of AtFAMA, PaFAMA and CrFAMA were aligned using ClustalW⁶⁴ with default settings in MEGA11⁶⁵. The region corresponding to the previously described angiosperm FAMA-specific sequence³ was inspected in the alignment. To assess conservation of this region, the alignment columns corresponding to the FAMA-specific region in the reference angiosperm FAMA sequence were extracted from the full-length alignment. The extracted region was exported as an amino acid alignment and used for pairwise sequence comparison. Pairwise amino acid distances were calculated in MEGA11 using the p-distance model, with gaps and missing data treated by pairwise deletion. Percent amino acid identity was calculated as follows:

% identity = (1 – p-distance) × 100.

Plant material and growth conditions

Arabidopsis thaliana Columbia-0 (Col-0) was used as the wild type, all transgenic lines and mutants were in the Col-0 genetic background. The *spch-3*, *mute-1*, *fama-1* were described previously⁶⁻⁸. Seeds of *pAtMUTE:AtMUTE-CFP* line were kindly provided from Dr. Dominique Bergmann.

Arabidopsis seeds were sown on half-strength Murashige and Skoog (MS) medium (Prod. No: M0221.0100, Duchefa Biochemie), adjusted to pH 5.75 and solidified with 0.8% plant agar (Prod. No: P1001.1000, Duchefa Biochemie). After stratification for at least two days at 4 °C, plates were incubated at 23 °C under long-day condition (16 h light/8 h dark).

Norway spruce seeds were obtained from the Natural Resources Institute Finland (Luke). One seed lot was used for temporal gene-expression analyses, whereas a separate seed lot was used for morphological analyses of stomatal complexes (Fig. 1) and whole-needle imaging (Supplementary Fig. 1a).

Norway spruce seeds were sown on a mixture of peat and vermiculite (4:1) in plates and stratified at 4 °C for at least one week. The plates were then transferred to 20 °C under continuous light conditions. After germination, the seedlings were transferred to individual pots and supplied weekly with Neko fertilizer diluted 1:1,000 (KASVI-RAVINNE, NPK 7-2-2, Neko, (<https://www.neko.fi/>)). The day on which plates were transferred to the growth chamber was defined as day 0.

Surface-sterilized spores of *Ceratopteris richardii* were obtained from Carolina Biological Supply Company (<https://www.carolina.com>). Spores were suspended in sterile Milli-Q water and spread onto half-strength MS medium (Prod. No: M0221.0100, Duchefa Biochemie), adjusted to pH 5.75 and solidified with 0.8% plant agar (Prod. No: P1001.1000, Duchefa Biochemie) with spreader. Without stratification, the plates were incubated at 23 °C under long-day condition (16 h light/8 h dark). Approximately one-month-old gametophyte was moved to fresh half-strength MS medium and partially submerged in sterile Milli-Q water to promote fertilization. The day on which plates were transferred to the growth chamber was defined as day 0.

Proembryogenic masses (PEMs) of Norway spruce embryogenic cell line 11:12:04 initiated from seeds provided by the Swedish Forest Research Institute in 2011 were used for the spruce transformation. Prior to transformation, the cell line was propagated in vitro on semi-solid ½ LP medium supplemented with 10 µM 2,4-dichlorophenoxyacetic acid (2,4-D), 4 µM benzyl adenine (BA), 1% sucrose and 0.35% gelrite in darkness at 21-22°C and subcultured onto medium of the same composition every 2-3 weeks⁶⁸.

Plasmid construction and plant transformation

The coding sequence of *PaSM* (1383 bp) and *PaFAMA* (1428 bp) were amplified from cDNA prepared from frozen needles of *Picea abies* clone Z4006 (Köttjö spruce). RNA was extracted as described below. The *CrSM* genomic sequence (2566 bp) was amplified from genomic DNA isolated from *C.fern* sporophyte leaves, as described in the genomic DNA extraction section.

All plasmids used for cross-species complementation analysis were generated utilizing Multisite Gateway Technology. For first-entry clone, *pAtSPCH* and *pAtMUTE* plasmids. *AtFAMA* promoter, containing approximately 3kb upstream of the start codon, was amplified from genomic DNA of *Arabidopsis* Col-0 using previously reported primers⁷ in pDONR221/Zeo. Second-entry clones contained the *PaSM*, *PaFAMA* coding sequences or *CrSM* genomic sequence without the stop codon in pDONR221/Zeo. The *CrFAMA* coding sequence (1077 bp) was synthesized by Biomatik (<https://www.biomatik.com/>) into pDONR221/Kan based on the publicly available *C. richardii* sequence Ceric.24G025300.1 (genome version 2.1; Phytozome 14 (<https://phytozome-next.jgi.doe.gov/>)). Third-entry clones contained triple Venus and the OCS terminator, p2R3a-3xVenusYFP-OcsT, was described in Siligato et al⁶⁶. Multisite Gateway LR reactions were performed using pFRm43GW or pHm43GW as the destination vector.

Each expression vector was introduced into *Agrobacterium tumefaciens* by electroporation. The transformed *Agrobacterium* cultures were centrifuged, and the pellets were resuspended in a solution containing 5% sucrose, and 0.05% Silwet. The *Agrobacterium* suspension was applied to the shoot apical meristem of *Arabidopsis spch*, *mute*, or *fama* heterozygous mutants.

For spruce transformation, the hygromycin resistance gene *HptII* under the control of the maize ubiquitin promoter and the pea *RbcS* terminator was amplified from the p6oUZm-ActOs-Gusi vector (<https://dna-cloning.com/binaries/>) with the U078 and U077 primers flanked respectively with the F and G Greengate overhangs designed by Lampropoulos et al.⁶⁷ (Supplemental Table 1, 2). The amplified PCR product was subsequently subcloned into the pGGF000. The *PaSM* (1383 bp) and *PaFAMA* (1428 bp) coding sequences were fused at their C termini to the SRDX repression domain in pGGC000 vector³¹. The *PaSM-SRDX* and

PaFAMA-SRDX fragments were amplified from unpublished Multisite Gateway constructs containing 35S:*PaSM-SRDX* and 35S:*PaFAMA-SRDX*, respectively. Consequently, each fusion contained a 27-bp attL2-derived sequence between the coding sequence and the SRDX sequence. GreenGate assembly was used to place either *PaSM-SRDX* or *PaFAMA-SRDX* under the control of the CaMV 35S promoter and the RbcS terminator. Each expression cassette was assembled together with the HptII selection cassette into the pGGZ003 destination vector (Supplementary Table 2). The resulting constructs were introduced into Norway spruce by *Agrobacterium*-mediated transformation as described below (see “*Agrobacterium*-mediated Norway spruce transformation”).

***Agrobacterium*-mediated Norway spruce transformation**

PEMs were prepared for *Agrobacterium* transformation 10-14 days after subculturing by resuspending 3 g of PEMs in 20ml liquid ½ LP medium containing 100 µM acetosyringone and then inoculated for 3 days on a rotary shaker at 110 rpm in the dark at 20-21°C. Unless otherwise specified, the pH of all media used for transformation and following plant production was adjusted to 5.8 before autoclavation.

A dense culture of *Agrobacterium tumefaciens* containing the vector with the gene of interest were scraped from LB agar plates containing the appropriate selection antibiotics and resuspended in infiltration buffer containing 10 mM MgCl₂, 10 mM MES (pH 5.5), and 100 µM acetosyringone, then incubated for 1.5–3 h on a rotary shaker at 110 rpm in the dark at 20–21 °C.

The flask containing 20ml of resuspended PEMs was diluted 1:2 with ½ LP medium containing 100µM acetosyringone and the induced bacterial suspension to reach a final OD600 of 0.5 for 1-1.5h by co-cultivation on a rotary shaker at 110 rpm in darkness at 20–21 °C.

After co-cultivation, the PEMs suspension was filtered onto a paper disc (70 mm Whatman no 1 filterpaper, Cytiva) transferred to a 9-cm Petri dish with semi-solid ½ LP medium supplemented with 100 µM acetosyringone and incubated for 2 days in darkness at 21-22°C.

Selection of putatively transformed PEMs was performed on semi-solid ½ LP medium supplemented with 400 mg/L timentine and hygromycin in darkness at 21-22°C with subcultures every 2-3 weeks until growing clumps of PEMs appeared. The hygromycin concentration used for selection was 0.5 mg/L for the first 2 weeks followed by 2.5 mg/L. The number of PEMs-clumps that had reached 3-4 mm in diameter was recorded and the clumps isolated to be established as putatively transgenic cell lines on semi-solid ½ LP medium for further propagation and plant production. Putative transformed PEMs were verified by genotyping PCR and qRT-PCR as described below.

The verified transformed spruce embryogenic tissue was transferred from semi-solid ½ LP medium to semi-solid DKM maturation medium⁶⁸ containing 3% sucrose, 0.5 g/L casein hydrolysate, 0.35% gelrite and cultivated for 7 days in the darkness at 21–22 °C. The cultures were then transferred to semi-solid DKM maturation medium supplemented with 29 µM ABA and cultured for 3 weeks, followed by subculturing onto fresh maturation medium supplemented with 29 µM ABA for an additional 3 weeks under the same conditions. Mature somatic embryos were manually picked and dried for 2 weeks under high humidity, in darkness at 21-22°C then germinated in squared 10 cm petri dishes on semi-solid AD1 germination medium⁶⁹ containing 3% sucrose, 0.35% gelrite and 0.5 g/L casein hydrolysate. Petri plates with germinating somatic embryos were placed for one week in dark at 21-22°C followed by a constant red light (wavelength: 660 nm; TL-D 18W/15, Philips, Stockholm, Sweden) at 5 µmol/m²/s at 21-22°C for 1-2 weeks. The Petri plates with geminants were gradually moved to constant white, fluorescent light (Fluora L 18W/77, Osram, Johanneshov, Sweden) at 55-60 µmol/m²/s at 22°C until fully developed with root and shoot^{69,70}. Developed germinants (after approximately

2 months under light) were acclimatized in a peat:perlite (80:20) composition under 24 h light conditions (Valoya LED lamps) at 110-120 $\mu\text{mol}/\text{m}^2/\text{s}$ at 21-22°C. The spruce plants were fertilized once per week using RIKAs (SW, Horto).

RNA extraction

For cloning of *Picea abies* genes, total RNA was extracted from approximately 100 mg of frozen tissue using a CTAB-based method modified from Chang et al⁷¹. Tissue was ground to a fine powder in liquid nitrogen and suspended in 750 μl of extraction buffer containing 2% (w/v) CTAB, 2% (w/v) PVP, 100 mM Tris-HCl (pH 8.0), 25 mM EDTA and 2 M NaCl, supplemented immediately before use with 2% (v/v) β -mercaptoethanol. Samples were incubated at 65°C for 1–3 min and extracted twice with an equal volume of chloroform:isoamyl alcohol (24:1, v/v). After centrifugation at 13,000 rpm for 10 min at room temperature, the aqueous phase was collected, and RNA was precipitated overnight at 4°C by adding one-quarter volume of 10 M LiCl. RNA was collected by centrifugation at 13,000 rpm for 15 min at 4°C and dissolved in nuclease-free water. RNA was further precipitated with 0.1 volume of 3 M sodium acetate and two volumes of absolute ethanol for at least 2 h at –20°C. The RNA pellet was washed with 70% ethanol, briefly air-dried and dissolved in nuclease-free water.

For qRT-PCR analysis of spruce needles, eight approximately one-month-old seedlings were pooled for each biological replicate. Needle samples were collected from four regions: the basal half of 0.2–0.3 cm needles, the basal half of 0.4–0.6 cm needles, the distal half of 0.4–0.6 cm needles, and the distal one-third of 0.8–0.9 cm needles. These samples were designated as stages 1, 2, 3, and 4, respectively. The predominant stomatal developmental stage in each sample group was determined by confocal microscopy. Stage 1 was characterised by prevalent ACDs, although some differentiating and dividing GMCs were also observed in this region (Fig. 3A). Stage 2 consisted mainly of differentiating and dividing GMCs, with some ACDs also present (Fig. 3B). Stage 3 was dominated by maturing stomata; GMC divisions were occasionally observed, whereas ACDs and GMC differentiation were absent (Fig. 3C). Stage 4 consisted predominantly of maturing or mature GCs. For verifications of putative transformed spruce PEMs, an approximately 5 × 5 × 5 mm piece of proembryogenic mass was collected from each independently transformed line. RNA was extracted using modified versions of the CTAB protocol described above with reference to Pavy et al⁷². For needle samples, the extraction buffer was supplemented with 0.5 g l⁻¹ spermidine and 2% (v/v) β -mercaptoethanol. For proembryogenic mass samples, the buffer was supplemented with 0.5 g l⁻¹ spermidine, and β -mercaptoethanol was replaced with 2 M DTT added immediately before use at final concentration 40 mM. Frozen samples were ground in liquid nitrogen and mixed with 750 μl of extraction buffer preheated to 65°C. Samples were incubated at 65°C for 10 min, with intermittent vortexing, and extracted twice with 500 μl of chloroform:isoamyl alcohol (24:1, v/v). Following each extraction, samples were centrifuged at 21,000 × g for 5 min at room temperature, and the upper aqueous phase was transferred to a new tube. RNA was precipitated by adding an equal volume of 7.5 M LiCl containing 50 mM EDTA and incubating at –20°C for at least 1 h or overnight. RNA was collected by centrifugation at 21,000 × g at room temperature, washed with 80% ethanol, briefly air-dried and dissolved in sterile Milli-Q water.

cDNA synthesis and qRT-PCR

RNA samples were treated with DNase I (Thermo Scientific, EN0521), and first-strand cDNA was synthesized using an oligo(dT)₁₈ primer and a First Strand cDNA Synthesis Kit (Thermo Scientific, K1612) according to the manufacturer's instructions. qRT-PCR was performed using HOT FIREPol® EvaGreen® qPCR Mix Plus with passive reference dye with ROX (Solis BioDyne, 08-24-00001). For developmental-stage-specific expression analysis, qRT-PCR was performed using three biological replicates and three technical replicates. Expression levels were normalised to the internal control *Actin* and are presented relative to stage 1. Transgene expression

in spruce transgenic lines was verified by qRT-PCR with one biological and technical replicate. Expression levels were normalised to the internal control *Actin* and are presented relative to WT. Relative gene expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method.

Genomic DNA extraction

C.fern sporophyte leaf (approximately 0.5×0.5 cm) was flash frozen with liquid nitrogen and was ground with frozen mortar and pestle. With the frozen material, we extracted DNA using DNeasy Plant Mini Kit (Qiagen, Cat no. / ID. 69104) following the provided protocol.

For verification of putatively transformed spruce PEMs, frozen PEM samples were allowed to thaw at room temperature for 5–10 min and suspended in 700 µl Edwards extraction buffer (200 mM Tris-HCl, pH 7.5, 250 mM NaCl, 25 mM EDTA, pH 8.0, and 0.5% SDS). The tissue was homogenised using a pestle. Samples were centrifuged at maximum speed for 10 min at room temperature, and 600 µl of the supernatant was transferred to a new tube containing an equal volume of isopropanol. DNA was precipitated overnight at –20 °C. The entire precipitated sample was transferred to a HiBind DNA Mini Column (DNACOL-01, Omega Bio-tek) and centrifuged at maximum speed for 30 s. Columns were washed twice with 700 µl DNA Wash Buffer containing ethanol (SPW Buffer, SPW-200, Omega Bio-tek), with centrifugation at maximum speed for 30 s after each wash, according to the manufacturer's instructions. Columns were subsequently centrifuged empty at maximum speed for 2 min to remove residual ethanol. DNA was eluted by applying 50 µl elution buffer pre-warmed to 60 °C to the centre of the column, incubating for 2 min at room temperature, and centrifuging at maximum speed for 1 min. Extracted DNA was used as a template for genotyping PCR to test for *Agrobacterium* contamination and to confirm the presence of the *PaSM* or *PaFAMA* transgene (See Supplementary Table 1 for primers).

Sample preparation and imaging

For differential interference contrast (DIC) microscopy imaging, plants were fixed in ethanol and acetic acid (7:1) and kept in fixative at least overnight. Samples were rinsed with Milli-Q water several times and mounted in Hoyer's solution (7.5 g arabic gum, 5 ml glycerol, 103 g chloral hydrate and 30 ml Milli-Q water). After the tissues became transparent, cotyledons were observed using a Leica DM6B light microscope. 20×objective lens was used for imaging.

For confocal microscopy of *Arabidopsis* samples, seedlings were submerged in 0.1% (v/v) SCRI Renaissance Stain 2200 (SR2200) in 1× phosphate-buffered saline (PBS) using a centrifuge to stain cell wall. For propidium iodide (PI) staining, seedlings were submerged into 1 mg/mL PI solution for several seconds, followed by immediate wash with Milli-Q water. 20×objective lens was used for imaging.

For cross-species complementation analyses, unless otherwise indicated, all quantitative analyses and imaging were performed using plants homozygous for the respective mutant allele. T3 or T4 transgenic generations were used. Sample sizes are indicated in the corresponding plots.

Stomatal density was calculated as the number of stomata per unit epidermal area. Stomatal index was calculated as $SI = S / (S + E) \times 100$, where *S* is the number of stomata [including/excluding abnormal stomata, as specified] and *E* is the number of other epidermal cells.

To describe wild-type spruce needle ontogeny, needles were excised from seedlings and fixed under vacuum in 4% paraformaldehyde (PFA) in 1×PBS containing 0.05% (v/v) Triton X-100. After one hour of vacuum treatment, the needles were washed several times with 1×PBS. For longitudinal imaging, the needles were transferred to 0.1% (v/v) SR2200 in ClearSee⁷³. For cross-section imaging, needles were embedded in 4%

agarose, and 200- μ m-thick cross-sections were prepared using a Leica Microm 650V vibratome. The sections were then transferred to 0.1% (v/v) SR2200 in ClearSee. 20 $\times$ objective lens was used for imaging.

To analyse the morphology of transgenic spruce lines, seedlings were fixed under vacuum for approximately 15 minutes in 4% PFA in 1 $\times$ PBS containing 0.05% (v/v) Triton X-100. Larger seedlings were cut into several pieces before fixation. After vacuum treatment, samples were stored in the fixative at 4 °C until preparation for observation. Needles were cut longitudinally halfway through with a razor blade under a stereomicroscope and immersed in 100 mM NaOH. Samples were then incubated at 65°C for 5–30 min, and rinsed several times with distilled water. Mesophyll cells and vascular tissues were removed as thoroughly as possible. The remaining needle epidermal tissues were immersed in 0.1% (v/v) SR2200 in ClearSee.

Stained samples were imaged with a Leica Stellaris 8 confocal microscope or a Leica DM6B fluorescence microscope. The number of epidermal cells separating adjacent stomata was quantified from stitched images over the longest continuous region in which each stomatal file could be reliably traced. For morphological analysing spruce transgenic lines, z-stack images were acquired. the optical section in which the pore reached its maximum length was selected. For guard cell length and width measurements, the optical section in which the guard cells reached their maximum dimensions was selected. Pores and GCs were manually outlined in ImageJ, and pore length and GC length were measured as the maximum Feret diameter, whereas GC width was measured as the minimum Feret diameter. Sample sizes are provided in Supplementary Table 3. 20 $\times$ or 10 $\times$ objective lens was used for imaging.

Immunolabelling and image analysis

Immunolabelling was performed largely as previously described⁷⁴. Briefly, mature or maturing needles were excised from fixed seedlings and washed twice with 1 $\times$ PBS. The needles were then embedded in 4% agarose dissolved in 1 $\times$ PBS, and 200- μ m-thick cross-sections were prepared using a Leica Microm 650V vibratome.

The sections were blocked in blocking buffer consisting of 2% (w/v) bovine serum albumin (BSA) in 1 $\times$ PBS containing 0.1% (v/v) Tween-20 for at least 30 min at room temperature with gentle agitation. Primary antibodies, LM19 or LM20 (PlantProbes, University of Leeds), were diluted 1:200 in blocking buffer, and the samples were incubated in the diluted primary antibody solution overnight at 4°C. The next day, the samples were washed five times with blocking buffer.

Samples were then incubated overnight at 4°C with secondary antibody, anti-rat IgG Alexa Fluor 488, diluted 1:400 in blocking buffer. After incubation, the samples were washed five times with 1 $\times$ PBS and cell walls were stained with 0.05% (v/v) SR2200 in 1 $\times$ PBS. Samples were observed using a Leica Stellaris 8 confocal microscope.

Fluorescence signal intensities were quantified in Fiji by using the segmented line tool with spline fit to cover the signal of subsidiary cell wall at the interface with the guard cell. Mean gray value was measured and to compare signal intensities of the individual lines, values were normalised to the mean gray value of the corresponding antibiotic resistance control embedded in the same section.

Cryo-EM

All cryo-SEM samples were prepared by means of the Quorum PP3010T Cryo-SEM preparation system. Firstly, the cut leaves were mounted on an aluminum stub by low temperature glue (Colloidal Graphite + O.T.C compound) and then plunged into slush nitrogen. After that, the deep-frozen samples were transferred into a pre-chamber at -140 °C, where a 5 nm thick platinum coating was deposited for reducing charging effect. Finally, the samples were loaded into the cryo-stage at 140 °C inside the JEOL JIB-4700F.

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Author Contributions

AV and MZI conceived and designed the research. MZI performed most of the experiments. KE contributed to the quantification of the cross-species complementation lines. AFMV performed phylogenetic analyses. VS performed the immunolabeling experiments and associated quantification. SG participated in experimental design. SJ, TD, UE, and ON optimized the spruce transformation protocol and generated the transgenic spruce lines. AV and MZI wrote the manuscript with input from all authors.

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Fig. 1 Conserved and distinct features of stomatal development in Norway spruce.

a, Schematic representation of stomatal development in a young Norway spruce needle near the vegetative meristem. Stomatal development proceeds from the base of the needle toward the tip. **b-f**, Detailed structures of young spruce needles. **Upper panels** show cross-sections; **Lower panels** show lateral views. **b**, Symmetric division of stomatal precursor cells. **c**, The divided protodermal cells differentiate into an elongated GMC and a round polar SC. The cell adjacent to GMC subsequently develops into lateral SMC. **d**, The GMC divides symmetrically to produce a pair of GCs, while the lateral SMC divides asymmetrically to generate lateral SCs. **e**, After GMC division, GCs begin to sink into the hypodermal layer. **f**, GCs are located at the hypodermal layer. Pink, yellow, green and blue indicate stomatal precursor cell, GMC, GC, and lateral SC, respectively; in **b**, these colours are shown as semi-transparent overlays. Blue asterisk and “P” indicate lateral SMC and polar S, respectively. White, SR2200. Scale bars, 10 µm.

Fig. 2 Genomes of ferns and gymnosperms contain a single SPCH/MUTE-like and a FAMA-like gene.

a, Maximum-likelihood bHLH phylogenetic tree of subfamily 1a. Species abbreviations are as follows: Asp, *Alsophila spinulosa* (fern); Ath, *Arabidopsis thaliana* (dicot); Atr, *Amborella trichopoda* (basal angiosperm); Cri, *Ceratopteris richardii* (fern); Gbi, *Ginkgo biloba* (gymnosperm); Mpo, *Marchantia polymorpha* (liverwort); Osa, *Oryza sativa* (monocot); Pab, *Picea abies* (gymnosperm); Pla, *Pinus lambertiana* (gymnosperm); Pme, *Pseudotsuga menziesii* (gymnosperm); Ppa, *Physcomitrium patens* (moss); Sgi, *Sequoiadendron giganteum* (gymnosperm); Smo, *Selaginella moellendorffii* (lycophyte). Background colour shows each subclade: purple, fern-specific subclade; green, FAMA/SMF subclade; turquoise, SPCH/MUTE (SM) subclade; yellow, gymnosperm-specific subclade; red, TEC1/6 and MYC67/70 containing subclade. *Arabidopsis* bHLH3c genes were used as outgroups and are shown in pink. **b**, Comparison of domain structures of tracheophyte bHLH1a proteins from the SM and FAMA subclades. Each bHLH1a contains bHLH domain (light gray) and a conserved C-terminal SMF domain (dark gray). AtSPCH and PaSM have MAPKTD or putative MAPKTD, shown in red, although the putative MAPKTD in PaSM is truncated. AtMUTE has MUTE specific domain (blue), whereas CrSM has neither MAPKTD nor MUTE specific domain. FAMA orthologues contain a putative FAMA specific domain (green) and a putative bHLH domain extension (yellow). For putative FAMA specific domain and putative bHLH domain extension, colour intensity indicates amino acid identity relative to *Arabidopsis* sequence. From the N terminus, amino acid identities of the corresponding regions were 72.7%, 77.8% and 50.0% in PaFAMA, and 13.6%, 50.0% and 35.7% in CrFAMA. Gray rectangles are MAPK3/6 target motifs or SnRK2 motifs previously characterised in earlier studies^{28,75}. Gray dashed rectangles are putative MAPK3/6 or SnRK2 target motifs predicted from amino acid sequences, based on the consensus motifs PxS/TP for MAPK3/6 and RxxS or SxxxxE for SnRK2. Blue circles indicate experimentally characterised or putative MPK3/6 phosphorylation residues. Red circles indicate experimentally characterised or putative SnRK2 phosphorylation residues.

Fig. 3 PaSM and PaFAMA show temporally distinct gene expression during Norway spruce stomatal development.

a-c, Characterisation of stomatal developmental stages in developing Norway spruce needles. Stage 1 is characterised predominantly by ACDs (**a**), stage 2 by GMC differentiation and division (**b**), and stage 3 by maturing GCs (**c**). Stage 4 represents a later stage containing more mature GCs. Schematic diagrams adjacent to the plots summarize the major stomatal developmental events represented by each stage. **d**, qRT-PCR analysis of *PaSM* and *PaFAMA* expression at each developmental stage. Expression levels were normalised to *Actin* and are shown relative to stage 1. *PaSM* was preferentially expressed at stage 1, with little or no expression at stages 3 and 4, which are characterised by maturing and mature stomata. *PaFAMA* was

predominantly expressed at stage 2, with comparable expression levels at stages 1, 3, and 4. Bars indicate mean relative expression, and error bars indicate $\pm$ s.e.m. Individual points represent biological replicates.

Fig. 4 Both *PaSM* and *PaFAMA* complement multiple steps of *Arabidopsis* stomatal development.

Cross-species complementation analysis using *Arabidopsis thaliana*. The schematic illustrates the progression of stomatal development from early lineage entry to mature guard cell formation. **a**, DIC images of 10-day-old abaxial cotyledons of Col-0, *spch-3* mutant, *pAtSPCH:PaSM/spch-3*, and *pAtSPCH:PaFAMA/spch-3*. **b**, Number of stomata per abaxial cotyledon in 5-day-old *pAtSPCH:PaSM/spch-3* seedlings and 10-day-old *pAtSPCH:PaFAMA/spch-3* seedlings. **c**, Confocal images of 10-day-old abaxial cotyledons of Col-0, *mute-1* mutant, *pAtMUTE:PaSM/mute-1*, and *pAtMUTE:PaFAMA/mute-1*. **d**, SD in 10-day-old abaxial cotyledons of *pAtMUTE:PaSM/mute-1* and *pAtMUTE:PaFAMA/mute-1*. **e**, Confocal images of 10-day-old abaxial cotyledons of *fama-1* mutant, *pAtFAMA:PaSM/fama-1*, and *pAtFAMA:PaFAMA/fama-1*. The red box indicates the region enlarged in the inset in *pAtFAMA:PaSM/fama-1*. An optical section from the z-stack reveals a pore-like structure within the stomatal tumour. **f**, Number of stomatal tumours having pores per total stomatal tumour in abaxial cotyledon in 10-day-old *pAtFAMA:PaSM/fama-1* seedlings. SI on the abaxial side of cotyledons in 10-day-old *pAtFAMA:PaFAMA/fama-1* seedlings. SI was calculated using either normal stomata only or both normal stomata and “abnormal stomata”. For **b**, **d**, and **f**, statistical significance was assessed by the Steel-Dwass-Critchlow-Fligner test; different letters indicate statistically significant differences between groups ($p < 0.05$). Box plots show the median and interquartile range, with whiskers extending to values within 1.5 $\times$ the interquartile range. Individual data points are shown as beeswarm plots. Cell walls were stained with SR2200 (**c,e**). Red asterisks, green triangles, blue asterisks, and white asterisks indicate stomata with aberrant morphology, single GCs, small cells and “abnormal”, respectively. Scale bars, 500 μ m in *pAtSPCH:PaFAMA/spch-3* in **a** and 10 μ m in all other images in **a**, **c**, and **e**.

Fig. 5 Both *CrSM* and *CrFAMA* show complementation capacity similar to Norway spruce stomatal genes.

a, DIC images of 10-day-old abaxial cotyledons of Col-0, *spch-3* mutant, *pAtSPCH:CrSM/spch-3*, and *pAtSPCH:CrFAMA/spch-3*. *pAtSPCH:CrSM/spch-3* showed a phenotype resembling *AtMUTE* overexpression⁷. **b**, SD in 5-day-old abaxial cotyledons of *pAtSPCH:CrSM/spch-3*. Number of stomata in 5-day-old abaxial cotyledons of *pAtSPCH:CrFAMA/spch-3*. **c**, Confocal images of 10-day-old abaxial cotyledons of Col-0, *mute-1* mutant, *pAtMUTE:CrSM/mute-1*, and *pAtMUTE:CrFAMA/mute-1*. **d**, Stomatal density in 10-day-old abaxial cotyledons of either *pAtMUTE:CrSM/mute-1* or *pAtMUTE:CrFAMA/mute-1*. **e**, Confocal images of 10-day-old abaxial cotyledons of Col-0, *fama-1* mutant, *pAtFAMA:CrSM/fama-1*, and *pAtFAMA:CrFAMA/fama-1*. **f**, Number of cell division per *fama* tumour in 10-day-old adaxial cotyledons of *pAtFAMA:CrSM/fama-1*. SI of abaxial cotyledon in 10-day-old *pAtFAMA:CrFAMA/fama-1* seedlings. Only normal stomata were quantified. For **b**, **d**, and **f**, statistical significance was assessed by the Steel-Dwass-Critchlow-Fligner test; different letters indicate statistically significant differences between groups ($p < 0.05$). Box plots show the median and interquartile range, with whiskers extending to values within 1.5 $\times$ the interquartile range. Individual data points are shown as beeswarm plots. Cell walls were stained with SR2200 (**c,e**). Red asterisks, white bracket, green triangles, blue asterisks, and white asterisks indicate stomata with aberrant morphology, pairs of stomata, single GCs, small cells and “abnormal stomata”, respectively. Scale bars, 10 μ m in **a**, **c**, and **e**.

Fig. 6 *PaSM* and *PaFAMA* display overlapping and distinct function in stomatal development.

a, Approximately one-month-old seedling images of antibiotic-resistant control, *35S:PaSM-SRDX* and *35S:PaFAMA-SRDX* lines. **b**, Density plots show the frequency distribution of the number of cells between

adjacent stomata. **c**, Pore length/GC length ratio of wild type, antibiotic-resistant control, 35S:*PaSM-SRDX* and 35S:*PaFAMA-SRDX* lines. **d**, GC length/width ratio of wild type, antibiotic-resistant control, 35S:*PaSM-SRDX* and 35S:*PaFAMA-SRDX* lines. **e**, Immunolabelling images of wild type, antibiotic-resistant control, 35S:*PaSM-SRDX* and 35S:*PaFAMA-SRDX* lines. LM19 and LM20 were used to detect distinct HG epitopes. **f**, Quantification of LM19 and LM20 signal intensity in the SC wall at the interface with GC. $n \geq 20$ subsidiary cells per line, from three independent experiments using three different plants. For **b–d**, Statistical significance was assessed using the Kruskal–Wallis test followed by Dunn’s multiple comparisons test against the antibiotic-resistant control. Asterisks indicate significant differences compared with the antibiotic-resistant control: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Box plots show the median and interquartile range, with whiskers extending to values within 1.5× the interquartile range. Individual data points are shown as beeswarm plots. For **f**, Statistical significance was assessed using a linear mixed-effects model (F-test for the fixed effect of line; LM19: $P = 0.005$, LM20: $P = 9.76 \times 10^{-7}$) followed by pairwise comparisons (Tukey method, no additional adjustments). Different letters indicate statistically significant differences between groups ($P < 0.05$). Scale bars, 1 cm in **a**, 20 μm in **e**.

Supplementary Fig. 1 Spruce needles contain one to three stomatal files on each side.

a, Longitudinal view of a young Norway spruce needle near the vegetative meristem. A 0.4 cm needle from a one-month-old seedling was imaged. **b**, Confocal image of cross-section of 1 cm needle from 4.25-month-old Norway spruce seedling. Red circles indicate the stomatal cells. Gray: SR2200. Scale bar: 1 mm in **a**, 50 μm in **b**.

Supplementary Fig. 2 *PaSM* and *PaFAMA* expression in *Arabidopsis* stomatal mutants causes diverse stomatal and epidermal phenotypes.

a–i, The abaxial surface of 10-day-old cotyledons of Col-0, *pAtSPCH:PaSM/spch-3*, *pAtSPCH:PaFAMA/spch-3*, and *pAtSPCH:PaSM/spch-3* × *mute* were imaged by microscopy camera, Dino-Lite microscope. The *spch* mutant genotype was not determined in *pAtSPCH:PaFAMA/spch-3*, whereas the other lines were homozygous for the *spch* mutation. The *mute* mutant background was homozygous in *pAtSPCH:PaSM/spch-3* × *mute*. **a–ii**, Cryo-EM images of 4-day-old or 5-day old abaxial cotyledons of *spch-/+*, *spch-/-*, and *pAtSPCH:PaSM/spch-3*. **b**, Confocal images of 2-day-old abaxial cotyledons of Col-0, *spch-3* mutant, *pAtSPCH:PaSM/spch-3*, and *pAtSPCH:PaFAMA/spch-3*. In *pAtSPCH:CrFAMA* lines, most epidermal cells showed intracellular signal with propidium iodide, possibly due to temporary changes in membrane permeability, differences in cell wall, membrane structure, cuticle, or cell death. **c**, Ratio of cell types at 10-day-old abaxial cotyledon of *pAtMUTE:PaSM/mute-1* and *pAtMUTE:PaFAMA/mute-1*. **d**, Ratio of cell types at 10-day-old abaxial cotyledon of *pAtFAMA:PaFAMA/fama-1*. **e**, Ratio of cell types of “abnormal stomata” at 10-day-old abaxial cotyledon of *pAtFAMA:PaFAMA/fama-1*. White dashed lines indicate the boundary between epidermal and mesophyll cells. Magenta, propidium iodide. Scale bar, 10 μm in **B**.

Supplementary Fig. 3 Additional characterization of stomatal phenotypes in *Arabidopsis* lines complemented by *CrSM* and *CrFAMA*.

a, DIC images of 10-day-old abaxial cotyledon of *pAtSPCH:CrSM/spch-3* weak phenotype line. Stomatal clusters were observed. **b**, Confocal images of the abaxial cotyledon epidermis of 2-day-old *pAtSPCH:CrSM/spch-3* and *pAtSPCH:CrFAMA/spch-3*. **c**, Microscope camera images acquired using a Dino-Lite microscope showing the abaxial surface of 10-day-old cotyledons of *pAtSPCH:CrSM/spch-3* and

pAtSPCH:CrFAMA/spch-3. The *spch* mutant background was homozygous. **d**, Confocal images of abaxial cotyledons from 2-day-old *pAtMUTE:AtMUTE-CFP* seedlings and 10-day-old *pAtMUTE:CrSM-3x venus/mute-1* seedlings. **e**, Number of pairs of stomata at 10-day-old abaxial cotyledon of *pAtMUTE:CrSM/mute-1*. **f**, Ratio of cell types at 10-day-old abaxial cotyledon of *pAtMUTE:CrSM/mute-1* and *pAtMUTE:CrFAMA/mute-1*. **g**, Confocal images of *fama* tumours in abaxial cotyledons from 10-day-old *fama-1* and *pAtFAMA:CrSM/fama-1* seedlings. **h**, SI on the abaxial side of cotyledons in 10-day-old *pAtFAMA:CrFAMA/fama-1* seedlings. SI was calculated using both normal stomata and “abnormal stomata”. **i**, Ratio of cell types at 10-day-old abaxial cotyledon of *pAtFAMA:CrFAMA/fama-1*. **j**, Ratio of cell types of stomata with extra divisions at 10-day-old abaxial cotyledon of *pAtFAMA:CrFAMA/fama-1*. For **e** and **h**, statistical significance was assessed by the Steel-Dwass-Critchlow-Fligner test; different letters indicate statistically significant differences between groups ($p < 0.05$). Box plots show the median and interquartile range, with whiskers extending to values within 1.5× the interquartile range. Individual data points are shown as beeswarm plots. Cell walls were stained with SR2200 (**b,d,g**), except where shown in magenta, which indicates propidium iodide staining. Scale bar, 10 µm.

Supplementary Fig. 4 *PaSM* and *PaFAMA* alter aspects of spruce stomatal development.

a, One-month-old wild type seedling. **b**, qRT-PCR analysis of *PaSM* and *PaFAMA* expression levels in proembryogenic mass samples from 35S:*PaSM-SRDX* and 35S:*PaFAMA-SRDX* lines, respectively. **c**, Fluorescence microscopy image of spruce transgenic lines showing potentially aborted GMCs. **d**, Quantification of LM19 and LM20 signal intensity in the SC wall at the interface with GC. Statistical significance was assessed using a linear mixed-effects model followed by pairwise comparisons (Tukey method, no additional adjustments). Different letters indicate statistically significant differences between groups ($P < 0.05$). Scale bar, 1 cm (**b**), 10 µm (**c**).

Supplementary file 1 Information of the data origin and curation for species used in the phylogenetic analyses

Supplementary file 2 Amino acid sequences used in phylogenetic analyses in fasta format

Supplementary file 3 Alignment of the sequences in fasta format

Supplementary file 4 Phylogenetic tree in NEXUS format with bootstrapping values

Supplementary Table 1 List of primers

Supplementary Table 2 List of vectors for spruce transformation

Supplementary Table 3 Number of samples observed for spruce transgenic analysis

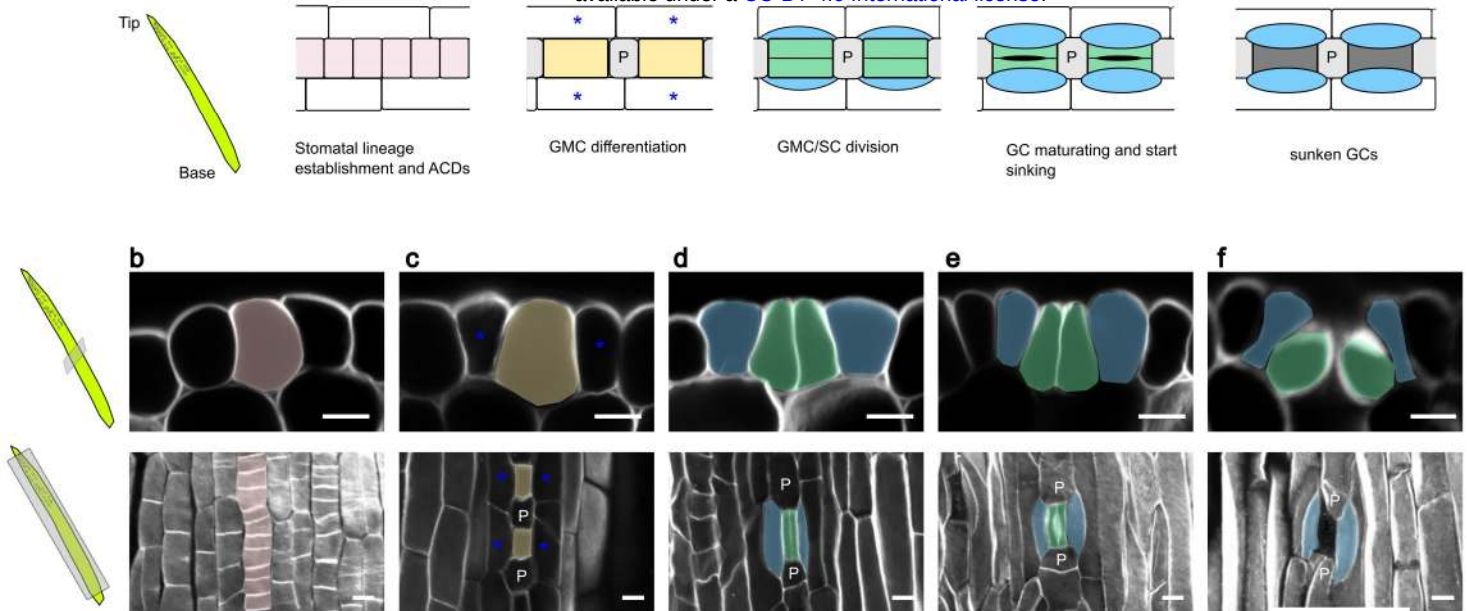


Fig. 1

a

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b

Sequence alignment of bHLH proteins (AtrSPCH, PaSM, CrSM) and their orthologs (AtrMUTE, AtrFAMA, PaFAMA, CrFAMA) across various species. The alignment shows conserved regions and substitutions. Red dots indicate specific substitutions, and blue dots indicate conserved regions.

Sequence alignment (AtrSPCH, PaSM, CrSM):

```

AtrSPCH 168 EVLSRPRVPSRPPSPVLSRKPPLSPRINHHQIHHLHLLPPISPRTPQPTSPYRAIPPQ
PaSM 317 EVLSRPRCSRPP-----PVSPRP-----LPASRTPQPGNPYR-----
CrSM 316 ESINPR-----MANKGSTPHGN-----

```

Sequence alignment (AtrMUTE, AtrFAMA, PaFAMA, CrFAMA):

```

AtrSPCH 228 LPLIPQPLRSYSSLASCSGLDPPYSPASSSSSSPSVSSNHESSVINELVANSKSALA
PaSM 377 -----P--KNIQSFPCT-----SIDSNFET--VKELVANSKSAVA
CrSM 376 -----VIDQLNLQGVKQELVASSRSEFA

```

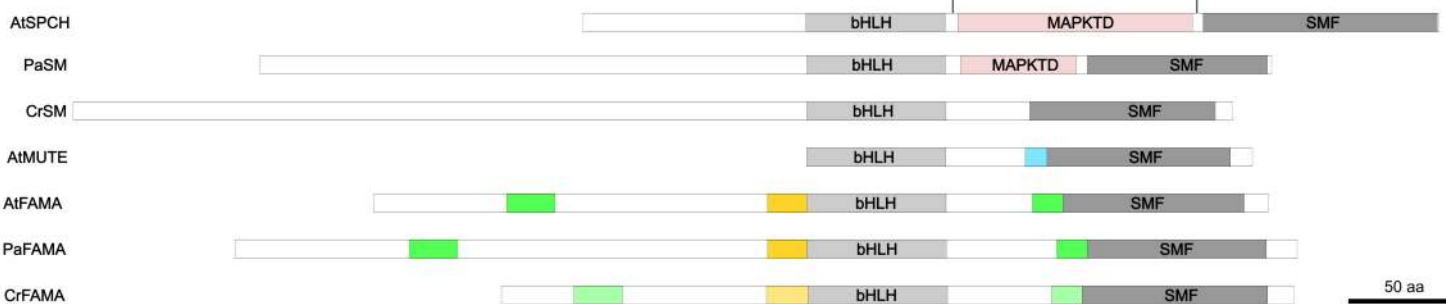


Fig. 2

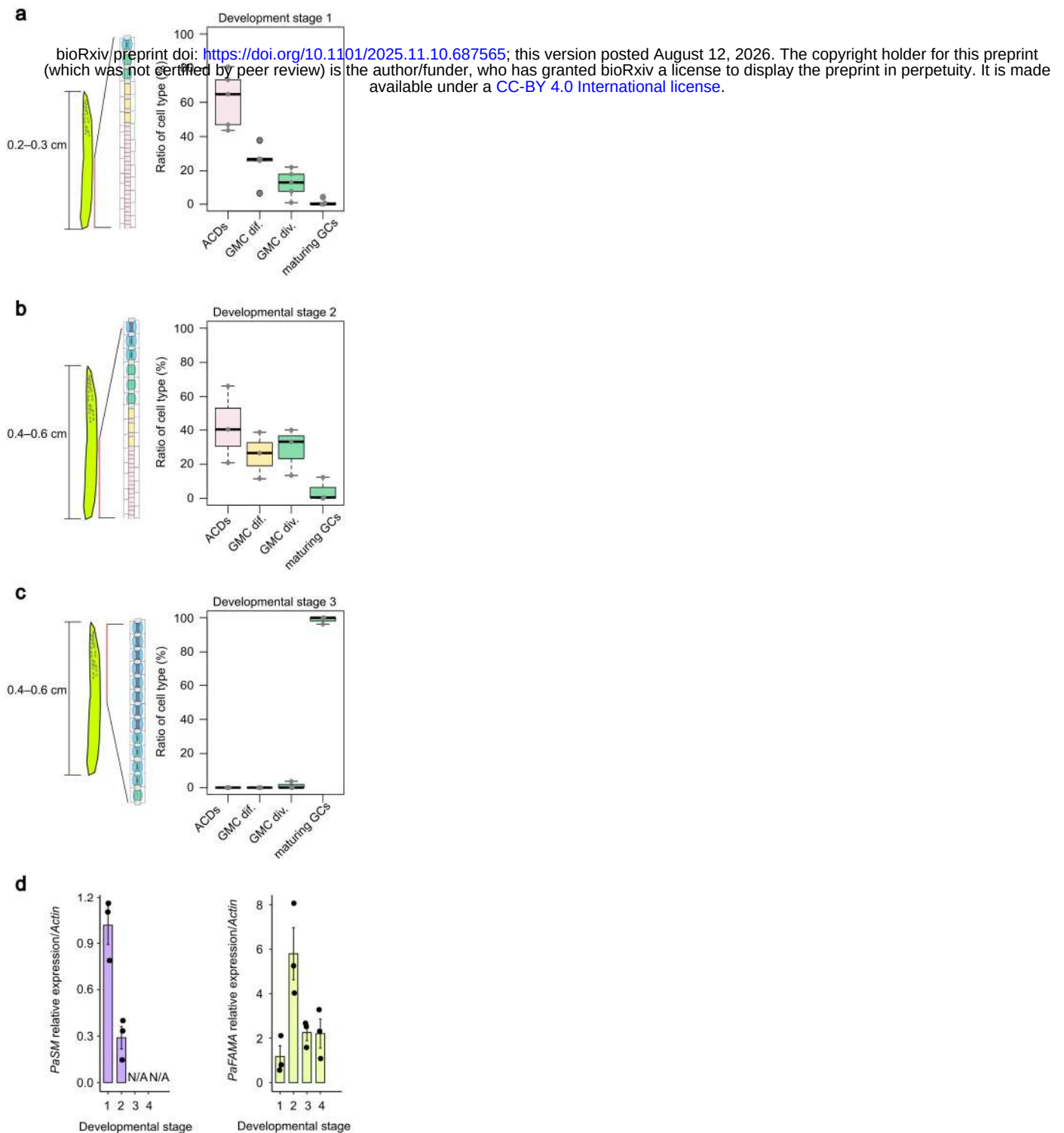
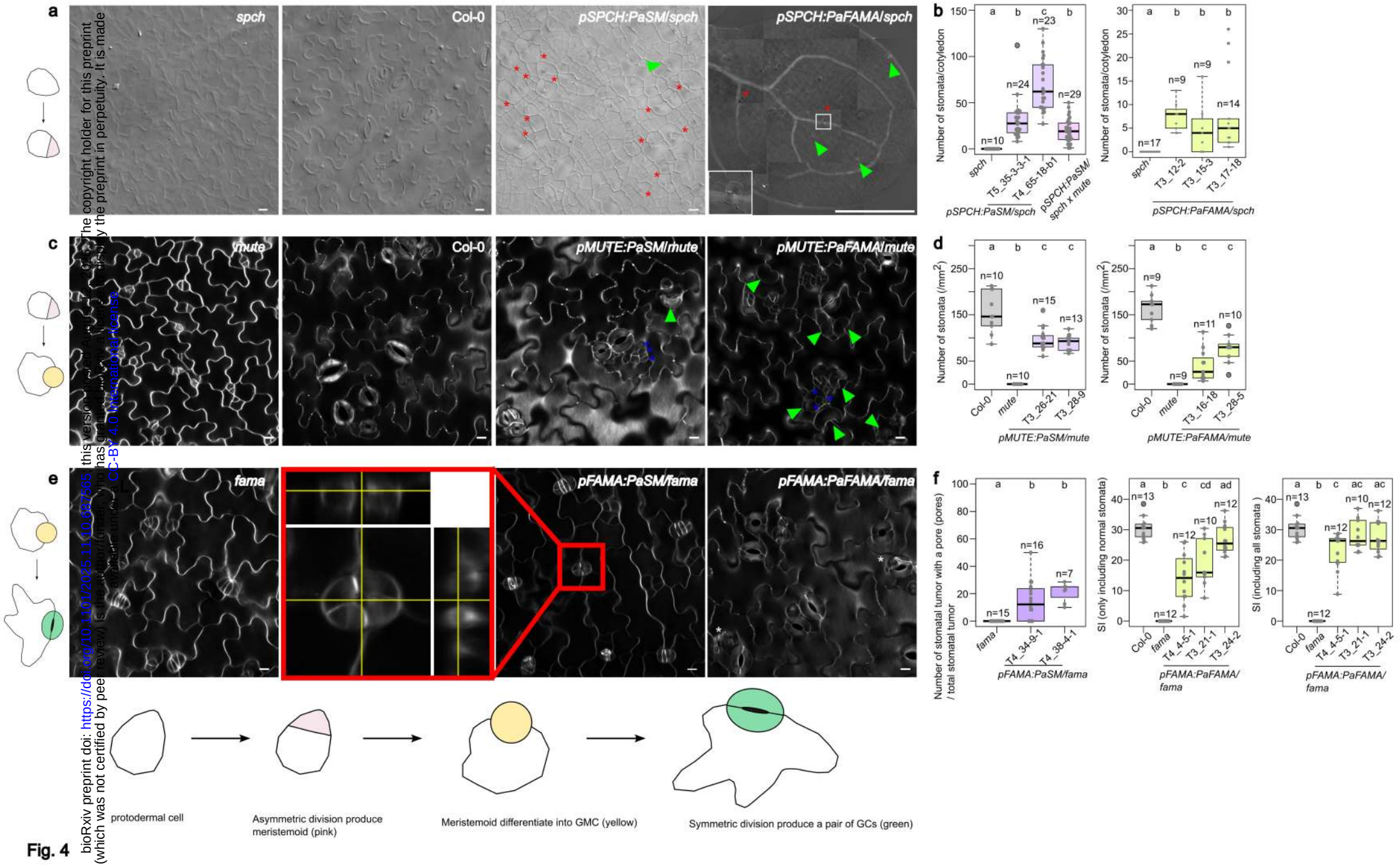
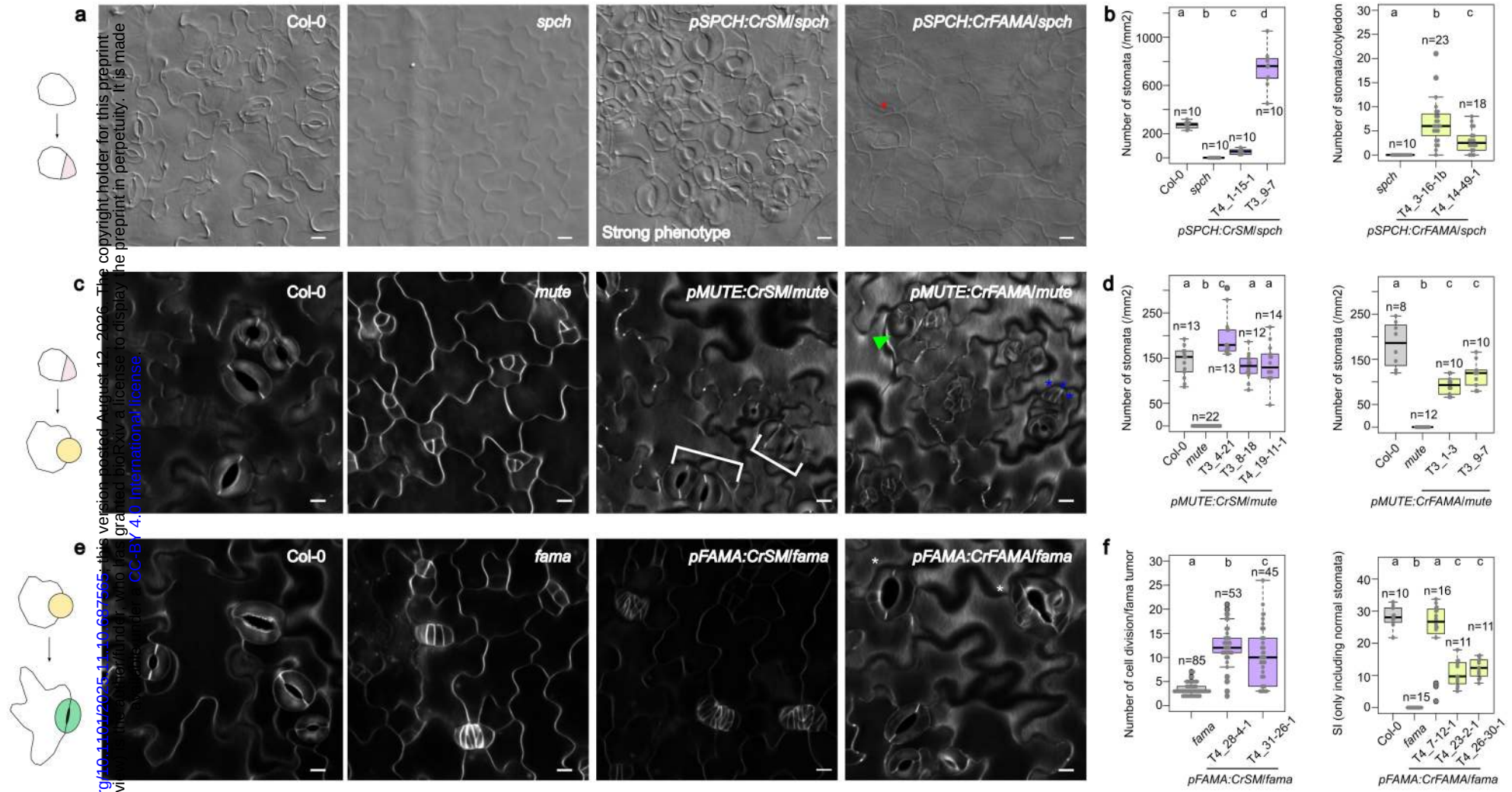


Fig. 3





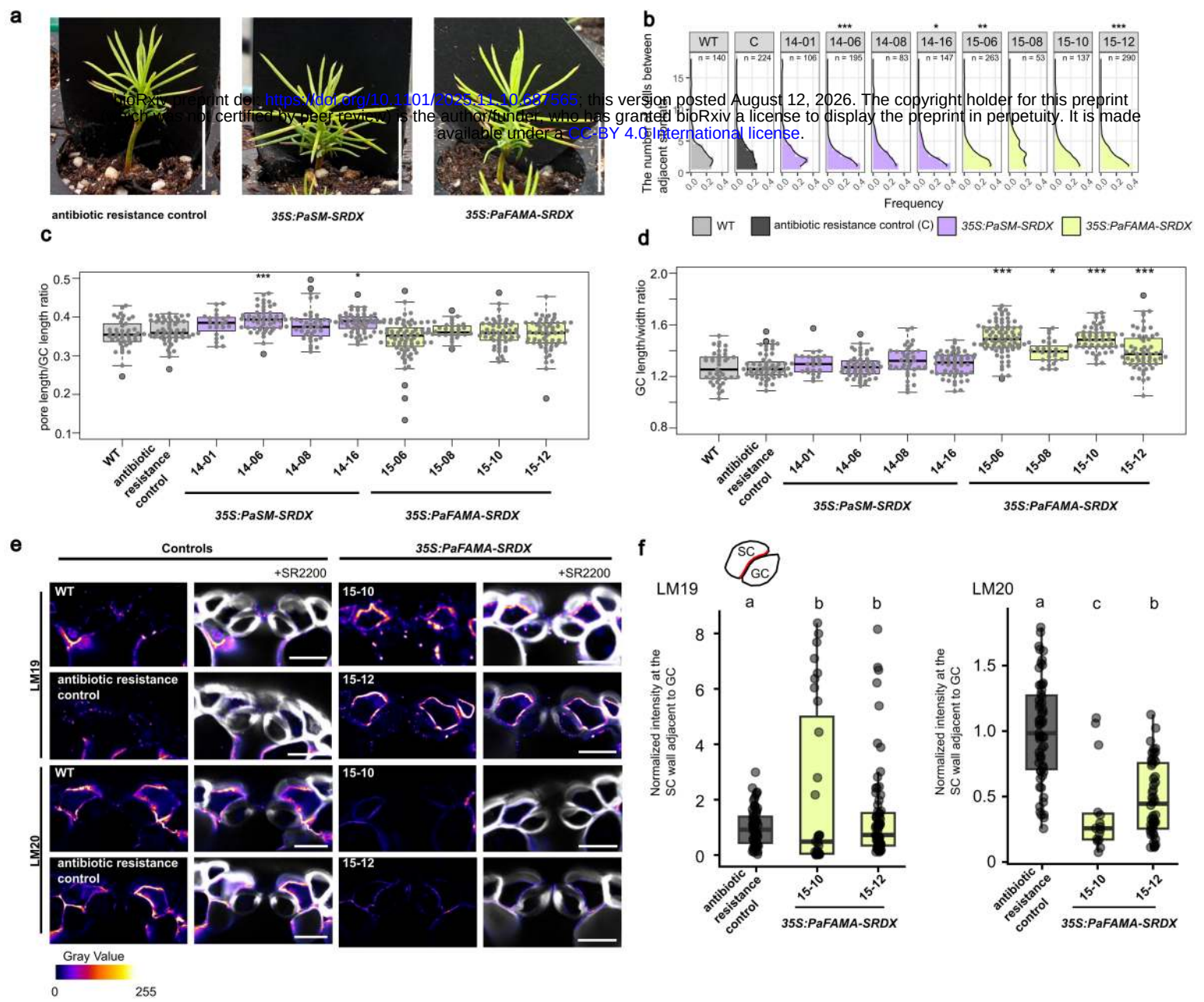


Fig. 6