Acquiring control: the evolution of stomatal signalling pathways

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Abstract

In vascular plants, stomata balance two opposing functions: they open to facilitate CO_2 uptake and close to prevent excessive water loss. Here, we discuss the evolution of three major signalling pathways that are known to control stomatal movements in angiosperms in response to light, CO_2 and abscisic acid (ABA). We examine the evolutionary origins of key signalling genes involved in these pathways and compare their expression patterns between an angiosperm and moss. We propose that variation in stomatal sensitivity to stimuli between plant groups are rooted in differences in i) gene presence/absence, ii) specificity of gene spatial expression pattern, and iii) protein characteristics and functional interactions.

Stomata: an evolutionary innovation

Adjustable stomata, which can open to enable CO_2 uptake and close to prevent water loss, represent a crucial plant adaptation to dry terrestrial environments. Stomata, comprising two guard cells that flank a central pore, are found on sporophyte tissues in nearly all land plants, from bryophytes (with the exception of liverworts) to angiosperms. It has been proposed that the stomata of bryophytes (mosses and hornworts) and vascular plants have different evolutionary origins, based on their absence in basal moss lineages and their association with intercellular spaces, which are thought to have multiple origins [see 1, 2, 3]. However, the alternative hypothesis of a single stomatal origin receives strong support from the shared homology of stomatal development genes between these plant lineages [4, 5]. This includes orthologs of the arabidopsis bHLH transcription factor *FAMA*, which share a conserved role in guard cell specification between moss and angiosperm species [6, 7]. For simplicity, we adopt the theory of a single stomatal origin herein.

Recent studies have revealed considerable differences in stomatal function between bryophytes and vascular plants. Bryophyte stomata, which are limited to sporangia, function predominantly to promote water loss for spore desiccation and develop mechanical restrictions that prevent stomatal closure when they mature [6, 8-11]. Fossils of extinct non-vascular plants with similar characteristics, indicate that these features are ancestral [9, 12]. In contrast to bryophyte stomata, vascular plant stomata remain flexible throughout development [11, 13], enabling them to close during unfavourable conditions like drought and restrict plant water loss. This suggests that, prior to lycophyte divergence, there was a shift in the role of stomata from promoting spore desiccation, to preventing water loss in vegetative tissues. In addition, stomata in vascular plants play a major role in CO_2 uptake, while the importance of stomata for CO_2 acquisition in bryophytes is currently debated [2, 9, 14].

In the last decade, interest in stomatal function in earlier-diverged plant lineages has gained considerable momentum. In particular, it has been debated whether or not these plant groups control stomatal aperture using the same mechanisms as angiosperms. Here we offer a new perspective. We integrate recent physiological results with evolutionary reconstructions for the key genetic components of the signalling pathways that control stomatal responses to light, CO_2 and **ABA (see Glossary**). To this end, we made use of recently released genome data from a charophyte alga [15], liverwort [16], and two ferns [17]. Additionally, we compare gene expression patterns between the angiosperm arabidopsis (*Arabidopsis thaliana*) and moss *Physcomitrella patens*, using publicly available microarray data [18]. This information is used to present a new model for the relative timing of key events during the evolution of guard cell signalling pathways.

Light-induced stomatal opening

Stomata open to fulfil function(s) in sporophyte desiccation (in bryophytes), and/or CO_2 acquisition (in vascular plants and possibly bryophytes). In bryophytes, there are limited reports (from one hornwort species and two closely-related moss species) that stomata open in response to white light [19-21]. However, others have reported that hornwort stomata lack light responses [22], highlighting the need for more research into stomatal opening in bryophytes. Stomatal responses to light have been studied in more detail in vascular plants. Two signalling pathways that control light-induced stomatal opening have been described in vascular plants: i) a blue light-specific pathway, and ii) a **photosynthetically active radiation** (**PAR**) pathway that overlaps with the CO_2-sensing pathway (described in the following section) [23, 24].

Guard cells sense blue light with **phototropins** (**PHOTs**) located in the guard cells (**Figure 1**)[25]. A specific stomatal response to blue light is observed in seed plants, ferns from early-diverged clades, and lycophytes [26], and thus likely evolved prior to lycophyte divergence (**Key Figure 2**). However, it has not yet been tested in hornwort and moss stomata, and thus the possibility that this response evolved prior to the divergence of bryophyte clades cannot be excluded. Blue light-induced stomatal opening has been lost in the largest living class of ferns, the Polypodiopsida [26, 27]. *PHOTs* have a general role in detecting blue light for diverse plant responses [28]. In line with this, they are expressed relatively non-specifically, in various cell types in arabidopsis [29], but within the leaf, *PHOT2* shows some degree of preferential expression in guard cells (**Figure 1**)[30]. It is likely that the *PHOT* genes originated in a green algal ancestor of land plants (**Key Figure 2**), and underwent multiple duplications separately in different plant lineages **(online Supplemental Information Figure S1, Table S1)**[31].

On exposure to blue light, PHOTs activate the **MAPKKKK BLUS1** in arabidopsis guard cells (**Figure 1**)**.** BLUS1, in turn, activates the **MAPKKK BHP**, which ultimately leads to the

activation of plasma membrane H^+-ATPases in guard cells [32-35]. In arabidopsis, *AHA1* is strongly expressed in guard cells and plays a major role in blue light-dependent stomatal opening [35]. H^+-ATPase activity causes hyperpolarization of the guard cell plasma membrane, which stimulates the uptake of K^+ via inward-rectifying Shaker channels [35, 36]. *AHA* genes are found in all plants, including algae (**online Supplemental Information Figure S2**)[37]. Stomatal opening in response to the fungal elicitor fusicoccin, which also activates H^+-ATPases [35, 38], has been reported in moss and hornwort models [19, 20], suggesting that H^+-ATPases have a conserved and ancient role in stomatal opening.

We find that both *BLUS1* and *BHP* likely arose from duplication events during angiosperm evolution, after the divergence of gymnosperms and the Amborellaceae, respectively (**online Supplemental Information Figures S3 and S4**). In arabidopsis, *BHP* shows a relatively general spatial expression pattern, suggesting that it may have additional functions in other plant tissues [34]. By contrast, *BLUS1* shows strong preferential expression in arabidopsis guard cells [30, 32]. In *P. patens*, none of the *MAPKKKK* genes from the same family as *BLUS1* show a sporophyte-specific expression pattern, with all at least weakly expressed in gametophytes, which lack guard cells (**Figure 1**). Altogether, this suggests that *BLUS1* may have evolved a specific role in angiosperm guard cells. However, further studies are needed to exclude the possibility that other related *MAPKKKK* genes fulfil a comparable role in other plant groups.

In arabidopsis, PHOTs also inhibit the activity of slow (S)-type anion efflux channels from the SLAC/SLAH family, to support stomatal opening [39]. This blue light-dependent inhibition involves two paralogous *MAPKKK* genes, ***CBC1*** and ***CBC2*** [40]. The *CBC* clade likely arose after a duplication event in a seed plant ancestor (**online Supplemental Information Figure S5**). The *CBC* genes are preferentially expressed in arabidopsis guard cells, similar to *BLUS1* (**Figure 1**). By contrast, related *P. patens MAPKKK* genes are strongly expressed in both

sporophytes and gametophytes **(Figure 1)**. It is possible that *CBCs* attained enhanced expression in guard cells after differentiation from other *MAPKKK* genes.

CO_2 signalling

In line with their role in CO_2 acquisition, angiosperm stomata open in response to low CO_2 (when CO_2 is limiting for photosynthesis), and close in response to high CO_2 (to avoid unnecessary water loss). It is likely that CO_2 functions as an intermediate signal in the stomatal opening response to PAR, which includes blue and red light; PAR fuels photosynthesis, thereby reducing CO_2 levels and triggering stomatal opening [41, 42]. PAR-induced stomatal opening is conserved between vascular plants, including ferns and lycophytes [26, 43]. However, the CO_2 responses in the clades may differ to some extent, as stomata open in response to low CO_2 levels in angiosperms in the dark, whereas this response requires light in gymnosperms, ferns and lycophytes [43, 44]. Angiosperm stomata close at high CO_2 levels, but there are conflicting results on whether other plant lineages share this response. Based on these findings, stomatal responses to high CO_2 are hypothesised to have evolved either i) prior to moss divergence [20, 45-47], or ii) in an early angiosperm [48-50].

CO_2 is converted to HCO_3^- in a reversible reaction catalysed by β-carbonic anhydrases (**βCAs**) in diverse plants, including algae [51]. CO_2 responses are reduced in arabidopsis *βca1* and *4* mutants [52, 53], and mutants for genes within the same clade in maize [54, 55], which suggests that guard cells likely sense changes in CO_2 via changes in HCO_3^-. Although the *βCA1*/2/3/*4* clade likely arose in a seed plant ancestor (**Key Figure 2**; **online Supplemental**

Information Figure S6), other *βCA* genes are likely to be capable of fulfilling a general role in CO_2 conversion in other plant groups.

It has been suggested that the arabidopsis SLAC1 anion efflux channel senses HCO_3^- directly [56, 57], facilitating stomatal responses to CO_2 [58], but (at odds with this hypothesis) a **MAPK** cascade is also important [59, 60]. Within the CO_2 signalling pathway, MPK4 and MPK12 inhibit the MAPKKK **HT1** at high CO_2 (HCO_3^-) levels [61, 62]. HT1, in turn, may act as a direct inhibitor of **OST1** [63], a **SnRK2** kinase with an important role in the activation of SLAC1 and the rapid (R)-type anion efflux channel QUAC1 [64, 65]. OST1 plays an important role in ABA signalling (as discussed in the next section), and basal OST1 activity is thought necessary for responses to elevated CO_2 [66]. HT1 can alternatively regulate the CBC proteins, which are shared with the blue light signalling pathway and also regulate SLAC1 [40]. The anion efflux through SLAC1 and QUAC1 channels depolarises the guard cell membrane and enables K^+ extrusion via the guard cell outward-rectifying potassium channel GORK [67], leading to stomatal closure (**Figure 1**)[see also 68].

The CO_2 signalling genes *MPK12*, *HT1* and *OST1* are all preferentially expressed in arabidopsis guard cells [30, 59, 69, 70], but no *P. patens* homologs of these genes show enhanced expression in sporophytes **(Figure 1; online Supplemental Information Figures S7-9)**. This suggests that specific roles and expression patterns for these genes in guard cells evolved after moss divergence**.** We find that *HT1* likely arose after a duplication event in an angiosperm ancestor (**online Supplemental Information Figure S8**). SnRK2 genes from diverse plants, including algae, can function similarly to AtOST1 [71], as discussed further in the next section. *MPK12* is thought to have arisen from duplication of an ancestral *MPK4* in the Brassicaceae [72]; in other angiosperms, *MPK4* genes are essential for CO_2 signalling, but also have a more general role, with involvement in other responses, including pathogen defence [73]. Several *P. patens* homologs (*PpMPK4a* and *PpMPK4b*) are similarly involved in immune

response signalling [74], however it is yet to be determined if these genes also play a role in any guard cell CO_2 responses.

ABA-induced stomatal closure

In accordance with a function in minimising water loss, stomata close in response to low air humidity and sustained drought in vascular plants [75-77]. In angiosperms, the stress hormone ABA plays a central role in both responses [30, 78-80]. Gymnosperms show stomatal closure in response to endogenous ABA levels and synthesise ABA after exposure to sustained dehydration stress/drought [76, 81, 82]. This suggests that ABA-dependent stomatal responses to drought stress were already present in an early seed plant ancestor [see 83]. However, ABA synthesis is slower in gymnosperms, occurring only after hours to days of sustained water stress, and gymnosperm responses to low air humidity are instead proposed to be **hydropassive** and ABA-independent [84, 85, cf. 86]. Thus rapid, ABA-mediated responses to air humidity likely evolved in angiosperms, only after gymnosperm divergence (**Key Figure 2**).

Reductions in stomatal aperture in response to exogenous ABA application have been reported in ferns [47, 87], a lycophyte [45], two moss species [20, 21], and a hornwort (*Anthoceros punctatus*) [19]. However, the latter report has been challenged by findings that the hornworts *A. punctatus* and *Phaeoceros laevis* lack stomatal responses to ABA [8], and there is now strong evidence that these stomata open once and are then incapable of closing [9, 11]. Thus far, only seed plants have been found capable of responding to the levels of ABA that the plant produces itself, during drought stress [76, 88-90]. Therefore, basic components required for ABA-signalling may have evolved early, prior to moss divergence, whereas it is likely that components that are important for ABA-induced stomatal closure arose later, in a seed plant ancestor (**Key Figure 2**).

ABA is detected by **PYR/PYL/RCAR** receptors [91-93]. Once the receptors bind ABA, they interact with Group A **PP2C** phosphatases and inhibit their activity [91, 92, 94]. In the absence of ABA in arabidopsis guard cells, PP2Cs repress OST1; thus binding of PP2Cs to ABA and the PYR/PYL/RCAR receptors releases OST1 from repression. OST1 then activates downstream targets, including SLAC1 and QUAC1 anion efflux channels [64, 65, 92, 95] (**Figure 1**). PYR/PYL/RCARs, PP2Cs and OST1-like SnRK2 kinases were likely present in the **most recent common ancestor (MRCA)** of green algae and land plants (**Key Figure 2; online Supplemental Information Figures S9-S11**)[71, 96]. Although PYR/PYL/RCAR genes are lacking from most **charophytes** [15, 97], a PYL gene was recently identified in *Zygnema circumcarinatum*, which is considered a closer relative to land plants than sequenced algal models *Chara braunii* and *Klebsormidium nitens* [96]. A general function for PYR/PYL/RCARs and Group A PP2Cs in ABA-signalling appears well conserved between vascular plants and bryophytes [16, 98]. However, diverse roles for ABA have evolved in plants [see 99, 100], including an early role in desiccation tolerance [101, 102], and later roles in spore and seed dormancy [103], and sex determination [104].

OST1 kinases from algae, bryophytes and vascular plants are all capable of activating arabidopsis SLAC1 in the heterologous *Xenopus* oocyte expression system [71, 104]. By contrast, SLAC channels from evolutionary distant groups of plants differ considerably with respect to their sensitivity to OST1. So far, SLAC1 homologs that are activated by native OST1 kinases have only been identified in moss and angiosperm models, but not in model algal, liverwort, lycophyte or fern species [71, 104]. In *P. patens*, the PpSLAC1 channel can be activated by PpOST1.2 [71]. Future studies are needed to clarify if this pair is expressed in *P. patens* guard cells, and capable of controlling stomatal aperture in the moss. In contrast to angiosperm *OST1* genes, which show a high degree of guard cell specificity [70, 105], current data suggests that *P. patens OST1* genes show a relatively non-specific expression pattern

(**Figure 1**). *P. patens SLAC/SLAH* homologs are also strongly expressed in tissues that lack stomata [68].

Concluding remarks and future perspectives

Although most of the gene families known to control stomatal movement probably evolved prior to the transition of plants to land, we find that some key gene clades are likely to have arisen later, during seed plant evolution. This includes *CBC* and *βCA1/2/3/4* clades in an early seed plant prior to gymnosperm divergence, *BLUS1* and *HT1* in an early angiosperm prior to divergence of the Amborellaceae, and *BHP* and *MPK4/11/12* later during angiosperm evolution. Even when genes are present in diverse plant groups, there can be differences in protein characteristics and interactions, affecting protein functionality, as has been found for SLAC proteins [71, 104]. Cell-specific expression patterns may also be an important characteristic to signify differences between plant groups. For genes that control stomatal movement in angiosperms, preferential expression in guard cells is a recurring feature and likely important for specific regulation of guard cell turgor. The timing of the emergence of this trait is an important question that remains to be answered. None of the *P. patens* genes discussed here show a sporophyte-specific expression pattern, with all genes also expressed in tissues that lack stomata. This suggests that signalling genes with specific roles in guard cells may have arisen later, after divergence of the mosses. However, guard cell isolation and expression profiling from diverse bryophytes, in addition to lycophytes and ferns, is needed. This work will benefit from the availability of hornwort genomes in the near future [106, 107].

As stomata are mainly found on leaves in vascular plants, most studies focus on their function in these tissues. However, stomata are also found on specialised tissue types (e.g. fern sporocarps [108], angiosperm floral nectaries and petals), and there may be differences in the

expression profiles of stomatal signalling genes associated with differences in stomatal function between tissue types [e.g. 109, 110] that remain to be explored. We still have many questions regarding the evolution of stomatal responses and the signalling pathways that control these (**see Outstanding Questions**), all of which will ultimately lead to a greater understanding of how these 'small pores with a global influence' operate.

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Glossary

ABA: abscisic acid, a stress hormone that plays an important role in stomatal closure in response to drought stress in seed plants.

AHA1: Autoinhibited H^+ P-type ATPase isoform 1 (also known as OPEN STOMATA2/OST2), a guard cell H^+-ATPase that plays a critical role in stomatal opening.

BHP: BLUE LIGHT DEPENDENT H^+-ATPASE PHOSPHORYLATION, a MAPKKK family protein involved in guard cell blue light signalling.

BLUS1: BLUE LIGHT SIGNALING1, a MAPKKKK family protein involved in guard cell blue light signalling.

βCA: β-carbonic anhydrase, an enzyme that catalyses the interconversion of CO_2 and HCO_3^-.

CBC1/2: CONVERGENCE OF BLUE LIGHT AND CO_2 1/2, two Arabidopsis paralogs within the MAPKKK family involved in guard cell blue light and CO_2 signalling.

Charophytes: a paraphyletic division of green algae that includes the closest living algal relatives of land plants.

HT1: HIGH TEMPERATURE 1, a MAPKKK family member involved in guard cell CO_2 responses.

Hydropassive: changes in guard cell turgor due to changes in apoplastic water potential in leaves.

MRCA: most recent common ancestor, the most recent individual from which a set of organisms are direct descendants.

MAPK/MPK: mitogen-activated protein kinase, a highly-conserved family of protein kinases that phosphorylates serine or threonine residues on target proteins.

MAPKKK: mitogen-activated protein kinase kinase kinase, a large family of serine/threonine-kinases, which includes BHP, CBC1/2 and HT1 from arabidopsis, each in separate subgroups.

MAPKKKK: mitogen-activated protein kinase kinase kinase kinase, a family of serine/threonine-kinases that includes arabidopsis BLUS1.

OST1: OPEN STOMATA1, a kinase from the SnRK2 family with a critical role in activation of SLAC1 anion channels for stomatal closure.

PAR: photosynthetically active radiation, light in the range of 400 to 700 nm in wavelength that supports photosynthesis.

PHOT: phototropin, a blue light-activated kinase.

PP2C: protein phosphatase type 2C, a subclass of serine/threonine phosphatases.

PYR/PYL/RCAR: PYRABACTIN RESISTANCE 1/PYR1-LIKE/REGULATORY COMPONENT OF ABA RECEPTOR, a family of ABA receptors.

SLAC1: SLOW ANION CHANNEL 1, an S-type anion efflux channel.

SnRK2: sucrose non-fermenting-1-related protein kinase 2, a plant-specific family of serine/threonine protein kinases.

Highlights

Recent findings reveal that stomata function differently in mosses and hornworts than in vascular plants, with bryophyte stomata promoting rather than preventing water loss. Important signalling genes that control stomatal opening and closure in response to changes in a plant's environment have been characterised in angiosperms. However, little is known about the evolutionary origins of these signalling pathways, and whether or not they are also present in bryophytes. Here we review recent findings in this field, and further examine the evolutionary origins and expression patterns of key signalling genes, using newly-available plant genomic and transcriptomic resources.

Outstanding Questions

Are separate stomatal opening responses to blue light and PAR present in mosses and hornworts, or did these arise in an early vascular plant, after divergence of bryophyte clades?

Do hornwort stomata open in response to low CO_2?

Do related genes fulfil the functions of seed plant-specific *βCA1/4* and *CBC* genes, and/or angiosperm-specific *BLUS1*, *HT1*, *BHP*, and *MPK4/12* genes in guard cells in other plant lineages, or do these represent novel components that have arisen during seed plant evolution?

Which genes are expressed in guard cells in earlier-diverged plant groups? When did key signalling genes become preferentially expressed in guard cells?

Are there differences in expression pattern between guard cells on leaves and those on other specialised tissue types (e.g. fern sporocarps, angiosperm petals/nectaries) present on the same plant?

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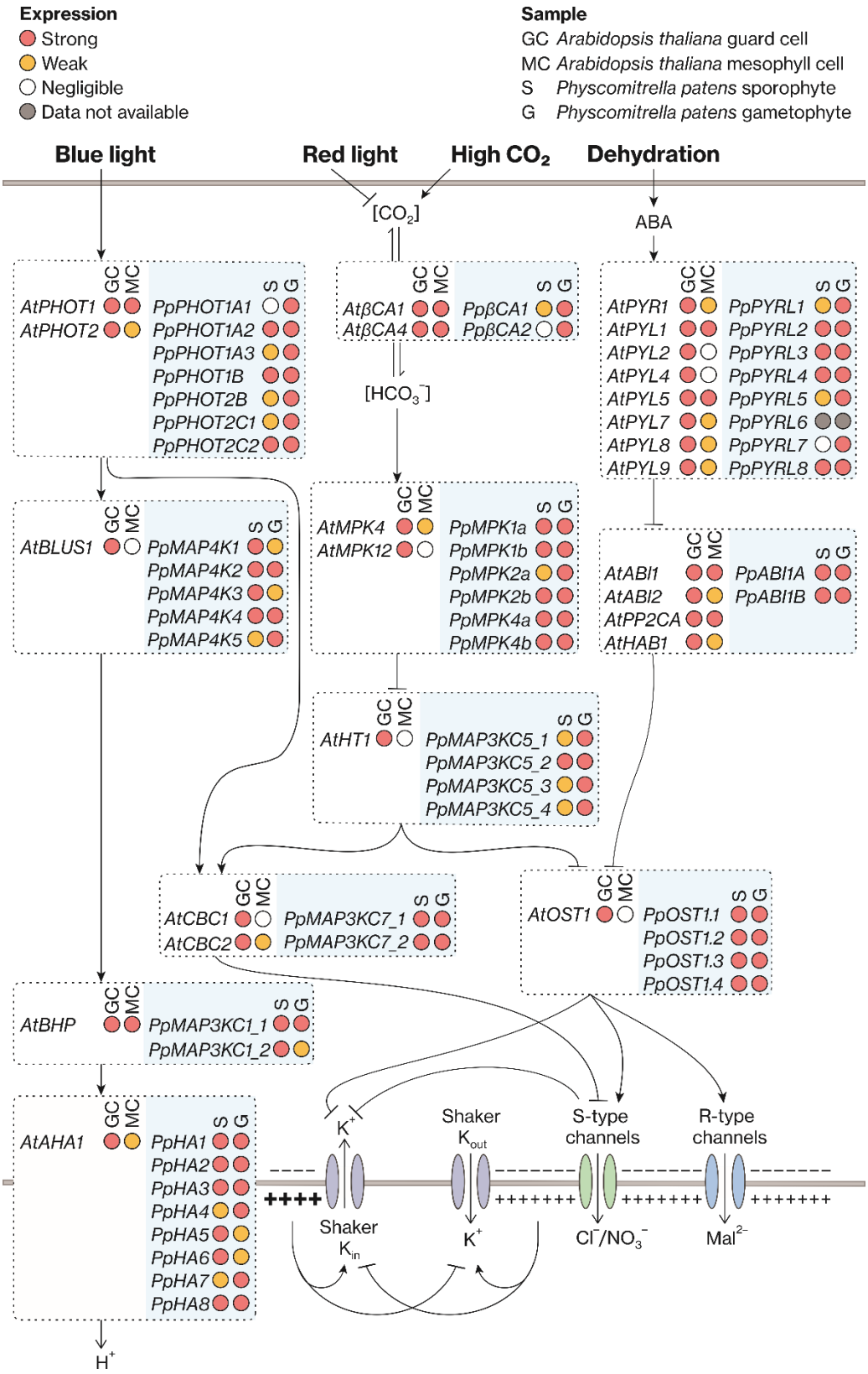

Figure 1. Guard cell expression of arabidopsis genes within the light-, CO_2-, and ABA-response pathways and homologous *Physcomitrella patens* genes.

The relative expression levels of arabidopsis stomatal movement genes in guard cells (GC) and mesophyll cells (MC) and their *P. patens* homologs in gametophyte (G) and sporophyte tissues (S), as indicated by coloured circles. Relative expression is shown as strong (50-100%, red), weak (6-49%, yellow) or negligible (0-5%, white), based on highest percent maximal expression using the microarray data for the arabidopsis water spray control samples of Yang *et al.* [111], and for *P. patens* from Ortiz-Ramírez *et al.* [18]. For *P. patens*, data is shown for stomata-bearing sporophyte tissues, occurring after peak expression of the guard cell specification gene and *FAMA* ortholog *PpSMF1* [6] (S2, S3 and M in ref. 18) and astomatal gametophyte tissues, comprising gametophore, rhizoids, caulonema, chloronema and archegonia tissues. Arabidopsis genes are interconnected by black arrows that indicate activation, or blunt-headed lines that indicate repression; unbroken grey lines represent the guard cell membrane. Only key arabidopsis genes thought to have a role within these pathways in guard cells are shown. AtPYR/PYL/RCAR family members are limited to those with highest expression in leaves/guard cells [93]. Inward- and outward-rectifying Shaker channels are abbreviated as Shaker K_{in} and K_{out}, respectively. See also Online Supplemental Information **Figures S1-S11, Table S1**.

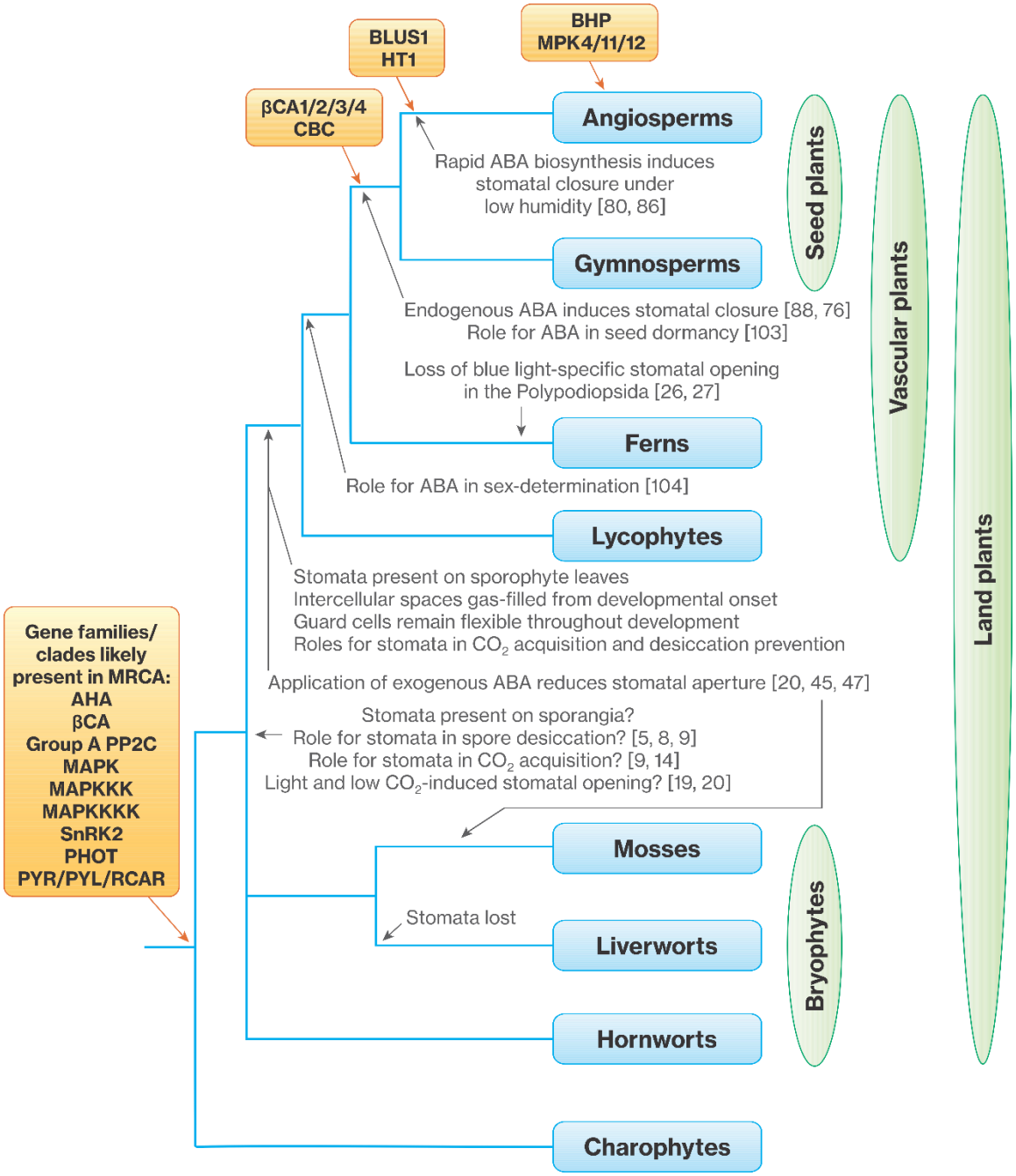

Figure 2. Predicted timing for key events during stomatal evolution.

The hypothesized timing of events is indicated on the current phylogeny for land plants (branch lengths not to scale), which recognizes current uncertainty in the relationships between bryophyte clades and vascular plants, but acknowledges strong support for a joint liverwort-moss clade [112, 113]. Question marks reflect uncertainty or disagreement in the literature. The hypothesis of a single origin for stomata (and associated loss in liverworts) is adopted (see [1] for alternatives), and charophytes are displayed as a monophyletic group for simplicity. Genes that were likely to have been present in the most recent common ancestor (MRCA) of algae and land plants are indicated. Genes that were likely to have arisen from a duplication event after divergence of the Amborellaceae are shown as occurring later in angiosperm evolution than those that are represented in *Amborella trichopoda*. See also Online Supplemental Information **Figures S1-S11**.