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On the origins of osmotically-driven stomatal movements

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Contents

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Summary

Stomatal pores with aperture that can be adjusted by changes in guard cell turgor have facilitated plant success in dry environments. We explore their evolutionary origins, considering recent findings from bryophytes. Unlike vascular plant stomata, which close to prevent water loss, bryophyte stomata become locked open to promote spore desiccation. We find that the families of ion channels, known to control stomatal movements in angiosperms, are ancient and represented across extant land plants. However, while angiosperm guard cells express specific ion channel genes, none are specifically expressed in stomata-bearing moss tissues. Given the evolutionary shift in stomatal function from promotion to prevention of water loss, we postulate that ion channels adopted guard cell-specific functions after the divergence of bryophytes.

Key words

Stomata, evolution, land plants, ion channels, hydroactive, turgor, osmotic adjustment, guard cell

I Introduction

Land plants likely evolved from a desiccation-tolerant green algal ancestor, which was unable to regulate water loss to the atmosphere. In contrast, some plant groups have become water management experts, capable of maintaining plant hydration in the driest environments. Adjustable stomatal pores represent an important adaptation enabling regulation of plant water loss. Stomata comprise a pore flanked by two guard cells, the turgor of which controls stomatal aperture; stomata open with increased turgor and close with turgor loss. These microvalves are found on sporophyte tissues throughout land plants, from bryophytes (except liverworts) to angiosperms (Fig. 1). The evolutionary origin/s of stomata in land plants remains uncertain, with divergent hypotheses of i) a single origin, or ii) multiple origins, proposed (e.g. Raven, 2002; Duckett & Pressel, 2018). Discussions are further complicated by uncertainty regarding the relationships between land plants (Puttick *et al.*, 2018; Rensing, 2018). For simplicity, we adopt the hypothesis of a single stomatal origin, supported by homology between guard cell development genes (Chater *et al.*, 2017).

Historically, angiosperms have been the predominant models in stomatal research. In angiosperms, stomatal movement is regulated via '**hydroactive**' osmotic adjustment of guard cell turgor (Box 1). These mechanisms for opening and closure rely on modification of guard cell ionic and organic contents, using plasma membrane ion channels, transporters and pumps. Alternatively, control of guard cell turgor can be '**hydropassive**', due to changes in leaf apoplastic water potential (Box 1). The origins of hydroactive mechanisms for stomatal control have been the focus of considerable debate. Opposing hypotheses predict that these mechanisms arose either i) early in a bryophyte ancestor, or ii) gradually in successive steps (e.g. Brodribb & McAdam, 2011; Chater *et al.*, 2011). We offer new perspective on this topic, by examining the evolution of key ion channels and considering their impact on stomatal control mechanisms over the past 400 million years. We find that extant land plants

share the presence of key ion channel families, but mosses seem to lack ion channels that are specifically expressed in guard cells. This suggests that the mechanisms required for osmotically-driven stomatal movement have been gradually acquired during land plant evolution.

II Stomatal form and biomechanics

Guard cells can be either 'kidney'- or 'dumbbell'-shaped (Fig. 2a-c). The kidney form was likely the earliest to evolve, as it is found across all plant lineages, including fossilised early land plants (Edwards *et al.*, 1998; Renzaglia *et al.*, 2017). Dumbbell-shaped guard cells, adjoined by two, larger subsidiary cells, are characteristic of the grasses and considered to represent a recent evolutionary advancement. The dumbbell form enhances the surface/volume ratio and, in combination with the flanking subsidiary cells, improves the speed of stomatal opening and closure, relative to dicots (Franks & Farquhar, 2007; Raissig *et al.*, 2017; Schäfer *et al.*, 2018).

The kidney-shaped guard cells of angiosperms open each stomatal pore by changing contour along the vertical/transversal axis, as well as by outward movement of dorsal walls into adjacent cells (Fig. 2d; Sharpe *et al.*, 1987; Franks & Farquhar, 2007). This lateral movement depends on radially-oriented fibres that strengthen the guard cell walls (Woolfenden *et al.*, 2017) and/or pectin-based mechanical restrictions at the polar ends of these cells (Carter *et al.*, 2017). Outward bending of the guard cells is restricted by the cell walls of neighbouring epidermal cells. Consequently, stomatal aperture depends on the turgor of both the guard cells and the counteracting adjacent cells. In most angiosperms, guard cells have a relatively small size compared to epidermal cells. This gives the epidermal cells a '**mechanical advantage**' over guard cells (Box 1; Sharpe *et al.*, 1987). As a result,

angiosperm stomata open hydropassively in response to rapid dehydration at low air humidity (Mott & Franks, 2001). This 'wrong way' response is not observed in lycophytes, ferns or gymnosperms (Brodribb & McAdam, 2011; McAdam & Brodribb, 2012), which suggests that epidermal cells lack a mechanical advantage in these plants.

The stomata of mosses and hornworts only move by changing their transversal shape (Fig. 2d; Paton & Pearce, 1957). Wall thickness and patterns of cuticularisation in fossilised stomata suggest that such changes in guard cell depth were the ancestral mechanism for pore opening (Edwards *et al.*, 1998). In contrast to vascular plant stomata, which remain flexible throughout development (Rui *et al.*, 2018), bryophyte stomata develop restrictions to movement when the guard cells mature (Merced & Renzaglia, 2013; Merced & Renzaglia, 2014; Merced, 2015). In hornworts, guard cells become differentially thickened, causing stomata to remain permanently open (Renzaglia *et al.*, 2017). Accordingly, hornwort stomata open once and become incapable of subsequent closure, even if guard cells completely lose turgor (Renzaglia *et al.*, 2017; Pressel *et al.*, 2018). Similar guard cell wall properties (thin outer walls that allow stomata to collapse open) in fossils from the Silurian and Early Devonian periods suggest that these traits are ancestral (Edwards *et al.*, 1998; Renzaglia *et al.*, 2017).

III Stomatal function

The primary role for stomata in mosses and hornworts is thought to be to facilitate the drying of sporangia (Duckett *et al.*, 2009; Pressel *et al.*, 2014; Chater *et al.*, 2016; Pressel *et al.*, 2018). In these bryophytes, stomata are found only on sporangia, and stomatal opening enables the initially liquid-filled intercellular spaces to dry, spores to desiccate and sporangia to dehisce (Duckett *et al.*, 2009; Chater *et al.*, 2016; Renzaglia *et al.*, 2017; Duckett &

Pressel, 2018; Pressel *et al.*, 2018). In line with this hypothesis, hornwort genera that lack stomata are adapted to grow in ecological niches that do not require spore desiccation (Pressel *et al.*, 2018); for example, stomata are lacking in *Notothylas*, which typically have water-dispersed spores (Glime, 2017). Fossils from the Silurian and Early Devonian periods show stomatal localisation on sporangia and similar guard cell wall properties to extant bryophytes, suggesting they shared a conserved function in spore desiccation (Edwards *et al.*, 1998; Renzaglia *et al.*, 2017).

Stomata are also present in the gametophytes of some extinct protracheophytes and early vascular plants, strongly suggesting that stomata in these early land plants had other functions in addition to spore desiccation (Edwards *et al.*, 1998). These stomata may have functioned in CO_2 acquisition, which is a major role in vascular plants and has also been proposed for bryophytes (Chater *et al.*, 2016; Renzaglia *et al.*, 2017). However, the importance of stomata for photosynthetic gas exchange in bryophytes has been questioned, as intracellular spaces are often lacking, or initially filled with liquid in hornwort and moss sporophytes (Duckett & Pressel, 2018). Alternatively, gametophytic stomata may have functioned as entry points for symbionts, similar to the gametophytic mucilage clefts of extant hornworts (Villarreal & Renzaglia, 2015).

In contrast to bryophyte stomata, vascular plant stomata are essential for preventing water loss, instead of promoting it. This prompts the question: how did these opposing functions evolve? Based on the fossil evidence above, it is likely that stomatal function changed in a vascular plant ancestor (Fig. 1). Thus, this change was probably associated with the relocation of stomata from sporangia to leaves, as major changes occurred in sporophyte form. The advantage of promoting water loss would have been lost for stomata on sporophyte leaves, and instead stomatal closure would have been advantageous in environments that

provided the selection pressure for other major water-saving plant innovations including the development of vasculature and roots.

The water-saving function of stomata is often discussed in connection with the drought stress hormone abscisic acid (ABA), which plays a key role in '**desiccation prevention**' (**Box 1**) by prompting stomatal closure. The evolution of this response has been the focus of considerable debate. Brodribb and McAdam found that ferns and lycophytes do not respond to endogenous ABA levels, and that drought induces stomatal closure by an ABA-independent, hydropassive mechanism (Brodribb & McAdam, 2011; McAdam & Brodribb, 2012). Similarly, recent findings show that several hornwort species lack a stomatal response to ABA (Pressel *et al.*, 2018). In contrast, others have reported that exogenous ABA reduces stomatal aperture in mosses, lycophytes and ferns (e.g. Chater *et al.*, 2011; Ruszala *et al.*, 2011; Hõrak *et al.*, 2017). This has led to the two theories that stomata acquired ABA sensitivity either i) early in evolution, or ii) only in a seed plant ancestor. In both scenarios, it is most likely that the stomatal ABA response evolved from an ancient role for ABA in **desiccation tolerance** (Box 1), which can be observed in moss protonemata (e.g. Pressel & Duckett, 2010), in addition to vascular plant sporophyte tissues (Giarola *et al.*, 2017). ABA has subsequently obtained diverse functions (see Sussmilch *et al.*, 2017), including spore and seed dormancy and sex determination (e.g. McAdam *et al.*, 2016; Moody *et al.*, 2016).

IV Evolution of guard cell ion channels

In angiosperms, hydroactive stomatal closure involves the efflux of anions, including Cl^- and NO_3^-, predominantly via SLOW ANION CHANNEL 1 (SLAC1)-type channels from the SLAC1/SLAC1 HOMOLOG (SLAH) family. Rapid (R)-type channels from the aluminium-activated malate transporter (ALMT) family extrude organic acids like malate, and outward-

rectifying Shaker K^+ channels release K^+. Conversely, the uptake of K^+, via inward-rectifying Shaker channels, plays a major role in hydroactive stomatal opening (see Hedrich, 2012). We examined available genome resources, including recently published genomes for the charophytic alga *Chara braunii* (Nishiyama *et al.*, 2018) and ferns *Azolla filiculoides* and *Salvinia cucullata* (Li *et al.*, 2018), for homologs of these ion channels. We found that these families are represented in charophytic algae, as well as diverse land plant lineages (Supporting Information Figs S1 and S2, Table S1), suggesting that these families were already present in an algal land plant ancestor. *C. braunii* appears to lack *SLAC/SLAH* genes (Nishiyama *et al.*, 2018), but their presence in *Klebsormidium* (Lind *et al.*, 2015), a genus thought to have diverged prior to *Chara* (Puttick *et al.*, 2018), suggests that this could be a secondary loss, or an artefact of current genome coverage.

The ability of ABA to activate guard cell SLAC1 channels, via the protein kinase OPEN STOMATA1 (OST1), is central for ABA-mediated stomatal closure (Geiger *et al.*, 2009; Schäfer *et al.*, 2018). In the *Xenopus* system, PpSLAC1 could be weakly activated by a *P. patens* OST1 ortholog (Lind *et al.*, 2015), but all algal, liverwort, lycophyte and fern SLAC1 orthologs tested could not be activated by native OST1 kinases (McAdam *et al.*, 2016). This suggests that the mechanism for SLAC1 activation may have arisen separately in moss and seed plant ancestors. Future investigation of the function and activation of SLAC1 and OST1 orthologs from an early-diverged moss, in addition to hornwort and gymnosperm species, will provide greater insight into their evolution and involvement in stomatal movement.

A recent electrophysiological study showed that guard cells from the ferns *Polypodium vulgare* and *Asplenium scolopendrium* operate voltage-dependent inward- and outward-rectifying K^+ channels with properties similar to angiosperm Shaker channels (Voss *et al.*, 2018). This is consistent with the presence of both clades of Shaker channels in sequenced fern models (Supporting Information Fig. S1). Although the importance of K^+ movement for

controlling hornwort and moss guard cell turgor has been questioned (Duckett *et al.*, 2009; Duckett & Pressel, 2018; Pressel *et al.*, 2018), we could record the activity of voltage-dependent inward and outward channels in moss guard cells from *Funaria* sp., with the hallmarks of voltage-dependent K^+ channels (L. J. Voss, M. R. G. Roelfsema, unpublished). However, while the inward-rectifying clade of the Shaker family is represented in model moss genomes, the outward-rectifying clade is absent, likely due to a secondary loss in mosses, since it is represented in the liverwort *Marchantia polymorpha* (Supporting Information Fig. S1**)**. Interestingly, the inward-rectifying clade of Shaker K^+ channels is absent in the lycophyte *Selaginella moellendorffii*, while the outward-rectifying clade is present (Supporting Information Fig. S1). The roles of missing Shaker channels might be fulfilled by a second K^+ channel family, the Big K^+-like (BK) channels, which resemble the large conductance animal BK channels. In animals, these channels allow potassium flow in response to membrane potential changes or a rise in cytoplasmic calcium level (Lee & Cui, 2010). Plant BK genes are present in bryophytes and earlier-diverged vascular plants, including the gymnosperm *Picea abies*, but they are absent in angiosperms (Supporting Information Fig. S2). The role of plant BK channels, and whether these are expressed in guard cells and fulfil any role in stomatal movement, remains to be investigated.

In *Arabidopsis*, key plasma membrane ion channels are expressed at higher levels in guard cells than other leaf cells (Fig. 3). This likely facilitates guard cell-specific turgor regulation and is relatively well conserved between angiosperms (e.g. Schäfer *et al.*, 2018). In the grasses, rapid shuttling of ions (particularly K^+) between the subsidiary and guard cells facilitates rapid stomatal movement (Raissig *et al.*, 2017; Schäfer *et al.*, 2018), and Shaker K^+ channel genes show specific expression patterns between these cell types (Büchsenschütz *et al.*, 2005). While little is known about the guard cell-specific expression of ion channel genes

in plant lineages other than the angiosperms, publicly-available microarray data allow the comparison of expression between stomata-bearing sporophytes and gametophytic tissues lacking stomata in the moss *P. patens* (Fig. 3a; Ortiz-Ramírez *et al.*, 2016). The guard cell-developmental gene *PpSMF1* (a *FAMA* ortholog) shows strong expression in an early stage of sporophyte development (Fig. 3b; Chater *et al.*, 2016). In contrast, none of the ion channel genes are truly sporophyte-specific in *P. patens*, since they are also expressed in gametophytic tissues (Fig. 3c; Supporting Information Fig. S2). Expression of some of these genes in sporophytes, leaves open the possibility that they may also be expressed in guard cells, especially *PpSLAC1* and *PpALMT3*, which both show strong expression at the same stage of sporophyte development as *PpSMF1* (Fig 3b and c). However, these genes are also expressed during gametophytic development, with strong expression in protonemata (both *PpSLAC1* and *PpALMT3*), gametophores (*PpSLAC1*) and/or rhizoids (*PpALMT3*). The expression in gametophytic tissues suggests a more general role in nutrient transport, rather than a specific role in stomatal movement. Future studies with a larger selection of early clades of land plants should reveal when ion channels were recruited to conduct guard cell-specific functions.

V Conclusions

While many gaps remain in our knowledge of stomatal evolution, recent studies have provided new insights into this process. In particular, new findings suggest that stomatal function underwent a major change: from promoting desiccation in bryophytes, to preventing water loss in vascular plants. The involvement of ion channels in stomatal closure is likely linked to their expression patterns; angiosperm guard cells specifically express key ion channel genes that play central roles in hydroactive stomatal movement. Future research should reveal if any of the uncharacterised genes we describe here encode true guard cell

channels that are regulated by signalling pathways that control stomatal movements. Given the important roles that stomata play in global water and carbon cycles, understanding the mechanisms controlling their movement, and how these evolved, remains a fascinating and important area for future discoveries.

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Author contributions

F.S., R.R. and R.H. wrote the manuscript. F.S. performed BLAST searches and phylogenetic analyses.

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Supporting Information:

Figure S1: Maximum likelihood phylogenies for ion channel families included in Fig. 3.

Figure S2: BK channels.

Table S1: Gene details.

Box 1 Definitions for key terms.

Hydroactive: adjustment of guard cell turgor through the uptake or release of ions, or synthesis of organic solutes in guard cells.

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

Mechanical advantage: an unequal relationship between the opposing forces generated by the turgor of epidermal cells and guard cells. In angiosperms, a mechanical advantage due to the differences in size of epidermal cells relative to guard cells causes a transient 'wrong way' stomatal response to a sudden drop in air humidity.

Desiccation tolerance: a strategy for plant survival during dry periods, involving mechanisms for withstanding loss of cellular water content, enabling cells to rehydrate successfully after a period of dormancy while desiccated. Mechanisms include accumulation of compatible osmolytes, stabilising compounds, and proteins, mediated by the drought stress hormone ABA.

Desiccation prevention: mechanisms for maintaining plant hydration in dry environments, including stomatal closure in combination with an impermeable cuticle.

Fig. 1 A model for the timing of key events during stomatal evolution. The evolution of key stomatal traits is indicated on the current model of land plant phylogeny (branch lengths not to scale), which recognises uncertainty in the relationships between bryophyte clades and vascular plants, but also acknowledges the strong support for a joint liverwort–moss clade (Wickett *et al.*, 2014; Puttick *et al.*, 2018). The hypothesis of a single origin for stomata (and associated loss in liverworts) is adopted; for alternatives see Rensing (2018). For simplicity, charophytes are displayed as a single monophyletic clade. Dashed lines reflect uncertainty or alternative hypotheses in the literature. Red arrowheads show stomatal location in stomata-bearing bryophytes.

Fig. 2 Stomatal forms and mechanics of movement. (a–c) Surface view of three major stomatal forms (from left to right, not to scale): (a) stoma comprising a single guard cell (seen in *Funaria* and *Physcomitrella* spp.); (b) stoma with kidney-shaped guard cells (seen in most land plants); (c) stoma with dumbbell-shaped guard cells that are flanked by subsidiary cells (seen in grasses). (d) Mechanics of stomatal opening. With increased turgor, guard cell shape changes primarily in depth and width in mosses and non-angiosperm plant lineages, while angiosperm guard cells also bend laterally into adjacent cells. Guard cells are shown in green.

Fig. 3 Ion channel function and expression patterns in moss models. (a) Diagram of cell/tissue types included in (b, c): *Arabidopsis* leaf with mesophyll cells (green) and guard cells (blue), and *Physcomitrella* gametophyte and sporophyte tissues. (b) Electronic

fluorescent pictographs (eFPs) depicting absolute expression levels of the *Arabidopsis* (At) guard cell transcription factor *AtFAMA* and its *Physcomitrella patens* (Pp) homolog *PpSMF1*. (c) eFPs depicting absolute expression levels of plasma membrane ion channel genes important for stomatal movement in *Arabidopsis* and their homologs in *Physcomitrella*. eFPs were generated using the *Arabidopsis* microarray data from the water-spray controls of Yang *et al.* (2008), and *Physcomitrella* microarray data of Ortiz-Ramírez *et al.* (2016). eFPs were adapted from outputs from ePlant (Waese *et al.*, 2017) and the *Physcomitrella* eFP browser at bar.utoronto.ca (Winter *et al.*, 2007; Ortiz-Ramírez *et al.*, 2016). Legend maxima for *Physcomitrella* genes also reflect expression in gametophytic protonemata and archegonia, spores, and protoplasts (not shown); strong expression (≥50% of maximum expression) in these tissues is indicated with text for each gene. Sequence details and phylogenetic relationships are shown in Supporting Information Table S1 and Fig. S1, respectively.

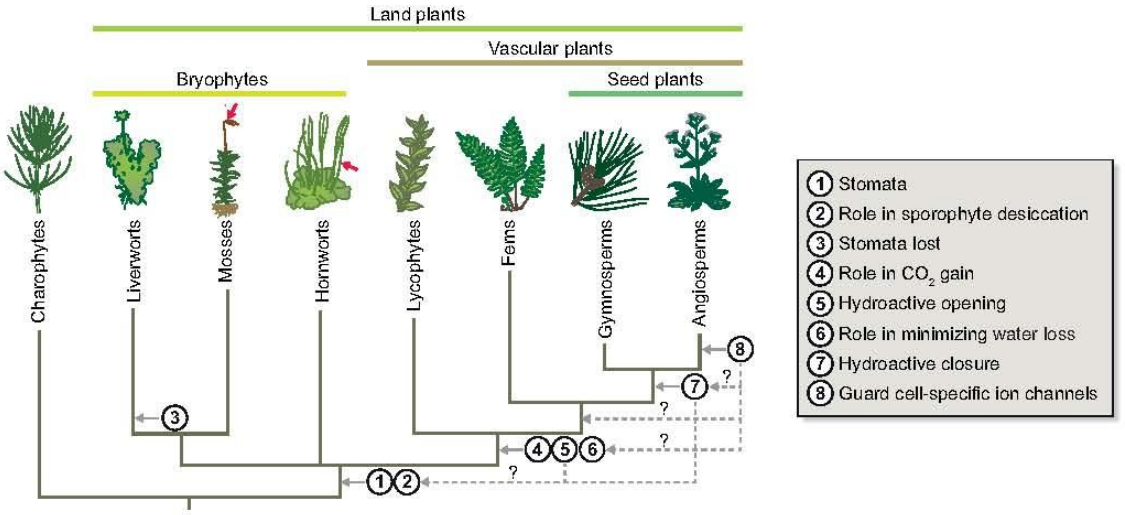

Figure 1

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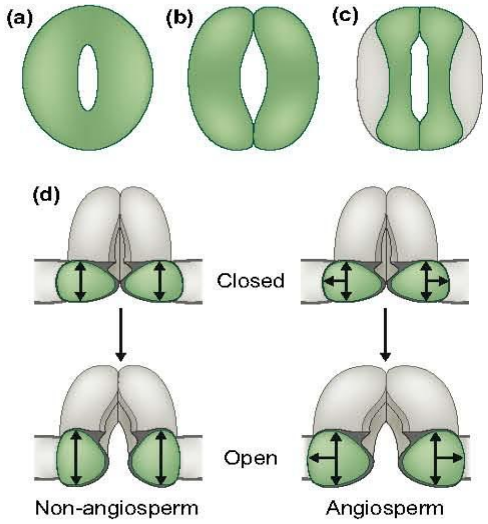

Figure 2

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Figure 3

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