Stomata: the holey grail of plant evolution

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The greatest cost associated with terrestrial photosynthesis is maintaining hydration in the presence of phenomenal evaporative forces from the atmosphere (Wong *et al.*, 1979). Without the capacity to maintain internal water reserves, vascular plants (tracheophytes) would never have escaped the soil boundary layer (Raven, 1977). Two key adaptations enable homoiohydry in vascular land plants: (i) a means to rapidly conduct large volumes of water over long distances via xylem and (ii) the ability to regulate water use by stomata (Raven, 1977). Xylem alone has long been credited for the evolutionary success of tracheophytes. Trees are only found in this clade, with most 'non-vascular' land plants (bryophytes) confined to the soil boundary layer and relying on vegetative desiccation-tolerance to survive drought (Proctor *et al.*, 2007). In contrast, stomata – which predate xylem in the fossil record and are found in most extant land plant clades, – are often relegated to a level of lesser importance for driving the evolution of homoiohydric land plants (Raven, 2002). We would argue that physiological data, particularly from bryophytes, challenge this conventional wisdom rooted in morphological observation, and suggest that the evolution of stomatal function was a critical innovation for the evolution of large plants.

WHY ARE BRYOPHYTES SMALL?

Tall growth maximises the capture of light vastly increasing individual productivity. In vascular plants there is abundant evidence that growing tall confers a selective benefit, from the adaptive advantage of a fast growth rate in forest tree seedlings (Walters & Reich, 2000), convergent evolution of woody growth in all major lineages (Stewart & Rothwell, 1983; Lens *et al.*, 2013), as well as competition for light explaining the evolution of canopy structures in forests (Falster & Westoby, 2003). A perplexing question then is why bryophytes have remained apparently immune to this competition, with most species growing within the substrate boundary layer and no evidence of extinct bryophyte trees? Today a majority of bryophyte species are highly adapted to ecological niches devoid of or with minimal competition from vascular plants, or indeed any other photosynthetic organism (Shaw & Renzaglia, 2004), making the idea of them competing with vascular plants for light moot. This argument is somewhat problematic considering that the bryophyte ancestor likely emerged prior to the appearance of vascular plants (Wellman *et al.*, 2003) (although there are no fossils of bryophytes in the Rhynie Chert, one of the oldest known macrofossil land plant assemblages), yet did not evolve to fill the ecological niche rapidly occupied later by early vascular plants. Reproductive limitations may play a role, with bryophytes relying on liquid water to transport their motile spermatozoids to the female egg (Glibert, 2000). Yet similar reproductive limits have been overcome in vascular plants (e.g. the evolution of pollen), and in bryophytes spermatozoids can travel vast distances in water (Pressel & Duckett, 2019) or by insect dispersal (Gibson & Miller-Brown, 1927). While the ecological specialization of most extant bryophytes renders solving the absence of tall bryophytes intractable, the Polytrichales provide a notable exception as the tallest bryophytes (species of *Dawsonia*, the tallest in this group, reach 0.7 m) (van Zanten, 1973) (Figure 1).

Recent work in these giants of the bryophyte world has found that the internal water-conducting hydroids in the moss *Polytrichum,* while of completely independent origin, are functionally homologous to xylem, being capable of transporting vast volumes of water under negative tension in the vegetative gametophyte (Brodribb *et al.*, 2020; Duckett & Pressel, 2020). This observation raises an intriguing conundrum, why are these tall mosses -that are capable of transporting water though a vascular system- still so small compared to vascular plants? In tracheophytes, stomata are found on the surface of leaves; in mosses, stomata – when present – are confined to the solitary spore capsule in the unbranched sporophyte (Paton & Pearce, 1957). The lack of a leaf more than one cell thick and with stomata in *Polytrichum* gametophytes means that, while water can be conducted to evaporating surfaces as effectively as in any tracheophyte, evaporation from leaves is poorly regulated. This poses no problem under humid conditions, however when vapour pressure deficit (VPD) increases, the excessive water loss, despite a thick cuticular and wax investiture, results in a negative water potential sufficient to cause embolism, ending water transport (Brodribb *et al.*, 2020). These observations suggest that stomata on leaves were indeed essential for the evolution of homoiohydric land plants, with stomatal closure at high VPD in vascular plants able to reduce significant declines in water potential and thereby prevent embolism (Brodribb *et al.*, 2017).

UNIQUE BRYOPHYTE STOMATA?

If the greatest limitation to *Polytrichum* competing with vascular plants is simply a lack of stomata on vegetative organs, then why do the leaves of Polytrichales not have stomata? No extant gametophytes have stomata today, yet stomata are found on stems below reproductive structures in both sporophyte and gametophyte generations of the extinct pre-vascular plant *Aglaophyton* (Edwards *et al.*, 1998), suggesting that the dominant life history stage of bryophytes is not in itself a limitation. We argue that while stomata are structurally superficially similar across all land plants – typically taking the form of two guard cells surrounding a pore – considerable evolution in stomatal function across land plant lineages is the reason why, although some bryophytes have highly elaborate vascular tissue, they do not utilize stomata to regulate leaf water loss. In contrast to tracheophytes that bear stomata on anatomically complex leaves and stems, bryophyte stomata are exclusively located on sporangia and contribute almost exclusively to a coordinated process that results in spore production and dispersal rather than to general assimilation (Renzaglia *et al.*, 2017; Duckett & Pressel, 2018). Among bryophytes, stomata are absent in all extant liverworts (Renzaglia *et al.*, 2007; Duckett & Pressel, 2018; Renzaglia *et al.*, 2020), an observation consistent with the maturation of the sporophyte within gametophyte protective tissue. Stomata on sporangia of mosses and hornworts, in contrast to tracheophytes, play an important role in promoting water loss for spore maturation and release (Lucas & Renzaglia, 2002; Duckett *et al.*, 2009; Pressel *et al.*, 2014; Field *et al.*, 2015; Chater *et al.*, 2016; Renzaglia *et al.*, 2017). Once open, mature bryophyte stomata are physically incapable of closing, rendering them useless for mitigating excessive water loss. The capsules of bryophytes are relatively short-lived compared to the subtending gametophytes, consequently the selective pressures to maintain water relations during the growing season that drove the evolution of complex stomatal opening and closing capacity and signals in tracheophytes did not play a role in bryophyte diversification.

Despite these compelling data there still remains a pervasive alternative view that when stomata first appeared, they were already in possession of the full suite of signalling and molecular operating machinery found in modern angiosperms and thus stomatal function was the same as in modern angiosperms (Chater *et al.*, 2011). In a recent example, Zhao *et al.* (2019) claim that the colonisation of land was enabled by an omnipresent chloroplast retrograde signal that closes all stomata during water stress. This paper is similar in conclusion to a body of literature dating more than a decade professing that all stomata respond to the hormone abscisic acid (ABA) (Chater *et al.*, 2011; Ruszala *et al.*, 2011; Cai *et al.*, 2017). Levels of this hormone increase in angiosperms when water status declines, triggering a signalling cascade that actively closes stomata (Geiger *et al.*, 2009; Lee *et al.*, 2009; Ma *et al.*, 2009; Park *et al.*, 2009; McAdam & Brodribb, 2015). Arguments in support of universal stomatal functional across all land plants deserve close scrutiny, as they imply stomata were irrelevant for plant adaptation, diversification or massive ecological transitions over the past 400 million years, and cannot explain why mosses with efficient hydroids such as *Polytrichum* have not capitalized on stomata to regulate leaf water loss.

QUESTIONING A UNIVERSAL STOMATAL RESPONSE TO CHLOROPLAST RETROGRADE SIGNALS

Observations of stomatal aperture responses in the moss *Sphagnum fallax* are central to the theory of Zhao *et al.* (2019) that a proposed chloroplast retrograde signal, 3’-phosphoadenosine 5’-phosphate (PAP) has closed stomata in response to water deficit for the past 500 million years. These observations are perplexing given that the stomata of *Sphagnum* species are highly distinct from those of other land plants, and have been described as pseudostomata (Duckett *et al.*, 2009; Merced, 2015). *Sphagnum* pseudostomata lack pores and subtending intercellular air spaces, and are covered by a calyptra throughout capsule development (Figure 2). The guard cells of *Sphagnum* never separate to form a discrete pore; they simply collapse when cell volume and turgor declines (Figure 2). Consequently, pseudostomata do not function in the dynamic regulation of gas exchange, as guard cell collapse is irreversible (Duckett *et al.*, 2009; Merced, 2015). Even if PAP drives guard cell re-joining in *Sphagnum* then the mechanism must have facilitated guard cell inflation, a converse function to the Zhao *et al.* (2019) model. It should also be noted that water-conducting cells are absent in *Sphagnum*.

While questions might arise surrounding the taxonomic validity of the moss used in the study by Zhao *et al.* (2019), even if another moss species, such as the most likely candidate *Funaria* (based on the single stomatal image provided), was used in their study, major differences in stomatal function between bryophytes and angiosperms further preclude any conclusion of universal mechanistic homology. Consistent with a role in sporophyte maturation and desiccation – a function that is antithetic to that of tracheophyte stomata – hornwort and moss stomata, including those of *Funaria* (Figure 2F), open and become locked in that state due to guard cell wall chemistry and architecture preventing subsequent closure (Merced, 2015; Merced & Renzaglia, 2017; Duckett & Pressel, 2018; Pressel *et al.*, 2018). Whereas mature stomata in angiosperms are responsive to a variety of environmental and endogenous cues including light intensity, water status, ABA, plasmolysis and physical damage, those of bryophytes remain unchanged (Duckett & Pressel, 2018; Pressel *et al.*, 2018). Also running contrary to functional congruence across land plants are considerable differences in stomatal numbers and sizes in bryophytes that are unrelated to taxonomy, ecology and genome sizes, and atmospheric CO_2 levels (Field *et al.*, 2015; Duckett & Pressel, 2018). Indeed, the loss of stomata in two hornwort clades and at least 60 times in mosses indicates that they are essentially disposable in bryophytes unlike their near universality in vascular plants (Renzaglia *et al.*, 2020).

In mosses and hornworts, ion changes in the guard cells have been found to occur concurrently with similar ion changes in epidermal cells (Duckett *et al.*, 2009; Duckett & Pressel, 2018). Consequently, we cannot conclude that the ion flux data presented by Zhao *et al.* (2019) were guard cell-specific without epidermal cell controls. Furthermore, the Zhao *et al.* (2019) model for universal stomatal closure by PAP does not consider evolution in ion channels or their guard cell-specificity (Sussmilch *et al.*, 2019a). These evolutionary transitions have occurred in ion channels that play a critical role in angiosperm guard cell movements: such as the absence of outward- and inward-rectifying Shaker potassium channel genes in bryophyte and lycophyte genomes (Gomez-Porras *et al.*, 2012; Sussmilch *et al.*, 2019a), respectively, and major differences in the activation of S -type anion channels across tracheophytes (McAdam *et al.*, 2016). Importantly, it is yet to be shown if chloroplast signals specifically change guard cell gene expression outside of angiosperms.

EVOLUTION OF STOMATAL FUNCTION IN TRACHEOPHYTES

While the behaviour of bryophyte stomata is undoubtably divergent from the behaviour of angiosperm stomata, it has recently been suggested that the ancestor of all land plants possessed stomata that functioned like those of the model, annual, angiosperm herb *Arabidopsis* and that bryophyte stomatal function is highly derived (Rich & Delaux, 2020). We would argue that evolution of stomatal responses across tracheophyte lineages challenges this view as well as the concept of a universal stomatal closure model by PAP. The *in situ* stomata of lycophytes and ferns respond to changes in leaf water status as highly predictable passive-hydraulic valves (Brodribb & McAdam, 2011). The stomata of angiosperms do not respond in this way (Buckley, 2019). Contrary to some reports that extremely high levels of exogenous ABA slightly reduces aperture in some fern and lycophyte species (Ruszala *et al.*, 2011; Cai *et al.*, 2017; Hõrak *et al.*, 2017), there is no evidence that endogenous ABA produced by a plant during drought, or any other endogenous metabolic signal like PAP, drives functional stomatal closure under drought stress in species from these lineages (Brodribb & McAdam, 2011; McAdam & Brodribb, 2012; Cardoso *et al.*, 2019; Cardoso *et al.*, 2020). These results suggest that the stomata of the ancestor of vascular land plants responded to leaf water status as passive hydraulic valves and the evolution of a functional stomatal response to ABA (driven by evolution in the interaction of key signalling proteins (Sussmilch *et al.*, 2019b)) arose in the common ancestor of the seeds plants, and was instrumental in the evolutionary success of this lineage of plants.

CONCLUSION

Retrograde signalling may be ancient, but like most plant hormone signalling pathways, neofunctionalization, diversity and cell specificity (e.g. action in guard cells) are likely to have evolved gradually through time (Sussmilch *et al.*, 2019b; Blázquez *et al.*, 2020; Cannell *et al.*, 2020; McAdam & Sussmilch, 2020), not in a single event 500 million years ago. The importance of PAP signals in regulating *Arabidopsis* stomatal response to water stress was established using mutants (Pornsiriwong *et al.*, 2017); based on current data, it is far from parsimonious to conclude that this signal closes the stomata of all land plants. Nevertheless, this work highlights the critical need to study how diversity in stomatal function has influenced the macroevolution of land plant lineages. This is indeed a critical future endeavour as there is evidence that evolution in these simple structures was instrumental not only in the evolution of homoiohydry and tall stature (Brodribb *et al.*, 2020), or anatomical adaptations that enabled survival during drought (Cardoso *et al.*, 2020), but also the ability of trees to survive in seasonally dry environments (Brodribb *et al.*, 2014), and leaves to attain high rates of photosynthesis (Rockwell & Holbrook, 2017). Furthermore, differences in stomatal function underlie differences in ecological strategies across tracheophytes, particularly with regards to light environment (Doi *et al.*, 2015) or soil water availability (Martínez-Vilalta & Garcia-Forner, 2017). While it is an impactful claim to state a single signal has ruled stomata for all of time (Zhao *et al.*, 2019) or that *Arabidopsis* physiological function reflects a land plant ancestral state (Rich & Delaux, 2020), such approaches to physiological evolution will never reveal why, for instance, with very similar xylem physiology (Brodribb et al., 2020) and a selective pressure to grow tall (McNickle *et al.*, 2016), *Polytrichum* does not overtop *Sequoia*.

FIGURES

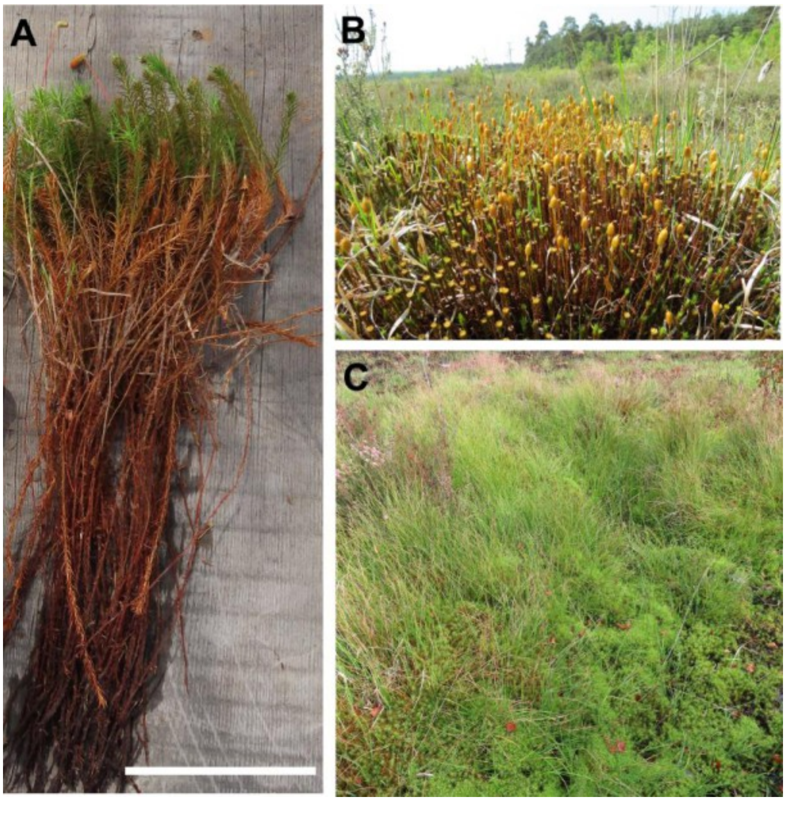

Figure 1. (A) *Polytrichum commune* Hedw. has an internal vascular system and is one of the tallest mosses (scale bar = 100 mm), yet this species is dwarfed by vascular plants (B and C). (B) Note the height of the surrounding forest in comparison to the *Polytrichum* bearing sporophytes in the foreground. (C) Hummocks of *Polytrichum* (most visible in the bottom right of the image) are often invaded and overtopped by tracheophytes, in this case moncots (seen in the top left of the image).

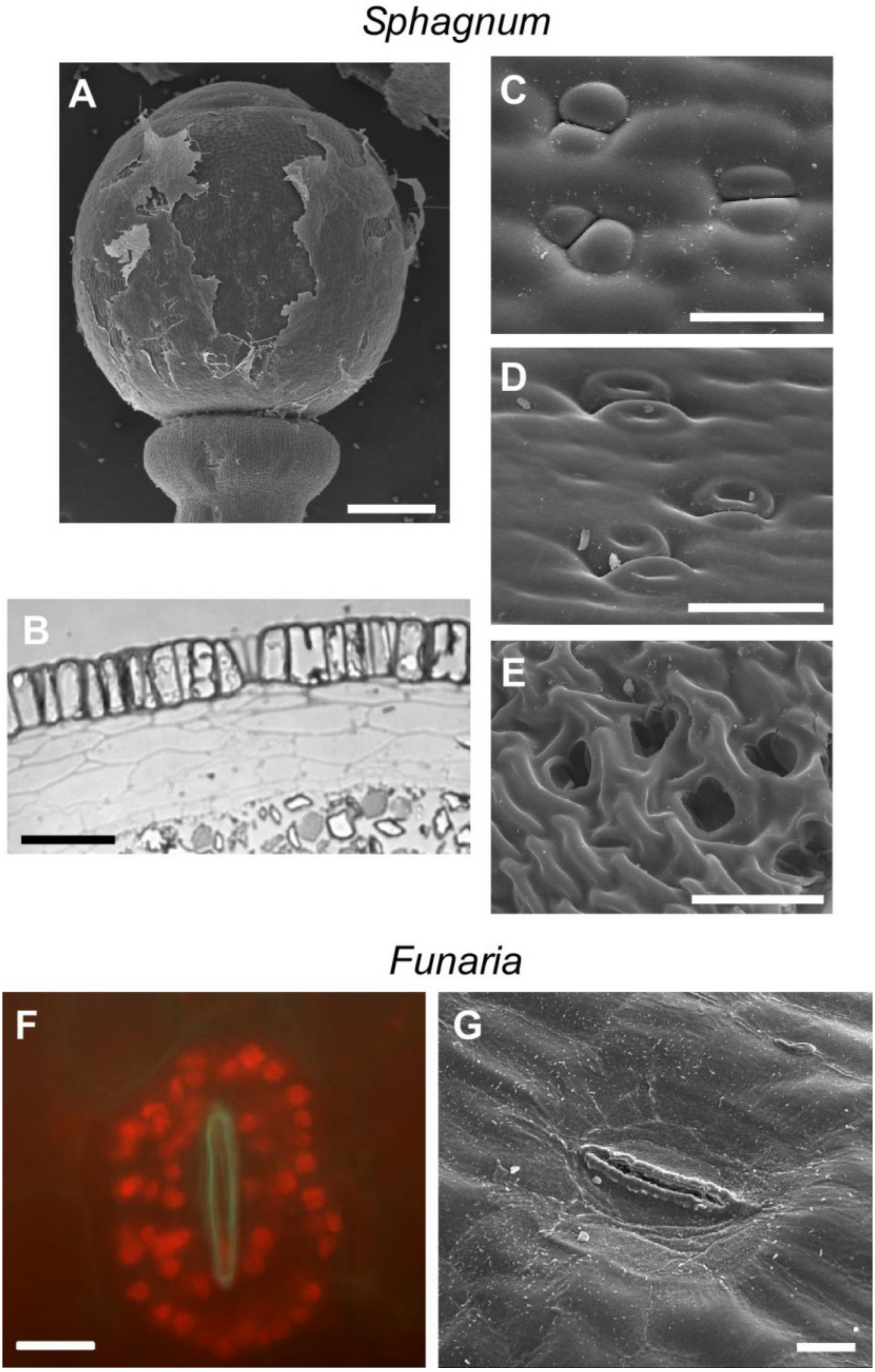

Figure 2. The pseudostomata of *Sphagnum* are anatomically and functionally unique amongst land plants. (A) Pseudostomata are found on the sporophyte capsule and are

covered in a calyptra that ruptures once the sporophyte has reached maturity (scale bar = 300 μm), (B) pseudostomata are not subtended by intercellular air spaces (scale bar = 75 μm). (C) Turgid pseudostomata can be found on a mature sporophyte under the calyptra. (D) As the sporophyte begins to dehisce the guard cells begin to lose turgor. (E) By the time the calyptra has ruptured and the capsule has dehisced the guard cells have shrunken apart at the top, appearing open (scale bars = 60 μm). The stomata of mosses outside the Sphagnopsida, like *Funaria hygrometrica* Hedw. (F), also open and become locked in that state due to a completely inflexible, thickened wall surrounding the pore (G) which renders them immobile (scale bars = 10 μm).

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