Research Update

Evolution of the stomatal regulation of plant water content.

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Abstract

Terrestrial productivity today is regulated by stomatal movements, but this has only been the case since “stomatophytes” became dominant on the land 390 million years ago. In this review we examine evidence for the function of early stomata, found on or near the reproductive structures of bryophyte-like fossils, and consider how this function may have changed through time as vascular plants evolved and diversified. We explore the controversial insights that have come from observing natural variation, rather than genetic manipulation, as a primary tool for understanding the function of the stomatal valve system.

Why is stomatal evolution important?

The variation in stomatal behaviour among extant plant groups has stimulated great interest recently across diverse fields of science. Behavioural differences in the responses of stomata to water stress within plant communities have been recognized as an important axis of variation in ecological strategy (Martínez-Vilalta and García-Forner, 2016; Meinzer et al., 2016), while systematic variation in stomatal size, density and response characteristics (Santelia and Lawson, 2016) among plant clades have important implications for interpreting plant-atmosphere interactions (Franks et al., 2012) (Franks this issue; Buckley this issue). Understanding how stomatal behaviour feeds into processes of plant selection is a fundamental goal in plant science, and much progress over the past three decades has been made towards this goal by applying the tools of genetic manipulation to the model angiosperm, Arabidopsis (Schroeder et al., 2001; Hetherington and Woodward, 2003; Kim et al., 2010; Hedrich, 2012) (De Angeli & Eisenach, this issue)(Jezek and Blatt, this issue). A very different, but potentially complementary approach, especially as we approach a post-genomics era, is the use of natural evolutionary variation as a lens through which the function of stomata can be viewed from a perspective of plant selection over evolutionary time. This novel evolutionary approach to understanding stomatal function presents huge potential for enhancing our understanding of not only the influence of stomatal behaviour on the colonisation of land by plants, but also the key processes that govern stomatal responses in modern species.

Stomatal evolution has become a focus of some debate in recent years (Brodribb et al., 2009; Brodribb and McAdam, 2011; Chater et al., 2011; Ruszala et al., 2011; Franks and Britton-Harper, 2016), largely initiated by evidence that although the stomata of ancient lineages of vascular plants opened similarly to angiosperms in response to light and CO₂, they close differently. In particular, the observation that stomatal conductance and transpiration of ferns and lycophytes does not decline significantly in response to ABA (Brodribb and McAdam, 2011), led to the theory that stomatal closure during water stress originated in early vascular plants as a passive response of guard cells to dehydration, and that the “active” closure mechanism, mediated by ABA, evolved much later in seed
plants. Debate about this theory has seen apparently contradictory evidence presented from researchers using diverse approaches. Genetic techniques are seen as the ideal means of resolving such debates, but so far this evidence has also provided confusing results about the presence and localization of necessary components required for ABA signalling in stomata (see below). In the face of equivocal physiological evidence, it is critical to consider stomatal evolution from first principles, in terms of how differences in stomatal regulation impacts plant performance. Here we focus on the evolution of stomatal regulation of plant water content, from the perspective of selection and adaptation, considering the functional role of stomata, and how this relates to variation in form, positioning and macroscopic function observable across the phylogeny of land plants. This macroscopic perspective is essential when considering core plant processes, such as stomatal action, because adaptive changes in function are expected to have profound and measureable effects on plant performance (McElwain, this issue).

The evolution of ABA responsiveness in land plants represents a fascinating example of how different perspectives can lead to profoundly different conclusions. For example we know that ferns are capable of very fast stomatal closure to >10% maximum aperture during dehydration, a necessary response to prevent damage to the plant (see below). The size, speed and shape of this closure response in ferns appears to be well explained by passive stomatal closure through guard cell dehydration, without any need for active processes of ion trafficking (Brodribb and McAdam, 2011). When combined with the fact that, unlike angiosperms, fern and lycophyte stomata do not respond to endogenous levels of ABA (McAdam and Brodribb, 2012), the conclusion is that ABA-mediated closure is not important in basal vascular plants seems robust. However reports regularly emerge of small stomatal responses in fern guard cells artificially exposed to ABA levels typically hundreds of thousands to millions of times higher than endogenous levels (Ruszala et al., 2011; Cai et al., 2017; Merilo et al. this issue)). The reasonable conclusion from these data is that fern guard cells react to exceedingly high levels of ABA, but the challenge remains to understand such observations in an evolutionary context.

Here we review recent discoveries made using an evolutionary approach to reconstructing stomatal function, and consider how this adds to the classical genetic manipulation approach in a model angiosperm that has dominated stomatal biology for the past three decades.

Developmental homology but functional divergence?

The opening and closing of stomata is a conspicuous feature of vascular land plant physiology (Darwin, 1898) and the presence of stomata on moss and hornwort sporophytes (Ziegler, 1987) as well as the epidermes of species from the oldest vascular land plant fossil assemblage, the 410 million year old Rhynie Chert (Edwards and Axe, 1992), suggest that these features are a critical ‘tool’ for terrestrial plant survival. Despite numerous losses of stomata in the bryophytes, including in the liverworts and a number of basal moss clades (Paton and Pearce, 1957; Haig, 2013), the structure and developmental genes that guide epidermal cell fate and ultimately the differentiation of guard cells appear to be ancient and highly conserved (Vatén and Bergmann, 2012; Renzaglia et al., this issue). This archaic developmental origin of stomata, raises the intriguing question of what selective pressure drove the evolution of these first adjustable pores in the earliest land plants? If the function of the earliest stomata was the same as in vascular plants, facilitating the dynamic optimisation of water use against carbon gain (Cowan, 1977), then common elements of the
stomatal control process likely evolved with these first stomata. This angiosperm-centric hypothesis has been long held as the best explanation for stomatal evolution (Haberlandt, 1886; Paton and Pearce, 1957; Ziegler, 1987; Chater et al., 2011), but has been recently challenged by key differences in the general behaviour and apparent role of early stomata as well as recent molecular and physiological evidence indicating a highly divergent functional role for stomata in these most basal stomata-bearing land plants (Haig, 2013; Pressel et al., 2014; Field et al., 2015; Chater et al., 2016; Renzaglia et al., this issue).

While the regulation of gas exchange by stomata to facilitate photosynthesis is a canon of plant physiology, based upon the well described behaviour of millions of stomata concentrated on the primary photosynthetic organs of derived vascular plants (Ziegler, 1987), the placement and number of stomata in non-vascular plants and the earliest, leaf-less vascular plants is vastly different (Figure 1). In the most basal land plant species there are no stomata on the primary photosynthetic organ, the gametophyte, with guard cells located solely on the reproductive sporophyte and, in mosses, on the apophysis at the base of the capsule (Haberlandt, 1886). This is similar to a number of the earliest vascular land plant fossils, particularly the cooksonioids, which are likely the common ancestor of extant vascular plants (Gonez and Gerrienne, 2010), and from which the first evidence of the oldest stomata is recorded (Edwards and Axe, 1992). These species had stomata (rarely more than 3) similarly clustered around the base of the reproductive sporangia (Edwards et al., 1998). There is compelling evidence that the main sporophyte axes of these plants were not capable of autonomous photosynthesis given their anatomy, and instead relied on primary photosynthesis occurring in a basal gametophyte (Boyce, 2008), much like extant bryophytes which have very low photosynthetic activity in the sporophyte (Paolillo and Bazzaz, 1968; Thomas et al., 1978).

Suggestions, based upon the frequency and positioning of the earliest fossil stomata, that ancestral stomata did not perform a photosynthetic role, are supported by observations of living examples of hornwort and moss stomata. In these plants the stomatal pore forms and opens only once then never closes (Pressel et al., 2014; Field et al., 2015; Renzaglia et al., this issue). This single opening event corresponds to a subsequent evaporation of the fluid-filled intercellular spaces in the sporangial tissue, facilitating the desiccation of the sporophyte prior to spore release. Additionally it has been proposed that enhanced transpiration caused by stomatal opening may also drive a strong transpirational flux of nutrients and photosynthates from the basal gametophyte into the developing sporophyte (Haig, 2013). Recently these observations have received considerable molecular support by a study in the moss species Physcomitrella patens in which a major delay in the dehiscence of capsules was observed in astomatal, guard cell developmental knockout mutants (Chater et al., 2016). Indeed, this lack of capsule dehiscence was the only reported functional difference between astomatal mutants and wildtype plants.

Ancient stomatal opening driven by photosynthesis in the guard cells

While a divergent role for stomata seems likely between basal land plants and more derived vascular plants, the observation that all stomata are apparently capable of opening suggests that a conserved opening mechanism may operate across stomatal-bearing land plants. The one possible exception seems to be the basal moss genus Sphagnum where stomatal opening is likely a derived mechanism, being triggered by the loss of guard cell turgor (Duckett et al., 2009). This conspicuous inversion of the normal positive relationship between aperture and guard cell turgor pressure in the stomata of...
Sphagnum however is not apparent in more derived mosses or hornworts, suggesting that, like vascular plants, stomatal opening in basal species other than Sphagnum requires an increase in guard cell turgor (Wiggans, 1921; Heath, 1938). In vascular plants particularly angiosperms, active metabolic processes essential for increasing guard cell turgor and opening the pore are well described, particularly in the light (Schroeder et al., 2001; Shimazaki et al., 2007)(Kinoshita, this issue). This opening process is driven by the hyperpolarisation of the guard cell membrane potential through the activation of the plasma membrane proton pump (H⁺-ATPase). Photosynthesis inside the guard cells (Zeiger and Field, 1982) provides a source of ATP (Tominaga et al., 2001; Lawson, 2009; Suettsugu et al., 2014), however in angiosperms this guard cell response alone is not sufficient to fully open stomata (Willmer and Pallas, 1974; Mumm et al., 2011; Chen et al., 2012). Photosynthesis in the mesophyll also provides a strong additional signal for opening (Roelfsema et al., 2002; Sibbernsen and Mott, 2010; Lawson et al., 2014) Santiella and Lawson, this issue potentially via an unknown aqueous signal (Fujita et al., 2013), possibly combined with starch metabolism (Horrer et al., 2016). Like vascular plants, bryophyte stomata contain chloroplasts (Butterfass, 1979; Lucas and Renzaglia, 2002). Indeed, the presence of chloroplasts in guard cells appears to be an ancestral trait, with distinctive colouring of the guard cells in comparison to epidermal pavement cells noted in rhytiophyroids (Edwards et al., 1998), suggesting that these earliest fossilised stomata similarly contained chloroplasts. While in angiosperms photosynthesis in the mesophyll appears to provide a major signal for driving stomatal opening in the light (Roelfsema et al., 2002; Lawson et al., 2014), in basal vascular plants, stomatal opening in the light can be driven by guard-cell autonomous photosynthesis alone, with rapid stomatal opening occurring in the isolated epidermis of leptosporangiate ferns (McAdam and Brodribb, 2012), which lack stomatal responses to blue light and hence open only by photosynthetetic processes (Doi et al., 2006; Doi and Shimazaki, 2008; Doi et al., 2015). This data indicates that a mesophyll signal for stomatal opening in the light likely evolved after the divergence of seed plants (McAdam and Brodribb, 2012). The ancestral ability of non-seed plant stomata to respond to light in the absence of the mesophyll may be due to the comparatively high number of chloroplasts in the guard cells of these species (Butterfass, 1979).

An association between guard cell photosynthesis and stomatal opening in the earliest stomatal-bearing land plants provides the ideal mechanism for actively triggering stomatal opening to desiccate the sporophyte. 390 million years ago however, the rise of extant clades of basal vascular land plants including the lycophytes, marked a major anatomical transition in land plants, namely the evolution of an independent, dominant sporophyte generation with dedicated photosynthetic organs covered in stomata. This evolutionary transition associating stomata with photosynthesis required a major change in the way land plants used stomata, from facilitating the desiccation of the sporophyte to maximising of photosynthetic gas exchange in the light, and the regulation of plant water status. Maximising leaf gas exchange for photosynthesis in the light likely required no evolutionary transformation in guard cell control, as this process could easily be achieved by allowing stomata to rapidly increase in turgor on exposure to light, presumably in the same way basal vascular land plants open stomata. The apparent involvement of the H⁺-ATPase in stomatal opening of the bryophyte Physomitrella appears to confirm a common mechanism with higher plants (Chater et al., 2011), although more work in bryophytes is required to confirm this potentially ancient mechanism. Extant lycophyte and fern stomata have the capacity to open rapidly in the light (Mansfield and Willmer, 1969; Doi and Shimazaki, 2008; McAdam and
Brodribb, 2012), with lycophyte stomata possessing both photosynthetically driven stomatal opening in red light as well as blue triggered opening, while leptosporangiate ferns (which comprise more than 96% of fern specific diversity (Palmer et al., 2004)) have lost blue light stomatal signalling (Doi et al., 2015). The reason behind this loss of blue light stomatal signalling in derived ferns is, as yet, unknown, but maybe due to a chimeric red-blue photoreceptor in leptosporangiate ferns, an adaptation associated with photosynthesis in low light environments (Kawai et al., 2003).

Stomata on the primary photosynthetic organ and the maintenance of homeohydry

Although a role for stomatal opening seems apparent across all land plants, the role and process of stomatal closure seems much less uniform, particularly if basal plants are considered. In vascular plants however, a primary stomatal function is the action to close the pore during water stress to reduce transpiration and maintain plant hydration and avoid damage to the plant vascular system (Fig. 2). The possible selective pressures driving the evolution of stomatal closure in the light is typically cited as; (1) being a mechanism for increasing water use efficiency (during stomatal closure at low humidity) or (2) as a means of protecting tissues from desiccation. It is hard to understand how selection for efficient water use can operate at the level of the individual plant due to the fact that water conservation inevitably provides more water for competitors (Cowan, 2002). The alternative, protective role, provides a much more convincing selective advantage to plants, and seems likely to underpin the evolution of stomatal responses in the light (Wolf et al., 2016). However, as mentioned above, several lines of evidence suggest that this action may not be an ancestral character in stomatal evolution, including the widespread capacity for desiccation-tolerance (Peñuelas and Munné-Bosch, 2010) in bryophytes and the fact that ancestral stomata probably aided rather than inhibited desiccation (of sporangia) in basal land plants (Caine et al., 2016). Desiccation-tolerance obviates the need to control evaporation from leaves, but was likely to be incompatible with the evolution of massive plants that began to dominate forests during the radiation of land plants (Kenrick and Crane, 1997). The reason for this is that the internal water transport system in all plants becomes cavitated during acute water stress causing leaves to be cut off from water in the soil (Tyree and Sperry, 1989). This type of damage can be easily repaired in small plants where capillary action can redissolve air embolisms in the vascular system after rain (Rolland et al., 2015), but larger woody plants are unable to repair cavitated xylem tissues by capillarity or root pressure (Charrier et al., 2016), and therefore xylem becomes irreversibly damaged during water stress (Brodribb and Cochard, 2009; Cochard and Delzon, 2013). Stomatal closure to protect the xylem from cavitation must have thus been an evolutionary prerequisite for the increase in plant size that occurred during the radiation of vascular plants. Indeed it has been demonstrated that the stomata of early vascular plants such as ferns close before the decline in hydraulic function associated with desiccation (Brodribb and Holbrook, 2003). New techniques that visualize the process of xylem cavitation, clearly demonstrate that a similar role of stomatal closure in protecting the xylem from cavitation during water stress (Fig. 2) is also common to gymnosperms and angiosperms (Brodribb et al., 2016; Hochberg et al., 2017). There are good examples of interaction between opening and closing signals to stomata during the early onset of plant desiccation, for example the opening of stomata at high VPD by exposure to low CO₂ (Bunce, 2006). However the closure mechanism during drying appears to override all other signals as plants approach turgor loss (Aasamaa and Sõber, 2011; Bartlett et al., 2016), and subsequent xylem cavitation (Hochberg et al., 2017).
The simplest and most effective means of closing a turgor-operated valve, such as the stomatal pore, when leaf water status declines is to establish a strong hydraulic connection between guard cells and surrounding leaf tissue, such that the hydration of the guard cells is physically linked to the hydration of the leaf. Such a linkage means that guard cells lose turgor as leaf water potential declines, passively closing the pore and dramatically reducing evaporation (Brodribb and McAdam, 2011). This extremely simple means of stomatal closure in response to declining leaf water status requires no metabolic input or complex signalling intermediates, and is well described in lycophytes and ferns (Lange et al., 1971; Lösch, 1977, 1979; Brodribb and McAdam, 2011; Martins et al., 2016). Stomatal responses to changes in vapour pressure deficit (or the humidity of the air) are thus highly predictable in these early vascular plants based on a passive model that links leaf turgor with guard cell turgor (Brodribb and McAdam, 2011; Martins et al., 2016). However, there is an important prerequisite for this passive mechanism to function correctly in these basal species; a minimal influence of epidermal cell mechanics on stomatal aperture, meaning that only guard cell turgor influences the aperture of the pore (Franks and Farquhar, 2007). This prerequisite is very much absent in angiosperms.

In angiosperms the passive response of stomata to rapid changes in leaf hydration is completely inverted, causing stomata to passively move in the wrong direction, opening when evaporation increases and closing as leaves hydrate. This peculiar characteristic arises because of a complicated mechanical relationship between guard cells and epidermal cells that results in “wrong-way” passive responses to leaf water content (Darwin, 1898; Iwanoff, 1928). An absence of this mechanical advantage in ferns and lycophytes appears consistent with passive control in these early clades, while cuticle analysis of the rhyniophytoids suggests that this lack of epidermal mechanical advantage is ancestral in stomatophytes. The stomata in these ancient plants apparently opened towards the periclinal wall of the guard cell (Edwards and Axe, 1992), much the same way as extant lycophytes and ferns (Franks and Farquhar, 2007; Apostolakos et al., 2010) for which there is no movement of the dorsal guard cell walls into the surrounding epidermal cells, a major contrast with modern angiosperms (Fig. 3).

Metabolically controlled stomatal closure in response to leaf water deficit

A passive linkage between leaf water status and guard cell turgor observed in extant basal vascular plants appears to be sufficient to prevent xylem cavitation during diurnal changes in evaporative demand (Martins et al., 2015). However, without more sophisticated mechanisms to reduce guard cell turgor and produce complete stomatal closure it has been hypothesized that passive closure does not provide a sufficiently tight stomatal seal capable of preventing ferns and lycophytes from rapidly reaching critical leaf water potentials when soil water is depleted during drought (McAdam and Brodribb, 2013). As a result, ferns and lycophytes in dry environments rely on either a high plant capacitance or low stomatal density (McAdam and Brodribb, 2013), desiccation tolerance (Hietz, 2010) and in some cases, rather cavitation resistant xylem (Baer et al., 2016) to survive drought. An active stomatal closing signal has the potential to restrict transpiration to rates approaching cuticular transpiration (Tardieu and Simonneau, 1998; Brodribb and Holbrook, 2003, 2004) providing the ability for species to preserve plant water even if leaves have low capacitance and large numbers of stomata. In seed plants the presence of an active regulator of stomatal responses to leaf water status is evident from diverse observations from the field (Schulze et al., 1974), under controlled conditions (Tardieu and Davies, 1993) and in electrophysiological
experiments on isolated guard cells (Grabov and Blatt, 1998; Pei et al., 2000; Raschke et al., 2003; Negi et al., 2008) (Jezek and Blatt, This issue) indicating the presence of a non-hydraulic signal driving stomatal closure. This signal is the phytohormone abscisic acid (ABA), which is synthesised as leaves lose turgor and which actively closes seed plant stomata (Mittelheuser and Van Steveninck, 1969; Pierce and Raschke, 1980; Davies et al., 1981).

**Co-option of an ancient and highly conserved ABA signalling pathway into the guard cells of seed plants**

The molecular signalling pathway eliciting an ABA response is well described from RCAR/PYR/PYL receptors, which in the absence of ABA bind to PP2Cs (Ma et al., 2009; Park et al., 2009), when ABA is present RCAR/PYR/PYL binding to PP2Cs is eliminated allowing PP2Cs to activate SnRK2 phosphorlylators (Yoshida et al., 2006). In seed plants, once activated by PP2Cs in the presence of ABA these SnRK2s phosphorylate guard cell specific membrane-bound anion channels (the best described of these are the S-type anion channels, SLACs) initiating the efflux of ions and consequently decreasing cell turgor and closing the stomatal pore (Geiger et al., 2009). There is an array of membrane bound channels and transporters that facilitate the efflux of most osmolytes once SnRK2s signal the presence of ABA and activate anion channels (Hills et al., 2012). The dominant role for SnRK2s in this ABA signalling cascade is strongly supported by experimental data. The phenotype of single gene mutants in the key SnRK2 for stomatal responses to ABA, OST1, are profound, displaying no sensitivity to ABA (Mustilli et al., 2002) or VPD (Merilo et al., 2013; Merilo et al., 2015). While other SnRK2-independent signalling pathways for ABA perception and SLAC activation have been suggested (Geiger et al., 2010; Brandt et al., 2012; Pornsiriwong et al., 2017), these are redundant and many either converge or require cross-talk with functional OST1 to activate anion channels (Brandt et al., 2015). Whether these alternative SnRK2-independent ABA signalling pathways play an adaptively relevant role in stomatal function is yet to be determined. The core SnRK2-centric ABA signalling pathway is highly conserved across land plants with all lineages, including those species without stomata, such as liverworts, displaying functional physiological responses to ABA (Ghosh et al., 2016), RCAR/PYR/PYL ABA receptors present in genomes (Hauser et al., 2011) and functional PP2Cs (Tougane et al., 2010). This highly conserved ABA signalling pathway is known to regulate desiccation tolerance mechanisms (Tougane et al., 2010), spore dormancy and sex determination in non-seed plants (McAdam et al., 2016), amongst other processes.

Three essential evolutionary steps (Fig. 4) were required for the co-option of the ancient ABA signal into a functionally relevant metabolic regulator of gas exchange in the earliest seed plants; (1) an operational SnRK2-SLAC pairing, (2) guard cell specific expression of this pairing, and (3) the ability to synthesise ABA over a time-frame relevant to stomatal responses. One or more of these requirements for ABA driven stomatal responses does not occur in non-seed plants. In lycophytes and ferns native SnRK2s are unable to activate native SLACs (McAdam et al., 2016), while a functional SnRK2-SLAC pairing, albeit weak, observed in *P. patens* (Lind et al., 2015) is not specific to the guard cells (Chater et al., 2011; Vesty et al., 2016), and likely plays a role in nitrate homeostasis. In well studied angiosperms there is a potent pairing of native SnRK2s and SLACs that are specifically expressed in guard cells (Li and Assmann, 1996; Geiger et al., 2009; Fujii et al., 2011). Whether this also occurs in the first group of land plants to possess functional stomatal responses to endogenous ABA, the gymnosperms, remains to be tested. The third requirement relates to the speed of ABA synthesis over a timeframe that matches apparent ABA-driven stomatal responses. In terms of
changing soil water deficit, this condition is met in all vascular land plants (Kraus and Ziegler, 1993; Hoffman et al., 1999; Kong et al., 2009; McAdam and Brodribb, 2013), but only in angiosperm does ABA synthesis appear to occur over a timeframe that corresponds to the dynamics of stomatal response to VPD (McAdam and Brodribb, 2015).

Diversity in the regulation of water use amongst seed plants is driven by differences in ABA metabolism

The first group of land plants to unequivocally respond to endogenous ABA, the gymnosperms, are able to synthesise ABA in leaves after at least six hours of sustained water stress (McAdam and Brodribb, 2014) and utilise these high levels to close stomata during drought (Brodribb et al., 2014). Evidence suggests that the earliest gymnosperms used ABA to prevent cavitation of the xylem when growing in seasonally dry environments (Brodribb et al., 2014), which is unlike fern and lycophyte species which appear incapable of dominating dry forest communities. While survival in dry environments likely provided the selective pressure to co-opt ABA signalling into the guard cells, the evolution of this trait appears to have become an important axis of variation in water use strategies and responses to leaf water status. Diversity in water use strategies stemming from differences in ABA metabolism is apparent in the gymnosperms. Species that have vulnerable xylem to cavitation synthesise high levels of ABA in order to close stomata and persist in seasonally dry environments (Brodribb and McAdam, 2013). By contrast, other conifer species produce cavitation-resistant xylem (Pittermann et al., 2010; Larter et al., 2015) that allows plants to survive when leaf water potentials drop below -4.5 MPa during drought. Once leaves reach this low leaf water potential stomata will close passively due to declining guard and epidermal cell turgor even in the absence of ABA (Brodribb et al., 2014; Deans et al., This issue). In these cavitation-resistant species, drought stress beyond this critical point leads to a decline in ABA levels to pre-stress values despite the plant experiencing extreme but recoverable water stress. This alternative strategy is also associated with anisohydric stomatal responses to drought, and is thought to prolong gas exchange and photosynthesis during drought (Brodribb and McAdam, 2013).

While ABA metabolism provides an important explanation for variation in stomatal behaviour within the gymnosperms, it also appears to explain differences between gymnosperms and angiosperms in their stomatal responses to water deficit. In gymnosperms, ABA synthetic rates are too slow to effectively regulate stomatal responses to changes in VPD. This is either due to a lack of specific enzymes late in the ABA biosynthetic pathway (Hanada et al., 2011; McAdam et al., 2015), or a general delay in the upregulation of critical rate-limiting enzymes for ABA biosynthesis (McAdam and Brodribb, 2015), both of which require further testing. The result of this slow ABA synthesis in gymnosperms is that conifer stomata have predictable and passive, non ABA-mediated responses to short term changes in VPD (McAdam and Brodribb, 2014), just like the two most basal lineages of vascular plants. In angiosperms however, ABA biosynthesis is extremely rapid (Christmann et al., 2005; Waadt et al., 2014; McAdam et al., 2016) and can be upregulated by a drop in leaf turgor over the time frame of minutes (Pierce and Raschke, 1981; McAdam and Brodribb, 2016). Thus angiosperms respond to VPD using ABA, as is evidenced by the change in ABA levels in angiosperm leaves in response to step changes in VPD (Bauerle et al., 2004; McAdam and Brodribb, 2015), while mutants in ABA biosynthetic and signalling genes have compromised stomatal responses to VPD (Xie et al., 2006; Merilo et al., 2013; McAdam et al., 2016). The result of this regulation of stomatal responses to VPD by ABA is that, unlike all other vascular land plant clades, the stomatal response to
VPD in angiosperms is often hysteretic and unable to be predicted by passive hydraulic processes (O’Grady et al., 1999; McAdam and Brodribb, 2015). Contributing to this hysteresis in stomatal response to VPD is an internal balance between the rates of ABA biosynthesis and catabolism, both of which are regulated in different tissues. While ABA biosynthesis in response to a drop in leaf turgor is hypothesised to occur very near to the vascular tissue (Kuromori et al., 2014), ABA catabolism in an Arabidopsis leaf is primarily controlled by two CYP707 genes, one expressed in vascular tissue, the other predominantly in guard cells (Okamoto et al., 2009). These two genes have different rates of expression in leaves that are exposed to high humidity, suggesting temporal variation in ABA catabolism across the leaf (Okamoto et al., 2009). Whether alternative expression profiles for these two key leaf ABA catabolism genes, or indeed ABA transport genes (Kuromori et al., 2011; Kanno et al., 2012) occurs across angiosperm species to explain reported differences in the sensitivity of species to VPD remains to be tested.

On-going questions in the field of stomatal evolution

Given the ever-growing multitude of genomic data from representative species spanning the land plant phylogeny, in silico analyses are becoming an increasingly popular means of discussing physiological evolution (Pabón-Mora et al., 2014; Yue et al., 2014; Chen et al., 2016). Stomatal evolution however provides some excellent examples of why gene phylogenies should always be used in combination with experimental studies of stomatal behaviour in situ. Evidence of highly conserved stomatal developmental genetics among land plants (Vatén and Bergmann, 2012; Caine et al., 2016) appears to support the long held notion of conserved stomatal function (Haberlandt, 1886; Paton and Pearce, 1957; Ziegler, 1987; Chater et al., 2011). However, a recent combination of molecular and whole plant experiments by Chater et al. (2016) suggests a divergent functional role for stomata in bryophytes. All that is required to transform the function of guard cells from passive to functionally ABA-sensitive is the relocation or concentration of an ancestral and highly conserved ABA signalling pathway into the guard cells (McAdam et al., 2016). However, this evolutionary transition could never be realised by in silico analysis of genomes alone, which instead find all stomata-bearing plants have the genetic capacity to perceive and respond to ABA, therefore providing the foundation for the assumption that all stomata respond to ABA. Whether recent in silico suggestions of major differences in the ABA biosynthetic pathway across land plant lineages (McAdam et al., 2015) explains differences in ABA synthetic rates (McAdam and Brodribb, 2014) remains to be tested.

There are a number of environmental signals regulating stomatal aperture that have not been discussed in this review for which either we have little regulatory understanding of, like inverted stomatal rhythms in CAM plants (Ting, 1987), or little functional understanding of, like stomatal responses to phytohormones other than ABA (Dodd, 2003). Future work to resolve these questions and place these responses in the context of evolution is essential. There remains much debate in the literature on the evolution of the stomatal response to CO2 (Brodribb et al., 2009; Franks and Britton-Harper, 2016). A common feature of all studies in this area is a conserved tendency to respond to CO2, particularly low CO2, which likely reflects a common photosynthetic signalling in all stomata (Brodribb et al., 2009; Franks and Britton-Harper, 2016). However, major differences across lineages in the way stomata instantaneously respond to CO2 in the dark (Doi and Shimazaki, 2008; Brodribb and McdAdam, 2013) as well as the influence of ABA on the sensitivity of stomata to CO2 (McAdam et al., 2011) reflect, as yet undescribed and potentially important, mechanistic differences
in the way stomata respond to more natural endogenous and environmental signals. Further work in this field is required to reveal these key mechanistic differences.

**Figure Legends**

**Figure 1.** Vast differences exist across land plant lineages in terms of stomatal density and size. Images of stomata-bearing epidermis from two unexceptional and highly representative species of respective lineages are presented to illustrate this phylogenetic difference. (Left panel) The epidermis of the sporophyte of a temperate, globally distributed, stomata-bearing hornwort species (*Phaeoceros carolinianus*), is the only organ of this non-vascular plant to bear stomata. Non-vascular plant species are characterised by a similar and extremely limited number of stomata (Paton and Pearce, 1957; Field et al., 2015). (Right panel) The epidermis of the angiosperm, canopy tree species (*Elaeocarpus kirtonii*) is the primary stomata-bearing organ of this and most other angiosperm species. Stomatal density in this leaf is approximately 230 stomata mm\(^{-2}\) which is quite modest for the leaves of an angiosperm tree (Franks and Beerling, 2009; Brodribb et al., 2013). Images were taken at the same magnification, scale bar = 100 µm.

**Figure 2.** Stomata in early vascular plants probably first closed to prevent cavitation of the vascular system, a function that appears to remain conserved until the present. Data here show the trajectory of stomatal closure (blue line) with respect to leaf water potential as three tomato plants were subjected to gradual soil drying (green, red and black crosses). Close to the water potential of complete stomatal closure, the process of xylem cavitation begins (three traces for leaves from three individuals are shown; black, red and green circles). Cavitation curves show the accumulation of cavitation events in the leaf veins of three tomato plants as they desiccate (data from Skelton et al. (2017)).

**Figure 3.** A schematic representation illustrating the difference in the main mode of guard and epidermal cell deformation during stomatal opening in non-angiosperms compared to angiosperms. The arrows indicate the direction of cell movement.

**Figure 4.** Top. Three key evolutionary transitions are required for ABA to regulate diurnal gas exchange in land plants. 1. The ability of native SnRK2 kinases (as in AtOST1 from *Arabidopsis thaliana*) to interact with, and activate, native anion channels (like the S-type anion channel AtSLAC1 from *A. thaliana*). Representative plots of a positive activation of *A. thaliana* proteins are taken from McAdam et al. (2016). 2. If native SnRK2s can activate native SLACs then the genes encoding these proteins must be uniquely expressed in guard cells, and this is the case in angiosperms (Li and Assmann, 1996), including *A. thaliana*, where AtOST1 is expressed in the guard cells as illustrated by the profound GUS staining of the promoter of AtOST1 in these cells (Fujii et al., 2007). 3. In order to regulate diurnal leaf gas exchange, foliar ABA levels must change over a timeframe that is relevant to the stomatal response to changes in VPD. Data for the gymnosperm *Metasequoia glyptostroboides*, taken from McAdam and Brodribb (2014), show strong increases in ABA level in branches that are dehydrated and maintained at specific leaf water potentials for a minimum of 6 hours. This contrasts with data from the angiosperm species *Pisum sativum*, taken from McAdam et al. (2016) which like most angiosperms displays a strong increases in ABA level after only 20 minutes following a doubling in vapour pressure deficit from 0.7 to 1.5 kPa. Bottom. Mapping these key transitions to a phylogeny of land plants, which assumes that mosses are sister to liverworts (Wickett et al., 2014) and divergent from hornworts, shows that only in angiosperms do all three of these essential
physiological and molecular requirements for diurnal gas exchange control by ABA occur. As
gymnosperms have functional stomatal responses to ABA (McAdam and Brodribb, 2014) and ferns
do not have guard cell specific expression of native \textit{SnRK2} or \textit{SLAC} genes (McAdam et al., 2016) it is
likely that seed plants were the first land plants to evolve a guard cell specific expression of these
genes. Only in angiosperms is ABA synthesis the same speed as the stomatal response to an
increase in VPD (McAdam and Brodribb, 2015).
Figure 1. Vast differences exist across land plant lineages in terms of stomatal density and size. Images of stomata-bearing epidermis from two unexceptional and highly representative species of respective lineages are presented to illustrate this phylogenetic difference. (Left panel) The epidermis of the sporophyte of a temperate, globally distributed, stomata-bearing hornwort species (*Phaeoceros carolinianus*), is the only organ of this non-vascular plant to bear stomata. Non-vascular plant species are characterised by a similar and extremely limited number of stomata (Paton and Pearce, 1957; Field et al., 2015). (Right panel) The epidermis of the angiosperm, canopy tree species (*Elaeocarpus kortonii*) is the primary stomata-bearing organ of this and most other angiosperm species. Stomatal density in this leaf is approximately 230 stomata mm⁻² which is quite modest for the leaves of an angiosperm tree (Franks and Beerling, 2009; Brodribb et al., 2013). Images were taken at the same magnification, scale bar = 100 μm.
Figure 2. Stomata in early vascular plants probably first closed to prevent cavitation of the vascular system, a function that appears to remain conserved until the present. Data here show the trajectory of stomatal closure (blue line) with respect to leaf water potential as three tomato plants were subjected to gradual soil drying (green, red and black crosses). Close to the water potential of complete stomatal closure, the process of xylem cavitation begins (three traces for leaves from three individuals are shown; black, red and green circles). Cavitation curves show the accumulation of cavitation events in the leaf veins of three tomato plants as they desiccate (data from Skelton et al. (2017)).
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