The Evolution of Mechanisms Driving the Stomatal Response to Vapor Pressure Deficit

Scott A.M. McAdam* and Timothy J. Brodribb

School of Biological Sciences, University of Tasmania, Hobart, Tasmania 7001, Australia

Stomatal responses to vapor pressure deficit (VPD) are a principal means by which vascular land plants regulate daytime transpiration. While much work has focused on characterizing and modeling this response, there remains no consensus as to the mechanism that drives it. Explanations range from passive regulation by leaf hydration to biochemical regulation by the phytohormone abscisic acid (ABA). We monitored ABA levels, leaf gas exchange, and water status in a diversity of vascular land plants exposed to a symmetrical, mild transition in VPD. The stomata in basal lineages of vascular plants, including gymnosperms, appeared to respond passively to changes in leaf water status induced by VPD perturbation, with minimal changes in foliar ABA levels and no hysteresis in stomatal action. In contrast, foliar ABA appeared to drive the stomatal response to VPD in our angiosperm samples. Increased foliar ABA level at high VPD in angiosperm species resulted in hysteresis in the recovery of stomatal conductance; this was most pronounced in herbaceous species. Increased levels of ABA in the leaf epidermis were found to originate from sites of synthesis in other parts of the leaf rather than from the guard cells themselves. The transition from a passive regulation to ABA regulation of the stomatal response to VPD in the earliest angiosperms is likely to have had critical implications for the ecological success of this lineage.

Plants continuously regulate transpiration by controlling the aperture of the stomatal pores on the surface of the leaf. The principal atmospheric determinant of stomatal aperture is the humidity of the air, which can be expressed as the vapor pressure difference between the leaf and the atmosphere. Stomatal responses to atmospheric vapor pressure deficit (VPD) have been well characterized across the diversity of vascular plant species (Darwin, 1898; Lange et al., 1971; Turner et al., 1984; Franks and Farquhar, 1999; Oren et al., 1999; Brodribb and McAdam, 2011; Mott and Peak, 2013), with stomata typically closing at high VPD and opening at low VPD. This comprehensive characterization has allowed for the development of highly effective empirical and mechanistic models of leaf gas exchange that provide robust predictions of the responses of transpiration to changes in VPD (Buckley et al., 2003; Katul et al., 2009; Damour et al., 2010; Medlyn et al., 2011). Despite the success of this modeling, the mechanism for the stomatal response to VPD remains poorly understood (Damour et al., 2010). Different hypotheses range from one extreme, whereby stomata respond passively through changes in leaf water content induced by the VPD or humidity perturbation (Lange et al., 1971; Mott and Peak, 2013), to the other extreme, whereby stomata close uniquely in response to the phytohormone abscisic acid (ABA; Xie et al., 2006; Bauer et al., 2013).

From the earliest recognition that stomata open and close by changes in guard cell turgor (Heath, 1938), there have been many attempts to link the passive changes in water status that occur during VPD or humidity transitions with stomatal responses to VPD or humidity (Lange et al., 1971; Mott and Peak, 2013). Studies have suggested that changes in atmospheric water content passively drive stomatal responses by changing bulk leaf water status, which in turn changes guard cell turgor (Oren et al., 1999), or alternatively by changing guard cell turgor directly (Mott and Peak, 2013). Models based on these entirely passive processes are highly effective in predicting steady-state stomatal conductance ($g_s$) in response to changes in VPD or humidity in angiosperms (Mott and Peak, 2013).

While hydraulic models provide robust predictions of steady-state $g_s$, they are less effective at predicting the dynamic responses of stomata to short-term perturbations, particularly with respect to the wrong-way responses that typically occur as transients (Buckley, 2005), as well as feed-forward behavior (Farquhar, 1978; Bunce, 1997; Franks et al., 1997; Tardieu and Simonneau, 1998; Ocheltree et al., 2014; compare with Mott and Peak, 2013). Although some of these models provide a pathway for incorporating the effect of ABA (Buckley, 2005), a lack of knowledge of ABA dynamics or action makes it difficult to integrate the influence of this active regulator of guard cell aperture into models. The stomatal behavior of single gene mutants (most notably the ABA synthesis and signaling mutants of Arabidopsis) strongly supports a role for ABA in mediating standard stomatal responses to changes in VPD. The stomata of these

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* Address correspondence to smcadam@utas.edu.au.

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mutants are known to have less pronounced responses to a reduction in relative humidity compared with wild-type plants (Xie et al., 2006). Recently, molecular work has shown that guard cells express many of the genes required to synthesize ABA (Okamoto et al., 2009; Bauer et al., 2013), with molecular proxies for ABA level also indicating that the biochemical activity of ABA in the guard cell may increase following short-term exposure of leaves to a reduction in relative humidity (Waadt et al., 2014). These findings suggest a role for ABA in regulating stomatal responses to VPD and have led some to the conclusion that ABA synthesized autonomously by the guard cells is the predominant mechanism for stomatal responses to increased VPD (Bauer et al., 2013).

Although the experimental evidence from molecular studies presents an argument for the role of ABA in the responses of stomata to changes in VPD, very few studies have quantified changes in ABA level in response to VPD. It is well established that ABA levels in leaves and guard cells can increase following the imposition of turgor loss or water stress (Pierce and Raschke, 1980; Harris et al., 1988; Harris and Outlaw, 1991). However, only a few studies have reported increases in foliar ABA level in response to high VPD (Bauerle et al., 2004; Giday et al., 2013), and none have investigated whether these observed dynamic changes or differences in ABA level were functionally relevant for stomatal control. In addition, no study has quantified the levels of ABA in guard cells during a transition in VPD.

Here, we investigate the relative importance of ABA for the stomatal response to VPD in whole plants, sampled from across the vascular land plant lineage. We provide, to our knowledge, the first functional assessment of changes in ABA levels driving stomatal responses to VPD as well as critically investigate the recent suggestion that stomatal responses to VPD are driven by an autonomous guard cell synthesis of ABA.

**RESULTS**

**Hysteresis in the Response of Angiosperm Stomata to VPD**

During a reversible sequence of VPD transitions (from 0.7 to 1.5 to 0.7 kPa), all species showed a pronounced reduction in $g_s$ in response to the increase in VPD to 1.5 kPa (Figs. 1–3). In all species, $g_s$ declined by between 50% and 70% when VPD was increased from 0.7 to 1.5 kPa (Supplemental Table S1). While stomatal closure at increased VPD was relatively similar across species, there were large differences between species in the recovery of $g_s$ on returning to 0.7 kPa, with only angiosperm species displaying hysteresis in the recovery of $g_s$ (Fig. 4). In the six diverse fern and conifer species, the rate of stomatal closure during a VPD transition from 0.7 to 1.5 kPa was the same as that for stomatal opening when the original
VPD was restored (Fig. 4), indicating a rapid, symmetrical stomatal response to VPD in these early branching vascular plant lineages. In contrast, in the two herbaceous angiosperm species, the rate of stomatal closure at high VPD was much greater than the rate of stomatal opening on returning to low VPD (Fig. 4). Stomatal responses to VPD in the woody angiosperm species (including the most basal angiosperm, *Amborella trichopoda*) were intermediate in terms of difference between the rate of stomatal closure at high VPD and opening at low VPD between the species from the basal vascular land plant groups and herbaceous angiosperms (Fig. 4).

**The Role of Foliar ABA Levels during a VPD Transition**

In the angiosperm species (Fig. 1) and two of the three conifers (Fig. 2), a concomitant increase in foliar ABA level was observed as $g_s$ declined when plants were exposed to a moderate increase in VPD from 0.7 to 1.5 kPa. In the two herbaceous angiosperm species, pea (*Pisum sativum*) and *Dahlia hybrida*, increasing foliar ABA levels alone were sufficient to account for the observed stomatal closure on exposure to a VPD of 1.5 kPa (Figs. 1 and 5). This functional level of foliar ABA is shown by the red horizontal line in Figure 1 and represents the ABA level required to produce the equivalent response of stomata assuming that ABA was uniquely driving the closure of stomata. This level was determined from the sensitivity of $g_s$ to exogenously applied ABA in the absence of water stress (Fig. 5).

In the two herbaceous species, returning to low VPD was accompanied by a very slow decline in foliar ABA levels as well as a similar, very slow hysteretic recovery of $g_s$ (Fig. 1). The very slow recovery in $g_s$ at low VPD in the two herbaceous species was reflected in higher water potential ($\Psi_l$) following the final transition in VPD to 0.7 kPa, compared with values measured at 0.7 kPa prior to the VPD transitions (Table I). In the most basal woody angiosperm species, *A. trichopoda*, rising foliar ABA levels similarly explained the observed decline in $g_s$ following a transition to high VPD, as predicted by the relationship between $g_s$ and endogenously applied ABA (Figs. 1 and 5). When VPD was returned to 0.7 kPa, foliar ABA levels returned slowly to their original levels, leading to significant hysteresis in the recovery of $g_s$ (Figs. 1 and 4). In the other woody angiosperm species, *Quercus robur*, exposure to high VPD resulted in a rapid increase in foliar ABA almost to a level sufficient to explain the depression in $g_s$ (Figs. 1 and 5). In Q. robur, declining ABA levels upon restoration of the initial VPD were also relatively rapid and were accompanied by no significant hysteresis in the response of $g_s$ ($P = 0.052$; Fig. 4).

To test whether the increase in foliar ABA levels in the leaves of angiosperm species at high VPD was due to an enhanced delivery of ABA from the xylem sap, driven by increased evaporation, or endogenous synthesis of foliar ABA, we measured ABA levels and $g_s$ in stems of *A. trichopoda* that were excised under water and exposed to a reversible transition in VPD. In branches of *A. trichopoda* excised under water, foliar ABA levels increased on exposure to a step increase in VPD after 20 min, and this increase in foliar ABA level corresponded with a reduction in $g_s$ (Fig. 6). The functional increase in foliar ABA level, in these excised stems, occurred despite xylem sap ABA concentrations being 90 times lower than levels in an intact plant under low-VPD conditions, approaching the detection limits of the ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS) apparatus (Fig. 6).

Unlike the angiosperm representatives, foliar ABA levels in the conifer species remained an order of magnitude too low to explain the significant stomatal closure observed in response to increased VPD; this was based on the sensitivity of $g_s$ to exogenously applied ABA (Figs. 2 and 4) and in *Metasequoia glyptostroboides* on the sensitivity of $g_s$ to ABA determined by three independent methods (isolated epidermis, exogenous feeding, and rehydration of branches with different levels of endogenously synthesized ABA; McAdam and Brodribb, 2014). In addition, changes in $\Psi_l$ in all conifer species were consistent with a passive control of stomatal aperture by leaf water status (Table I). All of the representative fern species showed rapid and pronounced stomatal closure in response to the imposed VPD transition, but no significant change in foliar ABA level was observed during the VPD transition (Fig. 3). All differences in foliar ABA level observed in the fern species were due to differences between leaves sampled rather than due to the VPD treatment imposed on the plants (Supplemental Fig. S1).

**Distribution of ABA in Angiosperm Leaves**

When ABA levels in the leaf of the herbaceous angiosperm pea doubled, during an increasing transition in VPD from 0.7 to 1.5 kPa (Fig. 1), the proportion of ABA in the abaxial epidermis declined by more than half, from 40% to just under 15% of the total ABA found in the whole leaf (Fig. 7A). When ABA levels in the epidermis were expressed relative to fresh weight of the sample, the increases in abaxial epidermal ABA during an increase in VPD significantly lagged behind the more pronounced increase in ABA levels that occurred in the remainder of the leaf (Fig. 7B; Supplemental Fig. S2). To test whether this increase in foliar ABA could reach the guard cells in this mutant with an extreme morphology (having an epidermis that is only connected to the leaf along major veins), we fed labeled [2H6]ABA into the transpiration stream. During the feeding of [2H6]ABA, we monitored leaf gas exchange and performed UPLC-MS analysis to investigate the presence of [2H6]ABA in the abaxial epidermis. Following feeding into the transpiration stream, the stomata of the *argenteum* mutant did indeed close, and [2H6]ABA could be readily identified in the epidermis immediately upon closure of stomata (Fig. 5D), despite the significant separation of the epidermis from the mesophyll.
DISCUSSION

Ancestral, Passive-Hydraulic Regulation of Stomatal Responses to VPD

In monitoring both foliar ABA levels and leaf gas exchange during VPD transitions, we show evidence for systematic differences between plant groups in the mechanisms that regulate the stomatal response to VPD in vascular land plants. Similar to previously described patterns in the responses of stomata to water stress across diverse species (Brodribb and McAdam, 2011; McAdam and Brodribb, 2014), we show that stomatal responses to VPD are characterized by passive-hydraulic regulation in ferns and conifers, whereby stomata respond as simple, hydraulic valves opening in response to an increase in leaf turgor and closing in response to a decrease in leaf turgor (we refer to this as the ancestral, passive-hydraulic regulation of the stomatal aperture). Unlike in lycophytes, ferns, and conifers, when angiosperm species are exposed to an increase in VPD initially stomata open, this transient wrong-way response is also passive but driven by the epidermis losing turgor faster than the guard cells (Mott et al., 1997; Franks and Farquhar, 2007). In this study, we pinpoint the origin of ABA-mediated stomatal responses to VPD to the earliest angiosperms.

Figure 2. Trajectories of $g_s$ (black circles; $n = 3$ leaves; mean ± se) and foliar ABA levels (white circles; $n = 3$; mean ± se), taken from the same leaves, as plants from three conifer species were exposed to a reversible sequence of VPD transitions from 0.7 kPa (blue) to 1.5 kPa (green) and returning to 0.7 kPa (blue). In all species for which an increase in foliar ABA level was observed at 1.5 kPa, the exogenously applied ABA level known to induce a similar reduction in $g_s$ as that observed at 1.5 kPa is presented as a red horizontal line (Fig. 3). FW, Fresh weight.

Figure 3. Trajectories of $g_s$ (black circles; $n = 3$ leaves; mean ± se) and foliar ABA levels (white circles; $n = 3$; mean ± se), taken from the same leaves (except in *P. esculentum*), as plants from three fern species were exposed to a reversible sequence of VPD transitions from 0.7 kPa (blue) to 1.5 kPa (green) and returning to 0.7 kPa (blue). FW, Fresh weight.
Lycophytes, ferns, and conifers have stomatal responses that conform to a passive-hydraulic model for $g_\text{s}$, that is independent of foliar ABA (Brodribb and McAdam, 2011; McAdam and Brodribb, 2014), and we show here that no functional increase in ABA level occurs in the leaves of fern and conifer species in response to changes in VPD. These results contrast with the conclusions reached by Ruszala et al. (2011) and Chater et al. (2011), who suggested that the stomatal response to ABA (and presumably, although not tested, the regulation of stomatal responses to VPD by ABA) had an ancient evolutionary origin at the base of the mosses. Interestingly, while we have suggested that the conifers represent the first extant lineage of vascular plants to possess a functional stomatal response to ABA (Brodribb and McAdam, 2011; McAdam and Brodribb, 2012a; compare with Ruszala et al., 2011), this earliest role for ABA in the regulation of stomatal aperture in gymnosperms can be attributed solely to enhancing stomatal closure during drought stress and probably not to functional stomatal responses to VPD, at least in well-watered plants (Brodribb and McAdam, 2013a).

**Foliar ABA Regulates the Stomatal Response to VPD in Angiosperms**

While the passive, hydraulic regulation of the stomatal response to VPD can be readily observed in the basal lineages of vascular land plants (Brodribb and McAdam, 2011; McAdam and Brodribb, 2014), angiosperm stomata are characteristically distinct from the stomata of other land plant groups. During reversible transitions in VPD, we identified a functionally and statistically significant increase in foliar ABA levels in three of the four angiosperm species examined (Fig. 1), and we show that this increase in ABA level is likely due to synthesis in the leaf rather than to increased delivery of ABA from the xylem sap (Fig. 5). This provides, to our knowledge, the first quantitative evidence that increases in foliar ABA levels in response to changes in VPD in angiosperms can exceed levels that will cause a similar degree of stomatal closure in the absence of water stress, and complements conclusions reached by the qualitative assessments of ABA signaling and synthesis mutants: namely, that ABA levels play a major role in delivering stomatal responses to VPD in angiosperms (Xie et al., 2006). Many of the past methods, especially the popular immunological methods, used to quantify foliar ABA levels (Walker-Simmons et al., 2000) and other hormones, from foliar auxin levels (Cohen et al., 1987) to steroids in baboon feces (Gesquiere et al., 2014), unless rigorously standardized against physiochemical methods, do not possess the resolution required to detect small but functionally significant increases in hormone levels, shown here to occur in angiosperm leaves during exposure to high VPD. This limitation in resolution may explain previous observations that ABA levels only noticeably increase when plants are exposed to water stress sufficient enough to cause turgor loss (Pierce and Raschke, 1980; Trejo and Davies, 1991; Wilkinson and Davies, 1997) and the paucity of data showing rapid ABA accumulation in leaves during an increasing VPD transition. While the increase in foliar ABA levels that we observed in angiosperm species with increasing VPD was functionally and statistically significant, there was a degree of variation between leaves in the canopy that may have been due to heterogeneity in stomatal density, foliar morphology or hydraulic conductance.

Interestingly, while we show here that foliar ABA appears to drive stomatal responses to changes in VPD in angiosperms, a number of studies have shown that the exposure of angiosperm leaves to low humidity is a critical prerequisite for delivering normal stomatal responses to ABA and that high humidity can desensitize stomata to ABA (Arve et al., 2013; Pantin et al., 2013b; Aliniaeifard et al., 2014). This interaction is more likely due to low humidity being a strong up-regulator of both ABA synthetic and signaling genes (Pantin et al., 2013b) than being due to changes in stomatal or leaf anatomy at high humidity (Aliniaeifard et al., 2014).

**Predominance of ABA from the Leaf Driving the Stomatal Responses to VPD in Angiosperms**

Qualitative molecular studies of ABA synthesis mutants have led to the hypothesis that ABA originating in
guard cells is solely responsible for driving the stomatal response to VPD (Bauer et al., 2013). We tested this hypothesis using the *argenteum* mutant of the angiosperm herb pea, which has fully functional stomata yet an epidermis that is substantially isolated from the mesophyll (Jewer et al., 1982). We observed a significant decline in the proportion of ABA in the adaxial epidermis when plants were exposed to increased VPD, suggesting that guard cell ABA synthesis in response to increased VPD is substantially dwarfed by the accumulation of ABA occurring in the remainder of the leaf (for mass-normalized ABA levels in the leaf and epidermis, see Supplemental Fig. S2). This concurs with a number of studies showing that the vascular tissue, particularly the phloem cells, have the genetic capacity to synthesize ABA as well as proteins capable of transporting ABA to the guard cells (Koiwai et al., 2004; Okamoto et al., 2009; Kuromori et al., 2010, 2014; Seo and Koshiba, 2011). We also found evidence that ABA can be transported from major sites of synthesis in the leaf to the epidermis even in a pea mutant where much of the normal liquid pathway for ABA flow between the vasculature and stomata is air filled (Fig. 7D). In this mutant, we observed a delayed increase in ABA accumulation in the abaxial epidermis in response to an increase in VPD, suggesting that the source of the epidermal ABA was probably derived from other tissue in the leaf. While there is an abundance of molecular evidence illustrating the biochemical capacity of guard cells to synthesize ABA (Christmann et al., 2005; Okamoto et al., 2009; Bauer et al., 2013), ABA accumulation in the leaf during a VPD transition occurs before levels increase in the epidermis or guard cells. These findings suggest that ABA synthesized by the leaf only, yet outside of the guard cells, may be the dominant signal for stomatal closure in response to changes in VPD. Using ABA signaling mutants, it has been suggested that increases in ABA level in the xylem sap can cause a reduction in leaf hydraulic conductivity and stomatal closure in the absence of stomatal sensitivity to ABA.

Table I. Leaf $\Psi_v$ (MPa; $n = 3$; mean ± s.e) at each equilibrium point before and after VPD transitions in all sampled species

<table>
<thead>
<tr>
<th>Group</th>
<th>Species</th>
<th>$\Psi_v$, Initial 0.7 kPa</th>
<th>$\Psi_v$, 1.5 kPa</th>
<th>$\Psi_v$, Final 0.7 kPa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fern</td>
<td><em>Pteridium esculentum</em></td>
<td>$-0.32 ± 0.04$</td>
<td>$-0.67 ± 0.02$</td>
<td>$-0.32 ± 0.05$</td>
</tr>
<tr>
<td>Fern</td>
<td><em>Marsilea hirsuta</em></td>
<td>$-0.17 ± 0.04$</td>
<td>$-0.58 ± 0.04$</td>
<td>$-0.26 ± 0.06$</td>
</tr>
<tr>
<td>Fern</td>
<td><em>Pyrosia lingua</em></td>
<td>$-0.61 ± 0.06$</td>
<td>$-0.95 ± 0.02$</td>
<td>$-0.71 ± 0.03$</td>
</tr>
<tr>
<td>Conifer</td>
<td><em>M. glyptostroboides</em></td>
<td>$-0.4 ± 0.04$</td>
<td>$-0.64 ± 0.04$</td>
<td>$-0.41 ± 0.03$</td>
</tr>
<tr>
<td>Conifer</td>
<td><em>Prumnopitys ladei</em></td>
<td>$-0.63 ± 0.04$</td>
<td>$-1.05 ± 0.1$</td>
<td>$-0.67 ± 0.01$</td>
</tr>
<tr>
<td>Conifer</td>
<td><em>Pinus caribaea</em></td>
<td>$-0.36 ± 0.05$</td>
<td>$-0.56 ± 0.03$</td>
<td>$-0.37 ± 0.04$</td>
</tr>
<tr>
<td>Woody angiosperm</td>
<td><em>A. trichopoda</em></td>
<td>$-0.38 ± 0.02$</td>
<td>$-0.7 ± 0.01$</td>
<td>$-0.36 ± 0.03$</td>
</tr>
<tr>
<td>Woody angiosperm</td>
<td><em>Q. robur</em></td>
<td>$-0.36 ± 0.02$</td>
<td>$-0.39 ± 0.02$</td>
<td>$-0.35 ± 0.01$</td>
</tr>
<tr>
<td>Herbaceous angiosperm</td>
<td><em>D. hybrida</em></td>
<td>$-0.4 ± 0.01$</td>
<td>$-0.7 ± 0.04$</td>
<td>$-0.3 ± 0.04$</td>
</tr>
<tr>
<td>Herbaceous angiosperm</td>
<td><em>P. sativum</em></td>
<td>$-0.3 ± 0.02$</td>
<td>$-0.42 ± 0.01$</td>
<td>$-0.12 ± 0.01$</td>
</tr>
</tbody>
</table>
Pantin et al., 2013a). This study suggests that foliar increases in ABA in response to increased VPD may lead to a hydraulically driven reduction in $g_s$ as well as hysteresis in the stomatal response to VPD. We found only a very small (less than 10%) mean reduction in leaf hydraulic conductivity (from evaporation and $\Psi_l$ data) in the two angiosperm herb species after VPD was returned to 0.7 kPa that would not be sufficient to cause the greater than 50% reduction in $g_s$.

Contribution of an Ancestral, Passive-Hydraulic Regulation of Stomatal Responses to VPD in Angiosperms

Foliar ABA level was able to account for the stomatal response to VPD, including the reduced rate of stomatal reopening after returning to low VPD in three angiosperm species; however, this was not the case in the woody angiosperm species $Q$. robur. The small increase in ABA level in response to the VPD transition, as well as the similar rate of the recovery of $g_s$ in this species, suggest that the ancestral, passive-hydraulic regulation of a stomatal response to VPD remains a significant influence over stomatal aperture in some angiosperm species. Whether this is also the case in angiosperm species during stomatal responses to very gradual transitions in VPD, which may not be severe enough to trigger ABA biosynthesis, remains to be tested. However, the presence of the ancestral, passive-hydraulic regulation of stomatal responses to VPD is supported by gas-exchange studies in the single-gene ABA signaling and synthesis mutants of Arabidopsis, all of which still show a degree of stomatal response to both increasing and decreasing VPD (Assmann et al., 2000; Xie et al., 2006). This had been attributed to an as yet unknown metabolic stomatal signal (Xie et al., 2006); however, it appears more likely that this partial response in the absence of a stomatal ABA signal is also due to passive-hydraulic regulation of the stomatal response to VPD in Arabidopsis.

Interestingly, a significant efflux of potassium ions from the guard cells of the ABA biosynthetic mutant, $aba3$, has been observed following a decrease in relative humidity (Bauer et al., 2013). This efflux of potassium ions in mutant plants exceeded that of wild-type plants; however, neither stomatal aperture nor $g_s$ was measured in that study. This observation further suggests that ABA alone is not entirely responsible for stomatal responses to VPD in angiosperms and highlights the importance of further investigation into the stomatal responses of ABA signaling and synthesis mutants to understand the relative importance of both mechanisms in delivering normal stomatal responses to changes in VPD in angiosperms.

A fundamental unanswered question remains as to what the selective advantage for evolving ABA-dependent stomatal responses to VPD is. This may lie in the similar selective advantages obtained by the evolution of additional active stomatal control mechanisms across the vascular land plant lineages, including a response to blue light (Doi et al., 2006), photosynthetic signaling from the mesophyll (McAdam and Brodribb, 2012b), and ABA (Brodribb and McAdam, 2011; compare with Ruszala et al., 2011) in seed plants as

![Figure 6](image-url)
well as a response to low CO₂ in the dark (Doi and Shimazaki, 2008) and high CO₂ in the light (Brodribb et al., 2009), possibly due to calcium signaling in the stomata, which appears to be unique to angiosperms (Brodribb and McAdam, 2013b). The evolution of these additional stomatal control mechanisms over the course of the last 400 million years has greatly enhanced the environmental signals that stomata can perceive and respond to (McAdam and Brodribb, 2012b). This increased complexity in the regulation of stomatal control mirrors the increasing ecological success of progressive lineages of vascular land plants.

CONCLUSION

We show that in evolutionary terms, the earliest response of stomata to VPD was a passive-hydraulic response that does not require foliar ABA. This response is currently found in the extant relatives of basal vascular land plants, including the lycophytes, ferns, and gymnosperms. However, a transition to an ABA regulation of the VPD response appears to have evolved very early in the angiosperm clade, as evidenced by the ABA dependence of the stomatal response to VPD in the most basal extant angiosperm, *A. trichopoda*. We provide evidence that bulk leaf ABA provides the signal for active stomatal responses to VPD in angiosperms.

Figure 7. A, Proportion of ABA from the whole leaf that is found in the abaxial epidermis (n = 3; mean ± se) and gs (red line taken from Fig. 1) during a sequence of VPD transitions from 0.7 kPa (blue) to 1.5 kPa (green) and returning to 0.7 kPa (blue) in the argenteum mutant of the herbaceous angiosperm pea. B, Comparison between the mass-normalized foliar ABA level and the abaxial epidermal ABA level from the same leaves (taken from Supplemental Fig. S2) following a step increase in VPD from 0.7 to 1.5 kPa. Times of sampling are shown; the red line represents a fitted linear regression, while the black line represents a 1:1 relationship between the two mass-normalized ABA levels (the two regressions are significantly different at P = 0.0018). For ABA levels plotted over time, see Supplemental Figure S1. FW, Fresh weight. C, Distinctive foliar morphology of the argenteum mutant (right) in comparison with a wild-type leaflet (left). The silvery coloring is due to a large air space between the epidermis and the mesophyll. Bar = 1 cm. D, Response of gs in a leaf of the argenteum mutant excised under water and fed labeled [2H₆]ABA into the transpiration stream (dashed red line). Chromatograms are from UPLC-MS analyses of the [2H₆]ABA channel (mass-to-charge ratio 269.2–159.1) with peaks from leaf tissue (green) and abaxial epidermal tissue (blue) prior to and after feeding (denoted by arrows); peaks are relative to sample mass, and red stars indicate the retention time for [2H₆]ABA.
MATERIALS AND METHODS

Plant Material and Experimental Conditions

Potted individuals of three morphologically and ecologically diverse fern species, the terrestrial species Pteridium esculentum (Dennstadiensiaceae), semi-aquatic Marsilea hirsuta (Marsileaceae), and epiphyte Pyrrosia lingua (Polypodiaceae), the conifers Metasequoia glyptostroboides (Cupressaceae), Pinus caribaea variety hondurensis (Pinaceae), and Prunus nigra var. latifolia (Podocarpaceae), the woody angiosperms Amborella trichopoda (Amborellaceae) and Quercus robur (Fagaceae), and the herbaceous angiosperm pea (Pisum sativum argenteum mutant [Fabaceae]) and Dahlia hybrida 'Cinderella' (Asteraceae) were used in this study. Plants were grown under controlled glasshouse conditions of 25°C/16°C day/night temperatures and 16-h photoperiod, with natural light supplemented by sodium vapor lamps to ensure a minimum 300 μmol quanta m⁻² s⁻¹ at the pot surface. All plants received weekly applications of liquid fertilizer (Aquasol; Hortico). Single individuals of the fern, conifer, and woody angiosperm species, or five even-aged individuals of the herbaceous angiosperm species (due to the limited number of leaves per plant), were placed in a growth cabinet (PGC-105; Percival Scientific) to acclimate for 1 week prior to experimentation. The temperature and photoperiod conditions in the growth cabinet were maintained the same as in the glasshouse (25°C/16°C day/night temperatures and 16-h photoperiod) with natural light and fluorescent lights ensuring a minimum 300 μmol quanta m⁻² s⁻¹ at the pot surface). During this acclimation period, however, a daytime VPD of 0.7 kPa (77% relative humidity) was sustained by the presence of containers of water and a 1-m² surface of wet hessian; temperature and relative humidity were monitored every 5 min during this period by a data logger (HOBO Pro Series; Onset). Following 1 week of acclimation, the simultaneous monitoring of leaf gas exchange, Ψ, and ABA levels was carried out approximately every 15 min (as described below), during a relatively fast step change in VPD. After an initial simultaneous measurement of leaf gas exchange, Ψ, and ABA levels, VPD was increased to 1.5 kPa (62% relative humidity) using a condensing dehumidifier (SecoUltra 00563; Olimpia-Splendid) in the growth cabinet. Temperature and relative humidity were monitored every 30 s during the experimental period by a humidity probe (HMP45AC; Vaisala) and temperature thermocouple connected to a data logger (CR10X; Campbell Scientific). A VPD of 1.5 kPa was maintained until leaf gas exchange had stabilized (60-90 min). VPD was then reduced to 0.7 kPa and maintained until leaf gas exchange had again stabilized. The relatively small and contained volume of air in the growth cabinet (3 m³) resulted in a relatively fast half-time for the VPD transition of 150 s following transitions between 0.7 and 1.5 kPa (with no hysteresis when VPD was returned to 0.7 kPa).

Leaf Gas Exchange and Ψ Measurements

During transitions in VPD, leaf gas exchange in three even-aged, fully irrigated leaves was measured approximately every 15 min using an infrared gas analyzer (LI-6400; LI-COR Biosciences). Conditions in the leaf cuvette were made as close as possible to those in the growth cabinet, with VPD regulated by a portable dew point generator (LI-630; LI-COR Biosciences). Leaves were enclosed in the cuvette, and instantaneous gas exchange was logged following stability in cuvette conditions (after approximately 30 s). Following gas-exchange measurements, the same leaf was then excised and immediately sampled for ABA quantification (see below). In the fern species P. esculentum, 6 cm² of leaf material could not be contained in the leaf cuvette due to the pinnate nature of the leaves; therefore, the same three pinnales were measured over the course of the experiment and neighboring tissue was sampled for foliar ABA quantification, after which leaf gas exchange was adjusted for leaf area in the cuvette.

Leaf Ψ values were assessed in three leaves at each equilibrium point before and after VPD transitions. Leaves were harvested and immediately weighed first in a damp paper towel, then aluminum foil, and bagged for the assessment of Ψ using a Scholander pressure chamber and microscope to precisely measure the balance pressure.

ABA Collection, Extraction, Purification, and Quantification

In all species except the angiosperm herb pea, samples harvested for ABA quantification were immediately weighed (~0.0001 g; MS204S; Mettler-Toledo) into 50-mL tubes, covered in approximately 15 mL of cold (0°C) 80% (v/v) methanol in water with 250 μg L⁻¹ (m/v) of added butylated hydroxytoluene (BHT), and transferred to −20°C. Foliar ABA levels in these samples were extracted, purified, and quantified by physicochemical methods using an added internal standard and UPLC-MS according to the methods of McAdam and Brodribb (2014).

Testing the Origin of the ABA Increase in the Leaf

To test whether the increase in foliar ABA level observed in angiosperm species was due to endogenous synthesis of ABA in the leaf or an enhanced delivery of ABA from the xylem sap because of an increase in evaporation, we measured foliar ABA level and g, in branches of A. trichiopoda excised under water. Three branches (with approximately seven leaves) were selected from the same plant used in the earlier experiments and excised under filtered, degassed water. Branches were acclimated to the growth chamber in which experiments were conducted (described above) for 24 h to ensure that ABA levels in the xylem sap were reduced. After this time, the branches were exposed to the above-described transition in VPD. Foliar ABA and g were measured simultaneously in a single leaf from each branch after 20 min of exposure to a VPD of 1.5 kPa, then again after the VPD was lowered to 0.7 kPa after a further 20 min. On completion of this experiment, branches were removed from the water and three branches of similar size were excised from the original plant. The remaining leaves on each branch were excised, and each branch was enclosed in a Scholander pressure chamber and placed in a Scholander pressure chamber and placed in a LMP1 chamber with 15.6 MPa of pressure applied to the branch. This pressure exuded xylem sap, and approximately 200 μL of sap was collected from each branch and ABA levels were quantified as described above, after determining the exact volume of sap expressed.

Fractional Distribution of ABA in the Leaf

Both foliar ABA and abaxial epidermal ABA levels were measured in pea. For these experiments, we used the argenteum mutant of pea, as it has fully functional stomata yet an epidermis that is only attached to the mesophyll along major veins (Jewer et al., 1982). The isolated epidermis of this mutant allowed for a rapid sampling of epidermal tissue from the same leaves in which leaf gas exchange was measured. Abaxial epidermis were entirely removed in a single peel and briskly placed in a 1.5-mL tube, weighed (~0.005 g), covered in cold 80% (v/v) methanol with added BHT, and transferred to −20°C (no more than 20 s elapsed from leaf excision to the covering of epidermis in cold methanol solution). ABA level was quantified from the remainder of the leaf, which was enclosed briefly (less than 30 s) in a damp paper towel. This tissue was weighed in a similar fashion, covered in cold 80% (v/v) methanol with added BHT, and also transferred to −20°C. To each 1.5-mL tube, a single stainless-steel ball bearing was added along with 15 ng of the internal standard [2H6]ABA. Samples were then homogenized using a cell lysis machine (Tissuclyser II; Qiagen), and ABA was extracted by storing samples overnight at 4°C, centrifuging for 5 min at 13,000 rpm, and drying the supernatant to completeness under vacuum. For all samples, endogenous ABA was quantified by UPLC-MS, following the methods of McAdam and Brodribb (2014). Both mass-normalized ABA levels were determined from each sample (as described above), and, as the entire extract from each sample was used to quantify ABA levels, the proportion of total foliar ABA found in the abaxial epidermis also was determined from the raw UPLC-MS peak areas. Total foliar ABA was calculated as the sum of peak areas from both the abaxial epidermal sample and the remaining leaf tissue sample.

Transfer of [2H6]ABA from the Transpiration Stream to the Epidermis in the argenteum Mutant of Pea

To assess whether ABA synthesized in the leaf could be transported to the epidermis and influence stomatal aperture, again we used the argenteum mutant of pea. Labeled [2H6]ABA was fed into the transpiration stream of an argenteum leaf through the stem excised under water while leaf gas exchange was monitored continuously. The experimental plant was 15 d old with three fully expanded leaves. The night prior to this experiment, the stem of the plant was excised under water and maintained in the dark until the following morning. At 8:30 AM on the day of the experiment, the most recent fully expanded leaf was excised in the cuvette of an infrared gas analyzer (LI-6400; LI-COR Biosciences), and environmental conditions in the cuvette were sustained for the duration of the experiment under the following conditions: air temperature of 22°C, light intensity of 1,000 μmol quanta m⁻² s⁻¹, and VPD regulated at 1.2 kPa (using a
portable dew-point generator [LI-610; LI-COR Biosciences]). When \( g_{s} \) had reached a steady-state maximum for at least 6 min, background levels of \([\text{H}_2\text{A}]\) in the abaxial epidermis and in the leaf tissue were assessed in a neighboring leaf by harvesting both tissues as described above. After this initial collection, \([\text{H}_2\text{A}]\) was added to the transpiration stream at a concentration of 1,000 ng mL\(^{-1}\) (the cut stem was approximately 5 cm from the lamina of the leaf in the cuvette). Leaf gas exchange was monitored until \( g_{s} \) had declined and reached stability after 35 min, following which the leaf in the cuvette was harvested for \([\text{H}_2\text{A}]\) quantification. \([\text{H}_2\text{A}]\) levels were assessed in both the abaxial epidermis and the remaining leaf tissue, using the harvest, extraction, purification, and quantification methods described above.

Stomatal Sensitivities to Exogenous ABA

In all species for which there was a systematic change in foliar ABA level during transitions in VPD, one of two methods was used to assess the sensitivity of stomata to exogenously applied ABA. In species that did not have pronounced hydropassive, wrong-way responses after excision under water and in which epidermal peels or stomatal apertures by microscopy were not possible (the conifer species \( P. \_ladisl \) and the woody angiosperm species \( Q. \_robur \)), stomatal sensitivity to ABA was assessed in branches that were fed exogenous ABA as described by McAdam and Brodribb (2014). Namely, leaf gas exchange was measured (as described above) in three branches that were excised under water and resin-filtered, deionized water. Leaves were equilibrated in the leaf cuvette until maximum and stable \( g_{s} \) was reached; then, an aliquot of concentrated exogenous ABA, prepared by dissolving crystalline ABA of mixed isomers (Sigma) in 1 mL of methanol and then diluting up to 250 mL with resin-filtered, deionized water, was added to the leaf water supply, increasing the concentration of ABA in the water to 2,500 ng mL\(^{-1}\). Periodically, as stomata closed, short shoots (of \( P. \_ladisl \) or neighboring leaves of \( Q. \_robur \)) were taken from the branch for foliar ABA quantification (see above). Each branch contributed at least five paired \( g_{s} \) and foliar ABA level data points. In the remaining species, the woody angiosperm \( A. \_trichopoda \) and the herbaceous angiosperms \( D. \_hydrilla \) and \( P. \_sempervirens \), stomatal sensitivity to ABA was determined by measuring the dose-dependent response of stomatal apertures (in viable guard cells from isolated epidermis) to exogenous ABA. Both of these methods are known to accurately reflect stomatal sensitivities to ABA, having both been tested against each other (and found to correspond) as well as against the sensitivity of stomata to endogenously synthesized ABA (McAdam and Brodribb, 2014).

Epidermides were prepared as described by Brodribb and McAdam (2013b). Following incubation in the light (200 μmol quanta m\(^{-2}\) s\(^{-1}\)) for 1 h in control buffer solution (50 mM KCl, 10 mM MES, and 0.1 mM CaCl\(_2\), pH 6.15), rendered nominally CO\(_2\) free following 1 h of bubbling with N\(_2\) gas), at least two epidermides were further incubated for 2 h in one of five incinerating concentrations (0, 20, 80, or 200 ng mL\(^{-1}\)) of exogenous ABA dissolved in the same buffer solution. Stomatal apertures \((n = 50\) minimum) were then observed. Brodribb and McAdam (2013b) described the methods that were used to prepare epidermides, assess guard cell viability, and perform the double-blind measurements of stomatal apertures. \( g_{s} \) from measured stomatal apertures at each concentration of ABA was calculated according to the formula given by Parlange and Waggoner (1970). Stomatal length was determined from the same images used to quantify stomatal apertures. Stomatal density was quantified from 10 images taken at random at a magnification of 20× from the epidermides used to quantify stomatal apertures. Stomatal pore density was measured from leaf cross sections of each species. To determine the relative importance of ABA-induced stomatal closure during VPD transitions, empirical relationships between \( g_{s} \) and exogenously applied foliar ABA level were established for each species (Supplemental Fig. S1), and exponential decay functions were fitted to these relationships to determine the foliar ABA level in the absence of water stress that would be sufficient to result in the observed decline in \( g_{s} \) during the VPD transition. For the conifer species \( M. \_ glyptostroboides \), the empirical relationship between \( g_{s} \) and foliar ABA level was taken from McAdam and Brodribb (2014).

Determining Changes in the Rates of Stomatal Responses to VPD

To provide a quantification of hysteresis observed during the response of stomata to a reversible transition in VPD, comparisons were made between the slopes of linear regressions fitted to the dynamic \( g_{s} \) data collected from 15, 30, and 45 min following a transition in VPD. The percentage change between the slope of stomatal closure compared with the slope of stomatal opening was calculated for each species. Analysis of covariance was then used to compare the two linear regressions using the AOV function in R (version 2.15.1).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. The trajectories of foliar ABA level in three leaves of the fern \( P. \_esculentum \).

Supplemental Figure S2. The trajectory of \( g_{s} \) and ABA level in both the leaf and epidermis of the \( argentum \) mutant of pea.

Supplemental Table S1. Percentage reduction in \( g_{s} \) following a doubling in VPD.

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Evolution of Stomatal Responses to VPD


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