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Research Article

Characterizing the breakpoint of stomatal response to vapor pressure deficit in an angiosperm

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Abstract

Vapor pressure difference between the leaf and atmosphere (VPD) is the most important regulator of daytime transpiration, yet the mechanism driving stomatal responses to an increase in VPD in angiosperms remains unresolved. Here, we sought to characterize the mechanism driving stomatal closure at high VPD in an angiosperm species, particularly testing whether abscisic acid (ABA) biosynthesis could explain the observation of a trigger point for stomatal sensitivity to an increase in VPD. We tracked leaf gas exchange and modeled leaf water potential (Ψ_l) in leaves exposed to a range of step-increases in VPD in the herbaceous species *Senecio minimus* Poir. (Asteraceae). We found that mild increases in VPD in this species did not induce stomatal closure because modeled Ψ_l did not decline below a threshold close to turgor loss point (Ψ_{tlp}), but when leaves were exposed to a large increase in VPD, stomata closed as modeled Ψ_l declined below Ψ_{tlp}. Leaf ABA levels were higher in leaves exposed to a step-increase in VPD that caused Ψ_l to transiently decline below Ψ_{tlp} and in which stomata closed compared with leaves in which stomata did not close. We conclude that the stomata of *S. minimus* are insensitive to VPD until Ψ_l declines to a threshold that triggers the biosynthesis of ABA and that this mechanism might be common to angiosperms.

Introduction

Stomata are highly dynamic, turgor-operated valves on the surface of leaves that respond to a suite of environmental signals to optimize the ratio of water loss for carbon gain (Raschke 1975, Cowan and Farquhar 1977). When stomata are open during the day, the most important regulator of dynamic changes in stomatal aperture is the vapor pressure difference between the leaf and the atmosphere (VPD) (Lösch and Tenhunen 1981, Schymanski et al. 2013). Stomata close when VPD increases, reducing excessive transpiration, and when VPD decreases, stomata will open. Determining the mechanism driving stomatal responses to VPD has been a concerted focus of stomatal biologists since the 1800s (Darwin 1898, Grantz 1990, Franks et al. 1997). Explanations in angiosperms are diverse and unresolved, ranging from a passive change in guard cell turgor (Lösch and Tenhunen 1981, Peak and Mott 2011) to direct sensing of atmospheric humidity (Lange et al. 1971) or hormonal control (Bauerle et al. 2004, Xie et al. 2006, McAdam and Brodribb 2016), or a combination of these (Bauer et al. 2013, Merilo et al. 2018).

The simplest mechanistic explanation for stomatal responses to changes in VPD is that guard cell turgor changes in concert with leaf turgor, so that when leaf turgor declines, as VPD increases, stomata passively close (Grantz 1990). This simple, hydraulically driven stomatal response to VPD is readily observed in species of nonangiosperm including lycophytes, ferns, and conifers (Lange et al. 1971, Brodribb and McAdam 2011, Deans et al. 2017, Cardoso et al. 2019). In most species from these lineages, stomatal responses to changes in VPD can be predicted with a high degree of accuracy using a biophysical model that assumes guard cell turgor changes in concert with leaf turgor (Cardoso et al. 2019). Stomatal responses to changes in VPD in most nonangiosperm species display no evidence of hysteresis and require no metabolic explanation (Gong et al. 2021). The passive regulation of stomatal responses to VPD in nonangiosperms

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is believed to occur because there is no mechanical advantage of the epidermis on stomatal aperture (Franks and Farquhar 2007). In angiosperms, stomatal aperture is not only a function of guard cell turgor but also the turgor of the epidermis (Raschke 1970, Buckley 2019), which exerts a mechanical advantage over the aperture of the pore. The presence of mechanical advantage means that stomatal responses to VPD in angiosperms are unlikely to be a simple function of changes in guard cell turgor (Buckley and Mott 2002, Buckley 2016), which is one of the reasons so many unreconciled, mechanistic explanations abound.

The stomata of many angiosperm species open to large apertures by displacing neighboring epidermal cells, delivering a higher rate of transpiration per pore compared with species from other lineages of land plants (Rockwell and Holbrook 2017, Westbrook and McAdam 2021). This mechanical interaction means that when leaf turgor declines at high VPD, angiosperm stomata open because of a loss of epidermal turgor. The consequence of this wrong-way stomatal opening is that a metabolic signal is required to drive right-way stomatal closure, unless epidermal turgor is lost (Buckley et al. 2003). The hormone abscisic acid (ABA) provides the most likely metabolic driver of stomatal closure at high VPD in angiosperms (McAdam and Brodribb 2015). ABA triggers stomatal closure by activating anion channels in the guard cell, which leads to a loss of cell turgor (Raschke et al. 2003, Geiger et al. 2009). ABA is synthesized in leaves as mesophyll cells approach turgor loss point (McAdam and Brodribb 2018) and has been found to increase in leaves at high VPD when stomata close (Bauerle et al. 2004, McAdam et al. 2016). There is considerable evidence that ABA is essential for driving stomatal closing responses to high VPD in angiosperms: stomata of severely ABA deficient mutants are open and unresponsive to VPD (Cernusak et al. 2019), so much so that these mutant plants can suffer lethal embolism at high VPD (Brodribb et al. 2021); and in wild-type plants, a rapid increase in ABA levels, triggered by active biosynthesis, occurs in leaves exposed to high VPD (Bauerle et al. 2004, McAdam et al. 2016). Furthermore, there is a body of literature that finds the stomata of some angiosperm species close only when VPD is raised beyond a threshold, or breakpoint, that is difficult to explain by a hydraulic mechanism (Sheriff 1977, Seversike et al. 2013, Choudhary et al. 2014, Riar et al. 2015, McAdam and Brodribb 2016, Schoppach et al. 2017, Sinclair et al. 2017, Sadok et al. 2019, Jafarikouhini et al. 2020, 2022, Bourbia et al. 2023). There are some contrasting results, not consistently observed across studies, that suggest stomatal responses to VPD in Arabidopsis (*Arabidopsis thaliana*) might be passive or have some passive contribution to the response, including the muted, but still present, stomatal response to a step-increase in VPD in single gene ABA biosynthetic and signaling mutants in this species (Merilo et al. 2018). There is also evidence in some angiosperm species for a continuous stomatal response to increasing VPD, with mild step-increases in VPD resulting in stomatal closure (Hall et al. 1975; Assmann and Gershenson 1991; Mott 2007).

In this study we had 2 aims: (i) to test whether the stomatal response to a step-increase in VPD in the intact leaves of the ruderal, herbaceous angiosperm species *Senecio minimus* Poir. occurs at a threshold leaf water potential (Ψ_l) and (ii) to test whether this threshold corresponds to a trigger for the synthesis of ABA. To do this, we monitored leaf gas exchange in leaves exposed to step-increases in VPD, and we modeled the dynamics of Ψ_l after the step-increases in VPD using key leaf hydraulic traits. If stomatal closure at high VPD is driven by ABA biosynthesis, we hypothesized that it would only occur when Ψ_l declined to a threshold at which ABA is synthesized, which would be approximately leaf turgor loss point (Ψ_{tlp}) (McAdam and Brodribb 2016). There are very few reports describing the influence of epidermal mechanics on stomatal responses to VPD (Buckley and Mott 2002), so we also sought to characterize the effect of VPD on the magnitude of the wrong-way stomatal response in this species.

Results and discussion

Steady-state stomatal responses to VPD

The steady-state stomatal response to VPD in *S. minimus* could be best predicted by an exponential decay function ($g_s = 0.46 \times e^{-0.9521 \times VPD}$), with stomata closing at high VPD (Fig. 1A). Similar exponential relationships between steady-state g_s and VPD have been widely documented across vascular land plant species (Morison and Gifford 1983, Mott and Parkhurst, 1991, Monteith 1995, Franks and Farquhar 1999) but provide little insight into whether these stomatal responses are passively or metabolically driven. In our experiments, all leaves in which we collected a steady-state g_s across a range of VPD were initially acclimated to a VPD of 1.2 kPa. In the 25 leaves in which measurements were made, the mean (±SE) g_s at a VPD of 1.2 kPa was 0.11 ± 0.06 mol m^{-2} s^{-1} (Fig. 1A). In 10 of the leaves measured that were exposed to a small step-increase in VPD (an increase <0.5 kPa), we observed that steady-state g_s did not decline after the VPD transition; in some leaves, steady-state g_s increased and remained at 0.15 mol m^{-2} s^{-1} after exposure to a mild increase in VPD (Fig. 1A). This lack of a consistent stomatal closing response to a mild step-increase in VPD could be visualized when g_s was plotted as a percentage change in response to the magnitude of the increase in VPD (Fig. 1B), where a threshold increase in VPD was found to trigger a reduction in g_s (Fig. 1B). Only when leaves acclimated to 1.2 kPa were exposed to an increase of at least 0.6 kPa in VPD did a generalized additive model indicate a significant reduction in g_s (Fig. 1B). This threshold VPD at which stomata closed is difficult to explain if stomatal responses to changes in leaf water status in *S. minimus* are under a purely, passive hydraulic regulation (Sheriff 1977), like those observed in species of nonangiosperm (McAdam and Brodribb 2015). In nonangiosperm species, there is no

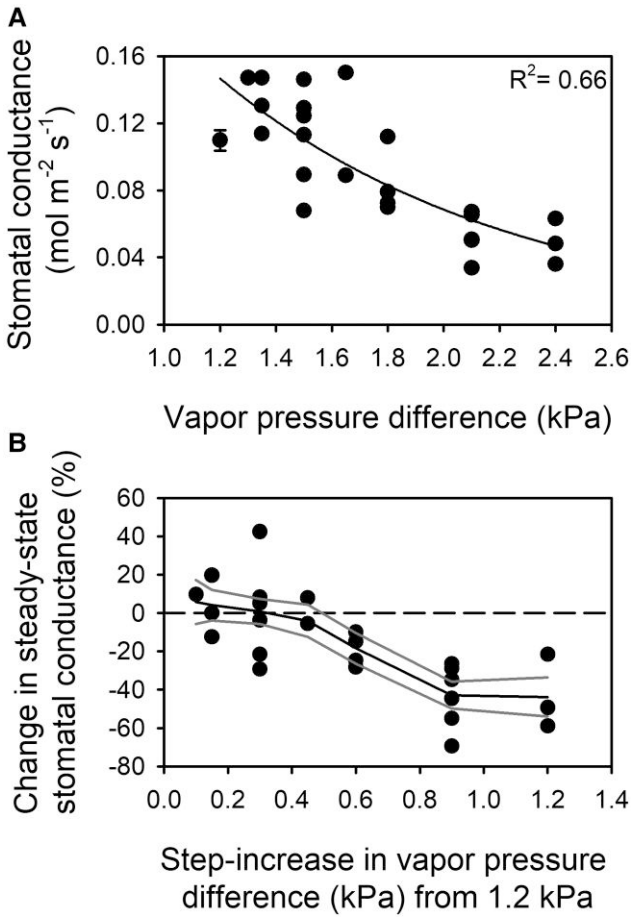

Figure 1. Stomata are relatively insensitive to small increases in VPD between the leaf and the atmosphere. **A)** Steady-state stomatal response to VPD in *S. minimus*. Each point represents a measurement from an individual leaf, except for the point at 1.2 kPa which is a mean (±SE) of 25 leaves measured under the same conditions. **B)** Change in steady-state stomatal conductance (g_s) after a step-increase in VPD in leaves initially acclimated to 1.2 kPa. The dashed horizontal line represents no change in g_s. The solid black line depicts a general additive model with the gray lines bounding it representing the SE of the model.

threshold at which stomata become sensitive to a step-increase in VPD (Brodribb and McAdam 2011) because these species have no epidermal mechanical advantage regulating stomatal aperture (Franks and Farquhar 2007), and therefore, there is no need for a hormonal or metabolic regulator of stomatal responses to drive the lowering of guard cell turgor following an increase in VPD (Buckley 2019). A threshold at which stomata are responsive to a step-increase in VPD in the angiosperm species *S. minimus* suggests that there is a trigger point, which we hypothesize is associated with the synthesis of the hormone ABA that must be crossed on exposure to a step-increase in VPD to drive stomatal closure. A threshold, or breakpoint, at which angiosperm stomata will respond to changes in humidity or VPD has also previously been reported in a number of angiosperm species (Sheriff 1977, Assmann and Gershenson 1991, Monteith 1995, Seversike et al. 2013, Choudhary et al. 2014, Riar et al. 2015, McAdam and Brodribb 2016, Schoppach et al. 2017, Sinclair et al. 2017, Sadok et al. 2019, Jafarikouhini et al. 2020, 2022, Bourbia et al. 2023).

Stomatal closure at high VPD occurs only when leaf turgor is transiently lost

We next sought to test whether an apparent trigger point for stomatal closure following a step-increase in VPD could be predicted by changes in Ψ_l following the VPD transition and whether stomatal responsiveness to a step-increase in VPD occurred when Ψ_l declined below Ψ_{tlp}, a cardinal Ψ_l at which mesophyll cells synthesize considerable levels of ABA (Pierce and Raschke 1980, Davies et al. 1981, McAdam and Brodribb 2018). We used a dynamic model to predict Ψ_l based on measurements of g_s, evaporation, mean leaf hydraulic conductance (K_{leaf}) at 1.2 kPa (2.0 mmol m^{-2} s^{-1} MPa^{-1}), and leaf capacitance (C_{leaf}) determined by pressure–volume curve analysis prior to Ψ_{tlp} (223 mmol m^{-2} MPa^{-1}) or after Ψ_{tlp} (739 mmol m^{-2} MPa^{-1}). We modeled Ψ_l dynamics following the transition in VPD for all gas exchange traces. In leaves exposed to a small step-increase in VPD, in which stomata did not close, we found that modeled Ψ_l did not drop lower than Ψ_{tlp} (Fig. 2A). In leaves exposed to a large step-increase in VPD in which stomata closed, the model suggested that both the magnitude of the step-change in VPD and the degree of the transient, wrong-way stomatal opening consistently resulted in predicted Ψ_l declining to a value below Ψ_{tlp} (Fig. 2B). The right-way stomatal closing response following the large transition in VPD resulted in a transient decline in modeled Ψ_l, with predicted Ψ_l recovering as stomata closed in all cases (Fig. 2B). This recovery of predicted Ψ_l on stomatal closure likely excludes the possibility that stomatal closure in response to the large step-change in VPD in *S. minimus* is passive and due to a loss of epidermal turgor. A loss of epidermal turgor is one of the few explanations for how plants with mechanical advantage can passively regulate stomatal closure when leaf water status declines. Once epidermal turgor is lost, stomata experience no mechanical advantage, and aperture becomes a function of guard cell turgor alone (Buckley 2019).

Modeled Ψ_l data suggested that in all leaves in which stomata closed on exposure to a high VPD, Ψ_l transiently declined below Ψ_{tlp} (Fig. 2C). To test whether a decline in Ψ_l below Ψ_{tlp} triggered the accumulation of ABA levels in the leaf, we measured ABA levels in leaves that had been exposed to step-increases in VPD in a large cuvette (the opaque conifer chamber LI-6400-22, in which a high flow rate [1,000 mL min^{-1}] was used and VPD transitions occurred rapidly). We exposed leaves to either a mild transition in VPD that did not induce stomatal closure or a larger transition in VPD that triggered stomatal closure. ABA levels were measured once g_s had reached a steady state; in all leaves, minimum Ψ_l during the VPD transition was modeled from gas exchange data. The larger conifer chamber has a volume of ~350 mL, so to

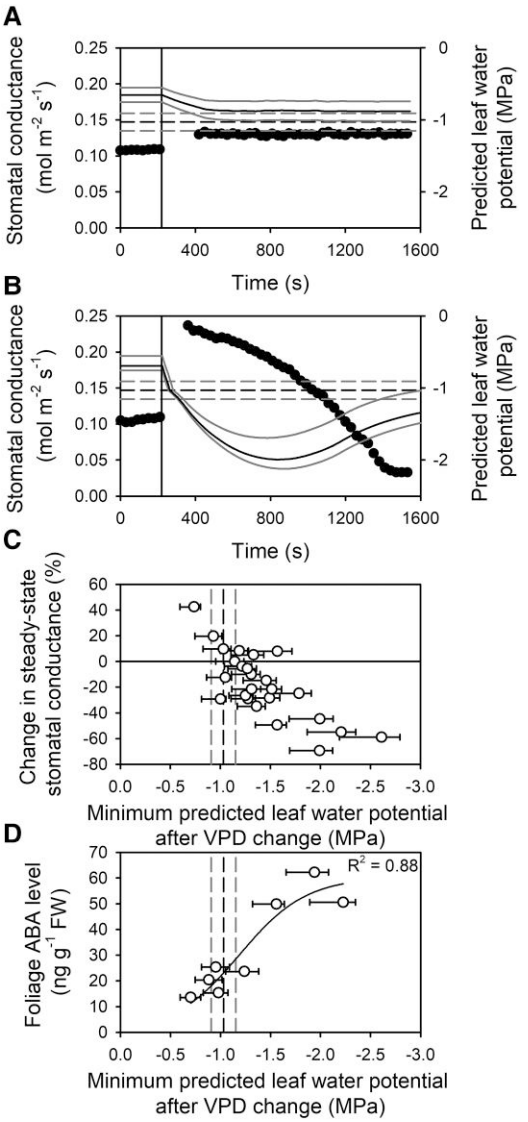

Figure 2. Stomata respond to a step-increase in VPD between the leaf and the atmosphere only when the increase is sufficient to cause transient leaf water potential (Ψ_l) to drop below bulk leaf turgor loss point (Ψ_{tlp}), triggering ABA biosynthesis. The response of stomatal conductance (g_s) to a **A)** small (1.2 to 1.35 kPa) and **B)** large (1.2 to 2.1 kPa) step-change in VPD; the change in VPD is denoted by the vertical line solid line (data logged during chamber equilibration after the VPD transition has been removed for clarity). Mean (solid black) bound by minimum and maximum (solid gray) modeled Ψ_l during the VPD transition are shown for each gas exchange trace. Black and gray dashed horizontal lines denote mean $\Psi_{tlp} \pm$ SE ($n = 5$). **C)** The relationship between the change in steady-state g_s after a step-increase in VPD and the minimum predicted Ψ_l experienced after the VPD transition. Black and gray dashed horizontal lines denote mean $\Psi_{tlp} \pm$ SE ($n = 5$); the solid horizontal line denotes no change in steady-state g_s after the change in VPD; error bars represent minimum and maximum modeled Ψ_l. **D)** ABA levels (ng fresh weight [FW]) measured in leaves after being exposed to either a small step-increase in VPD (<0.4kPa) that caused predicted Ψ_l to decline to mean $\Psi_{tlp} \pm$ SE (denoted by black and gray dashed vertical lines, respectively, $n = 5$) or in which predicted Ψ_l following the VPD transition declined to below mean Ψ_{tlp}.

trigger a rapid change in VPD, we swapped the intake air from a humid to a dry source. We found that ABA levels in leaves increased on exposure to high VPD when predicted minimum Ψ_l declined below Ψ_{tlp} (Fig. 2D). This data are similar to other studies in angiosperm species that have found there is an increase in leaf ABA level on exposure to high VPD when stomata close (Bauerle et al. 2004, McAdam and Brodribb 2015). These observations also confirm that while our modeling of Ψ_l transients following a VPD transition might have a level of inaccuracy, ABA levels increase in leaves in which transient Ψ_l declines below Ψ_{tlp}.

Our modeling of Ψ_l comes with a few caveats: there is known to be considerable variation between leaves in both maximum K_{leaf} and C_{leaf} (Blackman and Brodribb 2011, Loucos et al. 2017, Oliveira et al. 2022), both of which strongly influence the dynamics of Ψ_l, which may explain why in one leaf we predicted a decline in bulk Ψ_l below Ψ_{tlp} but observed no stomatal closure; this leaf may have had a higher K_{leaf} than others. Other factors not considered in our modeling include dynamic changes in K_{leaf} (Scoffoni et al. 2017), a trait that has been reported to decrease as soon as Ψ_l declines from 0. Recent observations in an herbaceous Asteraceae species suggest that dynamic changes in K_{leaf} may not be ubiquitous, with Bourbia et al. (2023) finding no evidence of VPD-driven declines in whole plant conductivity (K_{plant}) when stomata do not close. In our study, if K_{leaf} had declined at high VPD, modeled Ψ_l would drop well below Ψ_{tlp} under mild transitions in VPD that did not trigger stomatal closure. Without declines in K_{leaf}, our model predicted transient declines in Ψ_l that approached a threshold Ψ_l at which embolism occurs in a nontranspiring leaf of this species (Brodribb et al. 2016), although none of the leaves we measured showed any signs of leaf death after measuring. The low modeled Ψ_l may have been sustained in transpiring leaves without inducing embolism if the xylem of the minor veins had collapsed (Zhang et al. 2016). This greatly reduces the flux of water from major veins to the mesophyll protecting major veins from experiencing large declines in Ψ_l while resulting in considerable declines in mesophyll Ψ_l, which may have had a feedback on ABA biosynthesis (Zhang et al. 2023). How low mesophyll Ψ_l declined requires further investigation during an extreme transient increase in evaporative demand; some studies have suggested a period of time in which a degree of subsaturation of water vapor in the substomatal cavity may occur at high VPD (Cernusak et al. 2019, Wong et al. 2022). If this happened in our experiment, then at least some cells in the mesophyll may have experienced and survived very negative Ψ_l. Transient subsaturation of water vapor in the leaf would have also resulted in an underestimation g_s and of the degree of wrong-way opening after the VPD transition. This might explain the observed rising to maximum exponential—not linear—relationship between the magnitude of wrong-way stomatal opening after the step-increase in VPD and the size of the step-change in VPD (Fig. 3). Observations of stomatal aperture coupled with gas exchange following a VPD transition would resolve this unknown.

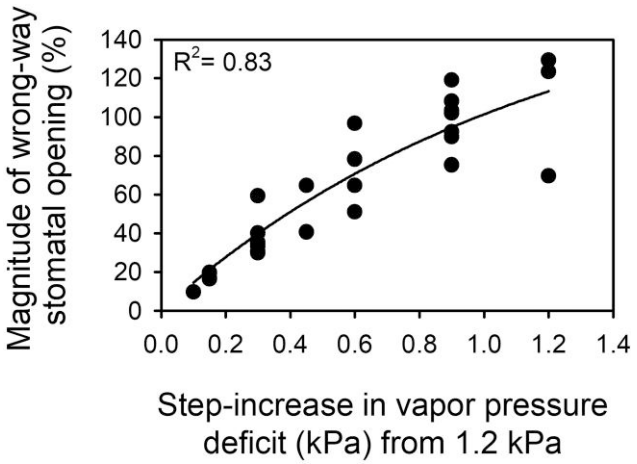

Figure 3. The relationship between the magnitude of wrong-way stomatal opening and the size of a step-increase in VPD imposed.

Wrong-way stomatal responses are a function of water status

One observation of g_s made following a VPD transition was that stomata tended to open in response to increased VPD even if they did not close because the transition in VPD was not sufficient to lower Ψ_l below the trigger point of response (Fig. 2A). This stomatal opening on exposure to high VPD is likely due to a passive, wrong-way opening that is a product of stomatal aperture being a function of both guard and epidermal cell turgor in angiosperm species (Buckley et al. 2011, Buckley 2019). We found that even when stomata closed in response to a large step-increase in VPD, there was a transient, wrong-way increase in g_s (Fig. 2B). The magnitude of this transient, wrong-way stomatal opening correlated with the magnitude of the step-increase in VPD (Fig. 3). We found that the largest transitions in VPD caused g_s to increase by more than 100% of an initial steady state value before right-way stomatal closure occurred (Fig. 3). Very few studies have documented the environmental determinants of the magnitude of the wrong-way response in angiosperms (Buckley and Mott 2002), and most have conducted experiments tracking the wrong-way transients following leaf excision (Powles et al. 2006, Westbrook and McAdam 2021). The progressive increase in the magnitude of wrong-way opening as the step-change in VPD increases suggests that the VPD transitions imposed in our experiment were not sufficient to cause a complete loss of epidermal turgor. Across diverse angiosperm species, the wrong-way response caused by a 1 kPa step-increase in VPD was found to be consistently smaller in magnitude than the wrong-way opening following leaf excision, further indicating that exposure to high VPD is not sufficient to cause a complete loss of epidermal cell turgor (Buckley et al. 2011). This differs from recent work in species from the fern family Marsileaceae, which have also evolved a mechanical advantage of the epidermis to allow stomata to open by lateral displacement into the epidermis (Westbrook and McAdam 2021). In these fern species, right-way stomatal responses are passive, and the magnitude of the wrong-way response diminishes at increasing VPD presumably because epidermal turgor is lower and rapidly lost as leaf water deficit increases.

Conclusion

We find that in the angiosperm species *S. minimus*, a threshold increase in VPD is required to trigger stomatal closure and that this threshold corresponds to Ψ_l transiently declining below Ψ_{tlp}. When transient Ψ_l declines below Ψ_{tlp}, ABA is synthesized and is likely the primary mechanism driving stomatal closure in response to increased VPD in this species (McAdam and Brodribb 2016). Our data suggest that angiosperm stomatal responses to increased VPD are a function of both epidermal cell turgor and ABA levels, with hormone levels being determined by leaf turgor dynamics. This is in contrast with the passive stomatal responses to VPD observed in most species of lycophyte, fern, and gymnosperm (Brodribb and McAdam 2011, Deans et al. 2017, Cardoso et al. 2019). A key question remains: why did angiosperms evolve stomatal responses to VPD regulated by hormones when species from sister groups have functional stomatal responses to VPD regulated by a simpler, passive mechanism? We would argue that epidermal mechanical advantage provides guard cells with the ability to open to larger apertures, as suggested by the evolution of these responses in species from the amphibious fern family Marsileaceae, which have the highest rates of leaf gas exchange measured in seed-free plants (Tosens et al. 2016, Westbrook and McAdam 2021), and that a threshold stomatal sensitivity to VPD in some angiosperm species, being a product of hormonal regulation of stomatal responses to water status, provides a competitive advantage by maximizing leaf gas exchange under conditions at which the risk of Ψ_l declining to lethal thresholds is minimal.

Materials and methods

Plant growth conditions

Two-year-old *S. minimus* plants were grown from seed (College Rd population (McAdam et al. 2011)) and used for all experiments. Plants were grown in a 5-L pot containing a 1:1:1 mix of Indiana Miami topsoil, sand, and propagation mix. Plants were grown under controlled environmental conditions (natural light, 18/23°C night/day temperatures) in the greenhouses at Purdue University, West Lafayette, Indiana. Plants were watered daily and received weekly applications of liquid fertilizer (Jack's Classic Petunia FeED, 20-6-22 N-P-K, JR Peters Inc. Allentown, PA, USA). Experimentation primarily took place in the winter months, from February to March, when plants were in a vegetative growth phase.

Stomatal responses to a step-increase in VPD

The stomatal response to a step-increase in VPD was measured in intact, fully expanded leaves using the small, lighted

fluorometer chamber of a LI-6800 infrared gas analyzer (LI-6800-01A, Li-Cor, Lincoln, NE). The conditions within the 6 cm^2 leaf area cuvette of the infrared gas analyzer were controlled at a constant CO_2 concentration of 400 μmol mol^{-1}, a light intensity of 1,000 μmol quanta m^{-2} s^{-1}, and a leaf temperature of 23°C. All leaves were enclosed in the cuvette and acclimated to a VPD of 1.2 kPa. All leaves selected for experiments had an initial g_s of ~0.11 mol m^{-2} s^{-1} in an attempt to minimize variation in hydraulic traits between leaves. Every 30 s, leaf gas exchange and cuvette conditions were automatically logged. The remainder of the plant outside of the cuvette was kept on the lab bench under room temperature and ambient lighting. Once all cuvette conditions and g_s were stable for at least 10 min, VPD of the air was increased by various increments ranging from 0.1 to 1.2 kPa. The VPD was then maintained until g_s remained constant (no change by more than 5 mmol m^{-2} s^{-1} for at least 15 min). After performing transitions for each leaf, leaves were excised from the plant and area in the cuvette was measured. Leaf gas exchange was adjusted for leaf area in the cuvette. Gas exchange data collected in the first 150 s following the transition in VPD using the LI-6800-01A leaf chamber were excluded due to disequilibrium in humidity between the reference and sample lines. In preliminary VPD transitions and in an empty LI-6800-01A, we found that 150 s was the maximum time it took for humidity to equilibrate following the largest transition in VPD (an increase of 1.2 kPa), with 90 s being the shortest time for humidity to equilibrate after a 0.1 kPa transition. To predict Ψ_l during these brief periods of humidity disequilibrium in the gas analyzer following the VPD change, we modeled g_s as a linear function of time using the gas exchange data collected immediately prior to the change in VPD immediately on humidity stability.

Pressure–volume curve analysis to determine Ψ_{tlp} and C_{leaf}

In five fully expanded leaves, mean Ψ_{tlp} and C_{leaf} before and after Ψ_{tlp} were determined by pressure volume curves (Tyree and Hammel 1972; Supplemental Fig. S1). Leaves were excised under water and rehydrated for 4 h, then weighed, and placed in a Scholander pressure chamber to measure Ψ_l. A microscope was used to precisely measure xylem balance pressure. Leaves were gradually dehydrated on the bench, and leaf mass and Ψ_l were periodically measured until at least three measurements were made after visible leaf wilting. After all measurements were made, leaf area and dry weight were recorded.

Determining K_{leaf} by evaporative flux

Three fully expanded leaves similar to those measured for gas exchange were used to determine K_{leaf}. Individual leaves were completely enclosed in an Opaque Conifer Chamber of an infrared gas analyzer (LI-6400-22, LI-COR) while still attached to the plant. The remainder of the plant outside of the chamber was kept on the lab bench under room temperature and ambient lighting. The chamber conditions were controlled at a constant VPD of 1.2 kPa, a light intensity of 1,000 μmol quanta m^{-2} s^{-1}, a carbon dioxide concentration at 400 μmol mol^{-1}, and a leaf temperature of 22°C. After gas exchange had reached a steady state (defined as less than a 3% change in g_s over 5 min), the chamber was opened and the leaf was rapidly excised from the plant, and Ψ_l was measured using a Scholander pressure chamber, after which leaf area was determined for correction of leaf gas exchange. K_{leaf} was determined for each leaf using Equation 1:

$$K_{leaf} = \frac{E}{0 - \Psi_l} \quad (1)$$

where E is evaporation determined using the gas analyzer and Ψ_l is measured in the leaf once gas exchange had reached a steady state.

ABA levels following VPD transition

Tissue in the gas analyzer cuvette from 8 leaves which were exposed to either a mild or large VPD transition was harvested once gas exchange had reached a steady state after the transition in VPD. Tissue from the leaf in the gas analyzer was weighed (±0.0001 g) and harvested immediately into −20°C 80% (v v^{-1}) methanol in water with added butylated hydroxytoluene (250 mg L^{-1}), roughly chopped, and stored at −20°C. Tissue was prepared for physicochemical quantification of ABA levels with an added internal standard using a liquid chromatography tandem mass spectrometry (Agilent 6460, QQQ LCMS) following the method of Cardoso et al. (2019).

Modeling the dynamics of Ψ_l

We used the dynamic model of Brodribb and McAdam (2011) to predict the dynamics of Ψ_l after a transition in VPD in all leaves measured. This model iteratively predicts Ψ_l dynamics as a function of evaporative demand, in this case determined by measured leaf gas exchange and VPD in the leaf cuvette according to Equation 2:

$$\frac{\partial \Psi_l}{\partial t} = \frac{K_{plant}}{C_{leaf}}(0 - \Psi_l) - \frac{g_s \mathrm{VPD}}{C_{leaf} P_{atm}} \quad (2)$$

where P_{atm} is the atmospheric pressure (Pa) and source Ψ_l in the stem is assumed to be 0 (this low Ψ_l in the stem is assumed because the plants were all well watered and the remainder of the canopy was not transpiring). We assumed the evaporative flux measurements of K_{leaf} were equivalent to K_{plant} under these conditions. We used C_{leaf} determined from pressure–volume curves either before or after Ψ_{tlp} depending on predicted Ψ_l. We used the highest and lowest measured values of K_{leaf} in this model to determine the upper and lower confidence intervals of the predicted Ψ_l.

Statistical analysis

A Student's *t* test was used to compare mean ABA levels between leaves which were exposed to a mild or large step-change in VPD. Modeling of Ψ_l was conducted in Microsoft Excel. The PVAST tool was used to determine C_{leaf} and Ψ_{tlp} from pressure volume curve data (Sack et al. 2022). Generalized additive models were performed using R (version 4.1.2) with the gam package.

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Author contributions

B.R.B. collected data with S.A.M.M., A.M. analyzed data and helped with experimental design, and S.A.M.M. designed the research and wrote the paper with contributions from all authors.

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. The relationship between leaf relative water content and leaf water potential in 5 leaves of *S. minimus*.

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Conflict of interest statement. None declared.

Data availability

The data underlying this article will be shared on request to the corresponding author.

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