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RESEARCH PAPER

Passive stomatal closure under extreme drought in an angiosperm species

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Abstract

The phytohormone abscisic acid (ABA) plays a major role in closing the stomata of angiosperms. However, recent reports of some angiosperm species having a peaking-type ABA dynamic, in which under extreme drought ABA levels decline to pre-stressed levels, raises the possibility that passive stomatal closure by leaf water status alone can occur in species from this lineage. To test this hypothesis, we conducted instantaneous rehydration experiments in the peaking-type species *Umbellularia californica* through a long-term drought, in which ABA levels declined to pre-stress levels, yet stomata remain closed. We found that when ABA levels were lowest during extreme drought, stomata reopen rapidly to maximum rates of gas exchange on instantaneous rehydration, suggesting that the stomata of *U. californica* were passively closed by leaf water status alone. This contrasts with leaves early in drought, in which ABA levels were highest and stomata did not reopen on instantaneous rehydration. The transition from ABA-driven stomatal closure to passively driven stomatal closure as drought progresses in this species occurs at very low water potentials facilitated by highly embolism-resistant xylem. These results have important implications for understanding stomatal control during drought in angiosperms.

Keywords: Abscisic acid (ABA), gas exchange, guard cells, turgor, water deficit, water potential.

Introduction

Stomata are mobile pores on the surface of leaves that regulate the exchange of water vapour from the plant with CO_2 from the atmosphere (Raschke, 1975). Unlike stomatal responses to light, CO_2, and photosynthesis which optimize gas exchange such that photosynthesis is maximized for a given rate of water loss (Wong *et al.*, 1979), the stomatal response to declining leaf water status restricts the majority of gas exchange with the atmosphere to conserve internal water reserves (Brodribb *et al.*, 2017). This is because most plants are unable to survive tissue desiccation. The sensitivity of stomata to soil water content and the effectiveness of stomatal closure during drought are critical in determining the survival time of an individual when available soil water is depleted (Martin-StPaul *et al.*, 2017). Consequently, considerable effort is put into understanding the specific environmental or physiological drivers of stomatal closure when soil water content declines (Tardieu and Davies, 1993; Abdalla *et al.*, 2021; Bourbia *et al.*, 2021; Verslues *et al.*, 2022). In terms of how stomata respond to declining soil water status, two mechanisms are recognized. The first is a direct, passive regulation of stomatal aperture by leaf water status (Rodriguez-Dominguez *et al.*, 2016; Cardoso *et al.*, 2019), and the other is an active trigger of stomatal closure, primarily through the hormone abscisic acid (ABA) (Tardieu and Simonneau, 1998; McAdam and Brodribb, 2012; Tombesi *et al.*, 2015), which is synthesized when mesophyll cells begin to lose turgor (Pierce and Raschke, 1980; Bacete *et al.*, 2022).

The relative importance of passive or active hormonal-driven processes for stomatal closure during drought vary across species, with the greatest difference being readily observed between seed and seed-free vascular land plants (McAdam and Brodribb, 2012, 2014; Gong *et al.*, 2021). In seed-free plants, stomatal closure during drought is passively driven by declines in leaf water status. Evidence for this simple, hydraulic regulation of stomatal closure comes from observations of rapid stomatal reopening on instantaneous rehydration of leaves or whole plants when stomata are shut by drought (McAdam and Brodribb, 2012; Cardoso *et al.*, 2019). When rehydrated, the stomata of species of seed-free plant reopen rapidly to maximum apertures as fast as the tissue rehydrates (McAdam and Brodribb, 2012; Cardoso *et al.*, 2019). This rapid reopening of stomata on the relaxation of leaf water potential provides compelling evidence for a passive mechanism driving stomatal closure because it occurs even when endogenous ABA levels in leaves are high, indicating that endogenous levels of this hormone play no role in closing the stomata in species from these groups during drought (Cardoso and McAdam, 2019).

In contrast to seed-free plants, in seed plant species in which the hormone ABA is synthesized during drought and levels are high in the leaf, similar rehydration experiments during drought fail to reopen stomata to maximum apertures (McAdam and Brodribb, 2012; Brodribb and McAdam, 2013). The degree of reopening of stomata in species of conifer, which do not have mechanical interactions between guard cells and epidermal cells, correlates with the level of ABA in the leaf at the time of rehydration (McAdam and Brodribb, 2014). These experiments, when combined with observations in ABA biosynthetic and signalling mutants in angiosperm species which have stomata that remain largely open during drought (Tal and Nevo, 1973; Xie *et al.*, 2006), with mutant plants dying rapidly when leaf water status declines (Brodribb *et al.*, 2021), make a compelling case for the importance of hormonal regulation of plant water status in seed plants.

In some species of *Cupressaceae* and *Taxaceae* (Brodribb *et al.*, 2014) and in extremely embolism-resistant species of *Fabaceae* (Nolan *et al.*, 2017; Yao *et al.*, 2021a, b) and *Lauraceae* (Mercado-Reyes *et al.*, 2023), ABA levels increase as soil water potential begins to decline, closing stomata. Once plants reach a water potential that approximates the bulk leaf turgor loss point, then ABA levels in the leaf begin to decline. Rapid rehydration experiments in species of the conifer genus *Callitris* (*Cupressaceae*) indicate that when ABA levels are low under extreme drought, the stomata reopen to maximum apertures as fast as leaf water potential relaxes (Brodribb and McAdam, 2013; McAdam and Brodribb, 2015). These experiments demonstrate that in *Callitris*, stomata transition from ABA-driven stomatal closure early during a drought to a passive regulation of stomatal closure once bulk leaf turgor is lost and ABA levels decline. The recent description of a peaking-type ABA dynamic in some species of angiosperm suggests that passive stomatal closure during drought might also be found in species from all lineages of vascular land plants.

There are a few reasons why passive stomatal closure during drought in angiosperms is not widely accepted. The first is that mutants of ABA biosynthesis and signalling do not close effectively during drought or when leaf water status changes (Brodribb *et al.*, 2021; Tulva *et al.*, 2023). This suggests that if there is a passive regulation of stomatal response to leaf water status in species from this group of land plants, it is minor or ineffective. Stomatal responses to short-term changes in leaf water status induced by vapour pressure difference between the leaf and the atmosphere (VPD) in angiosperms are not predictable by a passive–hydraulic model whereby guard cell turgor is linked to leaf turgor (Cardoso *et al.*, 2020; Binstock *et al.*, 2023). This contrasts with the stomatal responses to VPD in most non-angiosperm species of vascular plants (Brodribb and McAdam, 2011). The close mechanical interactions between guard cells and epidermal cells, which are unique to angiosperms and species of *Marsileaceae* (Westbrook and McAdam, 2020), mean that stomatal aperture does not decline when leaf water status declines, at least not until epidermal turgor is lost (Buckley, 2016, 2019). Finally, in species of *Caragana* (*Fabaceae*) in which a peaking-type ABA dynamic during extreme drought has been reported, the gaseous hormone ethylene has been suggested to close stomata during drought once ABA levels have declined (Yao *et al.*, 2021a).

To resolve whether the stomata of angiosperm species can be passively closed by leaf water status alone when soil water content declines, or whether species from this lineage always require a metabolic signal to promote stomatal closure, we conducted instantaneous rehydration experiments on branches of *Umbellularia californica* (*Lauraceae*) during a long-term drought to investigate stomatal control when water deficit is removed. We have recently described this species as having a peaking-type ABA dynamic during drought, which means ABA levels are low, and stomata are closed under extreme drought (Mercado-Reyes *et al.*, 2023). We hypothesize that if stomata are closed passively under extreme drought in this angiosperm species, then—on instantaneous rehydration—stomata will rapidly reopen to maximum apertures.

Materials and methods

Five-year-old plants grown in 5 litre pots containing a mix of Indiana Miami topsoil, ground pine bark, and sand (1:2:1 ratio) were used for experiments. Plants were grown under controlled greenhouse conditions under a 16 h photoperiod [supplemented by LED lighting providing a photosynthetic photon flux density (PPFD) of at least 150 μmol quanta m^{-2} s^{-1} at pot height] and a day/night temperature of 28/22 °C. Plants received daily irrigation when not under water deficit, and a monthly application of liquid fertilizer (Miracle-Gro® Water-Soluble All Purpose Plant Food, The Scotts Company LLC). Prior to imposing drought, leaf gas exchange was measured using an infrared gas analyser (Li-6800, Licor, Lincoln, NE, USA) in leaves from three branches. Conditions in the cuvette of the gas analyser were controlled to match the conditions in the greenhouse, with a chamber temperature set to 28 °C, the VPD set to 1.2 kPa, and incoming air was drawn from a buffer drum so that CO_2 in the cuvette matched ambient conditions in the greenhouse (~420 μmol mol^{-1}). Conditions in the cuvette were automatically logged every 30 s. Once acclimated to conditions in the cuvette and when gas exchange was stable, the branches were excised under water, and gas exchange recorded until stable. This enabled us to record maximum stomatal conductance in hydrated branches excised under water, accounting for any minor hydropassive stomatal closure that might occur on excision of angiosperm leaves under water (McAdam and Brodribb, 2012). Once gas exchange was stable, this value was used as the rate of stomatal conductance in unstressed branches that were fully hydrated. Leaf tissue was then harvested for leaf water potential determination using a Scholander pressure chamber followed by the physicochemical quantification of ABA levels using an added internal standard according to the methods of Mercado-Reyes *et al.* (2023).

A soil water deficit was imposed on the plants by withholding water. Periodically during drought, leaf water potential and foliage ABA levels were measured. Canopy conductance during the drought was determined gravimetrically by bagging pots and determining water loss rates over solar midday each day according to Mercado-Reyes *et al.* (2023). Once stomata had closed during drought, at a water potential at which ABA levels were known to be the highest during drought, a leaf was again enclosed in the cuvette of the gas analyser (using the conditions described above). A neighbouring leaf was taken for the determination of initial leaf water potential, which has minimal variation across the canopy when stomata are closed and before embolism has formed. After leaf gas exchange had stabilized, the branch was excised under water and instantaneously rehydrated (occurring over <10 min). Leaf gas exchange was logged until gas exchange had again stabilized. The leaf in the cuvette was then taken for determining a final leaf water potential and foliage ABA level. Because ABA levels were measured when all leaves were fully hydrated at the end of the rehydration experiment, ABA levels were expressed in terms of fresh weight. This experiment was repeated again once the levels of ABA in the leaf had declined to pre-stressed levels under long-term drought (~25 d after stomatal closure).

Results

In *U. californica*, stomata close during a long-term drought (Fig. 1A), and ABA levels display a classical peaking-type dynamic in which they increase early during drought then decline to pre-stressed levels as water potentials continue to decline (Fig. 1A). When leaves are instantaneously rehydrated, the degree of stomatal reopening was found to be associated with the level of ABA in the leaf (Fig. 1). When ABA levels were high in a leaf, at 6.33 μg g^{-1} FW when the plant was at a water potential of −3.15 MPa and stomata were closed, instantaneous rehydration did not cause stomata to significantly open despite water potentials relaxing to −0.54 MPa in 15 min (Fig. 1B).

Once water potential had declined to −3.83 MPa, ABA levels had declined to 3.85 μg g^{-1} FW, an intermediate level between the peak of ABA levels and pre-stressed levels, and—upon instantaneous rehydration—stomatal conductance rapidly increased to ~0.015 mol m^{-2} s^{-1}, which was less than half of stomatal conductance in leaves from plants that had not been exposed to drought and in which ABA levels were low (Fig. 1C). In branches in which ABA levels had declined to levels measured prior to drought (between 0.56 μg g^{-1} FW and 1.85 μg g^{-1} FW), instantaneous rehydration resulted in the rapid reopening of stomata to maximum conductance recorded in hydrated leaves measured in plants that had low levels of ABA prior to drought (Fig. 1D–F). In all of these leaves, leaf water potentials rapidly relaxed on rehydration (Fig. 1D–F).

Discussion

We show here that the stomata of the extremely drought-tolerant angiosperm species *U. californica*, which has a leaf P_{50} (or water potential at which 50% of xylem is embolized) of −7.54 MPa (Mercado-Reyes *et al.*, 2023), transitions to passive stomatal closure, from closure driven by the hormone ABA under long-term drought (Fig. 1). Our experiment, in which stomatal conductance was recorded during instantaneous rehydration of branches taken from drought-stressed plants, demonstrates that high levels of ABA in the leaf early in a drought are sufficient to keep stomata closed on rapid rehydration. This is similar to observations made across seed plant species which synthesize high levels of ABA during drought keeping stomata closed (McAdam and Brodribb, 2012, 2014).

We found that once ABA levels have declined to pre-stressed levels when plants approached −5 MPa, stomata become passively closed by low leaf water status alone. That the stomata of *U. calfornica* under these conditions could reopen rapidly to a conductance recorded in unstressed leaves suggests that neither ABA nor any other hormone or metabolic signal is keeping stomata closed during extreme drought in this species. If the osmotic potential of the guard cells was being actively lowered by the removal of ions from the stomata at this water potential, then stomata should not have been able to rapidly reopen to maximum conductance. This challenges the view that ethylene might be responsible for driving stomatal closure during drought in angiosperm species in which a peaking-type ABA dynamic has been observed (Yao *et al.*, 2021a).

Our observations in *U. californica* are very similar to work in the genus *Callitris*, in which instantaneous rehydration during a drought when ABA levels are lowest results in stomata reopening to maximum conductance (Brodribb and McAdam, 2013). It has been hypothesized that this ability to rapidly recover maximum rates of gas exchange is advantageous for species that grow in seasonally dry environments, allowing for the rapid and uninhibited recovery of photosynthetic capacity following rainfall (McAdam and Brodribb, 2015). We also observed that as ABA levels started to decline during drought, the residual ABA continues to keep stomata closed, with stomata not reopening to maximum conductance on instantaneous rehydration when ABA levels were half the level measured early in drought. This also mirrors work in *Callitris* in which there is a transition period between stomatal closure being exclusively driven by ABA to being driven by both low water status and ABA levels,

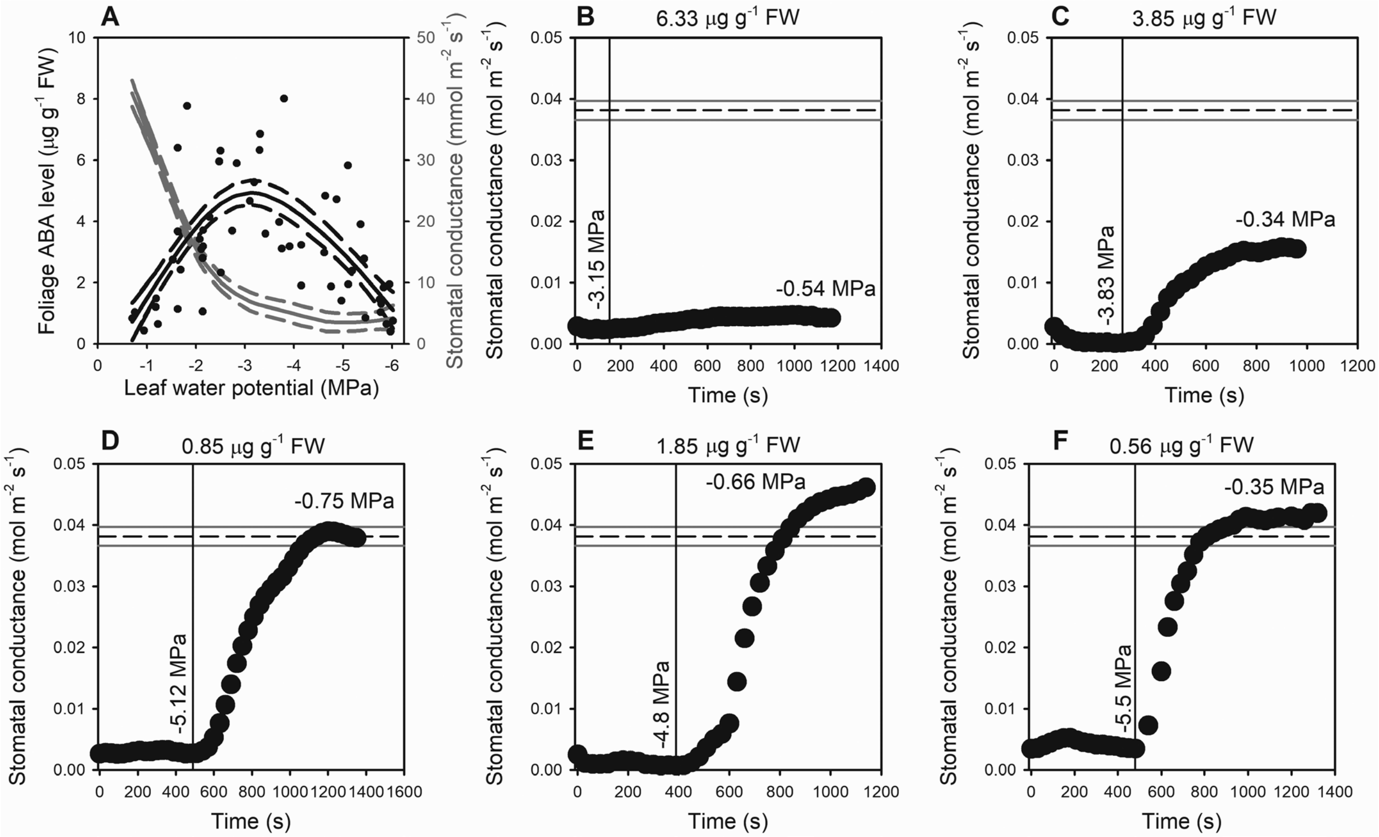

Fig. 1. Stomata transition from ABA-driven stomatal closure early during drought to passively driven stomatal closure under extreme drought in the angiosperm species *Umbellularia californica*. (A) The relationship between stomatal conductance (grey) and foliage ABA level (black) during drought in *U. californica*; data are generalized additive models (±SE) taken from Mercado-Reyes *et al.* (2023), from the same plants from which gas exchange data were collected. (B–F) The response of stomatal conductance (black dots) to instantaneous branch rehydration (denoted by the vertical line). The foliage ABA level measured in the leaf at the end of the gas exchange trace is written above each panel, and the water potential prior to rehydration is shown parallel to the line denoting rehydration. The water potential at the end of the gas exchange trace is also shown. The mean (n=5, ±SE) maximum stomatal conductance in unstressed branches that are excised under water with a mean ABA level of 0.93 ± 0.02 µg g^{-1} FW is shown as a vertical dashed line bound by the SE.

to finally being driven by low leaf water status alone (Brodribb and McAdam, 2013). Our results support a theory that ABA promotes early stomatal closure during drought but that as leaf water status declines sufficiently, then stomata can be closed passively in angiosperms (Rodriguez-Dominguez *et al.*, 2016). This differs from work in grape which suggests that declining water status passively closes stomata early in a drought and that ABA keeps stomata closed under long-term drought, although this conclusion comes from work on a species with xylem much more vulnerable to embolism formation (Tombesi *et al.*, 2015). The high level of ABA keeping stomata closed early during drought could be doing so by acting directly on guard cell anion channels (Geiger *et al.*, 2009), but this hormone can also act indirectly on stomatal closure by lowering leaf hydraulic conductance (Shatil-Cohen *et al.*, 2011). The speed with which leaf water potentials recover on instantaneous rehydration, when ABA levels were highest during drought, suggests that this indirect, hydraulic conductance pathway for stomatal closure via ABA is minor, if not absent, in this species.

Our work also suggests that the capacity to sustain high turgor pressures in guard cells in the light can be balanced by low plant water status. By demonstrating that stomata could reopen rapidly to maximum conductance in the light when instantaneously rehydrated after >25 d of being closed during drought, we conclude that the capacity to actively load ions into the guard cells in the light (via the H^+-ATPase) is not inhibited by low leaf water potential when ABA is not present (Pei *et al.*, 2022). Our data suggest that stomata are constantly loaded with osmolytes in the light under drought and that early during drought at relatively high leaf water potentials, ABA is responsible for the consistent removal of these ions from guard cells to keep stomata closed. However, under very low water potentials, the maximum loading of ions into guard cells is not sufficient to generate positive guard cell turgor pressure, thus ABA is no longer required to evacuate ions from the guard cells to keep stomata shut.

All data collected to date that report a peaking-type ABA dynamic during drought have shown that this phenomenon only occurs at very low water potentials (less than –4 MPa) and, consequently, a requirement is highly embolism-resistant xylem (Brodribb *et al*., 2014; Nolan *et al*., 2017; Yao *et al*., 2021a, b; Mercado-Reyes *et al*., 2023). Recent work in *U. californica* and *Callitris rhomboidea* suggests that ABA biosynthesis is deactivated at water potentials lower than the turgor loss point and requires sufficient time for the residual ABA to be conjugated for the levels in leaves to decline (Mercado-Reyes *et al*., 2023). Modelling suggests that passive stomatal control in angiosperms is possible once epidermal turgor is lost and the mechanical advantage of the epidermis is removed (Buckley, 2019). If epidermal turgor loss is assumed to occur at the same leaf water potential as the loss of bulk leaf turgor, then for most herbaceous plants in which embolism in the xylem forms at water potentials close to turgor loss point (Skelton *et al*., 2017), leaf death would supersede any observations of declines in ABA levels associated with passively closed stomata.

A key question that remains unanswered but emerges from our dataset is whether the stomata of angiosperms can be passively closed by declines in leaf water status when soil water status is high, for example when VPD increases (Merilo *et al*., 2017). It is unlikely that exposure to high VPD, when soils are saturated, is sufficient to cause a loss of epidermal turgor; thus, an active means of lowering guard cell turgor is required (Buckley, 2019; Binstock *et al*., 2023). Support for this is provided by evidence that ABA is synthesized rapidly in leaves exposed to high VPD (Bauerle *et al*., 2004; McAdam *et al*., 2016) and that when ABA signalling and synthesis is completely blocked, stomata do not respond to VPD (Fujii *et al*., 2011; Brodribb *et al*. 2021). Several studies, however, have reported that stomatal responses to VPD occur in single gene mutants in key ABA signalling and synthesis genes (Assmann *et al*,. 2000; Merilo *et al*., 2017), as well as in genetically altered plants in which ABA insensitivity is confined to guard cells (Yaaran *et al*. 2019), although these responses might be due to allelic leakiness or genetic redundancy in the pathways of ABA synthesis and response. We do not yet know what maximum turgor pressures are in the guard cells of open stomata in angiosperms compared with the stomata of other lineages of land plants and whether passive declines in leaf water status driven by high VPD would be sufficient to appreciably change the aperture of the pore, as hypothesized to occur in species of fern and lycophyte (Franks and Farquhar, 2007; Brodribb and McAdam, 2011).

Here we show that stomata of a highly embolism-resistant angiosperm species can be closed passively by low leaf water status alone under an extreme drought. Our results suggest that early during drought, stomata are closed by ABA but that they transition to being closed by low leaf water status alone when ABA levels begin to decline. Our observations of rapid stomatal reopening on instantaneous rehydration from a state of stomatal closure at very low water potentials suggests that the stomata of species from all lineages of vascular land plant have the potential to be passively regulated. Whether this passive regulation occurs at mild water potentials more akin to those experienced by herbaceous plants under drought, or when VPD increases in angiosperms, remains to be tested.

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Author contributions

SM: designing the experiments and writing the manuscript with the help of AM and CK; SM, CK, and JMR: data collection.

Conflict of interest

The authors declare no conflicts of interest.

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Data availability

All data are available on request from the authors.

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