Abscisic Acid Mediates a Divergence in the Drought Response of Two Conifers* and Scott A.M. McAdam

School of Plant Science, University of Tasmania, Hobart, Tasmania 7001, Australia

During water stress, stomatal closure occurs as water tension and levels of abscisic acid (ABA) increase in the leaf, but the interaction between these two drivers of stomatal aperture is poorly understood. We investigate the dynamics of water potential, ABA, and stomatal conductance during the imposition of water stress on two drought-tolerant conifer species with contrasting stomatal behavior. Rapid rehydration of excised shoots was used as a means of differentiating the direct influences of ABA and water potential on stomatal closure. Pinus radiata (Pinaceae) was found to exhibit ABA-driven stomatal closure during water stress, resulting in strongly isohydric regulation of water loss. By contrast, stomatal closure in Callitris rhomboidea (Cupressaceae) was initiated by elevated foliar ABA, but sustained water stress saw a marked decline in ABA levels and a shift to water potential-driven stomatal closure. The transition from ABA to water potential as the primary driver of stomatal aperture allowed C. rhomboidea to rapidly recover gas exchange after water-stressed plants were rewatered, and was associated with a strongly anisohydric regulation of water loss. These two contrasting mechanisms of stomatal regulation function in combination with xylem vulnerability to produce highly divergent strategies of water management. Species-specific ABA dynamics are proposed as a central component of drought survival and ecology.

By guarding the interface between plant and atmosphere, the stomata of land plants occupy a uniquely important role that connects diverse aspects of plant biology with atmospheric processes. Capitalizing upon the potential for stomata to be used to modify plant growth and survival, or as a tool for interpreting environmental change, requires a mechanistic understanding of how these tiny valves operate. Yet, an integrated understanding of stomatal control remains elusive. Foremost in this uncertainty is an explanation for how complex signals from the environment are translated into guard cell movement. A particularly challenging feature of stomatal behavior is the fact that environmental perturbation induces both physical and chemical responses within the plant and that turgor-regulated stomata are responsive to both signals. Disentangling these distinct contributions to stomatal conductance \( (g_s) \) has been made more complicated by the limited communication between molecular-scaled disciplines of mutant characterization and membrane transport biology and researchers at the larger scale of plant water relations and xylem transport. As a result, two contrasting views of stomatal control exist. Molecular biologists view stomata as osmotically regulated valves uniquely responsive to plant hormone levels and the resultant movement of ions across the guard cell membranes (Schroeder et al., 2001; Roelfsema and Hedrich, 2005). By contrast, most process-based models assume a direct influence of soil water content on stomatal aperture (Buckley, 2005; Damour et al., 2010).

The phytohormone abscisic acid (ABA) is seen as a cornerstone of stomatal function because it has been shown to trigger responses in guard cell membrane channels and transporters that cause a reduction in guard cell turgor, thereby closing stomata. ABA-mediated stomatal closure in seed plants (but not in ferns and lycophytes; Brodribb and McAdam, 2011) is broadly accepted as the explanation for stomatal closure during water stress (Zhang and Davies, 1989; Bauer et al., 2013); yet, there are very few studies that show a good correlation between the level of ABA and \( g_s \) during water stress in the field. The traditional explanation for this lack of a strong relationship suggests that ABA is a root-derived hormone that is delivered to the leaf in the transpiration stream (Zhang et al., 1987; Davies and Zhang, 1991) and hence that the xylem ABA flux, rather than the leaf level of ABA, should dictate the intensity of the stomatal response to soil drying (Tardieu et al., 1992; Tardieu and Davies, 1993). The flux-based model for ABA action in the leaf remains the most widely used interpretation of how stomata sense and respond to drying soil, despite the fact that there is mounting evidence for significant ABA synthesis in the leaf and guard cells, and short term responses to ABA that cannot be explained by xylem transport (Christmann et al., 2005; Lee et al., 2006; Georgopoulou and Milborrow, 2012). Furthermore, the ABA flux approach has never been successfully applied to explain variation.
in transpiration in trees (Sperry, 2000; Cochard et al., 2002), suggesting that there may be some benefit in reexamining some of the principles and assumptions used to link water stress, ABA, and transpiration.

Here, we examine the dynamics of stomatal closure, leaf ABA levels, and xylem tension during the gradual imposition of water stress upon two conifer species, *Pinus radiata* and *Callitris rhomboidea*, known for having contrasting stomatal responses to desiccation. Our primary aim is to separate the interacting effects of ABA and water tension on guard cell turgor pressure and stomatal diffusive conductance and hence to reveal the relative importance of water tension and ABA levels during drought as effectors of stomatal closure. Conifers are particularly suitable for identifying different closing signals because they do not appear to produce hydropassive stomatal movements (McAdam and Brodribb, 2012). This makes them ideal for examining the direct effects of ABA and water tension without the mechanical interactions between subsidiary cells and guard cells (Franks and Farquhar, 2007) that greatly complicate the mechanics of angiosperm stomatal movements. Both conifer species examined grow naturally in low rainfall habitats, but *P. radiata* is strongly isohydric (meaning that stomata close in a very narrow range of leaf hydration), while *C. rhomboidea* is anisohydric (meaning that stomata have a relatively low sensitivity to leaf hydration).

RESULTS

Dynamics of Leaf Water Potential, $g_s$, and ABA during Imposed Stress

The two conifer species, both adapted to seasonally dry environments, had fundamentally different stomatal responses and foliar ABA dynamics over increasingly negative leaf water potentials ($\Psi_l$) during a similar, prolonged period of drought stress (Fig. 1). In both species, $g_s$ declined with falling $\Psi_l$ following a sigmoidal trajectory; however, the range of $\Psi_l$ over which stomata closed varied widely between the two species (Fig. 1). *C. rhomboidea* displayed an anisohydric stomatal response to $\Psi_l$ with stomatal closure occurring over an extended $\Psi_l$ range (~1.2 to ~3 MPa), with $\Psi_l$ of individual plants continuing to decline over the prolonged period of drought to a minimum of less than ~7 MPa (Fig. 1). *P. radiata* had a strong isohydric stomatal response to drought stress, with 90% of stomatal closure occurring over a very narrow range of $\Psi_l$ (~1.3 to ~1.5 MPa) and individuals remaining at a relatively stable $\Psi_l$ between ~2 and ~2.5 MPa over an extended period of time (Fig. 1). The foliar ABA dynamics in *P. radiata* and *C. rhomboidea* were very different over the 35-d period of drought stress (Fig. 2). Foliar levels of ABA in *P. radiata* showed a rise to maximum after 33 d as $\Psi_l$ approached ~2.5 MPa and mean foliar ABA levels exceeded 2,000 ng g$^{-1}$ fresh weight (Figs. 1 and 2). Similarly in *C. rhomboidea*, foliar ABA level increased as stomata closed, reaching a peak level of around 1,200 ng g$^{-1}$ fresh weight at ~4 MPa (Fig. 1). However, peak ABA in *C. rhomboidea* occurred after only 15 d of water stress, after which, foliar ABA level progressively declined to reach predrought levels after 33 d of withholding water. At this point, stomata were completely closed and $\Psi_l$ was between ~6 and ~7 MPa (Figs. 1 and 2).

Poststress Recovery of Gas Exchange

The recovery of $g_s$ and transpiration followed different dynamics in the two conifer species following rewatering after 33 d (Figs. 1–3). In *C. rhomboidea*, low levels of foliar ABA at the end of the stress period allowed a rapid recovery of $g_s$ following rewatering, with stomata opening rapidly as plants hydrated (Fig. 3). No hysteresis in the relationship between $\Psi_l$ and $g_s$ was observed as rewatered plants recovered from
water stress (Fig. 1). In *P. radiata*, very high levels of foliar ABA resulted in hysteresis in the recovery of $g_s$ following rewatering, with stomata remaining closed until foliar ABA levels had declined after 48 to 72 h (Figs. 1 and 3). In *C. rhomboidea*, foliar ABA levels declined gradually over 2 to 3 d (Fig. 2). A strong relationship between $g_s$ and foliar ABA level was observed in *P. radiata*, with an exponential decline in $g_s$ observed with increasing ABA in both droughted and recovering plants (Fig. 2). This was not the case for *C. rhomboidea* plants, where there was no emergent relationship between $g_s$ and foliar ABA level during drought or recovery (Fig. 2).

Separating Effects of ABA and Desiccation

Rapidly rehydrating branches of water-stressed plants allowed stomatal closure due to the independent influences of ABA and $\Psi_t$ to be separated. Water-stressed shoots all had initial values of $g_s$ that were depressed to between 50% and less than 1% of the initial unstressed value. Rehydration of these shoots (to $>-0.6$ MPa) occurred over 1 to 3 min and led to different degrees of recovery of $g_s$ that was dependent on foliar ABA levels. In shoots with low levels of ABA, a rapid recovery of $g_s$ to prestressed values occurred, while a weak recovery of $g_s$ was evident in shoots with higher foliar ABA levels (Fig. 4A). Hydration of water-stressed shoots caused $\Psi_t$ in these excised shoots to rapidly rise to a point that was nonlimiting, according to data from droughted plants (Fig. 1), and hence, any sustained depression of $g_s$ could be attributed to the effects of ABA. Both species showed similar relationships between ABA and $g_s$ following rapid rehydration, expressed as a percentage of the maximum $g_s$ in each individual shoot (Fig. 4B). In both species, a log-linear relationship between rehydrated $g_s$ and foliar ABA level was observed, with $g_s$ recovering to only 20% of maximum when ABA levels were greater than 500 ng g$^{-1}$ fresh weight (Fig. 4B). Divergence between the species in terms of the percentage recovery of $g_s$ in shoots that had been excised and hydrated over the 33 d of water stress confirmed the contrasting ABA dynamics between species. In *P. radiata*, a minimal recovery of $g_s$ occurred after only 8 d of drought, indicating that high levels of ABA prevented stomata from reopening upon hydration. *C. rhomboidea* also showed an initial depression in the percentage recovery of $g_s$ after 8 d of water stress, but after 27 d, when ABA levels had fallen to prestress levels, the stomata of rapidly rehydrated excised shoots were able to rapidly recover between 82% and 99% of initial, unstressed $g_s$ (Fig. 5).

Figure 2. The trajectories of foliar ABA level over increasingly negative $\Psi_l$ during drought (red circles) and increasing $\Psi_l$ following rehydration and recovery (blue circles) in five individuals. The inserts depict the relationship of foliar ABA level and $g_s$ in the same individuals during drought and recovery.

Figure 3. Pooled data ($n = 5$, ± sd) of the recovery of whole-plant transpiration of droughted *C. rhomboidea* (black circles) and *P. radiata* (white squares) individuals following rewatering after 33 d of drought stress. The dotted line represents the day at which all individuals from both species were fully rehydrated; best-fit regressions were sigmoidal for *C. rhomboidea* ($r^2 = 0.88$) and linear for *P. radiata* ($r^2 = 0.93$).
Based upon the response of $g_s$ to instantaneous rehydration, it was possible to separate the closing influence of water tension and ABA during imposed water stress. The minimal recovery of $g_s$ upon rehydration of excised shoots in *P. radiata* indicated that stomatal closure was predominantly caused by high levels of foliar ABA during the entire period of water stress. By contrast, there was a dynamic shift in the influence of foliar ABA and $C_l$ in *C. rhomboidea* as water stress intensified (Fig. 6). Incipient stomatal closure under water stress in *C. rhomboidea* was largely due to increasing levels of foliar ABA, but as sustained water stress caused $\Psi_l$ to fall below $-5$ MPa, the influence of foliar ABA level on stomatal closure declined, and water tension became the predominant influence on $g_s$ and transpiration in *C. rhomboidea* (Fig. 6).

**DISCUSSION**

Superficially, the impact of water stress on our sample conifer species was identical and the same as virtually all nonmutant plants, whereby stomata close progressively as water stress intensifies. However, we found by teasing apart the influences of hydraulic tension and ABA during the imposition of water stress that the interactions between the stomata and the environment of these species were profoundly different. The contrasts in ABA dynamics and stomatal control in the two conifers examined here during soil drying introduce a new perspective on how plants respond to water stress and identify an important new source of diversity in plant water management. It is generally assumed that water stress induces a monotonic rise in ABA level in the xylem sap or leaf (Tardieu and Davies, 1993), thereby activating anion channels and depolarizing the guard cell membrane, causing a loss of guard cell turgor pressure and subsequent stomatal closure. This mode of action fits with the observations that in fully hydrated shoots of *P. radiata* and *C. rhomboidea*, stomata were highly sensitive to endogenous levels of foliar ABA and that water stress in both species caused an initial rise in ABA levels.

![Figure 4](image-url) **Figure 4.** A, Example kinetics of $g_s$ in two droughted *C. rhomboidea* branches with closed stomata that were rehydrated, one with low levels of endogenous foliar ABA and the other with high levels of endogenous foliar ABA (as noted in nanograms per gram), illustrating the dependence of stomatal reopening to maximum conductance (gray line) on the level of foliar ABA. B, The relationship between the percentage recovery of $g_s$ and foliar ABA level in branches that were dried on the bench for varying periods of time then rehydrated in *C. rhomboidea* (circles) and *P. radiata* (squares). Each point represents a different branch dehydrated for a different time (between 10 min and 24 h), such that a large range of water potentials and ABA levels were produced prior to rehydration.

![Figure 5](image-url) **Figure 5.** The relationship between the percentage recovery of $g_s$ in rehydrated leaves from branches taken from plants over an extended period of drought stress (indicated in days since the withholding of water) in *C. rhomboidea* (black circles) and *P. radiata* (white squares); lines represent means of data from a single branch taken each from three individuals and rehydrated at each time point ($\pm$ SD).
measuring gs during rapid rehydration of excised shoots, thereby separating the closing influence of ABA from the direct influence of apoplastic water tension on guard cell turgor. Rehydrating excised shoots also removed the potential influence of xylem-borne ABA, enabling us to focus uniquely upon the action of ABA levels present in leaves.

_P. radiata_ produced what would be considered a typical stomatal response (De Diego et al., 2012), whereby a rapid increase in foliar ABA prevented stomata from opening as water stress intensified and also prevented stomata from reopening when droughted leaves were excised and rapidly rehydrated (Fig. 5). However, this ABA-dominated mode of drought response in _P. radiata_ did not occur in _C. rhomboidea_, which was found to shift from ABA-dependent stomatal control to water potential-dependent stomatal closure during severe, but recoverable, water stress (Fig. 6). This idea was previously mooted as an explanation for the lack of hysteresis in the relationship between gas exchange and leaf hydration during recovery of many conifer species from water stress (Brodribb and Cochard, 2009), and the combined observations here of declining ABA levels and increasing stomatal responsiveness to rehydration in highly water-stressed _C. rhomboidea_ plants provides very strong support for divergence between _Callitris_ and _Pinus_ spp. in the way ABA influences the response to drought.

The idea that apoplastic water potential can sufficiently influence guard cell turgor to cause stomatal closure independently of ABA is an expected consequence of the hydromechanical stomatal control model proposed by Buckley et al. (2003). Despite this, the role of water tension tends to be dismissed by the bulk of stomatal physiologists who study processes at the genetic and membrane scale (Roelfsema and Hedrich, 2005). The counterview, that stomata only respond significantly to metabolic controls such as the plasma membrane H\(^+\)-ATPase and ABA-sensitive anion channels, is based upon the assumption that turgor pressures of guard cells in the light are too high to be significantly influenced by \(\Psi_1\) directly. This position seems reasonable when it is considered that the relatively small changes in \(\Psi_1\) (in the range of 1–2 MPa) typically observed to close stomata are insufficient to directly draw down guard cell turgor to a point where stomatal closure occurs. With the operational range of guard cell turgor pressures in the range of 4 to 6 MPa (Franks et al., 1998), it follows that an active reduction of guard cell turgor is the only way to significantly modify stomatal aperture. This may be the case for herbaceous species that tend to dominate molecular-scale research, but this assumption is not correct for woody plants, particularly those growing in drier environments. _C. rhomboidea_, for example, is able to comfortably recover from water potentials below ~6 MPa (Brodribb et al., 2010), and the species can survive for long periods at water potentials low enough to close stomata without the need for osmotic modification of the guard cell. Remarkably, we found that once leaf hydration in _C. rhomboidea_ declined to a point where \(\Psi_1\) should be low enough to close stomata independently of ABA (approximately ~4 MPa), the levels of ABA in the leaf declined dramatically (Fig. 2), leading to a transfer of the closing action from ABA early in drought to water potential after 18 d without water (Fig. 6). Hence, we found that the most stressed plants of _C. rhomboidea_ had the same or even lower levels of foliar ABA than unstressed individuals. We speculate that this peak and decline in ABA may be common in woody plants that can sustain low \(\Psi_5\) and that this behavior may be partially responsible for a confusing lack of correlation often observed between levels of water stress and leaf ABA in woody plants.

The effects of contrasting stomatal control mechanisms in _Callitris_ and _Pinus_ individuals are seen most clearly during recovery from water stress (Figs. 1 and 3). Due to the presence of very high levels of ABA in the leaves of severely stressed _P. radiata_ plants, stomata...
were not able to completely reopen for several days following rewatering due to residually high levels of ABA, despite the fact that leaves were fully hydrated. Severely stressed *C. rhomboidea* plants, on the other hand, had low levels of ABA and were thus able to immediately reopen stomata as soon as leaves or soil were rehydrated (Figs. 1 and 3). The switch from ABA-limited to hydraulically limited stomatal aperture during water stress provides *C. rhomboidea* with a water management strategy whereby plants can immediately benefit from any rainfall event unencumbered by hormonal limitation of $g_s$. Such a strategy fits well with the ecological characteristics of this species, being a shallow-rooted tree that grows most commonly in areas of low and sporadic rainfall (Harris and Kirkpatrick, 1991). Importantly, however, this strategy must be linked with cavitation-resistant xylem, such that $\Psi_f$ can fall to low levels without compromising the integrity of the water transport system. *Callitris* species have been shown to be highly resistant to water stress-induced xylem cavitation, surviving water potentials of around −8 to −10 MPa before suffering major impairment of xylem function (Brodribb et al., 2010). *Pinus* species, by contrast, tend to be much more vulnerable to xylem cavitation (Mitchell et al., 2012), meaning that water potential during nonlethal water stress must remain above −4 MPa in a range that is unlikely to close stomata without the aid of ABA-induced depolarization of the guard cell plasma membrane. It is likely, therefore, that the dynamics of ABA release or synthesis during water stress are linked closely with other stress tolerance characteristics and particularly xylem vulnerability.

Another important consequence of different ABA dynamics during drought is the possible effect on the timing of stomatal closure as water stress intensifies. Contrasts in the sensitivity of $g_s$ to changes in water potential and ABA have been observed among species (Tardieu and Simonneau, 1998), and recently, these different behaviors have been linked to differentiation of drought survival strategies in trees. Isohydric species are those that operate within a very narrow range of water potentials, with stomata closing early during drought, thereby maintaining plant hydration, but compromising carbon supply from photosynthesis early during stress. Alternatively, anisohydric species are able to maintain a degree of stomatal opening as water stress intensifies (Tardieu and Simonneau, 1998), allowing them to maintain gas exchange longer during drought exposure, potentially extending carbon supply further into an extended drought event (McDowell et al., 2008). A possible benefit of diminishing ABA levels during sustained drought may be that the final stage (the last 10%) of stomatal closure is delayed, thus maintaining carbon balance and producing the typical anisohydric stomatal response seen in *C. rhomboidea* (Fig. 1). The rapid rise in ABA levels with intensifying water stress observed in *P. radiata* would ensure complete stomatal closure at a $\Psi_f$ threshold very close to −1.5 MPa (Fig. 2) and a typical isohydric stomatal response to water stress. These patterns differ from the model of Tardieu and Simonneau (1998), who suggested that anisohydric stomata would be uniquely responsive to ABA dynamics, while isohydric species should respond to a combined function of ABA and $\Psi_f$. Although Tardieu and Simonneau (1998) recognized the interaction of ABA and water potential, their measurements were different to ours because they measured ABA fluxes in the xylem due to the fact that roots were considered to be the main source of ABA. Based upon their observations, Tardieu and Davies (1993) produced an empirical model whereby water potential exponentially amplified the effect of xylem ABA flux on stomatal aperture. Our data suggest that foliar ABA and apoplastic water potential have an additive effect on the guard cell aperture in conifers. Foliar ABA appears to have an exponential effect on the turgor and aperture of guard cells (Fig. 4), while apoplastic water potential has a linear influence on guard cell turgor. Hence, as the ratio of ABA to water potential changes, so too does the relative importance of each parameter in determining stomatal aperture (Fig. 6).

The substantial differences observed here in the dynamics of foliar ABA level and linked stomatal behavior in these two conifers reveal an important new axis of variation in the way plants respond to drought, centered around ABA physiology. We already know that there is considerable variation in the way species respond to this ubiquitous water stress hormone, with $g_s$ in early vascular plants shown to be insensitive (Brodribb and McAdam, 2011), while even among wild-type angiosperms such as some *Populus* species and hybrids, there are reports of ABA insensitivity (Furukawa et al., 1990; Braatne et al., 1992). Understanding diversity in the dynamics of ABA production during water stress among land plants holds great potential for explaining the evolution and ecology of different drought strategies as well as providing a new resource that could be exploited to select and modify plant behavior.

**MATERIALS AND METHODS**

*Plant Material, Growth Conditions, and Quantifying Gas Exchange*

Two drought-tolerant conifer species from different families, *Callitris rhomboidea* (Cupressaceae) and *Pinus radiata* (Pinaceae), were selected to observe the differences in stomatal response to $\Psi_f$ and foliar ABA level during water stress and recovery. Individuals were all the same age at the time of experimentation (approximately 3 years old) and had comparable leaf areas. *C. rhomboidea* plants were grown from seed collected from within their natural range, and *P. radiata* was grown from commercially available seed of composite provenance. All plants were grown in a sandy-loam soil (particle composition: 85% sand, 12% clay, and 3% silt) in 3.7-L pots (24-cm deep with a 15-cm diameter). Plants were watered daily to full soil capacity, unless undergoing drought. All plants received regular applications of 3- to 4-month slow-release fertilizer (14.6:1.13:6 N:P:K ratio, Osmocote). Growth conditions were 16-h days (supplemented and extended in the morning and evening by sodium vapor lamps ensuring a minimum 300–500 μmol quanta m$^{-2}$ s$^{-1}$ at the leaf surface during the day period) and 23°C/15°C day/night temperatures. Relative humidity was maintained at approximately 50% by a dehumidifier with integrated humidity sensors (model SeccoUltra 00563, Olimpia-Splendid).
Temperature and humidity were logged for the duration of the experiment using a HOBO Pro Series data logger (Onset). Maintaining a constant air temperature and relative humidity over the midday period during which transpiration was measured restricted vapor pressure deficit to a narrow range, meaning that logged temperature and humidity data could be used to convert measured transpiration to $g_\text{s}$, following the quantification of leaf area of each individual. Although leaf temperature was not monitored continuously, the glasshouse cell was well stirred, and temperature differences between leaf and air were never more than 1°C in either species with open or closed stomata. Leaf area was quantified by first determining leaf mass per unit area (grams per square meter) for each species using a subsample of hydrated leaves which were scanned (300 dots per inch, LiDE, Canon) for leaf area and weighed after drying for 72 h at 70°C. Leaf area of each individual was quantified as the quotient of total leaf dry weight (grams) and leaf mass per unit area of each individual collected and dried on completion of the experiment.

Drought and Recovery

Five individuals from each species were used to observe the relationship between foliar ABA level, leaf gas exchange, and $\Psi_f$ over an extended period of drought and subsequent recovery. Pots were double bagged to prevent evaporative water loss from the soil and shaded with a covering of aluminum foil. Midday (11:30 AM to 12:30 PM) whole-plant transpiration was quantified gravimetrically by weighing plants on a precision balance (approximately 0.02 g) (Mettler-Toledo PC5002-S). Immediately following midday measurements of transpiration, a small branch ($C.\text{rhomboidea}$) or two fascicles ($P.\text{radiata}$) were removed from each individual, immediately wrapped in damp paper towel, and bagged for $\Psi_f$ and foliar ABA quantification. $\Psi_f$ of the excised tissue was assessed using a Scholander pressure chamber and microscope to precisely measure xylem balance pressure, following which the tissue was immediately weighed for ABA extraction, purification, and quantification (see below). Drought stress was initiated by withholding water such that every second day over a period of 8 d, individuals were rewatered with one-half of the volume of water transpired during the preceding 2-d period. Water was thereafter withheld. Upon stomatal closure, transpiration, $\Psi_f$, and foliar ABA were quantified every 3 d for 24 d. After 9 and 18 d following stomatal closure, three individuals were rewatered with one-half of the volume of water transpired following stomatal closure to maintain an extended period of drought stress at a stable, non-thermal $\Psi_f$ (approximately 5.5 MPa for $C.\text{rhomboidea}$ and approximately 2.25 MPa for $P.\text{radiata}$). After 24 d following stomatal closure, all individuals were rewatered to soil water capacity and transpiration, $\Psi_f$, and foliar ABA quantification were assessed every day until transpiration had reached predrought levels.

The Effect of ABA on Stomatal Opening in Fully Hydrated Leaves

The effect of ABA on stomatal opening in fully hydrated leaves was examined in branches dehydrated in the laboratory as well as an additional three individuals of each species subject to an equivalent period of drought stress as the plants described above. These water stress treatments were used to induce different levels of endogenous ABA in leaves, after which shoots were hydrated rapidly over the period of minutes to determine the impact of ABA, independent of $\Psi_f$. During this period of stress, stomatal closure was the product of an unknown combination of both ABA and $\Psi_f$, but by alleviating the influence of $\Psi_f$ by rapidly rehydrating excised shoots, it was assumed that the remaining depression of $g_\text{s}$ could be attributed to ABA alone.

Unstressed, fully hydrated plants of both species were taken into the laboratory at 10 AM and 11 AM, where initial, maximum leaf gas exchange was quantified using an infrared gas analyzer (LI6400, LIcor). To ensure maximum leaf gas exchange, the cuvette conditions of the infrared gas analyzer were controlled at a constant vapor pressure difference of 1.2 kPa, light intensity of 1,200 μmol quanta m$^{-2}$ s$^{-1}$, CO$_2$ concentration of 390 μmol mol$^{-1}$, and leaf temperature was maintained at 22°C. Every 1-min leaf gas exchange and cuvette conditions were automatically logged. Measurements continued until a stable maximum $g_\text{s}$ was reached (stability was defined as less than a 5 mmol m$^{-2}$ s$^{-1}$ change in $g_\text{s}$ over 10 min). Following the quantification of maximum $g_\text{s}$, tissue in the cuvette was marked, and the branch, with at least 15 cm of stem below the measured leaves, was excised and allowed to dry on the bench. Individual branches were dried over a range of 10 min to 24 h to ensure a maximum range in accumulated, endogenous, foliar ABA and $\Psi_f$. Following the period of dehydration, the marked leaf area was again enclosed in the infrared gas analyzer cuvette, and several adjacent needles on the same shoot were taken for quantification of foliar ABA (see below). Following equilibration to cuvette conditions, the periderrm at the excised end of the stem was removed to approximately 5 cm from the cut end (to ensure no occlusion of the xylem by exuded sap), and the shoot was resect at a number of times under water to rapidly rehydrate the tissue through the xylem. Leaf gas exchange and cuvette conditions were automatically logged every 1 min during rehydration until $g_\text{s}$ again reached stability. Shoots were considered rehydrated when gas exchange had stabilized and $\Psi_f$ was greater than −0.5 MPa.

A similar approach was used to determine relative effects of ABA and $\Psi_f$ on stomatal aperture during soil drying. In this case, three plants were exposed to a prolonged period (34 d) of intensifying water stress. Maximum, unrestrigned leaf gas exchange was measured using an infrared gas analyzer using the same conditions as described above, with the measured tissue in the cuvette marked and tagged. These potted whole plants were droughted as described above, with midday transpiration and $\Psi_f$ recorded daily. On day two (prior to stomatal closure), day eight (first day of stomatal closure), day 27 (when ABA levels were approximately one-half of maximum in $C.\text{rhomboidea}$), and day 34 of drought (when ABA levels had returned to minimum in $C.\text{rhomboidea}$), foliar ABA level and leaf gas exchange was again measured using the marked tissue on one of the initially measured branches and the branch was excised and rehydrated under water as described above; data were again logged until $g_\text{s}$ had reached stability (Fig. 4A).

Quantifying the Relative Contribution of ABA and Leaf Water Potential on $g_\text{s}$ during Drought

To quantify the effects of the two stomatal closing signals, foliar ABA level, and $\Psi_f$, during drought, the data of $g_\text{s}$, foliar ABA level, and $\Psi_f$ collected from the first plants of each species that were droughted and recovered were used. In $C.\text{rhomboidea}$, the linear relationship between foliar ABA level and the percentage recovery of $g_\text{s}$ from the three droughted plants that were used to quantify the recovery of $g_\text{s}$ following instantaneous rehydration over drought was used to estimate an expected percentage reduction in $g_\text{s}$ in the droughted plants according to their quantified ABA levels (expected percentage $g_\text{s} = -0.0579$ × foliar ABA level + 108.05; Supplemental Fig. S1). In $P.\text{radiata}$, the exponential declining regression (expected percentage $g_\text{s} = 118.29 \times e^{0.0846 \times \text{foliar ABA level}}$) was used (Supplemental Fig. S1). Following the quantification of the observed percentage reduction in $g_\text{s}$, the percentage of observed $g_\text{s}$ that could be attributed to foliar ABA level was subsequently quantified according to the following equation:

$$\% \text{ stomatal closure due to ABA} = \frac{100 \times G_{\text{ABA}}}{G_{\text{obs}}}$$

where $G_{\text{ABA}}$ is the percentage of maximum $g_\text{s}$ expected at the foliar ABA level measured and $G_{\text{obs}}$ is the total percentage reduction in $g_\text{s}$ observed relative to the initial (prestress) maximum $g_\text{s}$. The percentage contribution to stomatal closure by $\Psi_f$ was calculated as the remainder, i.e. 100% minus the percentage of stomatal closure due to ABA. The implicit assumption here, that ABA-enhanced anion leakage from guard cells proportionally reduces their turgor pressure at all values of $\Psi_f$ is supported by observations of stomatal closure by ABA in excised leaves of $C.\text{rhomboidea}$ (Brodribb and McAdam, 2011). Other scenarios may also be plausible (Fardieu and Simonneau, 1998), and hence, the calculation here expresses a maximum ABA effect.

ABA Extraction, Purification, and Quantification

ABA extraction, purification, and physicochemical quantification by ultra-performance liquid chromatography were undertaken according to the methods of McAdam and Brodribb (2013), with the following modifications to the solidsate extraction purification step using a 600-mg strong anion exchange cartridge (Maxi-Clean, Grace Davison Discovery Sciences). An aliquot of 25 mL of each sample was taken, to which 50 μL of 1 M NaOH solution was added, increasing the pH above 8. The aliquot was then passed through a preconditioned SAX cartridge, following which the endogenous ABA and added [3H]ABA internal standard were eluted from the cartridge in 30 mL of 2% acetic acid in methanol (v/v). This eluate was dried under vacuum at 37°C.

Supplemental Data

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

Supplemental Figure S1. Relationships between foliar [ABA] and stomatal opening in droughted plants.
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