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Fern and Lycophyte Guard Cells Do Not Respond to Endogenous Abscisic Acid

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Stomatal guard cells regulate plant photosynthesis and transpiration. Central to the control of seed plant stomatal movement is the phytohormone abscisic acid (ABA); however, differences in the sensitivity of guard cells to this ubiquitous chemical have been reported across land plant lineages. Using a phylogenetic approach to investigate guard cell control, we examined the diversity of stomatal responses to endogenous ABA and leaf water potential during water stress. We show that although all species respond similarly to leaf water deficit in terms of enhanced levels of ABA and closed stomata, the function of fern and lycophyte stomata diverged strongly from seed plant species upon rehydration. When instantaneously rehydrated from a water-stressed state, fern and lycophyte stomata rapidly reopened to predrought levels despite the high levels of endogenous ABA in the leaf. In seed plants under the same conditions, high levels of ABA in the leaf prevented rapid reopening of stomata. We conclude that endogenous ABA synthesized by ferns and lycophytes plays little role in the regulation of transpiration, with stomata passively responsive to leaf water potential. These results support a gradualistic model of stomatal control evolution, offering opportunities for molecular and guard cell biochemical studies to gain further insights into stomatal control.

INTRODUCTION

The concept that abscisic acid (ABA) closes stomata in land plants is a fundamental principle of plant physiology established from studies that span all scales of plant function, from whole organism to individual guard cell biochemistry and physiology (Schroeder et al., 2001; Wilkinson and Davies, 2002; Kim et al., 2010). The influence of ABA on stomatal aperture therefore provides one of the most important links between cellular physiology and large-scale processes, such as plant water use, photosynthesis, and drought responses. Recent evidence suggests, however, that generalizations regarding the cardinal role of ABA in stomatal control are not correct for all land plants and that the guard cells of primitive vascular plant clades are insensitive to ABA (Lucas and Renzaglia, 2002; Brodribb and McAdam, 2011). The existence of phylogenetic structure in the way different lineages of land plants respond to ABA not only provides an exciting perspective on plant functional evolution, but also has the potential to stimulate new and important insights into the function of ABA in higher plants.

The principal role of ABA is believed to be water conservation during drought stress (Wilkinson and Davies, 2002). Soil drought induces an increase in the concentration of ABA in the plant, triggering stomatal closure and consequently a reduction in water loss by transpiration (Wright and Hiron, 1969; Jones and Mansfield, 1970; Cornish and Zeevaart, 1986; Davies and Zhang, 1991; Christmann et al., 2005). In addition to closing stomata during drought, ABA is a vital component in a number of networked guard cell–signaling cascades that regulate stomatal aperture in response to a wide range of environmental stimuli (Schroeder et al., 2001; Galvez-Valdivieso et al., 2009; Wilkinson and Davies, 2009; Lee and Luan, 2012). Much of the mechanistic understanding of ABA-induced guard cell closure and membrane-specific biochemistry comes from ABA signaling and synthesis mutants (Imber and Tal, 1970; Lemichez et al., 2001; Mustilli et al., 2002; Assmann, 2003; Tallman, 2004; Xie et al., 2006; Geiger et al., 2010). Stomata in these mutants are unresponsive to environmental stimuli (Koornneef et al., 1984; Mustilli et al., 2002), remaining open during leaf and soil dehydration, resulting in their classic wilty phenotype (Leymarie et al., 1998; Young et al., 2006). The high sensitivity of ABA signaling or synthesis mutants to water stress has been fundamental in cementing the vital role of ABA in plant survival.

ABA mutants and the genes identified from these mutants have provided important information about the molecular and biochemical pathways regulating guard cell turgor (Pei et al., 1997; Pei et al., 2000; Geiger et al., 2010; Geiger et al., 2011) and the role of ABA in these processes, with numerous comprehensive reviews on the topic (Schroeder et al., 2001; Outlaw, 2003; Li et al., 2006; Kim et al., 2010). However, there are many unresolved issues associated with the performance of ABA mutants that prevent this approach from providing a comprehensive understanding of the physiological controls of guard cells. Conflicting evidence from ABA mutants has been unable to convincingly resolve the regulation of stomatal responses to changes in humidity, particularly the role of ABA in regulating wild-type responses to these small changes in leaf hydration (Assmann et al., 2000; Xie et al., 2006). Adding to this ambiguity are data suggesting that the responses of wild-type stomata to changes in humidity can be accurately predicted without any influence of metabolic signals, such as ABA (Peak and Mott, 2011). Furthermore, the interconnectedness of many biochemical pathways responsible for stomatal control makes it

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difficult to decipher particular guard cell metabolism and biochemistry responsible for stomatal responses to signals such as red light and photosynthesis (Lawson, 2009). As a result, there remains little integration between the well-documented guard cell membrane processes and the responses of stomata to changes in environmental conditions experienced in the field. In particular, data from hydraulic experiments raise uncertainty about the centrality of ABA in closing stomata during water stress in whole plants (Oren et al., 1999; Brodribb and Cochard, 2009). This disparity between the membrane-level understanding of stomatal control and whole-plant behavior widens considerably on consideration of current models predicting stomatal behavior, many of which pay little heed to an appreciable role of ABA in regulating stomatal responses (Buckley et al., 2003; Buckley, 2005; Damour et al., 2010). With the current array of guard cell–signaling mutants unable to resolve many of these issues, prospects for alternative genetic approaches to understanding guard cell signaling are highly appealing. One such alternative is to investigate natural genetic variation observed across the phylogeny of land plants.

The phylogenetic approach to examining stomatal control is a recently emerging possibility that provides a means of identifying novel pathways associated with the evolution of components in the stomatal control network and has the potential to bridge the gap between membrane-level processes and whole-plant function. Several major discoveries have demonstrated the power of a phylogenetic approach in the investigation of stomatal physiology, including evidence for absent phototropin-mediated responses of guard cells to blue light in the basal lineages of vascular plants (Doi et al., 2006; Doi and Shimazaki, 2008) and, more recently, evidence that the stomata of early-branching vascular plants are only equipped with very simple responses to light and water content (Brodribb et al., 2009; Brodribb and McAdam, 2011; Haworth et al., 2011). These studies suggest that during the 400 million years since the evolution of stomata (Edwards et al., 1998), the control of stomatal aperture has increased in complexity and that this is reflected in the characteristic stomatal behaviors of the different clades of living vascular plants (McAdam and Brodribb, 2012). Central to the theory of gradualistic stomatal control evolution is evidence that regulation of transpiration by ABA is a derived character, evolving after the divergence of seed plants (Brodribb and McAdam, 2011). This idea has been recently challenged by linked reports of ABA sensitivity in the bryophyte genus Funariaceae (Chater et al., 2011) and the lycophyte species *Selaginella uncinata* (Ruszala et al., 2011). In their challenge to the gradualistic model of stomatal control evolution, these authors conclude that stomata evolved 400 million years ago in possession of all of the signaling and control complexity seen in modern angiosperm stomata (Chater et al., 2011; Ruszala et al., 2011). Testing these diametrically opposed alternatives to the evolutionary history of stomatal function is imperative if we are to resolve the current impasses in our understanding of guard cell function in higher plants and the integration of guard cell membrane–specific processes with global models of transpiration.

Using different lineages of plants, we examine the effectiveness of ABA in regulating stomatal aperture under the condition for which ABA is believed to be centrally important: water stress. We specifically investigate the action of endogenous ABA in regulating stomatal aperture during and after water stress in representative species from ancient clades, such as ferns and lycophytes, compared with the more recently derived seed plant clade.

RESULTS

Stomatal Response to Endogenous ABA during Drought

In agreement with prevailing literature, we found that our diverse sample of vascular land plants had identical physiological responses to water stress (Figure 1). After the imposition of water stress, all species closed their stomata concomitant with an increase in the level of ABA and a decrease in leaf water potential (Figure 1). All species experienced a similar level of stress, with the decrease in leaf water potential, augmentation of ABA, and stomatal closure occurring over 3 to 7 d. Leaf ABA levels rose markedly in all species, ranging from a threefold increase in conifers to a 20-fold increase in the fern *Dicksonia antarctica* (Figure 1). Rapid rehydration of excised droughted leaves allowed us to separate the simultaneous stomatal closing signals produced by leaf dehydration and ABA augmentation. In all species, we found that after a period of minutes after excision under water, droughted leaves hydrated rapidly to water potentials equivalent to predrought levels while maintaining high endogenous ABA levels (Figure 2). High levels of ABA in the leaf strongly reduced the reopening of droughted seed plant stomata despite the restoration of predrought water potentials. Rehydrated stomatal conductances in our seed plant sample (*Pisum sativum*, *Callitris rhomboidea*, and *Pinus radiata*) were <20% that of prestressed leaves with low levels of ABA (Figure 2). The suppression of stomatal reopening when droughted leaves of seed plants were instantaneously rehydrated was the same regardless of whether the leaf was rehydrated in a 1000 ng mL^{-1} concentrated solution of ABA or deionized water (Figure 2).

In contrast with the stomata of seed plants, the stomata of the representative ferns (*Pteridium esculentum* and *D. antarctica*) and lycophyte (*Selaginella kraussiana*) were found to be insensitive to the high endogenous levels of ABA produced by water stress and were able to rapidly reopen to maximum apertures when the leaves were instantaneously rehydrated from a droughted state (Figure 2). This instantaneous reopening of fern and lycophyte stomata occurred in leaves with high endogenous levels of ABA that were cut in water as well as leaves with endogenous ABA augmented by the 1000 ng mL^{-1} solution of ABA used to rehydrate the leaves. The reopening of fern and lycophyte stomata during rehydration was rapid, occurring over less than 5 min, with stomatal conductances reaching predrought levels recorded in leaves from the same plants with very low levels of foliar ABA (Figure 2).

Given the lack of ABA sensitivity in the stomata of water-stressed ferns and lycophytes, we further investigated whether high levels of ABA augmented by water stress in ferns played any role in aiding plant recovery after water stress. We found that during water stress, the stomata of two representative fern species (*P. esculentum* and *D. antarctica*) were extremely sensitive to leaf water potential and not the concentration of ABA in

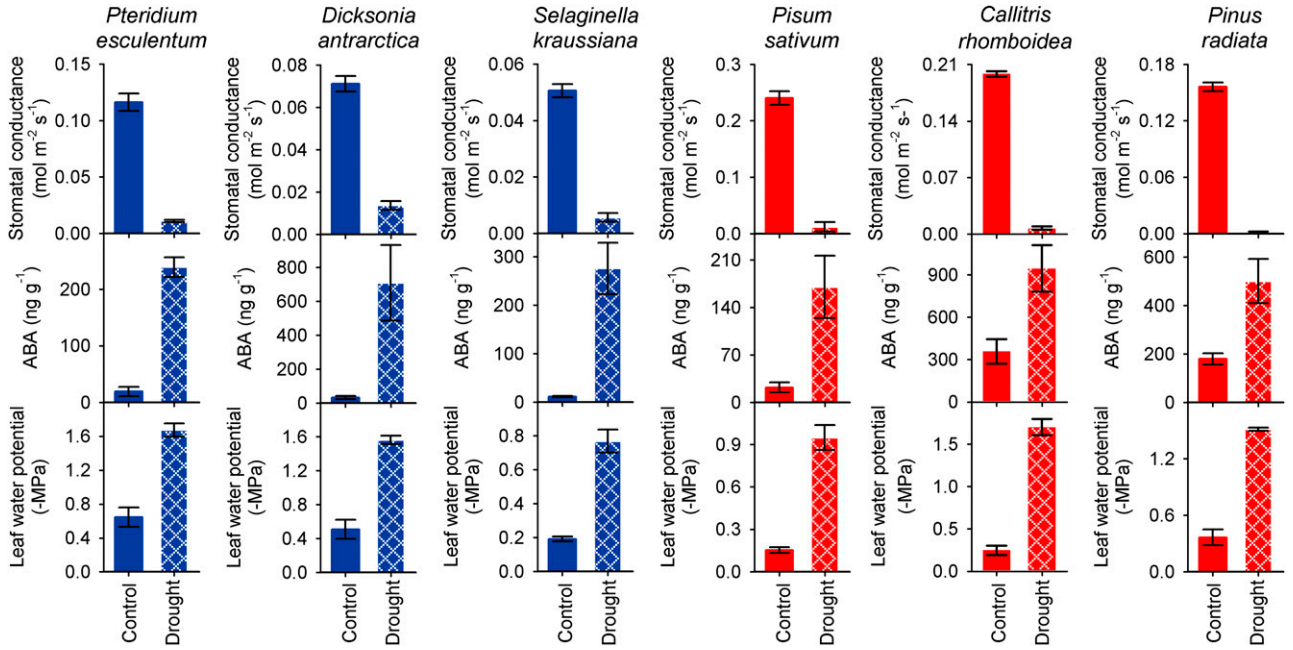

Figure 1. The Physiological Responses of Six Diverse Vascular Plant Species to Drought Stress.

In three seedless vascular plants, ferns (*P. esculentum* and *D. antarctica*) and a lycophyte (*S. kraussiana*) (blue bars), and three seed-bearing vascular plants, an angiosperm (*P. sativum*) and conifers (*C. rhomboidea* and *P. radiata*) (red bars), stomatal conductance (mol m^{-2} s^{-1}), foliar ABA level (ng g^{-1}), and leaf water potential (megapascal [MPa]) were measured in predrought controls (solid bars) and after exposure to a water stress that resulted in stomatal closure (hashed bars). Data represent means ± SE (n = 6).

the leaf (Figure 3). Furthermore, in these two fern species of contrasting growth habit and maximum transpiration rate, stomata closed predictably with the progressive dehydration of the plant (Figure 3). After recovery by rehydration of the soil, the stomata of both fern species reopened, following a trajectory that reflected passive stomatal control by leaf water potential, as evidenced by minimal hysteresis in the relationship between leaf water potential and stomatal conductance during whole-plant rehydration (Figure 3). The recovery of leaf gas exchange to predroughted levels after rewatering was not dependent on or influenced by the concentration of ABA in the leaf; ABA levels in the leaf increased during water stress but did not form any predictable relationship with stomatal aperture, especially after rewatering and recovery from stress (Figure 3).

Stomatal Response to Leaf Water Potential

Having demonstrated an insensitivity to water stress augmented endogenous ABA in fern and lycophyte stomata, we examined the instantaneous effects of rapid fluctuations in leaf water potential on stomatal aperture in excised leaves to see whether the responsiveness of fern and lycophyte stomata to leaf water potential occurred over short (<15 min) time scales. In two representative fern species (*P. esculentum* and *D. antarctica*) and a lycophyte (*S. kraussiana*), leaf excision in the air resulted in immediate and progressive stomatal closure as leaves dehydrated (Figure 4). The decrease in stomatal aperture as fern and lycophyte leaves dehydrated could be arrested and reversed by rehydrating the leaf in water, with stomata rapidly reopening (Figure 4). The response of fern and lycophyte stomata to leaf water potential was highly predictable over repeated cycling of leaf dehydration and rehydration (Figure 4). By contrast, the stomatal responses of representative seed plants (*P. sativum*, *C. rhomboidea*, and *P. radiata*) to changes in leaf water potential were unpredictable (Figure 4). In neither the angiosperm nor conifers did stomatal aperture track changes in leaf water potential over cycles of leaf dehydration and rehydration (Figure 4).

Suppression of Stomatal Opening by ABA

In the absence of an ABA-mediated stomatal response to water stress in ferns and lycophytes, we investigated whether exogenous ABA fed into the transpiration stream of representative ferns (*Astrolepis sinuata*, *P. esculentum*, and *Nephrolepis exaltata*) was able to suppress light-activated stomatal opening in hydrated leaves after a normal nocturnal dark period during which the stomata of all species closed (Figure 5). In two representative angiosperm species (*Oxalis corniculata* and *Lotus corniculatus*), exogenous ABA fed into the transpiration stream closed stomata and suppressed reopening in the light by >50% the next morning if the leaf was kept in the dark overnight (Figure 5). By contrast, ABA was not able to suppress light-activated stomatal opening in our photosynthetically diverse sample of fern species (Figure 5). Regardless of maximum stomatal apertures, the stomata of ferns were not stimulated to close when exogenous ABA was fed into the transpiration stream and were able to reopen in the light in the presence of high leaf ABA levels after nocturnal closure (Figure 5).

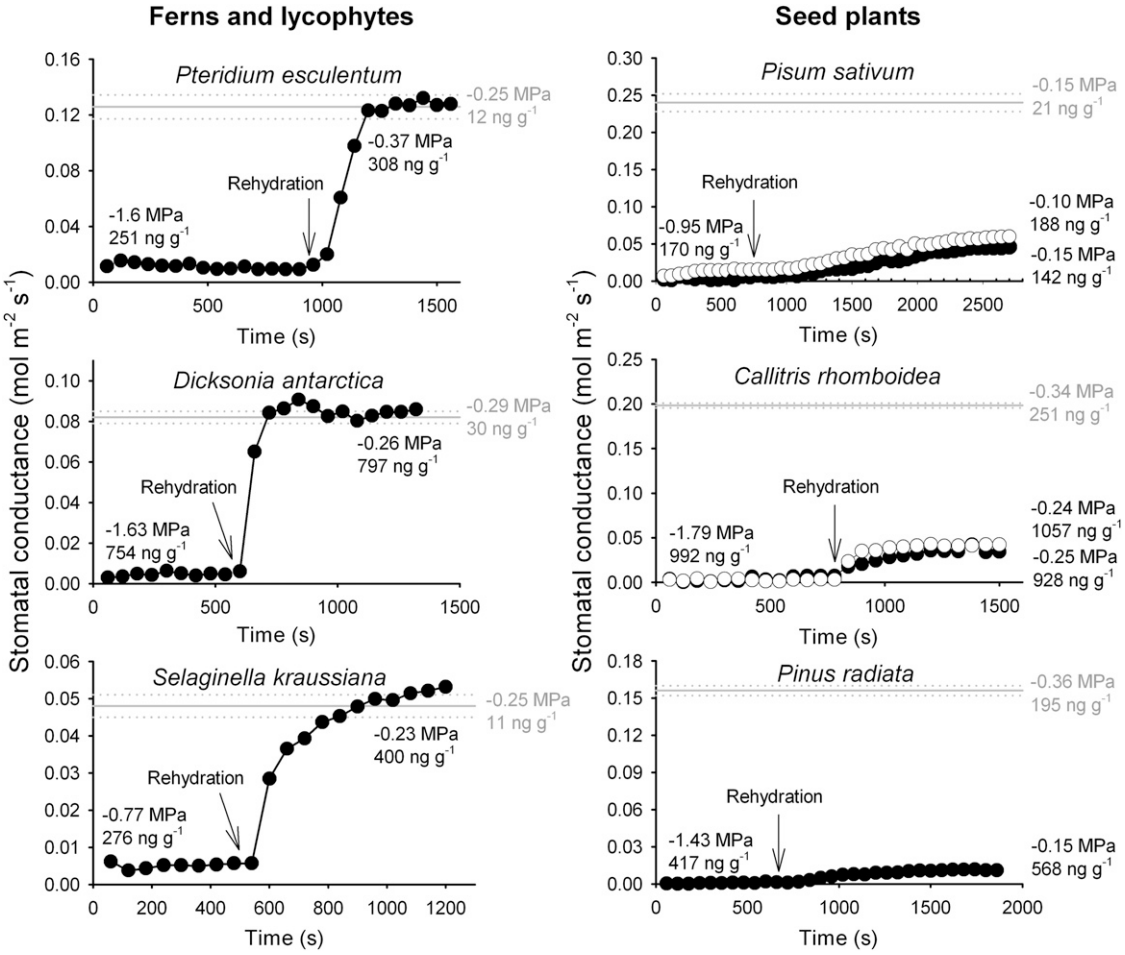

Figure 2. Unlike Seed Plants, Instantaneous Rehydration of Droughted Leaves Allows the Stomata of Ferns and Lycophytes to Reopen Despite High Endogenous Levels of ABA in the Leaf.

Prior to drought, stomatal conductance was measured on three leaves (mean represented by gray line; ±SE represented by dotted gray lines) in conjunction with leaf water potential (MPa) and foliar ABA concentration (ng g^{-1}) indicated in gray for each species. Plants were then droughted until stomata were closed, and leaf water potential and foliar ABA concentration were again measured. Stomatal conductance was measured on a leaf that was instantaneously rehydrated in water containing 1000 ng mL^{-1} of ABA (black circles) and in two seed plants in deionized water (white circles). After rehydration, leaf water potential and foliar ABA concentration were again measured. Representative time courses of stomatal conductance are presented for each species.

DISCUSSION

In agreement with the current paradigm of stomatal physiology, we found that diverse lineages of vascular plants responded similarly to water stress, with stomata closing in parallel with increasing levels of ABA in the leaf and decreasing leaf water potential (Figure 1). However, upon separating these two concurrent influences on stomatal closure, we found profound differences between lineages, showing that fern and lycophyte stomata were insensitive to high endogenous levels of ABA, responding uniquely to the water potential of the leaf in a manner consistent with stomata acting as passive hydraulic valves (Brodribb and McAdam, 2011). Such passive stomatal responses in ferns and lycophytes contrast starkly with seed plant stomata, which actively regulate leaf hydration during water stress via ABA (Ache et al., 2010). These contrasting behaviors indicate a profoundly different process of turgor regulation occurring in the guard cells of ferns and lycophytes than that of seed plants.

Contrasting Stomatal Responses to ABA across Vascular Plant Phylogeny

Water stress seems to invoke stomatal closure in different ways depending on a species' position in the plant phylogeny. In seed plants, ABA regulates stomatal aperture especially during and after water stress, yet according to our results, fern and lycophyte stomata respond passively to leaf water content and not ABA during changes in plant hydration (Figures 2 and 4). The lack of stomatal regulation by ABA in ferns and lycophytes is

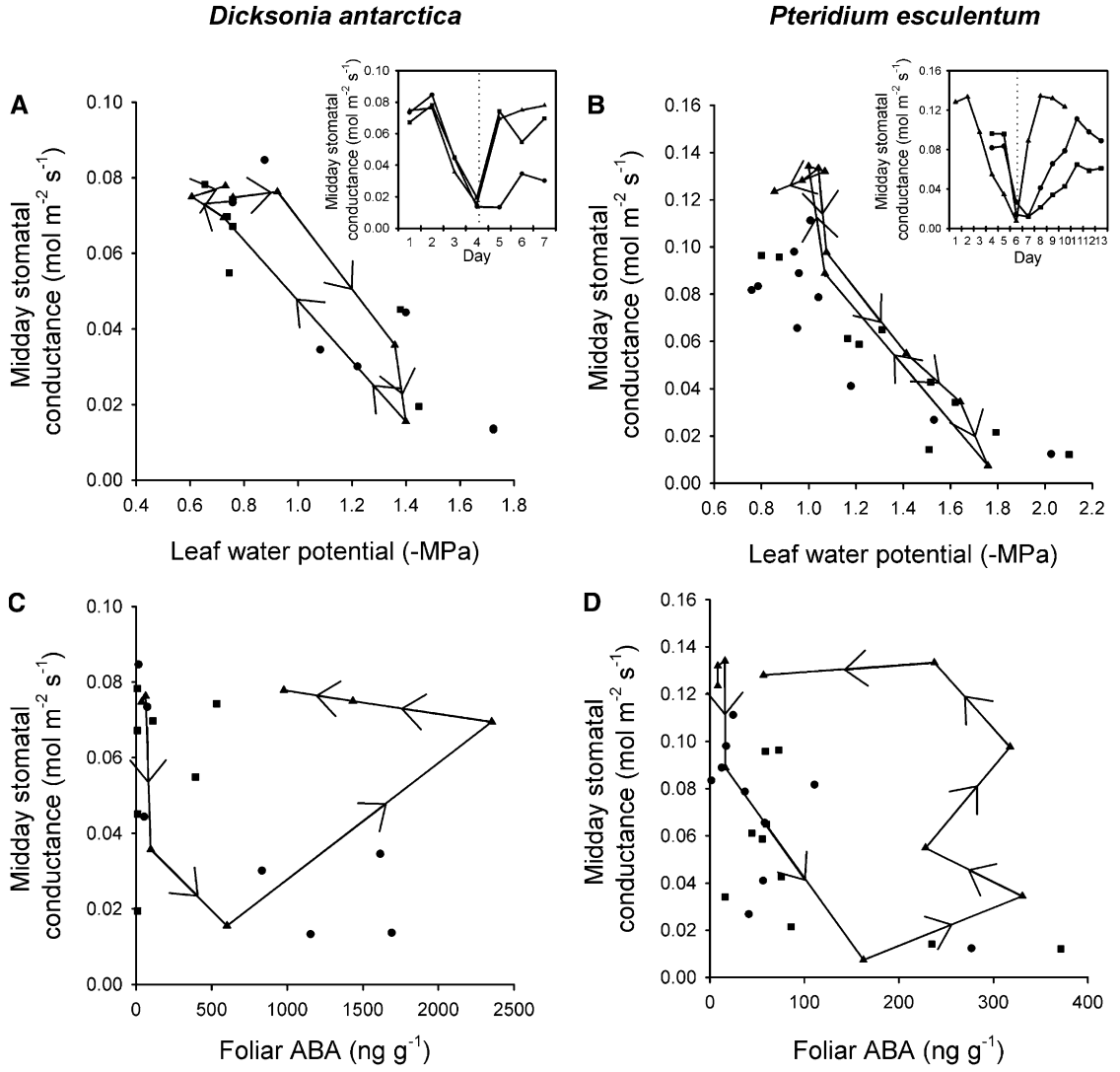

Figure 3. During Water Stress and Recovery, Stomatal Aperture in Ferns Is Controlled by Leaf Water Potential and Not the Concentration of ABA in the Leaf.

In two fern species, *D. antarctica* and *P. esculentum*, midday stomatal conductance was measured in conjunction with leaf water potential (**[A]** and **[B]**) and foliar ABA concentration (**[C]** and **[D]**) in three individuals (represented by different symbols) over a period of water stress and recovery by rewatering. Single lines with arrows connect leaf water potential and foliar ABA concentration for one individual of each species, with arrows indicating the progressive measurements during the drought and rehydration cycle. The inset depicts midday stomatal conductance over drought and recovery in the three individuals when water was withheld and the plants were stressed until stomata had closed; the dotted line represents the moment of rewatering and recovery from water stress.

surprising considering that all vascular land plants seem to synthesize ABA in response to water stress, including the ferns and lycophytes measured here (Figure 1). However, rehydrating water-stressed leaves with high levels of endogenous ABA provided a highly effective method for separating the influence of parallel changes in both leaf water content and ABA during drought stress on stomatal aperture. This technique revealed a dichotomy in stomatal control systems between ferns and lycophytes and seed plants (Figure 2), verifying the concept of passive stomatal control in ferns and lycophytes under natural conditions of water stress (Brodribb and McAdam, 2011).

Angiosperm stomata respond to water stress via a metabolic system of aperture control (Ache et al., 2010), and crucial to these responses is the presence of ABA in the leaf (Tardieu and Davies, 1992). In the seed plants sampled here, the effectiveness of endogenous ABA in reducing stomatal opening after the rehydration of droughted leaves supports extensive studies demonstrating the role of ABA in closing stomata during and after drought (Cornish and Zeevaart, 1986; Christmann et al., 2005; Lovisolo et al., 2008). In contrast with seed plants, we found that the stomata of ferns and lycophytes were able to rapidly and fully reopen when rehydrated, despite the presence

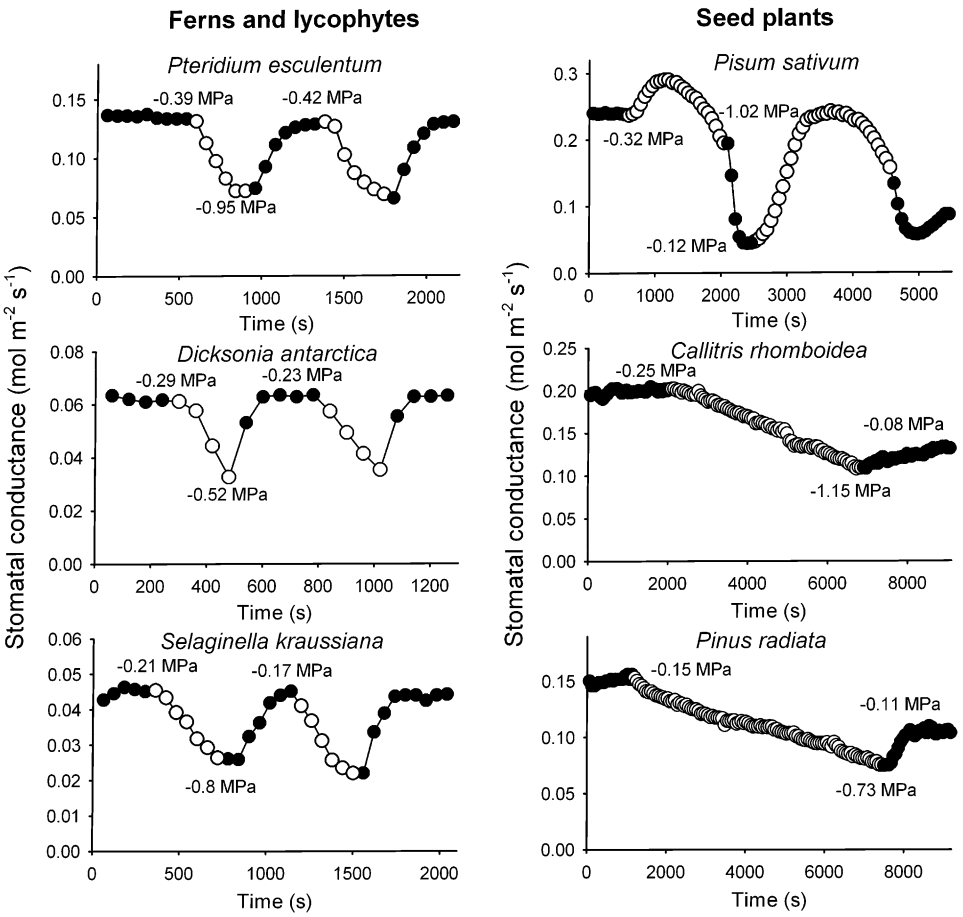

Figure 4. Stomatal Aperture Responds Directly to Leaf Water Potential in Excised Leaves of Ferns and Lycophytes but Not in Seed Plants.

Leaf gas exchange was initially measured on the leaf of a fully hydrated plant. Leaf water potential was measured, and then the leaf was excised and allowed to dehydrate (white circles); when stomata had closed by ~50%, the leaf was recut under water and allowed to rehydrate (black circles). This cycle of dehydration and rehydration was repeated with leaf water potentials recorded prior to every transition.

of high levels of endogenous ABA produced by water stress (Figures 1 and 2). Apparently, the only constraint for stomatal opening in the light in these early-branching clades of vascular plants is the hydration status of the leaf (Figures 2 and 5). The absence of an ABA effect on the stomata of ferns and lycophytes leads to predictable stomatal responses to leaf water potential during short-term (Figure 4) and long-term (Figure 3) water stress. The unpredictable responses of seed plant stomata to instantaneous changes in leaf hydration are indicative of interactions between metabolic, hydraulic, and mechanical effects on the guard cells as opposed to simple passive hydraulic control of stomatal aperture suggested for ferns and lycophytes (Figure 4) (Brodribb and McAdam, 2011). *P. sativum* stomata epitomize the unpredictable nature of angiosperm stomata. In this angiosperm, the mechanical advantage of epidermal cells over guard cells produces wrong-way responses (transient stomatal opening on dehydration and transient closure on rehydration), and these wrong-way transients are typically followed by right-way responses mediated by active changes in the osmotic balance between guard cells and epidermal cells (Figure 4) (Buckley, 2005). Like ferns and lycophytes, the gymnospermous seed plants (*C. rhomboidea* and *P. radiata*) showed no evidence of wrong-way stomatal behavior when their leaves were instantaneously dehydrated or rehydrated; however, the lack of correlation between leaf water potential and stomatal conductance in these species suggests a significant stomatal closing effect of ABA, even after short-term water stress (Figure 4).

These data strongly support the theory that fern and lycophyte stomata are insensitive to ABA and highly responsive to the water potential of the leaf. Brodribb and McAdam (2011) showed in nine species of ferns and lycophytes that stomata were insensitive to ABA when fed exogenously into the transpiration stream. Similarly, Ruszala et al. (2011) showed that the stomata on live leaves of the lycophyte *S. uncinata* were relatively insensitive to extremely high levels of exogenous ABA

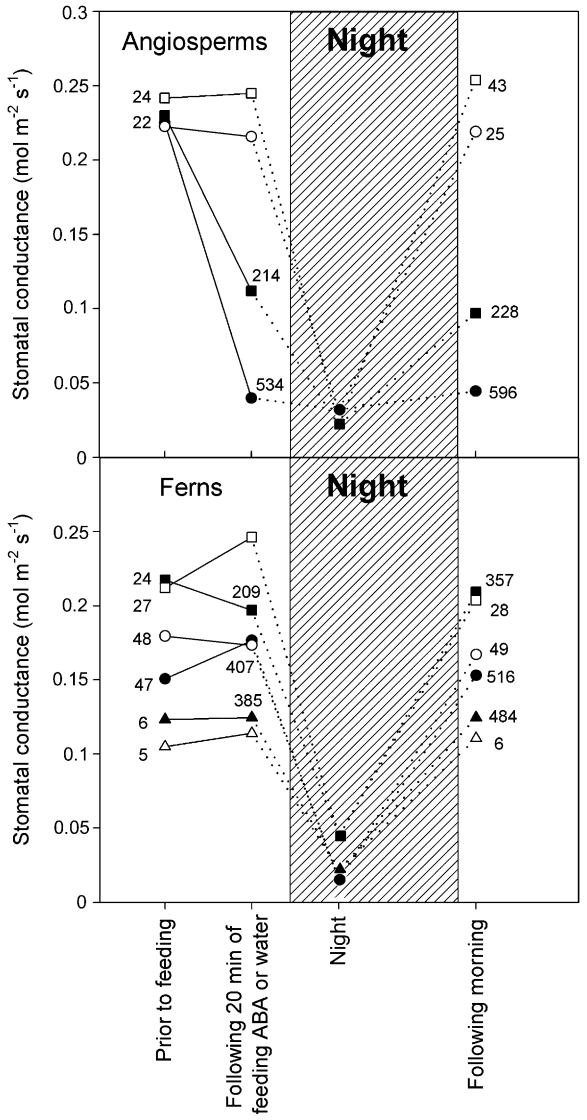

Figure 5. ABA Does Not Restrict Metabolic Stomatal Opening in the Morning When Fed into the Transpiration Stream of Ferns.

When ABA was fed into the transpiration stream (black symbols) of two angiosperm species, *O. corniculata* (squares) and *L. corniculatus* (circles), stomata closed within 20 min and, after 12 h of darkness, did not reopen to maximum apertures in the light the next day. Control leaves of angiosperms that were only fed water (white symbols) were able to open the next morning. When ABA was fed into the transpiration stream of three fern species, *A. sinuata* (squares), *P. esculentum* (circles), and *N. exaltata* (triangles), stomata did not close after 20 min and, the next morning, were able to open in the light, just like control leaves fed only water. Foliar ABA levels (ng g^{-1}) are shown at each time point of gas exchange measurement (except during the night) in the ABA-fed leaves and in both morning measurements of the control leaves.

(exceeding 260 000 ng mL^{-1}) in the xylem, resulting in a <15% reduction in stomatal conductance. Weak responses of stomatal aperture to very high levels of exogenous ABA have also been observed in the epiphytic fern *Platycerium bifurcatum*, for which foliar spray applications of ABA again resulted in a <15% reduction in gas exchange (Rut et al., 2008).

Gradualistic Evolution of Stomatal Control

The concept of gradualistic evolution of stomatal control components during the 400 million years since land plants evolved stomata (Brodribb and McAdam, 2011) is strongly supported by our observations of plants subjected to water stress. The contrasting stomatal behavior of water-stressed plants shown here provides compelling new evidence for ABA insensitivity in ferns and lycophytes, adding to previous studies using exogenous ABA (Rut et al., 2008; Brodribb and McAdam, 2011; Ruszala et al., 2011). Experiments in this study were specifically designed to observe stomatal responses to endogenous levels of ABA simulated by the types of water-stress conditions experienced in the field. By using living plants under water-stress conditions, we were able to observe stomatal responses under realistic endogenous ABA levels, avoiding the potentially confounding exposure of stomata to unrealistically high levels of exogenously applied ABA. In simulating the water-stress conditions under which guard cell closure is triggered, we found an absence of effective stomatal control by endogenous ABA in our sample of basal vascular plants, resulting in stomata that have contrasting environmental responses to seed plants (Figure 2). Therefore, we conclude that in the light, fern and lycophyte stomata open and close only in response to changes in leaf water potential (Figure 4). Our results do not support the recent conclusions of Ruszala et al. (2011) and Chater et al. (2011) that stomatal sensitivity to ABA evolved before vascular plants. The reason for the disparity between these studies may rest in the different measurement techniques used to quantify stomatal behavior. In this study, we measured stomatal diffusive conductance to water vapor on live plants to quantify stomatal aperture, whereas Ruszala et al. (2011) and Chater et al. (2011) based their conclusions on the common practice of calculating a mean stomatal aperture from measurements of large numbers of stomata on excised epidermal strips. We believe that directly measuring water loss from stomata provides a more sensitive and functionally meaningful index of stomatal opening than measurements of isolated stomata in vitro. Furthermore, many articles have indicated major limitations associated with direct stomatal observation on excised epidermes (Spence, 1987; Mott, 2009) as well as recently suggested modifications (Hubbard et al., 2012).

Our data support an evolutionary progression of stomatal control components in land plants, with modern seed plant stomata characterized by a number of metabolically controlled signals absent in the basal lineages of vascular plants, including phototropin-mediated blue light responses (Doi et al., 2006; Doi and Shimazaki, 2008), effective regulation of water use efficiency (McAdam and Brodribb, 2012), closure in response to high concentrations of carbon dioxide (Brodribb et al., 2009), and closure in response to exogenous (Brodribb and McAdam, 2011) and endogenous ABA (Figure 2). The gradualistic evolution of stomatal control components in land plants offers a great opportunity to further our understanding of guard cell membrane–specific processes as well as global models of transpiration over geological time.

Evolution of Stomatal Control by ABA and Relevant Genes

The absence of stomatal regulation by endogenous ABA synthesized in response to water stress in ferns and lycophytes

raises a significant question regarding the role of ABA in these basal lineages of vascular plants. Endogenous production of ABA by ferns and lycophytes during drought stress is likely a reflection of the important but lesser discussed role of ABA in enabling cell survival during stress events, as demonstrated across kingdoms and phyla (Shinozaki and Yamaguchi-Shinozaki, 2000; Zocchi et al., 2003; Karadeniz et al., 2006; Li et al., 2011). One possibility is that this ancient stress signal was coopted for enabling cellular survival in a terrestrial environment during the colonization of land by the earliest plants (Takezawa et al., 2011).

A key issue is the timing of the linkage between ABA and stomatal control. According to our data, the earliest occurrence of this stomatal control mechanism was ~50 million years after the evolution of stomata and the divergence of major plant groups, such as bryophytes, ferns, and lycophytes. Others posit an immediate combination of ABA into the functioning of the earliest stomata (Chater et al., 2011). Although this may seem adaptively reasonable, it assumes a conserved function of stomata as regulators of photosynthetic gas exchange. Such an assumption is far from certain, given that the earliest stomatal-bearing land plants are characterized by few stomata with an irregular distribution, being frequently observed associated with reproductive structures (Paton and Pearce, 1957; Edwards, 1996). These stomata are rarely associated with air-filled substomatal spaces and have a debatable functional capacity (Edwards et al., 1998; Lucas and Renzaglia, 2002); current opinion favors a role of these stomata in nutrient transport (Boyce, 2008; Ligrone et al., 2012), aiding evaporative thermoregulation (Raven, 2002), and driving the desiccation of sporophytic tissues (Duckett et al., 2009; Ligrone et al., 2012). Furthermore, these early stomatal-bearing land plants were ubiquitously confined to humid, wet environments (Edwards and Axe, 1992) and had very low rates of water loss, because of both internal anatomy and low stomatal densities (Konrad et al., 2000). Under these environmental conditions and morphological constraints, it seems highly unlikely that the earliest stomata evolved to regulate photosynthesis and plant hydration, and under such conditions, the evolution of an ABA-associated stomatal closure mechanism is improbable.

Despite there being no compelling argument for a selective pressure driving the evolution of ABA-regulated stomatal control in the earliest land-plant lineages, all of the relevant ABA signaling and synthesis genes as well as functioning proteins responsible for analogous seed plant stomatal control are present across the phylogeny of land plants, including the earliest stomatal-bearing lineages (Hanada et al., 2011). However, DNA sequence databases from lineages diverging prior to stomatal evolution, such as liverworts (Yamato et al., 2007), indicate the presence of all the major genes implicated by mutant analysis as essential for the regulation and sensitivity of seed plant stomata to ABA (including, *OPEN STOMATA1* [*OST1*], *ABA-INSENSITIVE1* [*ABI1*], and *GROWTH CONTROLLED BY ABA2* [*GCA2*]). In seed plant systems, with ABA governing rapid stomatal responses to environmental signals, it is not surprising that mutants for the genes involved in ABA sensitivity and synthesis invariably have phenotypes characterized by open stomata and wilty leaves. However, underlying this obvious stomatal phenotype and rapid response to ABA is a wide range of essential roles played by ABA in plant growth, survival, and reproduction, ranging from seed maturation and germination, phase change, stress tolerance (reviewed in Finkelstein et al., 2002), and in ferns and lycophytes, the sexual differentiation of gametophytes (Banks et al., 1993). Even the genes known to play unique roles in regulating the specific guard cell anion channels responsible for stomatal sensitivity to ABA in seed plants, such as the *SUCROSE NON-FERMENTING-RELATED PROTEIN KINASE2* (*SnRK2*) family, including *OST1*, play vital roles in the transduction of ABA signaling during seed dormancy and development (Nakashima et al., 2009), osmotic stress tolerance (Fujii et al., 2011), as well as other crucial non-ABA–related metabolic roles (Zheng et al., 2010). Indeed, on considering gene families associated with the metabolism and signaling of ABA across sequenced land-plant genomes, there is strong evidence for significant duplication events relating to morphological divergence (Hanada et al., 2011). Thus, it does not seem surprising that, despite the presence of homologous gene families definitively responsible for stomatal sensitivity to ABA in seed plants, fern and lycophyte stomata are insensitive to ABA (Figure 2). Although speculative, it seems likely that through one of the many duplication events occurring over the evolutionary history of the orthologous gene families related to ABA metabolism and signaling, a common ancestor of the modern seed plants coopted the ancient role of ABA as a stress signal into the rapid regulator of guard-cell–specific anion pumps.

METHODS

Responses of Vascular Plants to Drought and the Separation of the Effects of ABA and Water Potential on Stomatal Closure in Excised Leaves

The response to drought was observed in six diverse vascular plant species, including two ferns (*Dicksonia antarctica* [Dicksoniaceae] and *Pteridium esculentum* [Dennstaedtiaceae]), a lycophyte (*Selaginella kraussiana* [Selaginellaceae]), two conifers (*Callitris rhomboidea* [Cupressaceae] and *Pinus radiata* [Pinaceae]), and the angiosperm *Pisum sativum* (Fabaceae) wild-type line derived from the cv Torsdag (Hobart line 107). Six individuals of each species were grown in pots in the glasshouses of the School of Plant Science, University of Tasmania, Hobart, Australia under natural light, supplemented and extended to a 16-h photoperiod by sodium vapor lamps, ensuring a minimum 300 μmol quanta m^{-2} s^{-1} at the leaf surface throughout the day period. The temperature was maintained at 25°C during the day and 15°C at night. In all species, stomatal conductivity measurements were made at midday (1200 to 1300 h) using an infrared gas analyzer (Li-6400; Li-Cor) on healthy photosynthetic tissue (leaf cuvette conditions: 22°C, vapor pressure deficit between 1.1 and 1.2 kPa, 390 μmol mol^{-1} CO_2 concentration, and light intensity of 1000 μmol quanta m^{-2} s^{-1}) until stomata had closed to <20% initial conductances (between 3 and 7 d depending on the species measured). All gas exchange data were adjusted for leaf area in the cuvette on each measurement. Concurrently with every measurement of gas exchange, tissue was taken for ABA quantification (see below) and leaf water–potential measurement using a Scholander pressure chamber and microscope to precisely measure the xylem balance pressure. Measurements were made prior to drought, when all individuals were well watered; drought was then initiated by withholding water.

To examine the effects of ABA and water potential on stomatal closure across the land-plant phylogeny, three individuals from the six species listed above were selected. When the stomata of these individuals had closed to <20% initial conductances, plants were brought into the laboratory, and the leaf, branch, or pinnule from which the initial rates of gas

exchange prior to drought were measured were enclosed in the cuvette of the gas analyzer, and most of the leaves or pinnules on the same stem or rachis were excised; tissue again was taken for ABA quantification and water potential measurements, representing water potentials and ABA levels during peak water stress. Gas exchange and leaf environmental data were automatically logged every 1 min. After an acclimation period of at least 10 min in the cuvette, the stem or rachis was excised under water containing ABA at a concentration of 1000 ng mL^{-1} (in the angiosperm *P. sativum* and conifer *C. rhomboidea*, the experiment was repeated using water with no added ABA). The cut end of the stem was regularly recut to maximize water flow and avoid xylem blocking. Ten to 20 min after recutting, another branch or pinnule outside of the cuvette of the gas analyzer was excised and immediately wrapped in damp paper towel for immediate measurement of water potential and quantification of ABA, representing hydrated tissue with high levels of ABA.

Fern Responses to Soil Drying and Rewetting

To examine the response of ABA level and transpiration to water stress and subsequent recovery by soil rewetting in ferns, two phylogenetically disparate species with contrasting growth habits were selected (the understory tree fern from temperate rainforests, *D. antarctica*, and the rhizomatous, cosmopolitan fern from relatively dry habitats in full sun, *P. esculentum*). Each species was represented by three potted individuals acclimated to growth cabinet conditions under a 14-h photoperiod, with light supplied by mixed fluorescent and incandescent globes providing 600 μmol quanta m^{-2} s^{-1} at leaf height, 25°C/16°C day/night temperatures, and 50% relative humidity (controlled by a dehumidifier with integrated humidity sensors) (SeccoUltra 00563; Olimpia Splendid). Temperature and humidity were logged for the duration of the experiment using a HOBO Pro Series data logger (Onset). Whole-plant midday (1200 to 1300 h) gas exchange was monitored daily by weighing triple-bagged pots on a computer-interfaced balance to an accuracy of ±0.01 g (Mettler-Toledo PG5002-S). Transpiration was calculated as the loss of weight between measurements divided by the total leaf area. Leaf area was measured by scanning leaves before and after the experiment. In both species, drought resulted in varying degrees of leaf senescence (up to 20%) apparent the day after rewatering and recovery; whole-plant transpiration (expressed per square meter of leaf area) was corrected for this change in leaf area. Because vapor pressure deficit remained constant throughout the experimental period, whole-plant transpiration was proportional to stomatal conductance to water vapor.

Water stress was initiated by withholding water and was maintained until plants had reached at least 20% stomatal closure, after which pots were rewatered and maintained in a hydrated state until either transpiration had recovered to predrought levels or, in the case of individuals experiencing hydraulic dysfunction as a result of the drought, until transpiration ceased to increase for a consecutive 2 d. Immediately after midday transpiration measurements, a pinnule was removed from the same height each day on the plants, immediately wrapped in damp paper towel, and bagged for water potential measurements. After determination of leaf water potential, the pinnule was weighed and used for ABA quantification (see below).

Instantaneous Regulation of Stomatal Aperture by Water Potential

In the three fern and lycophyte species, *P. esculentum*, *D. antarctica*, and *S. kraussiana*, as well as the angiosperm *P. sativum* and conifers *C. rhomboidea* and *P. radiata*, the role of leaf water potential in regulating stomatal aperture was investigated in nondroughted leaves with low levels of ABA. Branches or pinnules attached to the plant were enclosed in the cuvette of a gas analyzer under the conditions described above, and data were automatically logged every 1 min. After an initial period during which leaf gas exchange equilibrated to conditions in the cuvette, tissue on the same branch or leaf was excised, immediately wrapped in damp paper towel, and bagged for water potential measurement. The branch or leaf bearing the tissue in the cuvette was excised, with ~30 cm of stem or rachis below the tissue in the cuvette, and allowed to dehydrate. In all species, gas exchange was monitored until stomata had closed by 50% of their initial conductance, after which tissue was collected from the same branch, immediately wrapped in damp paper towel, and bagged for water potential measurements (as above). The excised stem or rachis was recut under water and allowed to rehydrate. When gas exchange had reached stability after rehydration, tissue was again taken for water potential measurement. The cycle of dehydration to 50% stomatal closure and then rehydration was repeated. Gas exchange measurements were adjusted for leaf area.

ABA and Stomatal Opening in the Light

To test whether elevated levels of ABA in the leaf restricted stomatal opening in the morning in ferns, three species were selected spanning a spectrum of leaf gas exchange rate: (1) *Nephrolepis exaltata* (Lomariopsidaceae), with relatively low rates of gas exchange; (2) *P. esculentum*, with moderate to high rates of gas exchange; and (3) *Astrolepis sinuata* (Pteridaceae), with a high rate of gas exchange. Two angiosperms with similarly high rates of gas exchange were selected as controls: (1) *Lotus corniculatus* (Fabaceae) and (2) *Oxalis corniculata* (Oxalidaceae). Leaves of the ferns and stems of the angiosperms were excised under water, and, using marked fern pinnules or individual angiosperm leaves, steady state stomatal conductivity to water vapor was recorded using an infrared gas analyzer (conditions in cuvette as described above). Concurrently with every measurement of leaf gas exchange, the level of foliar ABA was quantified (see below) from an adjacent pinnule or leaf. Once initial rates of gas exchange were recorded, an aliquot of concentrated ABA was added to the water, resulting in a concentration of 1000 ng mL^{-1} ABA entering the transpiration stream. The leaf or stem was then allowed to transpire under light for 20 min, with the end of the stem or rachis under water regularly being recut, allowing for continual hydration and flow of ABA into the leaf. After 20 min on the same pinnule or leaf, stomatal conductivity was recorded, and an adjacent pinnule or leaf was taken for ABA quantification. The leaf or stem, while still taking up ABA from the water, was covered with a black bag and left in the dark overnight. Before dawn (~0600 h) the next morning, the same pinnule or leaf was again enclosed in the cuvette of the infrared gas analyzer under the above-described conditions, and steady state stomatal conductance was recorded before and after stomatal opening. At the same time, an adjacent leaf was taken for ABA quantification. Leaf gas exchange measurements were adjusted for pinnule or leaf area in the cuvette.

ABA Extraction, Purification, and Quantification

In all species, ~0.5 g of leaf tissue was used to quantify the foliar ABA level. ABA extraction and purification was performed according to the protocol of McAdam et al. (2011). Once the eluate was completely dried, ABA was taken up in 250 μL of 5% (v/v) methanol in 1% (v/v) acetic acid and centrifuged at 13,000 rpm for 3 min. After centrifugation, 100 μL of supernatant was taken for combined ultraperformance liquid chromatography (UPLC) and multiple reaction–monitoring tandem mass spectrometry analysis using a Waters Acquity H-series UPLC coupled to a Waters Xevo triple quadrupole mass spectrometer. A Waters Acquity UPLC BEH C18 column (2.1 mm × 100 mm × 1.7 μm particles) was used. The solvents were 1% (v/v) acetic acid in water (Solvent A) and acetonitrile (Solvent B) at a flow rate of 0.35 mL min^{-1}, with a gradient of 80% A: 20% B to 5% A: 95% B at 5 min, followed by reequilibration to starting conditions for 3 min. The column temperature was 45°C. A total of 10 μL of sample was injected. The mass spectrometer was operated in negative ion electrospray mode with a needle voltage of 2.7 kV; selected reaction

monitoring was used to detect ABA and [$^2H_6$]ABA. The ion source temperature was 130°C. The desolvation gas was nitrogen at 950 L h^{-1}. Cone gas flow was nitrogen at 50 L h^{-1}. The desolvation temperature was 450°C. The tandem mass spectrometry transitions monitored for ABA were *m/z* 263.2 to 153.1, 204.2, and 219.2; for [$^2H_6$]ABA, the transitions were *m/z* 269.2 to 159.1, 207.2 (three deuteriums were lost in this fragmentation), and 225.2. Cone voltage was 32 V in all cases. The collision energy was 18 V for the *m/z* 263.2 to 153.1 and 204.2 and corresponding deuterium-labeled channels and 16 V for the *m/z* 263.2 to 219.2 and corresponding deuterium-labeled channel. Dwell time was 50 ms per channel. Data were analyzed using Waters MassLynx and TargetLynx software. Quantitation was performed using *m/z* 263.2 to 153.1 and the corresponding deuterium-labeled channel. For all samples, the ratio of endogenous ion intensity to internal standard ion intensity was calculated. The product of this ratio and the amount of internal standard added was divided by the fresh weight of the tissue sample and adjusted for aliquot volume to determine the level of ABA per gram fresh weight in the leaf.

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AUTHOR CONTRIBUTIONS

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