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RESEARCH PAPER

Augmentation of abscisic acid (ABA) levels by drought does not induce short-term stomatal sensitivity to CO_2 in two divergent conifer species

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Abstract

The stomata of conifers display very little short-term response to changes in atmospheric CO_2 concentration (C_a), whereas the stomatal responses of angiosperms to C_a increase in response to water stress. This behaviour of angiosperm stomata appears to be dependent on foliar levels of abscisic acid (ABA_f). Here two alternative explanations for the stomatal insensitivity of conifers to C_a are tested: that conifers have either low ABA_f or a higher or absent threshold for ABA-induced sensitivity. The responsiveness of stomatal conductance (g_s) to a sequence of transitions in C_a (386, 100, and 600 μmol mol^{-1}) was recorded over a range of ABA_f in an angiosperm and two divergent conifer species. The different ABA levels were induced by a mild drought cycle. Although the angiosperm and conifer species showed similar proportional increases in ABA_f following drought, conifer stomata remained insensitive to changes in C_a whereas angiosperm stomata showed enhanced sensitivity with increasing ABA_f. The conifers, however, had much higher ABA_f prior to drought than the angiosperm species, suggesting that non-sensitivity to C_a in these conifers was due to an absent or inactive response/signalling pathway rather than insufficient ABA_f.

Key words: Abscisic acid, angiosperm, carbon dioxide, conifer, drought, stomata, stomatal conductance.

Introduction

Environmentally responsive stomata are a prerequisite for the function of leaves on land. The turgor pressure of guard cells, responding to environmental and physiological signals (Schroeder *et al.*, 2001), modulates stomatal aperture thereby regulating leaf water loss and carbon dioxide assimilation. Stomatal conductivity to the diffusion of gases (g_s) responds to atmospheric carbon dioxide concentration (C_a) across a wide diversity of angiosperm species (Linsbauer, 1917; Morison, 1985). This instantaneous response involves stomatal opening at low C_a and stomatal closure at high C_a, thus altering g_s. The signal for the stomatal response to C_a remains enigmatic, though it appears to originate in the mesophyll (Mott *et al.*, 2008).

The stomatal response to C_a, particularly stomatal closure in response to increasing C_a, is an important process in angiosperms because it provides a basis for the optimization of water use during photosynthesis (Farquhar and Sharkey, 1982). As a central component of water use optimization, the stomatal response to C_a has significant agricultural as well as ecological implications. The response of g_s to changes in C_a is therefore of immense significance to plants (Miller-Rushing *et al.*, 2009) and for atmospheric water balance (Betts *et al.*, 2007) as C_a continues to rise.

Although instantaneous stomatal responses to C_a have been recorded from a number of C_3 and C_4 angiosperm herbs and trees (Morison, 1987), the stomatal sensitivity of individual plants to changes in C_a can vary considerably depending on growth conditions and endogenous chemical signals (Raschke and Hedrich, 1985; Talbott *et al.*, 1996). Variation in stomatal response to C_a in intact leaves has

Abbreviations: A, CO_2 assimilation rate; ABA, abscisic acid; ABA_f, foliar abscisic acid level; C_a, atmospheric carbon dioxide concentration; E, transpiration; g_s, stomatal conductivity to the diffusion of gases; PPFD, photosynthetic photon flux density; Ψ_{leaf}, leaf water potential; VPD, vapour pressure deficit.

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been attributed to numerous physiological factors including abscisic acid (ABA) level (Raschke, 1975; Dubbe *et al.*, 1978; Eamus and Narayan, 1989), humidity or vapour pressure deficit (VPD) (Bunce, 1998; Talbott *et al.*, 2003), photosynthetic light response (Wong *et al.*, 1978; Messinger *et al.*, 2006), and levels of the ABA biosynthetic precursor zeaxanthin (Zhu *et al.*, 1998; Seo and Koshiba, 2002).

ABA was first demonstrated as an important regulator of stomatal responses to C_a in the asteraceous herb *Xanthium strumarium* L. (Raschke, 1975). Raschke (1975) found that the stomata of leaves from well-watered plants suspended in pure water did not respond to perturbations in C_a; however, following the addition of exogenous ABA to the transpiration stream, stomata were significantly sensitized to changes in C_a. ABA-induced sensitivity of stomata to C_a and the resulting effects on transpiration, assimilation, and water-use efficiency were quantified in five angiosperm species by Dubbe *et al.* (1978). The requirement of ABA for a stomatal response to C_a has subsequently been confirmed in many other angiosperm species including trees (Ridolfi *et al.*, 1996) and herbs (Eamus and Narayan, 1989; Bunce, 1998; Leymarie *et al.*, 1998). Current consensus recognizes ABA as a key modulator of stomatal sensitivity to C_a, with recent biochemical studies demonstrating that changes in C_a alter internal guard cell Ca^{2+} concentration (Young *et al.*, 2006), with ABA enhancing the sensitivity of guard cell anion channels and pumps to cytosolic Ca^{2+} concentration (Siegel *et al.*, 2009).

Recently, Brodribb *et al.* (2009) identified a broad phylogenetic pattern in the sensitivity of stomata to C_a, in which non-angiosperms (conifers, ferns, and a lycopod) showed very weak or absent responses to changing C_a. A similar lack of stomatal response to C_a in conifers was reported in a few *Picea* and *Pinus* species (Beadle *et al.*, 1979; Morison and Jarvis, 1983) as well as in long-term elevated CO_2 studies (Medlyn *et al.*, 2001). The physiological basis of this phylogenetic variation in stomatal sensitivity to C_a is unknown. Here two alternative explanations for stomatal insensitivity to C_a in conifers are investigated. The first is that conifer stomata are insensitive to C_a at all normal levels of endogenous foliar ABA (ABA_f) due to an absent or very high threshold for ABA-induced stomatal sensitivity to C_a. The second alternative is that conifers have low levels of ABA_f and therefore may not differ from angiosperms in stomatal responses to C_a when the ABA level is increased. Using mild drought treatments as an effective way of causing natural variations in ABA_f and thereby natural increases in stomatal sensitivity to C_a, the relationship between stomatal sensitivity to C_a and ABA_f is examined in two conifers and an angiosperm species.

Materials and methods

Plant material

Three species were selected for comparison, the ruderal angiosperm herb *Senecio minimus* Poir. (Asteraceae) acting as an angiosperm control, and two phylogenetically and functionally divergent conifer trees, the relatively slow-growing *Callitris rhomboidea* R.Br (Cupressaceae) and the fast-growing species *Pinus radiata* D.Don (Pinaceae). These three species had similar stomatal anatomy with only minimal encryption in the conifer species, a trait believed to have little effect on gas exchange (Roth-Nebelsick *et al.*, 2009).

Senecio minimus seedlings at the second full leaf stage were collected from within their natural range and grown individually in 1.3 l pots containing an 8:2:1 mix of composted pine bark, coarse river sand, and peat moss with added slow release fertilizer. *Callitris rhomboidea* individuals were grown from seed collected from within their natural range and potted in 2.0 l pots. They were ~2 years of age at the time of the experiment. *Pinus radiata* individuals (grown from commercially available seed of composite provenance) were 1 year of age at the time of the experiment and were also potted in 2.0 l pots.

Growth conditions and drought treatment

All plants were grown under controlled glasshouse conditions for at least 8 weeks prior to measurements. Growth conditions were 16 h days at 20 °C/15 °C day/night temperatures, receiving a maximum quantum photosynthetic photon flux density (PPFD) of 1300 μmol quanta m^{-2} s^{-1}. Natural light was supplemented by sodium vapour lamps to ensure a minimum 300–500 μmol quanta m^{-2} s^{-1} at the leaf surface throughout the day period. Relative humidity was maintained at 50% by a dehumidifier coupled to a humidity probe. Maintaining a constant air temperature and relative humidity restricted VPD to a narrow range, meaning that measured transpiration (E) was closely related to stomatal conductance. Plants were watered daily to full pot capacity when not under drought conditions. Measurements of ABA_f and gas exchange were carried out over a cycle where plants were initially well watered, then mildly droughted by withholding water and monitoring gas exchange (see below) until E fell to <25% of maximum. The plants were then rewatered and maintained at soil capacity. Leaf water potential (Ψ_{leaf}) was measured over drought cycles using a Scholander pressure chamber on single leaves (*S. minimus*), small shoots (*C. rhomboidea*), or fascicles (*P. radiata*), excised, and immediately wrapped in damp paper towel then aluminium foil, and finally bagged.

ABA extraction, purification, and GC-MS-MS quantification

ABA_f was quantified from between 1 g and 2 g of tissue from a single fully expanded leaf (*S. minimus*), small, scale-leaved, terminal branch (*C. rhomboidea*), or 8–10 needles (*P. radiata*). Extraction, purification, and gas chromatography–tandem mass spectrometry (GC-MS-MS) quantification of ABA_f were performed according to the methods of Jager *et al.* (2008) with the following modifications. After pre-conditioning, the Sep-Pak® C18 cartridge (Waters, Milford, USA) was washed with 15 ml of 20% (v/v) methanol in 0.4% (v/v) acetic acid and ABA was eluted with 15 ml of 45% (v/v) methanol in 0.4% (v/v) acetic acid. The eluate was taken up in 400 μl of methanol and methylated with 750 μl of a 1:10 dilution of (trimethylsilyl)diazomethane in diethyl ether for 30 min, following which ABA was taken up in 2×100 μl washes of diethyl ether, each time reduced to dryness under a nitrogen stream. The sample was then resuspended in 50 μl of chloroform prior to GC-MS-MS analysis. The ions monitored in the GC-MS-MS system were MS1 m z^{-1} 190, MS3 m z^{-1} 162 (endogenous ABA), and MS1 m z^{-1} 194, MS3 m z^{-1} 166 (internal standard [$^2H_4$]ABA). 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 ABA_f. All values are expressed in terms of leaf fresh weight.

Diurnal foliar ABA concentration post-drought

Three individuals of *S. minimus* and *C. rhomboidea* were used in a preliminary exploration of diurnal ABA_f following mild drought

recovery to determine the time of maximum and minimum ABA_f in both species. Diurnal variation in ABA_f was measured in *S. minimus* and *C. rhomboidea* on the day immediately following rewatering.

All pots were triple bagged to eliminate evaporative water loss from the soil and daily watering was withheld. Plants were weighed on a precision balance (±0.01 g, Mettler-Toledo XS6002S, Switzerland) between 12:00 h and 13:00 h, and transpiration ($g\ s^{-1}$) was recorded on each successive day following the initiation of drought. Drought continued until plants reached the above-described drought conditions. All three individuals of each species arrived at the prescribed level of drought on the same day and were rewatered at 05:00 h the following morning. On the day of rewatering, tissue was removed for ABA quantification from a single cohort of leaves at a similar stem height across all individuals to reduce the effect of age-related gradients in ABA_f (Soar *et al.*, 2004; Valdés *et al.*, 2004). Tissue was collected from all individuals at hourly intervals from 08:00 h to 13:00 h then at 90 min intervals until 17:30 h.

Leaf gas exchange measurements and the drought cycle

Three individuals from each species were used to determine the stomatal sensitivity to C_a under varying ABA_f over the course of a mild drought cycle.

A portable infrared gas analyser (Li-6400, Li-Cor Biosciences, Lincoln, NE, USA) was used to measure g_s ($mol\ m^{-2}\ s^{-1}$) over a sequence of transitions in C_a. Other variables within the leaf chamber of the Li-6400 were standardized during measurements (leaf temperature was maintained at 20 °C, PPFD at 1000 μmol quanta $m^{-2}\ s^{-1}$, and VPD automatically set at 1.3 kPa). Automatic setting of VPD resulted in small variations in air flow (±50 ml min^{-1}); however, during all measurements, major differences between inlet air VPD and the automatically set VPD were eliminated by manual adjustment of inlet air diverted through a desiccant column, thereby minimizing fluctuations in air flow. Leaf chamber C_a was controlled for the duration of all measurements by a gas injection system (Li-6400-01, Li-Cor Biosciences) regulating the concentration of CO_2 in the air supply line. At the start of measurements a single leaf (*S. minimus*) or collection of small terminal branches (*C. rhomboidea*) or needles (*P. radiata*) were arranged in the leaf chamber so that there were no leaves or stems overlapping. Leaves were allowed to equilibrate for 20 min in the chamber at current ambient C_a (386 μmol mol^{-1}), after which C_a was lowered to 100 μmol mol^{-1} for 20 min then increased to 600 μmol mol^{-1} for 20 min. Twenty minutes was sufficient time to establish a new stomatal steady state with <1% change in g_s per minute according to the dynamic responses in all three species (Brodribb *et al.*, 2009). During gas exchange measurements, g_s, assimilation (A), and leaf environmental traits were logged every 2 min. Following gas exchange measurement, all logged data were standardized against leaf area in the chamber.

On the day prior to the commencement of the drought cycle, gas exchange measurements were made twice on different leaves of the same individual at 09:30 h and 15:00 h for *S. minimus* or at both 12:30 h and 15:30 h for *C. rhomboidea* and *P. radiata*. The times at which gas exchange measurements were made were determined from the periods of maximum and minimum ABA_f from the post-drought diurnal ABA_f flux experiment to ensure maximum variation in ABA_f for each species. Tissue was harvested for ABA_f quantification 30 min into each gas exchange measurement cycle from a leaf, fascicle or branchlet adjacent to that undergoing gas exchange measurement.

Initial gas exchange measurements and drought commencement were undertaken on a separate day for each individual over no more than 3 d for each species. At the initiation of the drought treatment, plants were triple bagged and water was withheld. Plant water loss was determined gravimetrically as described above and, once the prescribed level of drought had been reached, individuals were rewatered at 05:00 h the following day. Assessment of stomatal sensitivity to C_a was only possible after rewatering and drought recovery when Ψ_{leaf} and g_s had increased to sufficient levels to allow gas exchange measurements to take place while ABA_f remained relatively high. When plants were experiencing drought, the strong interaction between g_s and Ψ_{leaf} made determining stomatal sensitivity to C_a impossible. Following rewatering, twice-daily assessment of C_a sensitivity and ABA_f was undertaken in all individuals on the first day, then a single individual per species was assessed over 4 d following drought recovery, to track stomatal sensitivity to C_a over a natural decline in ABA_f. Stomatal sensitivity was calculated as the slope of the linear regression between absolute values of g_s and C_a over the range 100–600 μmol mol^{-1} CO_2.

Statistical analysis

ABA_f and A data presented for both pre-drought and post-drought conditions are means and standard errors of means of three replicates per species. Means were compared using paired sample t-tests. One-way analyses of variance were used to assay differences between stomatal sensitivities at different sampling times over the drought cycle. A two-parameter single exponential rise to maximum curve was fitted to the sensitivity data of *S. minimus* over the ABA_f using SigmaPlot for Windows Version 8.02 (2002).

Results

Diurnal foliar ABA concentration post-drought

Distinctive patterns were observed in both the conifer and angiosperm species. In the angiosperm *S. minimus*, peak post-rewatering ABA_f (mean maximum 450 ng g^{-1}) occurred between 10:00 h and 11:00 h on the day following rewatering, dropping to lower values of ~120 ng g^{-1} after 14:30 h (Fig. 1A). In contrast, post-rewatering ABA_f in the conifer *C. rhomboidea* peaked at ~1700 ng g^{-1} by 13:00 h the day after rewatering, with relatively lower values, ~900 ng g^{-1}, measured at the start and end of the sampling period (Fig. 1B). The pattern of ABA_f post-drought in the conifer *P. radiata* was assumed to be the same as the diurnal pattern observed in *C. rhomboidea*, as the trend in *C. rhomboidea* was very similar to that of ABA flux to the leaves observed in stressed *Pinus sylvestris* L. individuals by Jackson *et al.* (1995) using radioimmunoassay ABA quantification methods.

Trends in E and Ψ_{leaf} over the drought cycle

Prior to drought stress all species had relatively high midday E (>0.005 g s^{-1}) and this level was maintained for 3 d (*S. minimus* and *P. radiata*) to 5 d (*C. rhomboidea*) after water was withheld (Fig. 2). Drought caused stomatal closure in all species, reducing E to <25% of initial E over 3–5 d depending on the species (Fig. 2). Minimum Ψ_{leaf} at 25% initial E was different for each species, ranging from between –0.7 MPa and –0.9 MPa in *S. minimus*, between –1.9 MPa and –2.1 MPa in *C. rhomboidea*, and between –1.7 MPa and –1.8 MPa in *P. radiata* (Fig. 2).

Following rewatering, a full recovery of Ψ_{leaf} to pre-drought levels took between 1 d and 2 d in all species, after which Ψ_{leaf} remained similar to or higher than pre-drought Ψ_{leaf} (Fig. 2). Following recovery from drought, E in *S. minimus* individuals increased to levels similar to pre-drought conditions by the fourth day (Fig. 2A). Transpiration

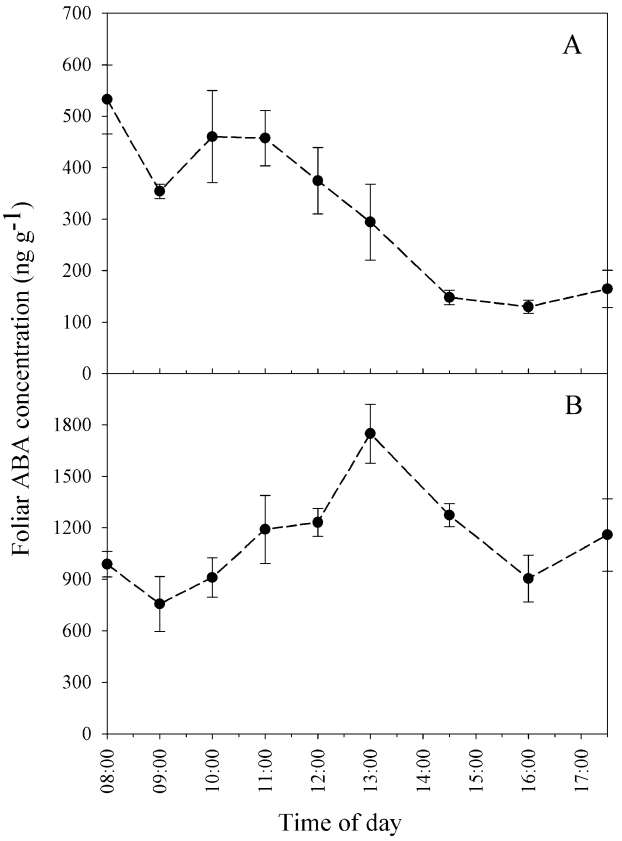

Fig. 1. Time course of foliar ABA concentration for *S. minimus* (A) and *C. rhomboidea* (B) on the day immediately following recovery from mild drought. Data points are means ±SE (n=3). Plants were rewatered at 05:00 h.

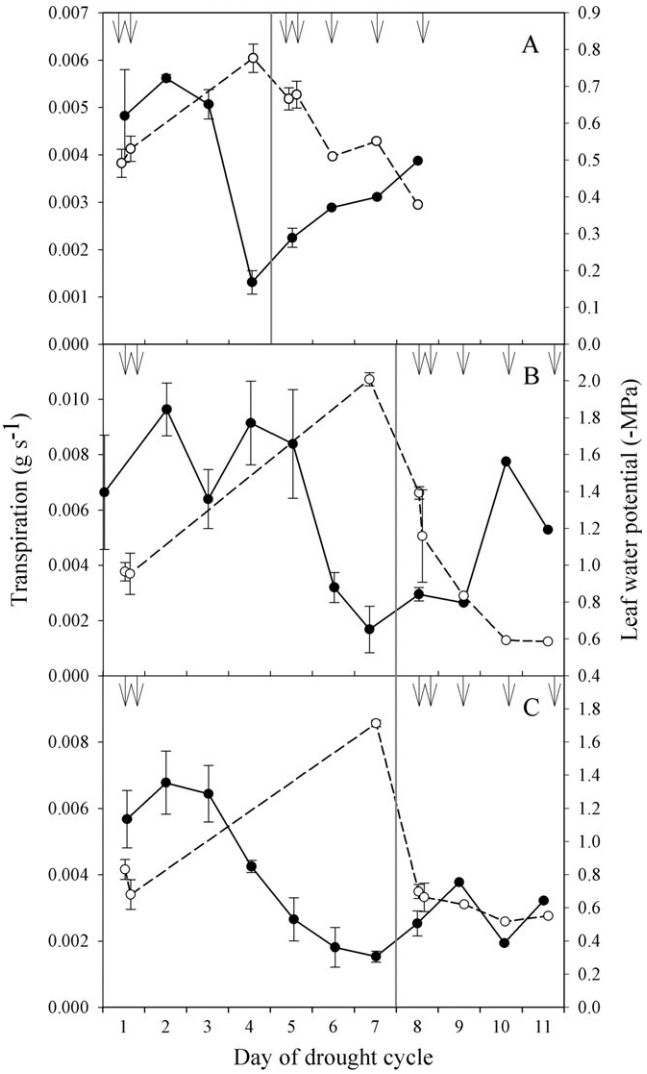

Fig. 2. Transpiration (solid line) and leaf water potential (Ψ_{leaf}) (dashed line) over the course of a mild drought cycle in *S. minimus* (A), *C. rhomboidea* (B), and *P. radiata* (C). Data points represent means ±SE (n=3) for all days except the final three when only one individual was represented. Water was withheld from day 1; rewatering occurred at the vertical line. Arrows mark times at which both ABA level and stomatal conductance were measured.

in *C. rhomboidea* remained low for 2 d following drought recovery, after which E returned to pre-drought levels (Fig. 2B). However, in *P. radiata* E only recovered slightly, remaining low, between 0.002 g s^{-1} and 0.003 g s^{-1}, over the 4 d post-drought recovery (Fig. 2C).

Stomatal sensitivity to C_a and ABA_f

The pattern of stomatal sensitivity to C_a (measured over the range of 100–600 μmol mol^{-1} CO_2), as ABA_f levels varied over the drought cycle, was noticeably different in the angiosperm *S. minimus* from that in the two conifer species (Fig. 3).

Following rewatering, the highest recorded ABA levels in all species occurred on the first day, after which levels gradually declined over the following 3 d (Fig. 3D–F). ABA_f increased on average at least 4-fold in all species; however, the pre-drought baseline ABA_f varied between species, with *S. minimus* displaying a relatively low pre-drought ABA_f (75 ng g^{-1}) compared with the two conifers *C. rhomboidea* (320 ng g^{-1}) and *P. radiata* (190 ng g^{-1}) (Fig. 3D–F).

In *S. minimus*, stomatal sensitivity to C_a was weak when ABA_f was comparatively low prior to drought stress (Fig. 3A). Stomatal sensitivity was highest in leaves immediately recovered from mild drought, when ABA_f was ~4 times higher than in pre-drought conditions (Fig. 3A). On the subsequent days following recovery from drought stress, stomatal sensitivity to C_a declined in parallel with ABA_f, although with some variability (Fig. 3A). The relationship between stomatal sensitivity of *S. minimus* to C_a and ABA_f was curvilinear, apparently saturating at ABA_f >500 ng g^{-1} (Fig. 3A). In contrast, the two conifer species *C. rhomboidea* and *P. radiata* showed little sensitivity to C_a despite a substantial enhancement of ABA_f in leaves of droughted plants (Fig. 3B, C). In both conifer species the stomatal sensitivity to C_a was not significantly affected by increasing ABA_f and was no different from the stomatal sensitivity prior to drought (P >0.05) (Fig. 3A–C). The two conifer species displayed a similar lack of stomatal response to C_a prior to and following drought (Fig. 4). Only

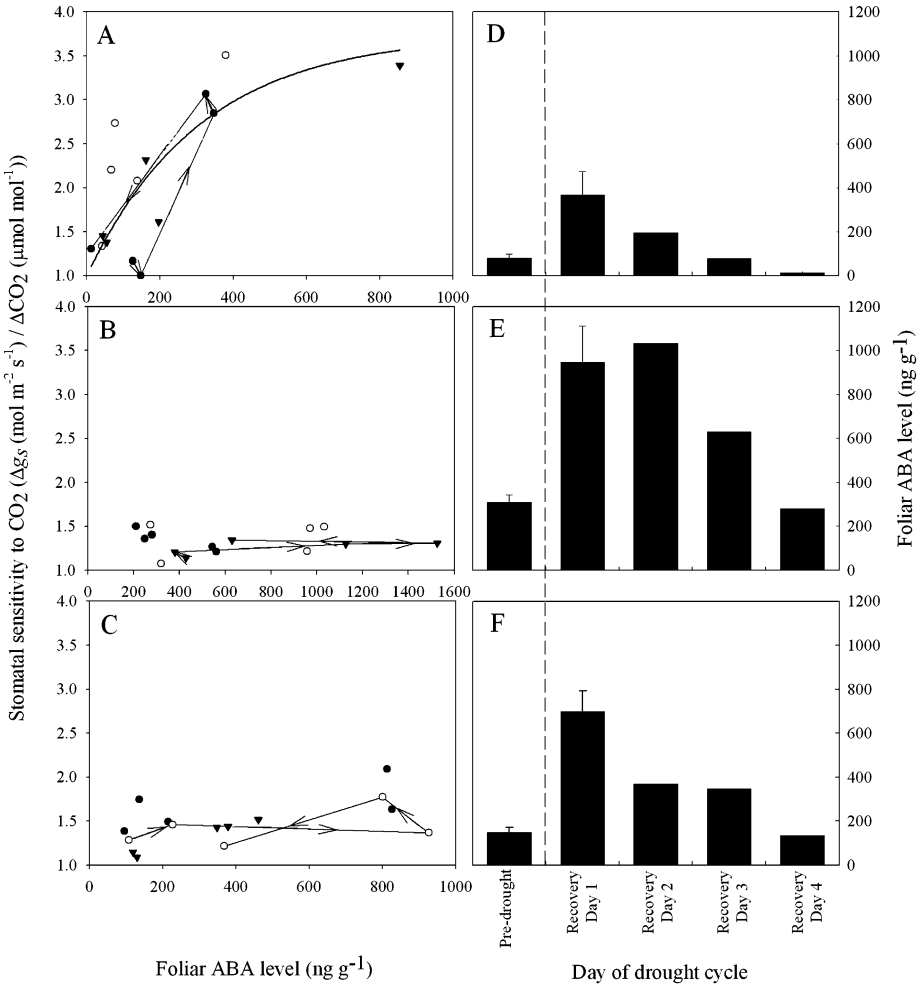

Fig. 3. Stomatal sensitivity to CO_2 relative to foliar ABA level in *S. minimus* (A), *C. rhomboidea* (B), and *P. radiata* (C). Three individuals are represented by different symbols, with a representative individual from each species linked with arrows indicating the transition from two initial well-watered states, two states on the initial day following rewatering, and a subsequent day post-drought recovery. A significant regression (R^2=0.57; P <0.01) described the sensitivity of *S. minimus* stomata to C_a [$C_a = 2.745\left(1e^{0.0032ABA_f}\right)$] as indicated by the solid line, although neither conifer species presented a significant relationship. Mean foliar ABA level ±SE (n=3) for pre-drought, and the 4 d post-drought recovery are also shown for *S. minimus* (D), *C. rhomboidea* (E), and *P. radiata* (F). Vertical dashed lines separate pre-drought unstressed ABA levels from levels following drought recovery.

the angiosperm *S. minimus* showed a pronounced change in the dynamics of the stomatal response to C_a following drought, when ABA_f was high (Fig. 4).

The effect of drought on A

Prior to the drought treatment, individuals of *S. minimus* and *C. rhomboidea* both had similar mean A (11.65±0.72 and 11.86±2.08 μmol m^{-2} s^{-1}, respectively) while *P. radiata* individuals had a slightly lower mean A (7.6±0.71 μmol m^{-2} s^{-1}) (Fig. 5). Mild drought stress reduced mean A in all species; in *S. minimus* and *C. rhomboidea* mean A was reduced by a similar degree (41% and 46%, respectively), while in *P. radiata* mean A was only reduced by 27% (Fig. 5). This reduction in mean A recovered in both conifer species by the third day following rewatering, and by the fourth day in the angiosperm *S. minimus* (Fig. 5).

Discussion

The stomata of the two conifer species *C. rhomboidea* and *P. radiata* were found to be insensitive to changes in C_a in spite of >4-fold increases in ABA_f induced by mild drought treatment (Fig. 3). This lack of an ABA-induced stomatal sensitivity in the two phylogenetically and ecologically disparate conifer species suggests a common state for conifers in general, strongly contrasting with the response of the representative angiosperm *S. minimus* in which stomatal sensitivity was largely dependent on ABA_f following recovery from drought (Fig. 3A, D). The stomatal responsiveness of *S. minimus* to C_a increased with levels of ABA_f, similar to that previously reported in the excised or ABA-injected leaves of other angiosperm species (Raschke, 1975; Dubbe *et al.*, 1978; Raschke and Hedrich, 1985; Ridolfi *et al.*, 1996; Bunce, 1998; Leymarie *et al.*, 1998).

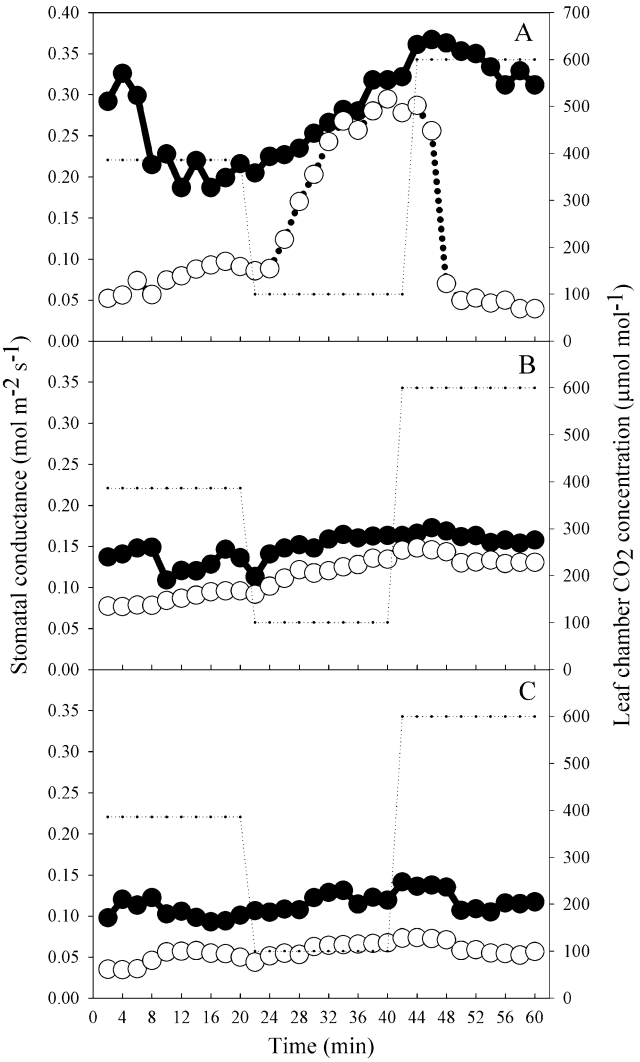

Fig. 4. Time-courses showing the responses of stomatal conductance (g_s) to step changes in CO_2 concentration (small dotted line) in a single representative individual of *S. minimus* (A), *C. rhomboidea* (B), and *P. radiata* (C) in the morning prior to the commencement of drought (filled circles) and the morning immediately following recovery from mild drought stress (open circles).

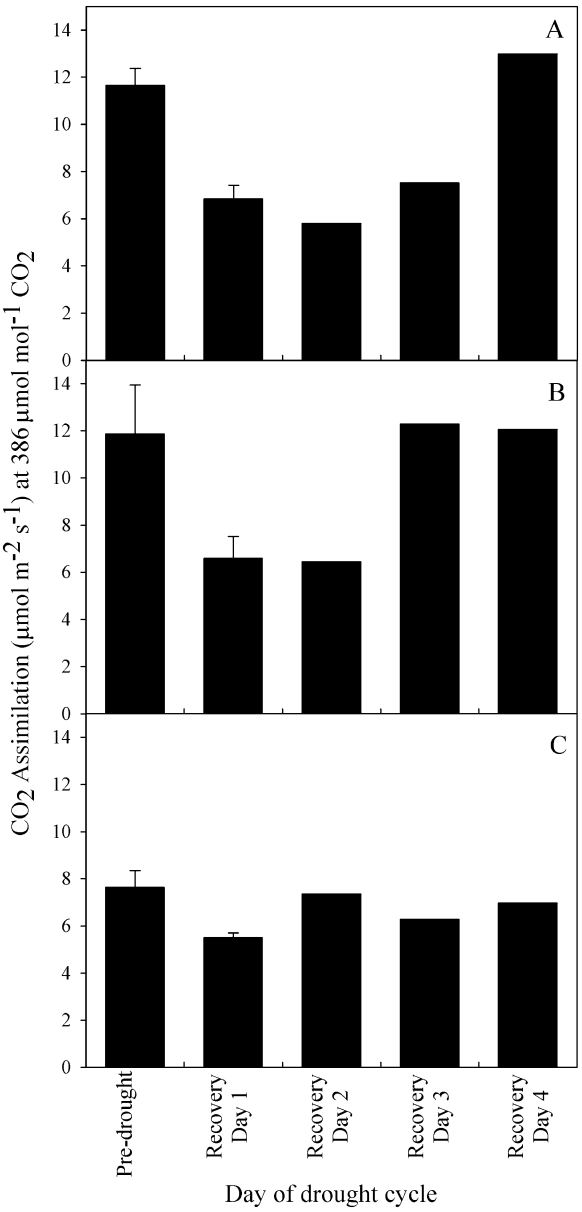

Fig. 5. Mean assimilation of CO_2 at ambient (386 μmol mol^{-1}) CO_2 ±SE over the mild drought cycle including prior to the drought, and the 4 d post-drought recovery in *S. minimus* (A), *C. rhomboidea* (B), and *P. radiata* (C). (n=3 for pre-drought and recovery day 1, n=1 for the remaining days following drought recovery).

There are four possible explanations that could account for the lack of stomatal sensitivity to C_a in conifers: (i) that ABA levels in conifers were too low to enhance sensitivity to C_a; (ii) that photosynthesis was severely down-regulated or damaged by drought in the conifer species; (iii) that the stomatal response to C_a in conifers is entirely absent; or (iv) that ABA has a more limited physiological role in coniferous species compared with angiosperms. The first three explanations are unlikely, for reasons discussed below.

The first explanation, that ABA levels in the conifers were insufficient to enable stomatal sensitivity to changes in C_a, is the most unlikely explanation. In this study both conifer species contained high levels of ABA_f and showed a similar 4-fold increase in ABA_f as a result of mild drought stress compared with the angiosperm species (Fig. 3). To date all angiosperm species reported are either always sensitive to C_a or show sensitivity induced by increases in ABA_f, unlike the two conifer species in this study (Raschke and Hedrich, 1985).

The possibility of reduced stomatal sensitivity in the conifer species due to a reduced photosynthetic capacity prior to or caused by mild drought is also unlikely (Fig. 5). The mesophyll plays a significant role in regulating the stomatal sensitivity to C_a in angiosperms (Mott *et al.*, 2008) with photosynthesis probably acting as a transducer, possibly from the mesophyll through a vapour phase (Sibbernsen and Mott, 2010). In all species the mild drought treatment that caused significant increases in ABA_f resulted in similar mild reductions in A (Fig. 5). The stomata of *S. minimus*

were most sensitive to C_a on the day immediately following drought recovery (Fig. 3A, D) when A in this species was 41% lower than on the day before the initiation of drought (Fig. 5). These results validate ABA as the primary cause of increased stomatal sensitivity to C_a in *S. minimus*, and that the reduced sensitivity of the two conifer species was not due to an initially very low A or a damaged photosynthetic system as a result of the mild drought (Fig. 5).

The third explanation that the pathway responsible for the C_a response is entirely absent in conifers and hence the sensitization of stomata by ABA never occurs is also unlikely. Small increases in g_s observed when conifer stomata are exposed to low C_a (Brodribb *et al.*, 2009) as well as an increased sensitivity of conifer stomata to C_a under increasing VPD (Bunce, 2007) suggest that the stomatal response to C_a in conifers is not entirely absent. Bunce (2007) reported that the stomatal insensitivity of the conifer *Picea sitchensis* (Bong.) to decreasing C_a (from 380 μmol mol^{-1} to 100 μmol mol^{-1}) was reversed to a significant but small degree by increasing VPD above ~1.4 kPa. This reported increase in sensitivity at high VPD was, however, much smaller than the relative increases in sensitivity observed in the angiosperm *Helianthus annuus* L. in which g_s at 100 μmol mol^{-1} CO_2 returned to maximum levels regardless of the VPD exposure (Bunce, 2007). The small increase in stomatal sensitivity to C_a in conifers when exposed to high VPD suggests that conifer stomata have the potential to respond to changes in C_a, but that this response is constrained by the lack of another regulating signal. The strong interaction between the two stomatal signals, ABA and C_a, in angiosperms is evident and suggests that the angiosperm-like regulation of stomatal control by ABA is absent in the two coniferous species.

A diminished role for ABA in conifers?

The possibility of a missing or inactive signalling pathway in the stomata of conifers leads to the final explanation for the contrast between the angiosperm and two conifer species: a fundamental difference in stomatal control by ABA between the two lineages. A limited number of studies have investigated ABA_f in conifers using physicochemical methods (Kraus and Ziegler, 1993; Hoffman *et al.*, 1999; Kong *et al.*, 2009). These studies all indicate that ABA_f in conifers, including Pinaceae (Kraus and Ziegler, 1993; Kong *et al.*, 2009) and Taxaceae (Hoffman *et al.*, 1999) species, were typically >400 ng g^{-1} fresh weight under well-watered conditions. This is in contrast to angiosperm species under similar unstressed conditions for which ABA_f is typically <100 ng g^{-1}. The large differential between conifers and angiosperms was also observed in this study, where mean ABA_f in unstressed *C. rhomboidea* was 320 ng g^{-1}, and in *P. radiata* 190 ng g^{-1}, compared with mean levels of 75 ng g^{-1}, in *S. minimus* (Fig. 3D–F).

The link between ABA_f and stomatal conductance has been widely documented in angiosperms from detached leaves (Raschke and Hedrich, 1985) as well as in ABA biosynthetic mutants fed ABA solutions (Pugliesi *et al.*, 1994). In all cases increasing ABA_f had the effect of closing stomata. Murphy and Ferrell (1982) found in the conifer *Pseudotsuga menziesii* (Mirb.) Franco a very weak relationship between ABA_f and E, apparent only in early summer. In the present study, values of E (Fig. 2A–C) and g_s (Fig. 4A–C) prior to drought were similarly high in the angiosperm and two conifer species despite the relatively high ABA_f in the two conifers (Fig. 3D–F). Additionally, by the fourth day following drought recovery ABA_f had returned to pre-drought levels in all species (Fig. 3D–F) but this recovery did not correspond to a full recovery in E (Fig. 2). Recovery of ABA_f (Henson, 1981; Liu *et al.*, 2001) and xylem ABA level (Loewenstein and Pallardy, 2002) to pre-drought levels prior to the recovery of E or g_s has been recorded in a number of angiosperm species and highlights a transient role for ABA in the control of stomatal conductance following drought. The E, g_s, and ABA_f results from this study point to the possibility of ABA levels in conifers having less of a control over stomatal aperture, a conclusion recently suggested in a study on conifer gas exchange recovery over a drought cycle by Brodribb and Cochard (2009).

The reduced stomatal response to ABA in conifers raises the question of how these plants function so effectively. One alternative that needs investigation is raised by the fact that stomata of conifers, unlike angiosperms, operate similarly to ferns with a large safety margin between Ψ_{leaf} at 50% closure and Ψ_{leaf} at the point of hydraulic failure (Brodribb and Holbrook, 2004; Brodribb and Cochard, 2009). This relatively large Ψ_{leaf} safety margin means cavitation and repair are very rare in vessel-less conifers compared with angiosperms, and hence the utility of ABA as a means of enhancing embolism repair (Lovisolo *et al.*, 2008) is also very limited.

Conclusion

The combination of a lack of ABA-induced stomatal response during drought (Brodribb and Cochard, 2009), stomatal insensitivity to C_a despite increases in ABA_f, and high levels of pre-drought ABA suggests that conifers have a relatively weak biochemical and physiological response to ABA. These differences between conifers and well-studied angiosperm species suggest missing or inactive biochemical pathways in the guard cells of conifers, placing an emphasis on the need for re-evaluation of generalizations about the role of ABA in plants.

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