Research

Evolution of stomatal closure to optimize water-use efficiency in response to dehydration in ferns and seed plants

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Summary

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- Plants control water-use efficiency (WUE) by regulating water loss and CO_2 diffusion through stomata. Variation in stomatal control has been reported among lineages of vascular plants, thus giving rise to the possibility that different lineages may show distinct WUE dynamics in response to water stress.
- Here, we compared the response of gas exchange to decreasing leaf water potential among four ferns and nine seed plant species exposed to a gradually intensifying water deficit. The data collected were combined with those from 339 phylogenetically diverse species obtained from previous studies.
- In well-watered angiosperms, the maximum stomatal conductance was high and greater than that required for maximum WUE, but drought stress caused a rapid reduction in stomatal conductance and an increase in WUE in response to elevated concentrations of abscisic acid. However, in ferns, stomata did not open beyond the optimum point corresponding to maximum WUE and actually exhibited a steady WUE in response to dehydration. Thus, seed plants showed improved photosynthetic WUE under water stress.
- The ability of seed plants to increase WUE could provide them with an advantage over ferns under drought conditions, thereby presumably increasing their fitness under selection pressure by drought.

Introduction

From root absorption to circulation up the stem and transpiration through the leaves, water transport and utilization are the foundation of survival of land plants (McAdam & Brodribb, 2012; Buckley *et al.*, 2017). Plants invest high amounts of energy to build a safe and effective water transport system (mainly comprised of the vascular bundles) (Brodribb *et al.*, 2017; Waseem *et al.*, 2021). Furthermore, plants have evolved a fully functional signalling network for the optimal adjustment of stomatal aperture, aiming to regulate water-use efficiency (WUE) (Xiao *et al.*, 2018), such that it may be maximal under a wide range of environmental conditions (Cowan & Farquhar, 1977; Brodribb *et al.*, 2009; McAdam & Brodribb, 2012; Yao *et al.*, 2021b).

Selective pressures throughout the history of land plants have been proposed as evolutionary drivers favouring a continuous increase in WUE (Brodribb *et al.*, 2009; McAdam & Brodribb, 2012; Gago *et al.*, 2014; Negin & Moshelion, 2016). Thus, differences in gas exchange efficiency are linked to systematic patterns of stomatal behaviour. For example, the stomata of

angiosperms close rapidly in response to increasing ambient CO_2 concentration, whereby instantaneous WUE (WUE_{ins}, defined as the ratio of photosynthesis rate (A) to transpiration rate (E)) increases significantly, relative to the early diverging lineages of ferns and lycophytes (Brodribb *et al.*, 2009). Furthermore, in seed plants, intrinsic WUE (WUE_i, defined as the ratio of A to stomatal conductance (g_s)) remains high and constant under fluctuating light intensities. However, the stomata of ferns and lycophytes are reportedly incapable of sustaining homeostatic WUE_i at nonsaturating light intensities. WUE_i decreases significantly in ferns and lycophytes, especially those that display the greatest changes in photosynthetic rates following the transition from high to low light intensity (McAdam & Brodribb, 2012). Both these studies indicated that more advantageous WUE control capabilities may have conferred a competitive advantage that contributed to the success of seed plants in modern ecosystems (Brodribb *et al.*, 2009; McAdam & Brodribb, 2012). Water deficit is the most common environmental stress affecting plants around the world, and is one of the primary selective forces in the plant evolutionary process (Bohnert *et al.*, 1995). However, whether seed plants show a greater capacity to optimize WUE in response to water deficit than ancient plant lineages, such as ferns, remains unknown.

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Angiosperms show greater stomatal conductance and transpiration and photosynthesis rates under well-watered conditions (Brodribb *et al.*, 2005; Brodribb & Feild, 2010; Carriquí *et al.*, 2015); further, under such conditions their stomata open beyond the optimum required for maximum A (Deans *et al.*, 2019). Conversely, when water supply is insufficient or when the transpiration rate exceeds water supply, the first line of defence against a decrease in internal water potential is to close 'excessive' stomatal opening, mainly by inducing an increase in foliar abscisic acid (ABA), which in turn reduces transpiration (Davies & Zhang, 1989; Dodd, 2013; Assmann & Jegla, 2016). Stomata are more sensitive to a decrease in internal water potential than photosynthetic biochemistry, and stomatal closure causes a drop in mesophilic CO_2, thereby increasing the concentration gradient for CO_2 diffusion and ultimately increasing WUE (Davies *et al.*, 2002; Álvarez *et al.*, 2011; Medrano *et al.*, 2015). The accumulation of ABA, which reduces stomatal closure when plants are subjected to a water deficit, is considered to be a key factor that can optimize WUE and shape plant adaptation to dry environments (Campitelli *et al.*, 2016; Negin & Moshelion, 2016). However, early-diverging vascular plant lineage ferns are characterized by low stomatal conductance and photosynthesis rates (Brodribb *et al.*, 2005; Brodribb & Feild, 2010; Carriquí *et al.*, 2015), which are *c.* 30% of those measured in angiosperms under well-watered conditions; furthermore, stomata do not open beyond the optimum as observed in well-watered angiosperms (Deans *et al.*, 2019). Consequently, a drought-induced decline in g_s would constrain photosynthesis greatly, A and g_s would decrease synchronously, and the species would show no improvement in WUE. Therefore, if seed plants are better equipped for significantly improving WUE in order to adapt to disturbances caused by water deficit and produce a more competitive outcome than ferns, the mechanism at play might be associated with a rapid reduction in 'excessive' stomatal conductance in response to elevated concentrations of ABA.

In the present study, we hypothesized that ferns and seed plants would show contrasting patterns of WUE during drought. In order to test this hypothesis, leaf water potential (ψ_{leaf}), A, g_s, E and ABA concentrations were examined in four ferns, five gymnosperms and four angiosperms subjected to drought stress conditions. Data obtained from this experiment were compared with data from previously published studies on WUE in phylogenetically diverse ferns, gymnosperms and angiosperms, and then analysed. The results supported our hypothesis that seed plants possess a higher stomatal capacity to upregulate WUE based on a rapid decrease in 'excessive' stomatal opening in response to elevated concentrations of ABA, compared with more ancient plant lineages (e.g. ferns), which are not equipped with 'excessive' stomatal opening.

Materials and Methods

Target species and growing conditions

Functionally and phylogenetically diverse vascular plant species, including four ferns (*Coniogramme intermedia*, *Polystichum* *neolobatum*, *Matteuccia struthiopteris* and *Athyrium sinense*), five gymnosperms (*Pinus tabuliformis*, *Pinus bungeana*, *Taxus chinensis*, *Metasequoia glyptostroboides* and *Ginkgo biloba*), and four angiosperms (*Populus davidiana*, *Euonymus bungeanus*, *Fraxinus chinensis* and *Sophora japonica*) were selected according to their habitats to test their WUE response to a water deficit. All the species were collected from the same site in the Xiaolongshan Natural Forest area in Tianshui City, Gansu Province, China (34°00′–34°40′N, 105°30′–106°30′E). *Matteuccia struthiopteris* and *A. sinense* are widely distributed in open and sunny habitats. They are mainly found growing by the creek and forest edges. *Polystichum neolobatum* and *C. intermedia* grow widely in shady habitats, while seed plants are widely distributed in forests in sunny, partially shaded or shaded habitats (Table S1), where the average temperature is 23°C during the day and 18°C at night, and the average relative humidity (RH) is 66% during the day and 86% at night in June and July ranges. Maximum natural photosynthetic photon flux density (PPFD) at midday during the summer is *c.* 300–400 µmol quanta m^{-2} s^{-1} for *P. neolobatum* and *C. intermedia* growing in shady habitats, and more than 1400 µmol quanta m^{-2} s^{-1} for the species growing in sunny habitats (data from local weather station; Chen *et al.*, 2019).

In April 2014, small uniform seedlings were transferred into pots (diameter 15 cm, height 22 cm) containing a mixture of humus soil, vermiculite, and perlite in a 9 : 2 : 1 ratio. These plants were grown at a nearby tree nursery where they received an unlimited water and nutrient supply, and similar light conditions as those found in their natural habitats. In June 2016, after *c.* 2 yr, potted plants were transferred to a glasshouse in the experimental station of the Yuzhong campus at Lanzhou University, China (35°51′N, 104°07′E). All environmental factors, except for irrigation water used in lieu of rainfall, were kept the same in an attempt to mimic natural conditions. The experimental setup was maintained at 23°C : 18°C, day : night with a RH of 66% : 86%, day : night. *Polystichum neolobatum* and *C. intermedia* received *c.* 0.25–0.33 of the natural light intensity under a shade net, while plants of the other species (two ferns and nine seed plants) received plenty of natural light. All plants were irrigated daily until the beginning of the experiment.

Exposure to drought stress

Towards the end of June, when the average height of the experimental seed plants ranged from 100 to 150 cm, and that of ferns ranged from 30 to 50 cm, drought stress was imposed on more than 10 individuals per species by withholding water supply for *c.* 3 wk for ferns and 3–5 wk for seed plants. During the dry period, ψ_{leaf}, A, g_s, E and leaf ABA concentrations were measured at 1–2 d intervals.

ψ_{leaf} and gas exchange measurements

The ψ_{leaf} values of four fully expanded upper leaves from different individuals of each species were measured between 07:30 h and 09:00 h Beijing Standard Time (BST) every 1–2 d, using a pressure chamber (Model 1000; PMS Instrument Co., Albany,

OR, USA), and following the precautions recommended by Fang *et al.* (2010). Briefly, leaves were collected and immediately placed in a sealable Whirl-Pak bag (Nasco, GA, USA), which had previously been exhaled to ensure 100% humidity. Following 20 min equilibration, ψ_{leaf} was measured.

After measurement of ψ_{leaf}, the leaves in close proximity to the leaves used for measuring ψ_{leaf} were used to measure A, g_s and E with a portable gas exchange instrument (LI-6400; Li-Cor, Lincoln, NE, USA) configured as an open system and equipped with a LED for a light source. These parameters were measured early in the morning, as preliminary data showed that the values for gas exchange parameters measured in the species under study were highest in those early hours. The measuring system was maintained at a temperature of 22°C in the leaf chamber, an ambient CO_2 concentration of 400 ppm, a vapour pressure difference between 1.1 and 1.4 kPa, and an air flow rate of 400 ml min^{-1}. Gas exchange was measured at 1200 µmol m^{-2} s^{-1} PPFD in all the species tested in this study, except for two shade ferns (*P. neolobatum* and *C. intermedia*), which were measured at a photosynthetic flux density of 400 µmol m^{-2} s^{-1}. The rates of A, E, and g_s for water vapour allowed the determination of WUE_{ins} (A/E) and WUE_i (A/g_s) as described by Fischer & Turner (1978) and Gago *et al.* (2014).

ABA measurement

Leaves in close proximity to the leaf used for measuring gas exchange were removed from each plant every 2–4 d after measurements of gas exchange. If, between two adjacent sampling points, ψ_{leaf} fell by < 0.3 MPa, the leaves were not sampled. The removed leaves were divided into two groups. Approximately half of these leaves were immediately weighed and dried to constant mass to determine the dry mass : fresh mass ratio. The remaining leaves were weighed immediately before freezing in liquid nitrogen and storing at −80°C to measure ABA concentrations.

Abscisic acid concentrations were determined following the methods described by McAdam & Brodribb (2015). Briefly, leaf samples were ground to a powder under liquid nitrogen and mixed with 5 ml of an 80% methanol solution (containing 0.02 M 2,6-di-tert-butyl-4-methyphenol). Labelled ABA (D6-ABA) was added to this mixture, and the resulting solution was maintained at 4°C for 12 h. Following centrifugation at 4°C and 10 000 *g* for 10 min, the supernatant was removed and the pellet was extracted twice with a mixture of methanol and water (4 ml). The collected supernatant was first concentrated with petroleum ether and then, using an ester phase, it was dried under a stream of nitrogen gas using a Termovap Sample Concentrator (Model HP5106GD; Shanghai Eastern Analytical Instruments Co. Ltd, Shanghai, China). The residue was dissolved in methanol and analysed for chromatographic purity by high-performance liquid chromatography (HPLC) using OrbiTrap Fusion Lumos (Thermo Fisher, San Jose, CA, USA). ABA concentration (ng g^{-1} DW) was calculated as ABA per leaf fresh mass × (leaf dry mass/leaf fresh mass) of the leaves adjacent to the leaves sampled for ABA.

Addition of exogenous ABA

Using a sharp knife, fronds were cut from three individuals of each fern species under double distilled water and rehydrated. Once the immersed fronds were fully hydrated, g_s was measured using the LI-6400 photosynthesis system (Li-Cor) under the conditions described earlier. After g_s had stabilized, ABA solution was added to the solution containing the immersed shoots in order to achieve a target concentration of 15 000 ng g^{-1}. Then, g_s was continuously measured for 90 min to test whether exogenously added ABA induced stomatal closure.

Database selection

To characterize WUE patterns that are widespread across vascular plants, we used 139 published reports providing data for A, g_s and E under well-watered and drought conditions for 339 species, including 202 angiosperm species from 44 families, 53 gymnosperm species from nine families, and 84 fern species from 30 families (Table S2). This literature was obtained from Google Scholar; keyword searches included 'water-use efficiency', 'photosynthesis and stomatal conductance', 'drought' or 'water stress'. The data points were obtained from tables and digitalized figures by Engauge Digitizer (Mark Mitchell, Baurzhan Muftakhidinov and Tobias Winchen *et al.*, 'ENGAUGE DIGITIZER software'; http://markummitchell.github.io/engauge-digitizer).

Statistical analysis

Treatment effects across the three lineages were evaluated using one-way ANOVA followed by Duncan's multiple range test to separate means. The differences between mean values in well-watered and moderately stressed plants within each lineage and from gymnosperms vs angiosperms were evaluated with independent-sample t-tests. All statistical analyses were performed with SPSS 15.0 (SPSS Inc., Chicago, IL, USA), and results were considered significant at $P < 0.05$. A regression analysis was performed using SIGMAPLOT 12.5 (Systat Software Inc., San Jose, CA, USA). The maximum-likelihood function that best fitted our data for each species was selected to construct vulnerability curves using the 'optim' function in R 3.1.0 (http://www.r-project.org; Burnham & Anderson, 2004; Scoffoni *et al.*, 2012). Four functions were tested, according to previous studies (Pammenter & Willigen, 1998; Scoffoni *et al.*, 2012): linear ($Kz = a\psi_z + b$), three-parameter sigmoidal $\left(Kz = \dfrac{a}{1+e^{-\left(\frac{\psi - x_0}{b}\right)}} \right)$, logistic $\left(Kz = \dfrac{a}{1+\left(\frac{\psi_z}{x_0}\right)^b} \right)$, and exponential function ($Kz = y_0 + ae^{-b\psi_z}$).

Kz in these functions denotes either A, g_s, or E, and ψ_z denotes leaf water potential. For each dataset, functions were compared using the Akaike information criterion (AIC), corrected for a low n. The function with the lowest AIC value (differences > 2) was chosen as the maximum-likelihood function. ψ_{leaf} at 50% loss of photosynthesis (AP_{50}), stomatal conductance (g_sP_{50}) and transpiration

(EP_{50}) were determined. The differences between AP_{50} and g_sP_{50} and between AP_{50} and EP_{50} within each linkage were evaluated with paired t-tests. Maximum A (A_{max}), g_s (g_{s-max}), or E (E_{max}) were the mean values measured under well-watered conditions.

Results

The nine seed plant species under study, especially the five angiosperms, showed higher mean maximum A, g_s and E values than the fern species, under well-watered conditions (Figs 1, S1–S3; Table S1). Decreasing ψ_{leaf} resulted in a gradual decrease in g_s across species (Figs 1, S1–S3). In all four fern species, A, g_s and E decreased synchronously with decreasing ψ_{leaf} (Figs 1, S1), thus indicating no difference between AP_{50} and g_sP_{50} (paired-samples t-test, $P = 0.356$; Table S1), or between AP_{50} and EP_{50} (paired-samples t-test, $P = 0.736$; Table S1). However, A, g_s and E responded differentially to decreasing ψ_{leaf} in all nine seed plant species, with g_s and E being more sensitive to such decreasing ψ_{leaf}, compared with A (Figs 1, S2, S3). Especially shortly after drought treatment initiation, g_s decreased sharply, while A remained relatively constant (Fig. 2b,c). Thus, the instantaneous slope of A vs g_s and A vs E curve increased as drought stress progressed (Fig. 2e,f,h,i). As a result, g_sP_{50} and EP_{50} were 0.29 ±

0.09 and 0.30 ± 0.06 MPa higher than AP_{50} among gymnosperms, and 0.27 ± 0.07 and 0.30 ± 0.09 MPa higher than AP_{50} among angiosperms, respectively (paired-samples t-test, $P = 0.006$–0.047) (Table S1). No significant differences in AP_{50}, g_sP_{50} or EP_{50} were observed between gymnosperms and angiosperms (independent-samples t-test, $P = 0.059$–0.078) across seed plant lineages (Table S1), indicating that the sensitivity of A, g_s and E to decreasing ψ_{leaf} values did not clearly diverge in the two groups tested herein.

In ferns, WUE_i and WUE_{ins} remained unaltered with decreasing ψ_{leaf} under drought stress (Figs 2d,g, S4, S5), as all fern species exhibited a synchronous decrease in A, g_s and E (Figs 2a, S1). However, WUE_i and WUE_{ins} were upregulated in seed plants (Figs 2, S4, S5), as shown by the more rapid decline in g_s and E than in A (Figs 2b,c, S2, S3). Further, WUE_i and WUE_{ins} showed 1.6- and 2.0-fold increases in the five gymnosperms, and 1.4- and 2.1-fold increases in the four angiosperms, respectively, from well-watered to moderate drought stress conditions. However, both WUE_i and WUE_{ins} either decreased or remained unchanged under severe drought conditions (Figs 2, S4, S5). Data from the present study and 339 species in previously published reports showed that angiosperms had lower WUE_i and WUE_{ins} values across the three lineages under well-watered
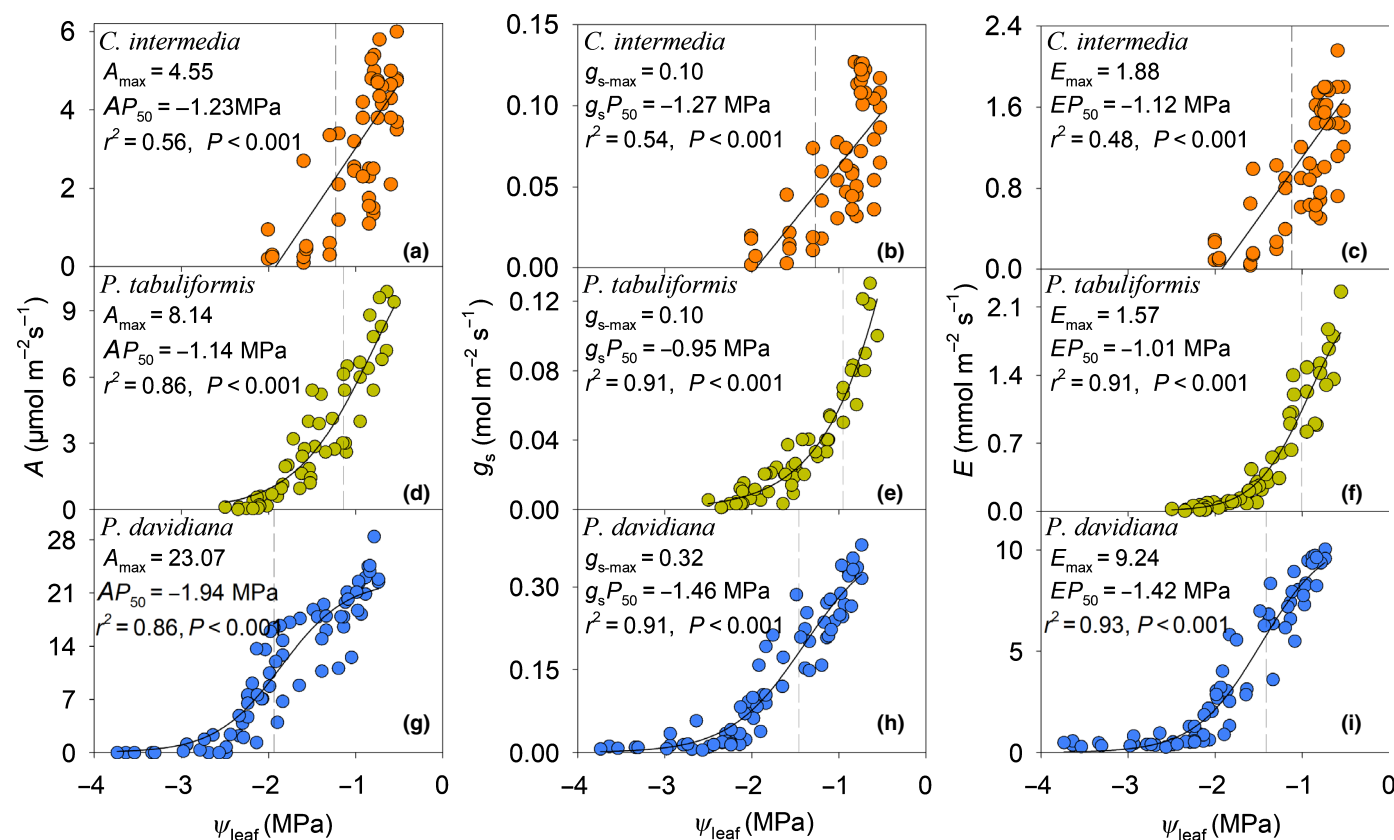

Fig. 1 The coordination of photosynthesis rate (A), stomatal conductance (g_s) and transpiration rate (E) with decreasing leaf water potential (ψ_{leaf}). Leaf photosynthesis (A) (a, d, g), stomatal conductance (g_s) (b, e, h) and transpiration (E) (c, f, i) decreased gradually with decreasing ψ_{leaf} in fern *Coniogramme intermedia* (a–c), gymnosperm *Pinus tabuliformis* (d, e, f) and angiosperm *Populus davidiana* (g–i). The vertical dashed line shows ψ_{leaf} at 50% loss of leaf photosynthesis (AP_{50}) (a, d, g), stomatal conductance (g_sP_{50}) (b, e, h) and transpiration (EP_{50}) (c, f, i). The line plots are the maximum likelihood function using a linear function (a–c), a three-parameter sigmoidal function (f–i), and a logistic function (d, e) according to the function with the lowest Akaike information criterion (see the Materials and Methods section). The correlation coefficient (r^2) and probability (P) of the fitted regressions are listed. Maximum values for A (A_{max}), g_s (g_{s-max}) and E (E_{max}), and the values of AP_{50}, g_sP_{50} and EP_{50} are given for each species.
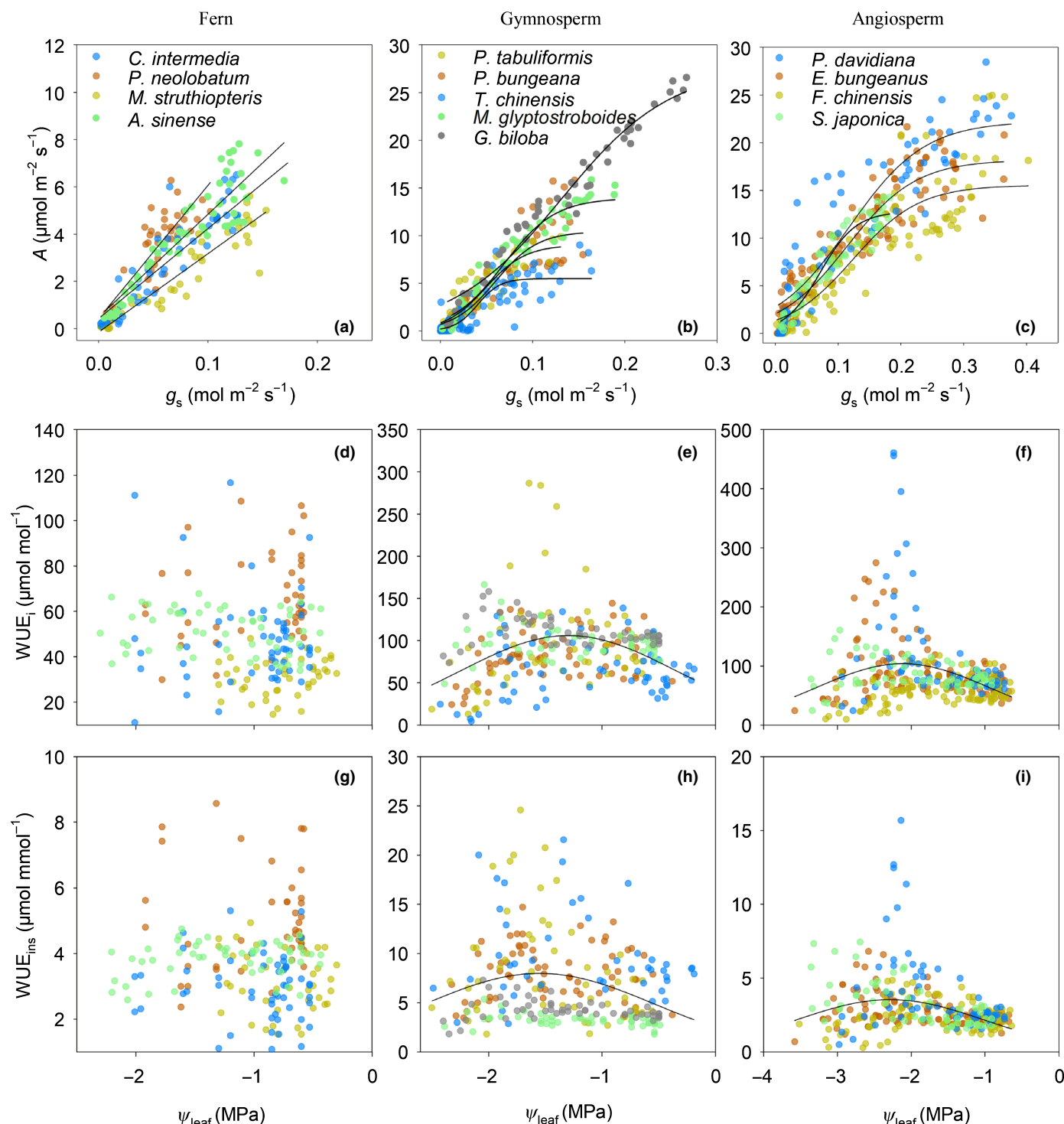
Fig. 2 The relationships between photosynthesis rate (A) and stomatal conductance (g_s), and between water-use efficiency (WUE) and decreasing leaf water potential (ψ_{leaf}). Photosynthesis (A) decreased linearly with decreasing stomatal conductance (g_s) in fern species (a), but initially slowly and then gradually with decreasing g_s in gymnosperm species (b) and angiosperm species (c). Water-use efficiency (A/g_s (WUE$_i$) and the A/transpiration (E) (WUE$_{ins}$)) did not change with decreasing ψ_{leaf} in ferns (d, g), but increased and then decreased in gymnosperms and angiosperms (e, h, f, i). The line indicates significant regressions ($P < 0.05$).

conditions (Fig. 3), whereas, under moderate drought stress, seed plants – especially angiosperms – significantly upregulated their WUE$_i$ and WUE$_{ins}$, with a significant 1.8- to 2.6-fold increase, in order to cope with water deficit. However, ferns did not

exhibit such responses (Fig. 3). WUE$_i$ and WUE$_{ins}$ of gymnosperm species were similar to those of angiosperm species at moderate drought stress (independent-samples t-test, $P = 0.112$–0.428; Fig. 3; Table S2).
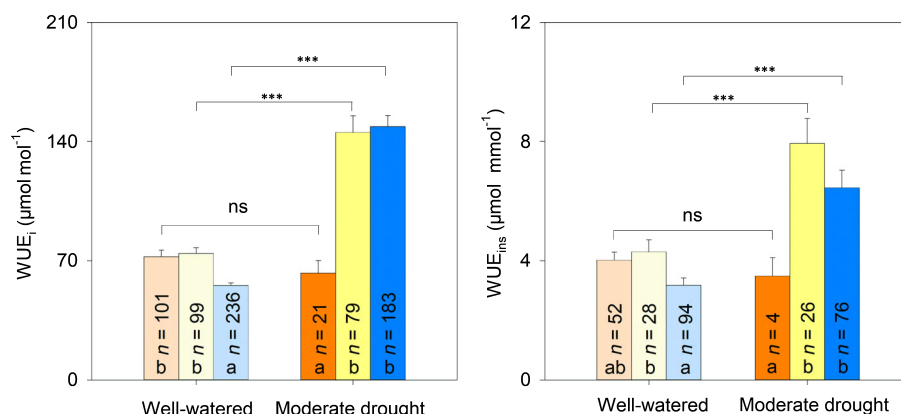
Fig. 3 Meta-analysis showing moderate drought-induced increasing water-use efficiency (WUE) in gymnosperms and angiosperms, but no in ferns. Intrinsic WUE (WUE_i) (the photosynthesis (A)/stomatal conductance (g_s)) and instantaneous WUE (WUE_{ins}) (A/transpiration (E)) in angiosperms (blue bars) and gymnosperms (yellow bars) increased significantly under moderate drought stress (light colour indicates well-watered, dark colour indicates moderate drought), while the WUE_{ins} and WUE_i in ferns (orange bars) did not increase (light colour indicates well-watered, dark colour indicates moderate drought) (data from Supporting Information Table S3). For WUE_i, $n = 236$ for well-watered angiosperm and $n = 183$ for angiosperm under moderate drought; the respective figures are $n = 99$ and 79 for gymnosperms and $n = 101$ and 21 for ferns. For WUE_{ins}, the values are, respectively, $n = 94$ and 76 for angiosperms, $n = 28$ and 26 for gymnosperms, and $n = 52$ and 4 for ferns. Mean values ($\pm$SE) labelled with an asterisk are significantly different ($P < 0.05$), and ns indicates no significant difference ($P \geq 0.05$) between well-watered and moderate drought treatments within each lineage. Mean values labelled with the same letter are not significantly different across three lineages under each treatment ($P \geq 0.05$).

The small increase recorded in leaf ABA concentrations in ferns under drought stress was not significant (Figs 4a, S6), indicating there was no correlation between g_s and ABA concentrations (Figs 4b, S6). Furthermore, no reduction in g_s was observed even when the ABA solution was applied to the leaf transpiration stream at a concentration of 15 000 ng ml^{-1} for 90 min (Fig. S7). By contrast, ABA concentrations in leaves of seed plants increased four- to 12-fold linearly or slowly initially and then rapidly with decreasing ψ_{leaf} (Figs 4c,e, S8, S9), thereby inducing an exponential decline in g_s (Figs 4d,f, S8, S9).

Discussion

Water deficit is the most widespread environmental stress factor that has shaped plant evolution (Adams *et al.*, 2017). Here, we investigated the WUE of phylogenetically diverse ferns and seed plant species in response to water deficit. Our results showed that seed plants have evolved the ability to upregulate WUE when exposed to drought stress conditions. By contrast, ferns seemed unable to upregulate WUE. Increasing WUE in seed plants was related to a slow drought-induced decrease in photosynthesis rate coupled to a rapid decrease in stomatal conductance and transpiration rate. This was seemingly a result of a decrease in 'excessive' stomatal opening observed during the early stages of drought in response to ABA accumulation in leaves. However, ferns, in which stomatal conductance was low and stomata did not open beyond the optimum point for maximum WUE even under well-watered conditions, exhibited a steady WUE in response to a decreasing leaf water potential. These data are consistent with previous reports of stable WUE in seed plants but reduced values in ferns and lycophytes under varying light intensities (McAdam & Brodribb, 2012). Further, WUE increased in seed plants, but not in ferns or lycophytes under increasing CO_2 concentrations (Brodribb *et al.*, 2009). Our findings underline the idea that the

upregulation of WUE in seed plants may offer a competitive advantage in environments with low water availability. Furthermore, our data strongly suggest that the evolution of stomatal function is not a highly conserved process but an evolutionary transition that enables WUE upregulation in response to water deficit.

The data from this study were combined with those of 339 phylogenetically diverse species from different habitats; the results of the analysis of all these data showed that angiosperms exhibited lower WUE compared with gymnosperms and ferns under well-watered conditions. However, WUE (including WUE_i and WUE_{ins}) was significantly upregulated under water-deficit in all seed plant species as a result of a slower rate of decline of A than of either g_s or E (Fig. 3; Brodribb, 1996; Fang *et al.*, 2011; Yao *et al.*, 2021b). Modelling of A and g_s in response to drought conditions has shown that WUE_i increases with drought stress (Misson *et al.*, 2004; Manzoni *et al.*, 2011). Thus, optimized WUE in response to water deficit is a basic adaptive trait of seed plants. In contrast with studies of the responses of seed plants to drought stress, studies on changes in WUE in ferns are significantly limited, with only 19 species having been investigated, including the data from the study reported herein (Table S3). All available data show no ability for upregulation of WUE in these species. Therefore, it can be argued that ferns lack the ability to increase WUE when a decrease in water supply occurs. We are aware that WUE_i and WUE_{ins} data in the meta-analysis in our study were obtained from plants growing under different conditions of vapor pressure deficit, and hence the lack of a rigorous framework for the comparison of WUE_{ins}. Nonetheless, WUE_i is comparable across the three plant lineages, and higher WUE_i in seed plants adds support to the fact that seed plants exhibit improved WUE under water stress, whereas ferns do not. The difference in WUE_i and WUE_{ins} between angiosperms and gymnosperms under drought stress across seed
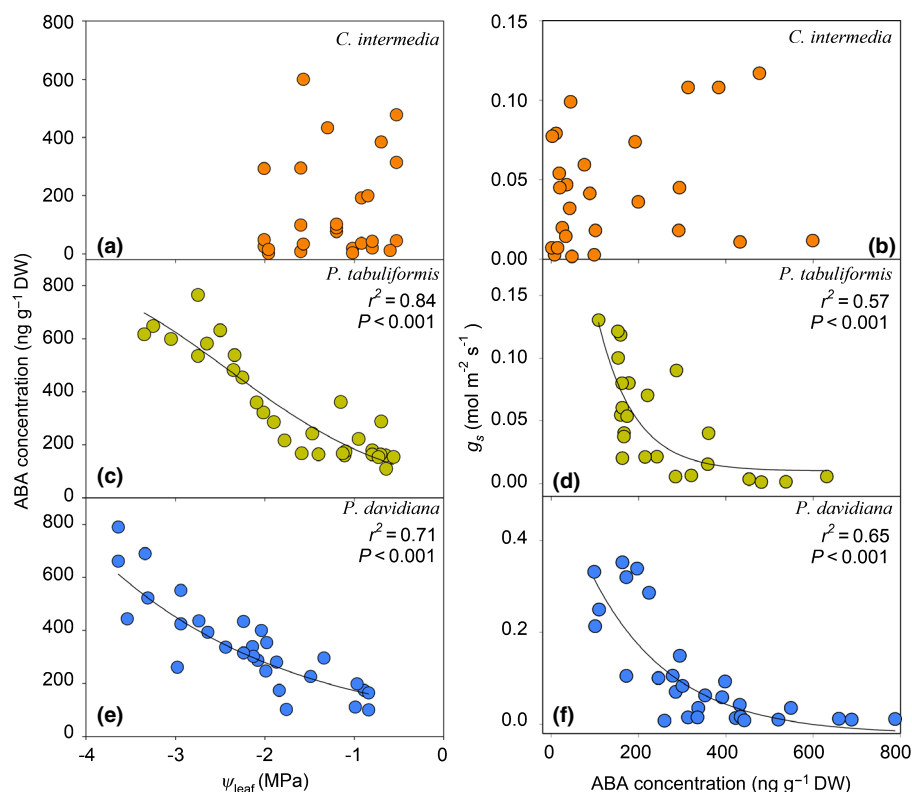
Fig. 4 The relationships between abscisic acid (ABA) concentration and decreasing leaf water potential (ψ_{leaf}), and between stomatal conductance (g_s) and increasing ABA concentration. ABA concentration increased significantly with decreasing ψ_{leaf}, and stomatal conductance (g_s) decreased significantly with increasing ABA concentration in gymnosperm *Pinus tabuliformis* (c, d) and angiosperm *Populus davidiana* (e, f), but the correlation was not observed in fern *Coniogramme intermedia* (a, b). The line plots are the maximum likelihood function using a three-parameter sigmoidal (c, e) and an exponential (d, f) function according to the function with the lowest Akaike information criterion value (see the Materials and Methods section). The line indicates significant regressions ($P < 0.05$), and the correlation coefficient (r^2) and probability (P) of regressions are listed.

species was not significant (Fig. 3). However, equal WUE values in gymnosperms may not mean they are equally as competitive in response to drought as angiosperms. Besides the increasing degree of WUE, the accumulated area of increasing WUE (consisting of a boundary by the regression line of increasing WUE and the horizontal line of WUE of well-watered plants) might be related to their competitive ability as well. Investigating the accumulation area of WUE in diverse gymnosperm and angiosperm species might provide novel insights into the evolution process of the gas exchange mechanisms across seed plants.

Increasing concentrations of ABA in the leaves of seed plants are reportedly associated with stomatal closure to reduce transpirational water loss at the expense of CO_2 inward diffusion for photosynthesis (Pantin *et al.*, 2013; Gago *et al.*, 2014; Lim *et al.*, 2015; McAdam & Brodribb, 2018; Yao *et al.*, 2021a). However, stomata of seed plants are more sensitive to ABA than to photosynthesis (Holaday *et al.*, 1992), as has been shown for deciduous trees (Aasamaa *et al.*, 2002) and shrubs (Yao *et al.*, 2021b), and as evidenced by a meta-analysis of 237 species (Gago *et al.*, 2014). Similar results were observed in *Tradescantia virginiana* treated with ABA (Franks & Farquhar, 2001). Even after addition of ABA to isolated mesophyll cells of *Phaseolus vulgaris*, *Lycopersicum esculentum* and *Nicotiana tabacum* at a concentration 19 times higher than the minimum concentration necessary

for stomatal closure, the increasing intracellular ABA concentration did not inhibit photosynthesis (Mawson *et al.*, 1981). These results indicate that ABA concentrations do not directly affect photosynthesis. Hence, the gap between stomatal and photosynthetic responses results in an increase in WUE. Indeed, the increase in WUE observed in angiosperms during drought is seemingly a result of their stomata opening beyond the optimum required for maximum *A* under well-watered conditions, and a rapid reduction of the 'excessive' stomatal opening induced by ABA accumulation under drought conditions, which implies that the decline in g_s has little effect on *A* (Deans *et al.*, 2019) upon drought stress initiation, whereby WUE increases. Vessel elements with greatly improved hydraulic efficiency (Feild & Brodribb, 2013) and leaves with greater vein density and lower leaf hydraulic resistance (Brodribb & Feild, 2010) might facilitate high rates of water transport and, hence, increasing transpiration (Deans *et al.*, 2019). Thus, angiosperms seemingly experience diminishing returns of *A* with respect to g_s (Deans *et al.*, 2019) when they are experiencing well-watered conditions, suggesting that maximizing *A* is a stronger selective force than WUE when water supply is unlimited. Here, we argue that the rapid decrease in 'excessive' stomatal opening in response to elevated ABA, as well as the slowly decreasing photosynthetic activity are the basis of WUE upregulation in plants (Hetherington & Woodward,

2003). Some studies have shown that ABA was synthesized slowly in some gymnosperm species (delay of hours) but rapidly in angiosperms (within minutes) after exposure to water stress (McAdam & Brodribb, 2015, 2016). In this study, drought stress was imposed over a period of 3–5 wk. Thus, we did not have the temporal resolution to observe these differences even if they occurred.

Ferns have vessel elements with low hydraulic efficiency (Feild & Brodribb, 2013), low leaf vein density and high leaf hydraulic resistance (Brodribb & Feild, 2010), all of which tend to constrain stomatal conductance (Franks, 2006; Brodribb et al., 2007; Boyce et al., 2009; Xiong et al., 2018; Deans et al., 2019), as indicated by the magnitude of g_s. The stomatal conductance was c. 30% of that observed in angiosperms under well-watered conditions (Brodribb et al., 2005; Brodribb & Feild, 2010; Carriquí et al., 2015). Thus, ferns with no stomata open beyond the optimum for maximum A exhibited higher WUE than angiosperms (Deans et al., 2019). A linear regression of A vs g_s was observed with decreasing ψ_{leaf}, hence the unchanged value for WUE during dehydration. Three lines of evidence support the lack of increase in WUE:

(**1**) Stomatal conductance of ferns under well-watered conditions is similar to the stomatal conductance of angiosperms that have suffered moderate to severe drought stress; a decrease in g_s would greatly constrain A, which would be similar to that observed in angiosperms under moderate or severe drought stress (Fig. 2).

(**2**) Stomatal closure is mainly regulated by passive hydraulics, not by ABA (Brodribb & McAdam, 2011). Under 70% RH, g_s in leaves of *Athyrium filix-femina* and *Dryopteris filix-mas* decreased in response to an ABA spray. By contrast, no decrease was observed under 30% RH (Hõrak et al., 2017). A marked decrease in stomatal opening in response to ABA was also observed in stomatal aperture assays of *Polystichum proliferum* and *Nephrolepis exaltata* (Cai et al., 2017). However, stomata of *A. filix-femina* and *N. exaltata* did not respond to endogenous ABA accumulation during drought stress (Cardoso et al., 2019; Cardoso & McAdam, 2019).

(**3**) Even under the assumption that stomata of some ferns would respond to ABA, stomata not opening beyond the optimum for WUE would result in no improvement in WUE.

In this study, the conditions in the glasshouse were very similar to those in the field, so as to avoid the possibility of an anomalous behaviour of ferns in the glasshouse. Therefore, we contend that an increase in WUE in seed plants confers an improvement in productivity per unit water lost in transpiration, thereby providing them with a competitive advantage under water deficit absent in ferns. Data obtained in this study are consistent with previous knowledge that fern species compete weakly and are less distributed in arid environments (Karst et al., 2005; Bickford & Laffan, 2006; Link-Pérez & Laffan, 2018).

In conclusion, our results suggest that the rapid decrease in 'excessive' stomatal opening in response to elevated ABA occurring after the establishment of early-diverging vascular plants, such as ferns, has enabled seed plants to greatly improve WUE and, consequently, their overall performance under drought conditions compared with their predecessors. Although a more effective

hydraulic mesophyll conductance is the basis for 'excessive' stomatal opening and a higher rate of photosynthesis in seed plants that would provide them with superior ability for growth to subsequently replace their predecessors (Brodribb et al., 2005; Brodribb & Feild, 2010; Gago et al., 2013; Carriquí et al., 2015), this study showed that the increase in WUE in seed plants was superior to that in ferns under changing water status, which seems to have contributed to the increased propagation of seed plants in arid regions (Dolan, 2001; Brodribb et al., 2007, 2012; Hetherington & Dolan, 2019). Altogether, our results provide a strong line of support to the theoretical framework of the evolution of plant species.

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Author contributions

X-WF and TJB designed the experiments and wrote the manuscript; M-HB and Y-JY prepared the figures in the manuscript; M-HB, Y-JY, Z-FN, HJ and X-DL collected most of the data. Y-JY and M-HB contributed equally to this work.

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References

Aasamaa K, Sõber A, Hartung W, Niinemets Ü. 2002. Rate of stomatal opening, shoot hydraulic conductance and photosynthetic characteristics in relation to leaf abscisic acid concentration in six temperate deciduous trees. *Tree Physiology* 22: 267–276.

Adams HD, Zeppel MJB, Anderegg WRL, Hartmann H, Landhäusser SM, Tissue DT, Huxman TE, Hudson PJ, Franz TE, Allen CD *et al.* 2017. A multi-species synthesis of physiological mechanisms in drought-induced tree mortality. *Nature Ecology Evolution* 1: 1285–1291.

Álvarez S, Navarro A, Nicolás E, Sánchez-Blanco MJ. 2011. Transpiration, photosynthetic responses, tissue water relations and dry mass partitioning in callistemon plants during drought conditions. *Scientia Horticulturae* 129: 306–312.

Assmann SM, Jegla T. 2016. Guard cell sensory systems: recent insights on stomatal responses to light, abscisic acid, and CO$_2$. *Current Opinion in Plant Biology* 33: 157–167.

Bickford SA, Laffan SW. 2006. Multi-extent analysis of the relationship between pteridophyte species richness and climate. *Global Ecology and Biogeography* 15: 588–601.

Bohnert HJ, Nelson DE, Jensen RG. 1995. Adaptations to environmental stresses. *The Plant Cell* 7: 1099.

Boyce CK, Brodribb TJ, Feild TS, Zwieniecki MA. 2009. Angiosperm leaf vein evolution was physiologically and environmentally transformative. *Proceedings of the Royal Society B* **276**: 1771–1776.

Brodribb TJ. 1996. Dynamics of changing intercellular CO_2 concentration (ci) during drought and determination of minimum functional ci. *Plant Physiology* **111**: 179–185.

Brodribb TJ, Feild TS, Jordan GJ. 2007. Leaf maximum photosynthetic rate and venation are linked by hydraulics. *Plant Physiology* **144**: 1890–1898.

Brodribb TJ, Feild TS. 2010. Leaf hydraulic evolution led a surge in leaf photosynthetic capacity during early angiosperm diversification. *Ecology Letters* **13**: 175–183.

Brodribb TJ, Holbrook NM, Zwieniecki MA, Palma B. 2005. Leaf hydraulic capacity in ferns, conifers and angiosperms: impacts on photosynthetic maxima. *New Phytologist* **165**: 839–846.

Brodribb TJ, McAdam SA, Jordan GJ, Feild TS. 2009. Evolution of stomatal responsiveness to CO_2 and optimization of water-use efficiency among land plants. *New Phytologist* **183**: 839–847.

Brodribb TJ, McAdam SA. 2011. Passive origins of stomatal control in vascular plants. *Science* **331**: 582–585.

Brodribb TJ, McAdam SA, Carins Murphy MR. 2017. Xylem and stomata, coordinated through time and space. *Plant, Cell & Environment* **40**: 872–880.

Brodribb TJ, Pittermann J, Coomes DA. 2012. Elegance versus speed: examining the competition between conifer and angiosperm trees. *International Journal of Plant Sciences* **173**: 673–694.

Buckley TN, Sack L, Farquhar GD. 2017. Optimal plant water economy. *Plant, Cell & Environment* **40**: 881–896.

Burnham KP, Anderson DR. 2004. Multimodel inference: understanding AIC and BIC in model selection. *Sociological Methods & Research* **33**: 261–304.

Cai SG, Chen G, Wang YY, Huang YQ, Marchant DB, Wang YZ, Yang Q, Dai F, Hills A, Franks PJ et al. 2017. Evolutionary conservation of ABA signaling for stomatal closure. *Plant Physiology* **174**: 732–747.

Campitelli BE, Des Marais DL, Juenger TE. 2016. Ecological interactions and the fitness effect of water-use efficiency: competition and drought alter the impact of natural MPK12 alleles in Arabidopsis. *Ecology Letters* **19**: 424–434.

Cardoso AA, McAdam SAM. 2019. Misleading conclusions from exogenous ABA application: a cautionary tale about the evolution of stomatal responses to changes in leaf water status. *Plant Signaling & Behaviour* **14**: 1610307.

Cardoso AA, Randall JM, Mcadam SA. 2019. Hydraulics Regulate Stomatal Responses to Changes in Leaf Water Status in the Fern *Athyrium filix-femina*. *Plant Physiology* **179**: 533–543.

Carriquí M, Cabrera HM, Conesa MÀ, Coopman RE, Douthe C, Gago J, Gallé A, Galmés J, Ribas-Carbo M, Tomás M et al. 2015. Diffusional limitations explain the lower photosynthetic capacity of ferns as compared with angiosperms in a common garden study. *Plant, Cell & Environment* **38**: 448–460.

Chen RF, Yang HY, XiH G, Zheng ZL. 2019. Analysis of microclimate characteristics in Xiaolongshan forest ecological station area. *Journal of Gansu Forestry Science and Technolgy* **44**: 5–8.

Cowan IR, Farquhar GD. 1977. Stomatal function in relation to leaf metabolism and environment. *Symposia of the Society for Experimental Biology* **121**: 471–505.

Davies WJ, Wilkinson S, Loveys B. 2002. Stomatal control by chemical signalling and the exploitation of this mechanism to increase water use efficiency in agriculture. *New Phytologist* **153**: 449–460.

Davies WJ, Zhang J. 1989. Chemical regulation of growth and physiology in drying soil: the case for abscisic acid. *Current Topics in Plant Biochemistry and Physiology* **1989**: 100–109.

Deans RM, Brodribb TJ, Busch FA, Farquhar GD. 2019. Plant water-use strategy mediates stomatal effects on the light induction of photosynthesis. *New Phytologist* **222**: 382–395.

Dodd IC. 2013. Abscisic acid and stomatal closure: a hydraulic conductance conundrum? *New Phytologist* **197**: 6–8.

Dolan L. 2001. Plant development: the benefits of a change of scene. *Current Biology* **11**: R702–R704.

Fang XW, Turner NC, Yan GJ, Li FM, Siddique KHM. 2010. Flower numbers, pod production, pollen viability, and pistil functionare reduced and flower and pod abortion increased in chickpea (*Cicer arietinum* L.) under terminal drought. *Journal of Experimental Botany* **61**: 335–345.

Fang XW, Turner NC, Li FM, Li WJ, Guo XS. 2011. *Caragana korshinskii* seedlings maintain positive photosynthesis during short-term, severe drought stress. *Photosynthetica* **49**: 603–609.

Feild TS, Brodribb TJ. 2013. Hydraulic tuning of vein cell microstructure in the evolution ofangiosperm venation networks. *New Phytologist* **199**: 720–726.

Fischer RA, Turner NC. 1978. Plant productivity in the arid and semiarid zones. *Annual Reviews of Plant Physiology* **29**: 277–317.

Franks PJ. 2006. Higher rates of leaf gas exchange are associated with higher leaf hydrodynamic pressure gradients. *Plant, Cell & Environment* **29**: 584–592.

Franks PJ, Farquhar GD. 2001. The effect of exogenous abscisic acid on stomatal development, stomatal mechanics, and leaf gas exchange in *Tradescantia virginiana*. *Plant Physiology* **125**: 935–942.

Gago J, Coopman RE, Cabrera HM, Hermida C, Molins A, Conesa MÀ, Galmés J, Ribas-Carbó M, Flexas J. 2013. Photosynthesis limitations in three fern species. *Physiologia Plantarum* **149**: 599–611.

Gago J, Douthe C, Florez-Sarasa I, Escalona JM, Galmes J, Fernie AR, Flexas J, Medrano H. 2014. Opportunities for improving leaf water use efficiency under climate change conditions. *Plant Science* **226**: 108–119.

Hetherington AJ, Dolan L. 2019. Evolution: diversification of angiosperm rooting systems in the early cretaceous. *Current Biology* **29**: R1081–R1083.

Hetherington AM, Woodward FI. 2003. The role of stomata in sensing and driving environmental change. *Nature* **424**: 901–908.

Holaday SA, Ritchie SW, Nguyen HT. 1992. Effects of water deficit on gas-exchange parameters and ribulose 1, 5-bisphosphate carboxylase activation in wheat. *Environmental and Experimental Botany* **32**: 403–410.

Hõrak H, Kollist H, Merilo E. 2017. Fern stomatal responses to ABA and CO_2 depend on species and growth conditions. *Plant Physiology* **174**: 672–679.

Karst J, Gilbert B, Lechowicz MJ. 2005. Fern community assembly: the roles of chance and the environment at local and intermediate scales. *Ecology* **86**: 2473–2486.

Lim CW, Baek W, Jung J, Kim JH, Lee SC. 2015. Function of ABA in stomatal defense against biotic and drought stresses. *International Journal of Molecular Sciences* **16**: 15251–15270.

Link-Pérez MA, Laffan SW. 2018. Fern and lycophyte diversity in the Pacific Northwest: patterns and predictors. *Journal of Systematics and Evolution* **56**: 498–522.

Manzoni S, Vico G, Katul G, Fay PA, Polley W, Palmroth S, Porporato A. 2011. Optimizing stomatal conductance for maximum carbon gain under water stress: a meta-analysis across plant functional types and climates. *Functional Ecology* **25**: 456–467.

Mawson BT, Colman B, Cummins WR. 1981. Abscisic acid and photosynthesis in isolated leaf mesophyll cell. *Plant Physiology* **67**: 233–236.

McAdam SA, Brodribb TJ. 2012. Stomatal innovation and the rise of seed plants. *Ecology Letters* **15**: 1–8.

McAdam SA, Brodribb TJ. 2015. The evolution of mechanisms driving the stomatal response to vapor pressure deficit. *Plant Physiology* **167**: 833–843.

McAdam SA, Brodribb TJ. 2016. Linking turgor with ABA biosynthesis: implications for stomatal responses to vapor pressure deficit across land plants. *Plant Physiology* **171**: 2008–2016.

McAdam SA, Brodribb TJ. 2018. Mesophyll cells are the main site of abscisic acid biosynthesis in water-stressed leaves. *Plant Physiology* **177**: 911–917.

Medrano H, Tomás M, Martorell S, Flexas J, Hernández E, Rosselló J, Pou A, Escalona JM, Bota J. 2015. From leaf to whole-plant water use efficiency (WUE) in complex canopies: Limitations of leaf WUE as a selection target. *The Crop Journal* **3**: 220–228.

Misson L, Panek JA, Goldstein AH. 2004. A comparison of three approaches to modeling leaf gas exchange in annually drought-stressed ponderosa pine forests. *Tree Physiology* **24**: 529–541.

Negin B, Moshelion M. 2016. The evolution of the role of ABA in the regulation of water-use efficiency: From biochemical mechanisms to stomatal conductance. *Plant Science* **251**: 82–89.

Pammenter NW, Willigen CV. 1998. A mathematical and statistical analysis of the curves illustrating vulnerability of xylem to cavitation. *Tree Physiology* **18**: 589–593.

Pantin F, Monnet F, Jannaud D, Costa JM, Renaud J, Muller B, Simonneau T, Genty B. 2013. The dual effect of abscisic acid on stomata. *New Phytologist* 197: 65–72.

Scoffoni C, McKown AD, Rawls M, Sack L. 2012. Dynamics of leaf hydraulic conductance with water status: quantification and analysis of species differences under steady state. *Journal of Experimental Botany* 63: 643–658.

Waseem M, Nie ZF, Yao GQ, Hasan M, Xiang Y, Fang XW. 2021. Dew absorption by leaf trichomes in *Caragana korshinskii*: An alternative water acquisition strategy for withstanding drought in arid environments. *Physiologia Plantarum*. doi: 10.1111/ppl.13334.

Xiao L, Yobi A, Koster KL, He Y, Oliver MJ. 2018. Desiccation tolerance in Physcomitrella patens: rate of dehydration and the involvement of endogenous abscisic acid (ABA). *Plant, Cell & Environment* 41: 275–284.

Xiong D, Douthe C, Flexas J. 2018. Differential coordination of stomatal conductance, mesophyll conductance, and leaf hydraulic conductance in response to changing light across species. *Plant, Cell & Environment* 41: 436–450.

Yao GQ, Li FP, Nie ZF, Bi MH, Jiang H, Liu XD, Wei Y, Fang XW. 2021a. Ethylene, not ABA, is closely linked to the recovery of gas exchange after drought in four *Caragana* species. *Plant, Cell & Environment* 44: 399–411.

Yao GQ, Nie ZF, Turner NC, Li FM, Gao TP, Fang XW, Scoffoni C. 2021b. Combined high leaf hydraulic safety and efficiency provides drought tolerance in *Caragana* species adapted to low mean annual precipitation. *New Phytologist* 229: 230–244.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 The correlation of A, g_s, E with decreasing ψ_{leaf} in ferns.

Fig. S2 The correlation of A, g_s, E with decreasing ψ_{leaf} in gymnosperms.

Fig. S3 The correlation of A, g_s, E with decreasing ψ_{leaf} in angiosperms.

Fig. S4 The correlation of WUE_i with decreasing ψ_{leaf} in three lineages.

Fig. S5 The correlation of WUE_{ins} with decreasing ψ_{leaf} in three lineages.

Fig. S6 The correlation of ABA with decreasing ψ_{leaf}, and of g_s with ABA in ferns.

Fig. S7 Response of g_s to exogenous ABA in ferns.

Fig. S8 The correlation of ABA with decreasing ψ_{leaf}, and of g_s with ABA in gymnosperms.

Fig. S9 The correlation of ABA with decreasing ψ_{leaf}, and of g_s with ABA in angiosperms.

Tables S1 Summary of physiological traits, and results of one-way ANOVAs and t-tests for the difference across three lineages, between gymnosperms and angiosperms and between AP_{50} and g_sP_{50}, AP_{50} and EP_{50} within each lineage.

Tables S2 Summary of WUE_i and WUE_{ins}, and results of one-way ANOVAs and t-tests for the difference across three lineages and between gymnosperms and angiosperms.

Tables S3 Dataset of WUEi and WUEins of seed plants and ferns under well-watered conditions and moderate drought stress.

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