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LETTER

Stomatal innovation and the rise of seed plants

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Abstract

Stomatal valves on the leaves of vascular plants not only prevent desiccation but also dynamically regulate water loss to maintain efficient daytime water use. This latter process involves sophisticated active control of stomatal aperture that may be absent from early-branching plant clades. To test this hypothesis, we compare the stomatal response to light intensity in 13 species of ferns and lycophytes with a diverse sample of seed plants to determine whether the capacity to optimise water use is an ancestral or derived feature of stomatal physiology. We found that in seed plants, the ratio of photosynthesis to water use remained high and constant at different light intensities, but fern and lycophyte stomata were incapable of sustaining homeostatic water use efficiency. We conclude that efficient water use in early seed plants provided them with a competitive advantage that contributed to the decline of fern and lycophyte dominated-ecosystems in the late Paleozoic.

Keywords

Ferns, lycophytes, plant evolution, stomata, water use efficiency.

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INTRODUCTION

The vast volume of water transpired from the leaves of terrestrial vegetation plays a critical role in maintaining both the global water cycle and climatic stability (Hetherington & Woodward 2003). However, for plants themselves, the parallel flow of water out of leaves during photosynthesis represents one of the greatest costs associated with life on land. Even when soil water is abundant, the price of replenishing transpired water is high, including major investments in roots for water acquisition and an internal vascular network dedicated to water transport (Raven & Handley 1987; Tyree & Sperry 1989; Raven & Edwards 2001; Pittermann 2010). The combination of high transport costs and a finite availability of water in soils places a large selective pressure on plants to economise water use (Raven 1993). The most readily observable adaptation to this pressure is the evolution of adjustable stomata that allow plants to actively regulate transpiration in response to changes in environmental conditions (Raven 2002). By regulating transpiration in response to light and atmospheric dynamics, stomata enable plants to minimise water loss for a given rate of CO_2 uptake during photosynthesis (Wong *et al.* 1979; Hari *et al.* 1999).

To optimise photosynthetic gain relative to transpirational losses (water use efficiency), stomata must regulate the porosity of the leaf to both CO_2 and H_2O (Cowan 1977; Cowan & Farquhar 1977; Farquhar & Sharkey 1982). Indeed, it has been shown that, in response to a wide range of environmental conditions, stomata appear to operate in an optimised fashion, regulating leaf porosity such that water use remains highly efficient under changing environmental conditions (Cowan 1977; Cowan & Farquhar 1977). The principles of dynamic optimisation of stomatal aperture in angiosperms are fundamental to the way we understand stomatal responses to the environment (Farquhar & Sharkey 1982), and have been generally applied to the modelling of gas exchange at scales ranging from the leaf to canopy (Buckley *et al.* 2003; Konrad *et al.* 2008; Dewar *et al.* 2009; Damour *et al.* 2010; Katul *et al.* 2010; de Boer *et al.* 2011). Indeed, it seems likely that the evolutionary pressure to improve the gas exchange ratio of $CO_2 : H_2O$ was a likely selection pressure canalising the function of stomata in the early vascular land plants, 400 million years ago (Raven 2002). However, the regulation of water use efficiency by stomata requires a complex feedback control of photosynthesis and transpiration (as seen in modern seed plants) and the applicability of this model across evolutionary time has only recently been considered. Curiously, recent advances in our understanding of stomatal control in early-branching vascular plant lineages suggest that the integrated metabolic stomatal control necessary for optimal regulation of stomatal aperture may not exist in more ancient plant clades such as ferns and lycophytes (Hollinger 1987; Doi *et al.* 2006; Doi & Shimazaki 2008; Brodribb *et al.* 2009; Brodribb & McAdam 2011; Haworth *et al.* 2011).

To maintain high water use efficiency under changing light conditions, stomata must track the leaf assimilation rate (A) and respond to maintain homeostasis in the exchange ratio of $CO_2 : H_2O$ (Wong *et al.* 1979). The mechanism of this control is poorly understood, but is known to involve an interaction between the mesophyll and stomata providing a feedback between stomatal opening and photosynthetic rate (Wong *et al.* 1979; Lee & Bowling 1992; Mott *et al.* 2008). However, in the two most basal lineages of extant vascular plants, the lycophytes and ferns, stomata have weak or no active metabolic responses to increased CO_2 concentration in both the light and dark (Doi & Shimazaki 2008; Brodribb *et al.* 2009; Ruszala *et al.* 2011), the phytohormone ABA (Brodribb & McAdam 2011; Ruszala *et al.* 2011) and phototropin-mediated blue light (Doi *et al.* 2006), and are extremely sensitive to leaf water status, acting as passive hydraulic valves in the light (Brodribb & McAdam 2011). Daytime stomatal aperture in the basal lineages of vascular plants is controlled by leaf water status rather than metabolic signalling, and this raises the possibility that these clades may not be able to maintain high water use efficiency under diurnal fluctuations in light intensity.

Through a reduction in water wastage, dynamic stomatal optimisation confers an important improvement in productivity per unit water lost as transpiration compared with a non-optimised condition (Cowan 1977). Hence, the possibility that this important feature of stomatal control is only present in some groups of vascular plants would have major implications in terms of explaining competitive

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outcomes between major plant lineages during land plant evolution, and for modelling atmosphere-plant canopy interactions through geologic time (Hetherington & Woodward 2003; Berry *et al.* 2010). Investigating such a possibility, herein, we test the hypothesis that the stomatal capacity to dynamically conserve high water use efficiency is a derived condition that evolved within seed plants. We examined the stomatal response to light in a wide phylogenetic and functionally divergent selection of fern and lycophyte species, and compared these with seed plants (angiosperms and gymnosperms) to test for basic differences in the way these plant groups regulate water loss.

MATERIALS AND METHODS

Species examined

The response of stomata to changes in light intensity were examined across a functionally and phylogenetically diverse selection of vascular plant species, including 7 angiosperms, 5 gymnosperms, 11 ferns and 2 lycophytes (Table S1). Direct observations of stomatal aperture on leaves, isolated epidermis and xenografts (cross-species transfers of epidermis to mesophyll) were made with five representative species including two angiosperms, a gymnosperm, a fern and a lycophyte species, selected to span the phylogeny of vascular plants (Table S1). All individuals were grown in pots in the glasshouses of the School of Plant Science, University of Tasmania, Hobart, Australia, where they received a maximum natural photosynthetic photon flux density (PPFD) of 1300 μmol quanta m^{-2} s^{-1} at the leaf level, and natural light was supplemented with sodium vapour lamps to ensure a minimum 300–500 μmol quanta m^{-2} s^{-1} at the leaf surface throughout the day period, 25 °C/15 °C day/night temperatures, with stomatal aperture observation experiments undertaken over late spring-early summer, November and December 2010, and leaf gas exchange parameters over summer, December to March 2011. All plants received 3-month applications of a slow-release fertiliser. In all species, the most recent fully expanded photosynthetic tissues were chosen for experiments.

Gas exchange over transitions in light intensity

Two protocols were used to test stomatal responses to transitions between high (1000 μmol quanta m^{-2} s^{-1}) and low (100 μmol quanta m^{-2} s^{-1}) light intensities. In a sub-sample of seven fern and lycophyte species, we examined stomatal dynamics for 1 h after light transitions from dark to high light, and high to low light intensity. The purpose of these long observation periods was to ensure that equilibrium stomatal conductance was maintained after each light transition. These measurements established that 30 min was sufficient time to achieve equilibrium stomatal conductance (< 1% change over 3 min), and hence we examined a second, larger group of 7 angiosperms, 5 gymnosperms, 11 ferns and 2 lycophytes allowing 30 min equilibration between transitions from dark to high light to low light. Leaf-level gas exchange was measured using an infrared gas analyser (Li-6400; Li-Cor, Lincon, NE, USA) with leaves enclosed in a cuvette maintained at a constant 22 °C, vapour pressure deficit (VPD) was maintained between 1.2 and 1.4 kPa and CO_2 concentration in the air kept at ambient concentrations (390 μmol mol^{-1}). Fern and lycophyte species were brought into the laboratory the night prior to experimentation, watered, bagged in a plastic bag and kept in a dark cabinet. To reduce the effects of diurnal variations in evaporative demand and circadian rhythms on g_s measurements individual leaf measurements were initiated no later than 07:30 h, with only one series attempted per day. To prevent changes in plant hydration, leaves outside the leaf cuvette were kept bagged and damp throughout the duration of the experiment.

Stomatal aperture observation over light transitions

Light microscopy was used to examine the response of stomatal aperture of live leaves, isolated epidermes and xenografts (see Appendix S1) to light intensity in a sub-sample of five species selected to span the phylogeny of vascular plants (Table S1). Plants were brought into the laboratory the night before experimentation, the selected branch or leaf was enclosed in a black polyethylene bag, and the plant was placed in a dark room. Isolated epidermes were prepared on damp blotting paper under a stereomicroscope, with illumination provided by a green fluorescent light (photosynthetic photon flux density (PPFD) < 1 μmol quanta m^{-2} s^{-1}), by completely removing the upper epidermis and mesophyll using a razor blade, and this method is known to result in isolated epidermis that has a high number of viable stomata capable of sustaining responses to changes in environmental conditions for more than 4 h (Mott *et al.* 2008; Sibbernsen & Mott 2010). Epidermes were then mounted on a bridge of damp blotting paper placed in a 5 mL solution of 50 mM KCl, 0.1 mM $CaCl_2$, 10 mM 2-(N-morpholino)ethanesulfonic acid, pH 6.8 in the Perspex base of a controlled environment chamber. Air flow through the chamber was supplied by an industrial compressed air cylinder (CO_2: 420 ± 10 μmol mol^{-1}) passing at a flow rate of 310 mL min^{-1} through an ADC HG-1 water vapour generator (Hodderson, UK) at the highest setting for water vapour generation heating the ferrous sulphate columns to 34.2 °C, resulting in a VPD close to zero in the air passing through the chamber. Temperature in the chamber was monitored using a fine wire thermocouple and logged to a Campbell Scientific CR10X data logger (chamber air temperature: 22 ± 2 °C). The required light intensity in the chamber was supplied from above using a Schott KL1500 fibreoptic light source, and monitored at the leaf or epidermal level in the chamber using a Walz 2060-M 1.5 mm diameter microquantum sensor (Heinz Walz GmbH, Effeltrich, Germany) that was calibrated regularly against a Li-Cor LI190SZ quantum sensor (Lincoln, NE, USA). Live leaves were secured in the chamber with the attached stem or leaf base extending out of the chamber between rubber seals, and the remaining leaves on the stem were removed and the plant covered in a black plastic bag to reduce any negative hydraulic influence from the rest of the plant on the leaf in the chamber.

Once the leaf, epidermis or xenograft was secured in the chamber on the stage of a Zeiss Axiolab light microscope (Oberkochen, Germany), the stage light was illuminated, and using a ×20 long-working distance objective (LD Epiplan ×20/0.4, Carl Zeiss, Oberkochen, Germany) four live, viable stomata, were selected within close proximity of each other, and their positions relative to veins and easily recognisable epidermal features were noted. Viability of stomata was visually assessed using established criteria (Rogers *et al.* 1981). Only symmetrical guard cells containing smooth, round chloroplasts and surrounded by intact epidermal cells were used. Stomatal viability was confirmed in all samples by an opening response to guard cell illumination. The selection of a small population of closely neighbouring viable stomata enabled dynamic tracking of stomata over the entire experimental period, and as a technique has been shown to offer the least variability in measurements of stomatal

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aperture from isolated epidermis (Gorton *et al.* 1989; Mott *et al.* 2008). The leaf, epidermis or xenograft was left in darkness for a 40-min acclimation period in the chamber. Lights were then turned on illuminating the epidermis initially at 1000 μmol quanta m^{-2} s^{-1} for 30 min, then 100 μmol quanta m^{-2} s^{-1} for 30 min, and finally 2 h and 35 min of darkness; during this time, the aperture of the four selected stomata was regularly measured at predetermined intervals using illumination from the microscope stage light on a Nikon Digital sight DS-L1 camera (Tokyo, Japan) attached to a ×2.5 magnification tube, this measurement of aperture took less than 1 min for all four stomates. The treatments for each species were repeated, so that data represent eight individual live and viable stomata. A Li-6400 was used to observe the gas exchange responses of g_s and A in the five species examined to a similar series of transitions in light intensity (see Appendix S1).

RESULTS

Instantaneous response of stomata to light

Changes between high-light intensity (1000 μmol quanta m^{-2} s^{-1}) and low-light intensity (100 μmol quanta m^{-2} s^{-1}) produced a similar range of photosynthetic responses in all species (mean percent change in assimilation rate ± SEM, seed plants: 56.03 ± 2.69%; ferns and lycophytes: 48.58 ± 3.00%, $P > 0.1$, single factor ANOVA), but the stomata of ferns and lycophytes were found to be much less dynamic in their responses to light than seed plants (Figs 1, 2 and S1). Differences between seed plants and ferns and lycophytes were very clear during transitions between high light and low light whereupon seed plant stomata always closed significantly more than fern and lycophyte stomata (mean percent reduction in stomatal conductance (g_s) ± SEM, seed plants: 56.68 ± 2.8%; ferns and lycophytes: 11.9 ± 4.2%, $P < 0.001$, single factor ANOVA, Figs 1, 2 and S1). The mean rate of stomatal closure when expressed as a percentage of initial rate was significantly slower in the fern and lycophyte species (mean per cent change in g_s 0.033 ± 0.025% s^{-1}) compared with the seed plants (0.081 ± 0.040% s^{-1}) ($P < 0.05$, single factor ANOVA), although there was considerable overlap between groups, and in terms of absolute rates of change in g_s, no statistical difference between the two groups was identified ($P > 0.05$, single factor ANOVA) (Table S2). During transitions from darkness to low-light intensity, there was no significant difference in the mean stomatal opening speed of fern and lycophyte species compared with seed plants (the fastest response of all species being observed in the fern *Marselia hirsuta;* Table S2). Despite similar response dynamics, the stomata of ferns and lycophytes opened 95% more than seed plants when exposed to low light from darkness (mean for ferns and lycophytes: 0.114 ± 0.008 mol m^{-2} s^{-1} compared with 0.063 ± 0.006 mol m^{-2} s^{-1} in seed plants), despite no significant difference in assimilation rate between groups (Fig. S2). Differences in stomatal behaviour between the groups of vascular plants were not associated with incompetent stomatal closure or high rates of cuticular water loss (Fig. S3), as demonstrated by the fact that mild-

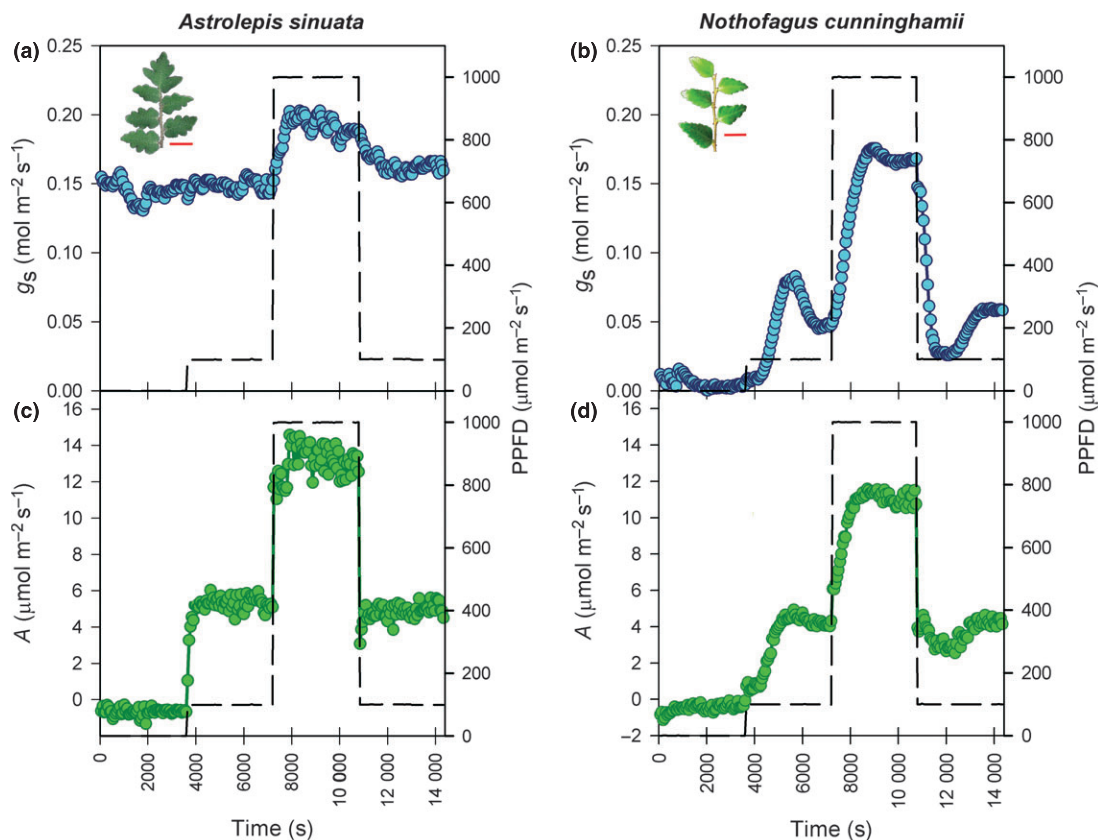

Figure 1 Ferns display a reduced stomatal response to transitions in light intensity (PPFD) without evidence of a feedback between assimilation and stomatal conductance, commonly observed in seed plants. Leaves of the representative fern *Astrolepis sinuata* (a and c) and angiosperm *Nothofagus cunninghamii* (b and d) exposed to the same series of 1 h transitions in PPFD (dashed line), stomatal conductance (a and b) and assimilation (c and d) were recorded every 1 min. Insert depicts foliage of the respective species (scale bar = 1 cm).

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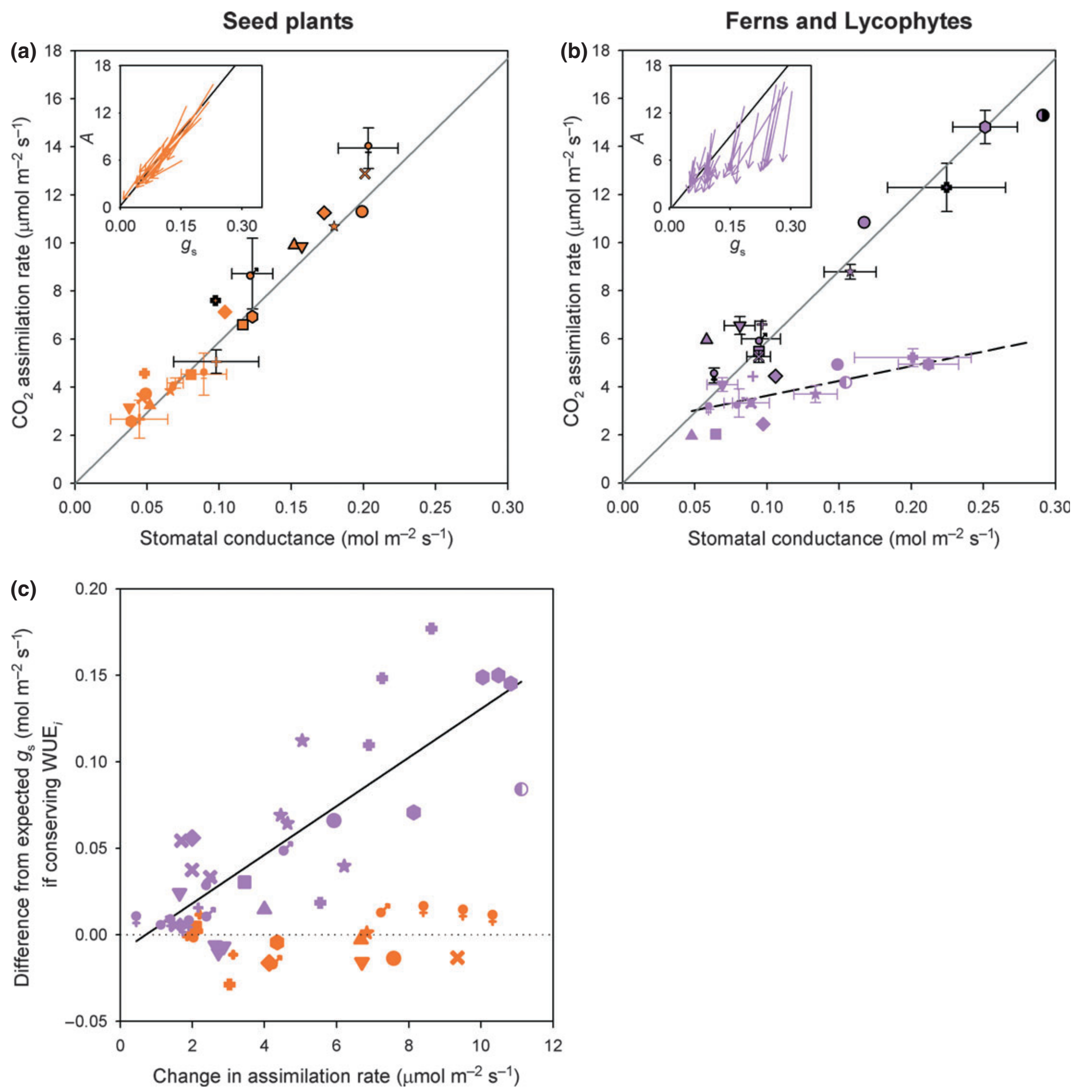

Figure 2 Reduced stomatal response to decreasing light intensity in ferns and lycophytes (purple symbols) results in water wastage relative to seed plants (orange symbols). Under saturating light (1000 μmol quanta m^{-2} s^{-1}; all black-bordered symbols) the ratio of assimilation (*A*): stomatal conductance (g_s) was similar in all species including seed plants (a) and ferns and lycophytes (b) (grey regression line reflecting conservative intrinsic water use efficiency (WUE_i) in all vascular species R^2 = 0.81). However, only seed plants were able to maintain high WUE_i at low light intensity (100 μmol quanta m^{-2} s^{-1}; unbordered symbols). In contrast, ferns and lycophytes wasted water by producing stomatal conductances significantly higher than the value required to conserve WUE_i (shown by the proportional regression line). The responses of individual leaves (insert graphs; arrowed lines connect data from before and after transitions from high to low light intensity) demonstrate how seed plants (a) (orange) maintain a proportional relationship between g_s and assimilation rate, whereas ferns and lycophytes (b) (purple lines) do not close stomata sufficiently to conserve high WUE_i at low light. (c) Water wastage (deviation from the proportional regression in Fig. 2a,b) in fern and lycophyte species was correlated with the change *A* during the transition from high to low light (see Table S1 for a key to the symbols and corresponding species).

water stress produced a rapid stomatal closure in all fern and lycophyte species resulting in low minimum leaf conductances to water vapour (< 0.010 mol m^{-2} s^{-1} in all species except the semi-aquatic species *M. hirsuta*) (Fig. S3). However, hydrated leaves of ferns and lycophytes produced significantly higher stomatal conductances than seed plants in the dark ($P < 0.001$, single factor ANOVA, Fig. S1).

Regulation of water use efficiency

Reduced responsiveness of fern and lycophyte stomata to light when compared with seed plants markedly affected the ability of these plants to maintain high water use efficiency at non-saturating light intensity (Fig. 2). Under saturating light, a linear relationship between assimilation rate and g_s in all species indicated that intrinsic water use efficiency (WUE_i; A/g_s) was conservative among the entire vascular plant sample (Fig. 2a,b). In contrast, significant differences in WUE_i between the fern and lycophyte species and seed plants emerged when leaves were measured at a non-saturating light intensity of 100 μmol quanta m^{-2} s^{-1} (Fig. 2b). Stomatal closure in angiosperms and gymnosperms maintained constant high WUE_i after the transition from high to low light ($P > 0.05$, single factor ANOVA, Fig. 2a); however, in fern and lycophyte species, WUE_i was markedly lower at low light compared with high light ($P < 0.001$, single factor ANOVA, Fig. 2b). Reduced WUE_i in ferns and lycophytes was the greatest for species that displayed the largest changes in assimilation rate following

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the transition from high- to low-light intensity (Fig. 2c). Hence, in fern and lycophyte species that were close to photosynthetic saturation at low light, there was little change in WUE_i when light intensity was lowered from 1000 to 100 μmol quanta m^{-2} s^{-1}, whereas in species with photosynthetic rates > 8 μmol CO_2 m^{-2} s^{-1}, the transition to low light resulted in wasteful water losses ranging from 74% to 212% above the level required for constant WUE_i (Fig. 2c). Very similar results were obtained when light intensities were increased from dark to 100 μmol quanta m^{-2} s^{-1} (Fig. S2), and in this case, the fern and lycophyte species opened stomata far beyond the point required to maintain high WUE_s resulting in a similar wastage of water as plants exposed to a transition from high to low light. Under the same conditions, seed plants only opened sufficiently to reach the point where WUE_i was maintained at the same high level achieved under high light (Fig. S2).

Mesophyll-stomatal feedback

When stomata were isolated from the leaf mesophyll, the behaviour of seed plant stomata changed, whereas the representative fern and lycophyte species showed little impediment of function (Figs 3 and S4). When examined *in situ* on the leaf, the stomata of the seed plants examined changed by > 4 μm during transitions between high and low light (Figs 3 and S4), with a similar dynamic to that observed in g_s (Figs S4 and S5). However, when stomata were isolated from seed plant leaves, they responded to increases in light intensity, but failed to close in response to any decrease in light intensity after the initial stomatal opening (Figs 3 and S4). Normal stomatal function in seed plants could be restored in isolated stomata if epidermal strips were reattached to mesophyll, even if this mesophyll was from a fern species (Fig. S6).

Unlike seed plants, fern and lycophyte stomata responded identically, regardless of whether they remained attached to the leaf or isolated from the mesophyll ($P > 0.05$, paired two-tailed *t*-tests; Figs 3 and S4). *In situ* and isolated stomata of the fern *Dryopteris cycadina* and lycophyte *Selaginella kraussiana* opened rapidly in response to a transition from dark to high light, increasing aperture from < 3 to > 6 μm in both species (Figs 3 and S4). Following the transition from high to low light stomatal aperture from both isolated epidermis and live leaves did not decrease significantly over 30 min (Figs 3 and S4), but the transition to dark caused a closure dynamic similar to that observed from g_s measurements (Figs 3 and S5).

DISCUSSION

In contrast to seed plants, we found that a diverse sample of fern and lycophyte species were unable to maintain a high instantaneous water use efficiency following transitions in light intensity (Fig. 2). Among our sample of two lycophyte clades and early and late branching fern clades (including sun and shade dwelling species) (Table S1), no species was capable of regulating stomata to prevent wastage of water as photosynthetic conditions changed. Therefore, we conclude that although fern and lycophyte stomata have a clear response to red light (Doi *et al.* 2006), only seed plants possess a feedback control between assimilation rate and stomatal aperture that enables leaves to maintain a constant and high ratio of $CO_2 : H_2O$ exchange under changing photosynthetic conditions (Wong *et al.* 1979). These data add to recent studies suggesting that important evolution in the function of stomata occurred after their first appearance > 400 million years ago (Doi *et al.* 2006; Brodribb *et al.* 2009), challenging the view that stomatal physiology has remained conserved since the Devonian period (Beerling & Franks 2009; Chater *et al.* 2011).

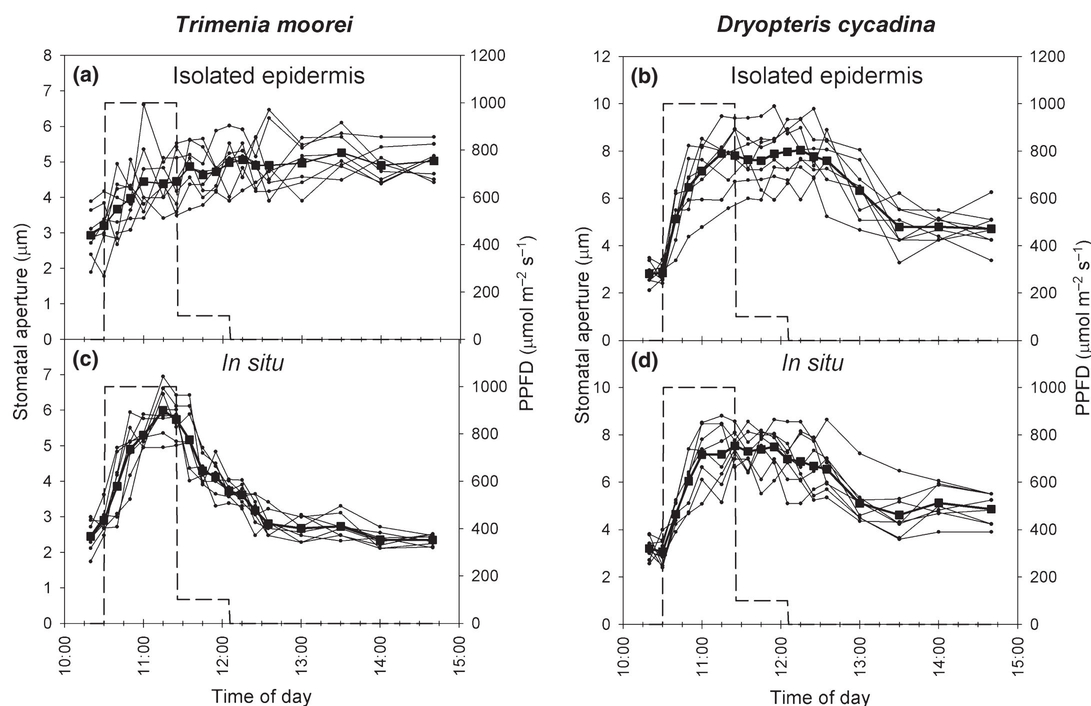

Figure 3 The response of stomatal aperture to a sequence of light intensities (dashed line) on isolated epidermes (a and b) and intact leaves (c and d) of the basal angiosperm *Trimenia moorei* (a and c) and fern *Dryopteris cycadina* (b and d). Small circles on thin lines represent individual stomata, and large squares on the thick line represents the average ($N = 8$). The stomata of *T. moorei* (and other seed plants) became insensitive to reductions in light intensity, once isolated from the leaf, whereas *D. cycadina* and lycophyte stomata behaved identically whether they were connected or excised from leaves (see Fig. S4 for other species).

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Our data strongly support the hypothesis that fern and lycophyte species lack an important component of stomatal control that is present in seed plants, rather than the alternative possibility that fern and lycophyte stomata are simply too slow or leaky to achieve optimal control of transpiration (Franks & Farquhar 2007). Although the mean rate of stomatal closure in the fern and lycophyte species examined was slower in terms of percentage change in conductance than seed plants, there was no difference in the rates of stomatal opening (Table S2), and yet during both stomatal opening and closure, we found that fern stomata were more wasteful of water compared with seed plants (Figs 2 and S2). Stomatal dysfunction and/or cuticular leakiness were additionally ruled out as factors that may have explained a lack of water economy in fern and lycophyte species. As shown here and previously, fern and lycophyte species have high stomatal sensitivity to changes in guard cell turgor (Brodribb & McAdam 2011) and very effective closure in response to desiccation (Fig. S3). In the absence of other explanations for the sub-optimal behaviour of fern and lycophyte stomata, we conclude that a critical component of the stomatal control process that responds to a feedback signal from mesophyll photosynthetic rate to the guard cells in seed plants is not present in fern and lycophyte species. Such a feedback mechanism is required for the optimal control of water loss; it has long been recognised that the presence of the mesophyll is required for normal responsiveness of stomata to changes in irradiance (Mouravieff 1956, 1957) and that the feedback signal responsible for this arises in the photosynthetic tissue of the leaf and is transmitted to the stomatal guard cells (Lee & Bowling 1992, 1995; Sibbernsen & Mott 2010). Our data indicate that this mesophyll-guard cell signal is absent in fern and lycophyte species, because removing stomata from the mesophyll had no effect on stomatal function in fern and lycophyte species, whereas in seed plants, we found that excised stomata lost the ability to respond optimally to light [Figs 3 and S4; see also Mott *et al.* (2008)].

The capacity of stomata to maintain high leaf water use efficiency under changing light conditions appears to have evolved after the divergence of ferns, < 360 million years ago (Pryer *et al.* 2004), and coincides with a major evolutionary pulse of metabolic stomatal control processes in the early seed-bearing vascular plants (Fig. 4). Combining our data with recent discoveries about the stomatal physiology of early-branching land plants, a reconstruction of the major transitions in the functional evolution of stomatal control based upon broad patterns preserved among extant representatives of ancient lineages is now possible (Fig. 4). Six extant land plant lineages possess stomata, the sporophytes of two non-vascular bryophyte groups (mosses and hornworts), two spore-bearing vascular plant lineages (lycophytes and ferns) and two seed-bearing vascular plant lineages (gymnosperms and angiosperms) (Ziegler 1987). Stomata in the two bryophyte groups, although not widely examined, appear to encompass a large diversity of morphologies and functions (Paton & Pearce 1957; Lucas & Renzaglia 2002; Duckett *et al.* 2009), and are often not involved in water conservation (Garner & Paolillo 1973; Hartung *et al.* 1987; Lucas & Renzaglia 2002; Duckett *et al.* 2009). Evidence of stomatal control by ABA is equivocal in bryophytes, with weak stomatal responses to ABA reported in the single-celled stomata of Funariaceae species when measured *in vitro* (Garner & Paolillo 1973; Chater *et al.* 2011), but contradictory data have been shown for mosses and hornworts (Paton & Pearce 1957; Lucas & Renzaglia 2002).

A canalisation of stomatal physiology seems to have occurred following the divergence of vascular plants, whereupon stomata became uniquely involved in water conservation and desiccation prevention (Lucas & Renzaglia 2002; Raven 2002; Duckett *et al.* 2009; Brodribb & McAdam 2011). Stomata of the basal lineages of vascular plants, the lycophytes and ferns, are characterised by an opening response to photosynthetically active red light (Doi & Shimazaki 2008; Lawson 2009) (Fig. 3), but transpiration rates are insensitive to physiologically relevant concentrations of ABA (Brodribb & McAdam 2011; Ruszala *et al.* 2011), with stomata controlled passively by leaf water content during the day (Brodribb & McAdam 2011). The evolution of seed-bearing vascular plants in the Paleozoic era coincides with a significant evolutionary shift in stomatal function, with increased metabolic control of stomata (Fig. 4). Key changes include a transition from the passive hydraulic regulation of leaf water status to an active process of stomatal regulation that is highly sensitive to the phytohormone ABA

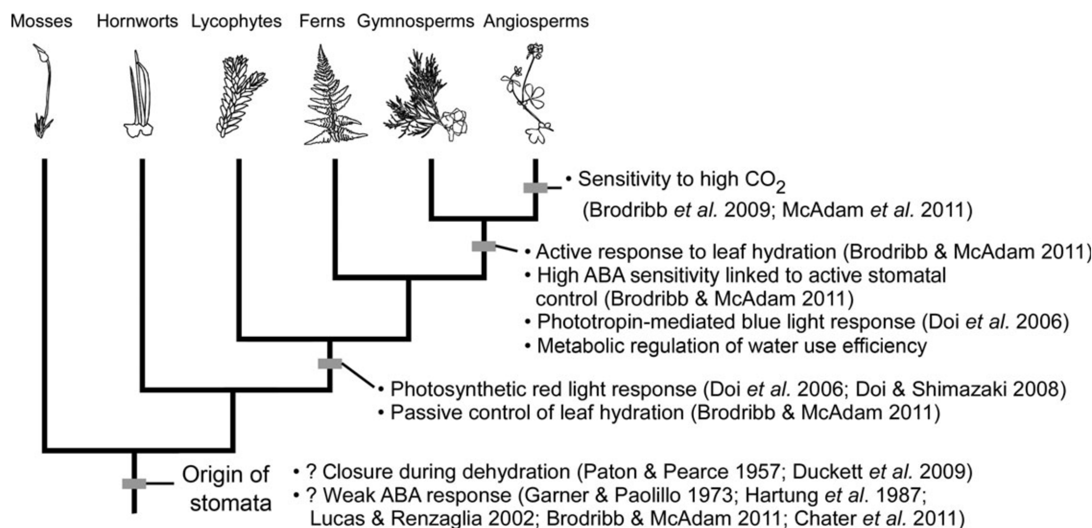

Figure 4 Reconstructed evolution of stomatal control processes based on extant representatives of major plant clades. Evolution of metabolic regulation of high water use efficiency occurs coincidently with other key functional metabolic innovations, suggesting a major evolutionary transition of stomatal physiology following the divergence of seed plants.

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(Brodribb & McAdam 2011). Associated with this shift towards a metabolic control of stomatal aperture appears to be the development of guard cell specific, phototropin-mediated responses to blue light (Doi *et al.* 2006) and, as we show here, the capacity to integrate signals from the mesophyll to dynamically optimise water use (Fig. 2). A final step in the trend of increasing complexity of stomatal physiology appears to be the evolution of high stomatal sensitivity to elevated CO_2 after the divergence of the angiosperms (Brodribb *et al.* 2009; McAdam *et al.* 2011). It should be noted that this reconstruction is based on extant representative species, and hence, it cannot identify the precise origin of the metabolic regulation of high water use efficiency because many seed plant clades are now extinct, preventing a functional reconstruction of these critical groups (Doyle & Donoghue 1992; Mathews 2009). Although the stomatal function of extinct leaves cannot yet be established, the pattern within extant groups is rather clear, and this pattern provides a new perspective on the functional evolution of stomata in land plants (Fig. 4).

The major transition in stomatal physiology reconstructed here as occurring with the evolution of seed plants during the Paleozoic (Fig. 4), would have enabled seed plants to greatly improve diurnal water use efficiency during photosynthesis when compared with their predecessors. The resultant increase in productivity per unit water loss must have conferred a significant competitive advantage to early seed plants. Importantly, however, the size of the water use advantage enjoyed by seed plants during daily variations in light intensity is dependent upon the maximum rate of photosynthesis. Under non-saturating light, ferns with high photosynthetic rates are at a distinct disadvantage compared with photosynthetically equivalent seed plants (Fig. 2b), whereas ferns with low photosynthetic maxima remain close to optimal water use, because photosynthesis remains saturated during light transitions. The increasingly wasteful use of water in ferns with higher rates of photosynthesis (Fig. 2a) may partially explain why fern and lycophyte species were never able to evolve leaves with a high capacity for photosynthesis as seen in seed plants (Brodribb & Feild 2010). In addition, this may account for the success of ferns in the shaded forest understory and their rarity as canopy dominants (Page 2002; Karst *et al.* 2005). Thus, our data provide evidence for a sporophyte-driven hypothesis [as opposed to the traditional gametophyte-sensitivity hypothesis (Watkins *et al.* 2007)] to account for the ecological limitations of ferns and the rise of seed plants.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1 The response of stomatal conductance and assimilation to transitions in light intensity observed over 60 minutes in a selection of six fern and lycophyte species.
Figure S2 Ferns and lycophytes have reduced water use efficiency following increases in light intensity from darkness to low levels.
Figure S3 Night-time and cuticular conductances in 13 fern and lycophyte species.
Figure S4 Stomatal aperture responses from isolated epidermis and live leaves to changes in light intensities in three additional species; the eudicot Lotus corniculatus, gymnosperm Ginkgo biloba and lycophyte Selaginella kraussiana.
Figure S5 Comparative gas exchange measurements over transitions in light intensity in the five species used to observe responses in stomatal aperture.
Figure S6 The response of stomatal aperture to changes in light intensity in an epidermal xenograft of an angiosperm epidermis on the mesophyll of a fern.
Table S1 Description of the species used in the study.
Table S2 Rates of stomatal opening and closure in fern, lycophyte and seed plant species.

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