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Evolutionary innovations in the stomatal control of vascular plants

By

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Statement of co-authorship of published work

The following people and institutions contributed to the publications of work undertaken as part of this thesis:

Scott A. M. McAdam, School of Plant Science:	Candidate, primary author of all chapters
Timothy J. Brodribb, University of Tasmania:	Co-author of all papers and manuscripts, first named author on chapter 2 (see explanation below).
Greg J. Jordan, University of Tasmania:	Co-author of paper comprising Chapter 4
John J. Ross, University of Tasmania:	Co-author of paper comprising Chapter 4

Brodribb TJ, McAdam SAM (2011) Passive origins of stomatal control in vascular plants. *Science* **331**(6017), 582-585.

The results presented in Chapter 2 of this thesis were published in this paper. Although the candidate was not the first author of the paper, he collected all of the data, analysed the results and wrote Chapter 2 in its entirety (under the advice of his supervisors); the figures are the only component of this publication directly used in this chapter. Timothy Brodribb assisted with the conceptualisation and technical implementation of the study.

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This paper comprises the entirety of chapter 3. Scott McAdam was the primary author (70%), Timothy Brodribb assisted with the conceptualisation and technical implementation of the study, as well as assisting with the writing of the manuscript (30%).

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This paper comprises the entirety of chapter 4. Scott McAdam was the primary author (70%), Timothy Brodribb, John Ross and Gregory Jordan assisted with the conceptualisation of the study, as well assisting with the writing of the manuscript (combined 30%).

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Abstract

Stomata are the numerous pores on the leaves of land plants. These pores occur between two adjustable guard cells. Guard cells are primarily responsible for regulating the aperture of the stomatal pore through changes in turgor pressure. Studies in angiosperms have demonstrated that the control of guard cell turgor pressure is metabolic, regulated by a diverse array of guard cell ion pumps. A range of environmental and endogenous signals activate these ion pumps including the hormone, abscisic acid (ABA), photosynthetic rate, red light and CO_2 concentration. As such all of these signals are instrumental in regulating efficient water use. However, until the work undertaken in this thesis, it was largely unknown whether this metabolically regulated stomatal signalling observed in angiosperms evolved once with the first stomata over 400 million years ago, or whether the various components of this complex system were assembled over the course of land plant evolution. The aim of my thesis was therefore to investigate the evolution of stomatal control and its possible ecological implications in the four extant lineages of vascular plants (lycophytes, ferns, gymnosperms and angiosperms). Using a phylogenetic approach, I examined the responses of stomatal conductance, aperture and morphology from representative species to exogenous and endogenous ABA, water stress, changes in the rate of photosynthesis and CO_2 concentration.

The stomata of ferns and lycophytes were insensitive to both exogenous and endogenous ABA. High concentrations of exogenous ABA fed into the transpiration stream of a diversity of fern and lycophyte species did not cause stomata to close despite very high levels of ABA in the leaf. In addition the stomata of drought stressed seed plants close with high levels of endogenous ABA in the leaf; however fern and lycophyte stomata from droughted leaves that were instantaneously rehydrated, rapidly reopened to pre-droughted levels despite the high levels of endogenous ABA in the leaf. In spite of this lack of control by ABA, the stomata of ferns and lycophytes were sensitive to changes in leaf water status, with highly predictable stomatal behaviour, passively responding to leaf water balance in the light. These results suggest that active stomatal regulation by ABA in response to changes in leaf hydration evolved after the divergence of ferns from an ancestral passive-hydraulic stomatal control system.

Unlike angiosperms, the stomata of conifers display very little short-term response to changes in atmospheric CO_2 concentration. Also, the sensitivity of angiosperm stomata to CO_2 concentration is regulated by foliar ABA level, but increased ABA levels following drought stress did not augment the stomatal sensitivity to CO_2 concentration in conifers.

The capacity of guard cells to actively optimise water use is another crucial component of the metabolic control mechanisms of seed plant stomata. I examined the possibility that this stomatal capacity was also derived in seed plants by comparing the stomatal response to light intensity in 13 species of ferns and lycophytes with a diverse sample of seed plants. While seed plant stomata were capable of maintaining a high ratio of photosynthesis to water use at different light intensities, fern and lycophyte stomata were unable to sustain similar ratios at low light intensities. The behaviour of stomata on excised epidermis indicates that the reason for this difference is the evolution of a feedback signal from photosynthetic tissue to the guard cells, unique to seed plants.

I further investigated the adaptive mechanisms adopted by a diverse morphological and ecological sample of ferns and lycophytes with passively controlled stomata that enable survival in response to water stress. Ferns and lycophytes survive prolonged soil drought stress by making significant changes to physiology (by increasing tolerances of low relative water content) and morphology (by increasing the volume of available leaf water). These adaptations are integrally governed by a passive stomatal response to leaf water status and not metabolic stomatal control by ABA as seen in seed plants. A passive stomatal control in the light has influenced the adaptations adopted by ferns and lycophytes over 360 million years of competition and persistence with the dominant seed plant lineages.

My results therefore show that the suite of metabolic stomatal control mechanisms found in angiosperms did not evolve synchronously in the earliest stomatal bearing land plants. The results from this thesis expand our understanding of stomatal function in modern land plants; offer physiological explanations for the rise and fall in dominance of the different lineages of vascular plants over geological time and may explain differences in the ecological strategies employed by the diversity of extant and extinct land plants.

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CHAPTER 1

General Introduction

Over a century and a half of research on angiosperms has built up a set of powerful models of how plants control stomatal apertures and regulate gas exchange. These models have been instrumental in predicting stomatal behaviour at a cellular level in aid of understanding and modifying plant survival and growth. However, recent work has challenged the idea that the mechanisms underpinning these models apply to all groups of land plants (Doi *et al.* 2006; Doi and Shimazaki 2008; Brodribb *et al.* 2009). This leads to a fascinating field of research into the evolution of stomatal control.

Leaf gas exchange is intimately regulated by the movement of guard cells opening and closing the stomatal pore. A diverse assemblage of models attempt to predict stomatal conductance (see Damour *et al.* 2010). These models include those requiring extensive parameterisation (Jarvis 1976), economic models that predict stomatal behaviour based upon simple optimization criteria (Cowan 1977; Cowan and Farquhar 1977) and models that predict stomatal conductance based on changes in solute concentration in the guard cells (Chen *et al.* 2012). At their core all models have two simply defined, yet inherently complex processes that act to regulate guard cell turgor and stomatal aperture (Damour *et al.* 2010). These two processes are the passive regulation of guard cell turgor by leaf water status (Buckley and Mott 2002; Buckley 2005) and the active control of guard cell osmotic potential by the transport of ions across cell membranes (Schroeder *et al.* 2001; Shimazaki *et al.* 2007; Lawson 2009).

Passive regulation of water loss

Many models of whole leaf transpiration dedicate substantial influence to a passive regulation of stomatal aperture by leaf turgor (Buckley 2005). This is due to two lines of evidence: the relationship between stomatal aperture and guard cell turgor elegantly demonstrated across a diversity of vascular land plant stomata (Meidner and Bannister 1979; Franks *et al.* 1995; Franks *et al.* 1998; Franks and Farquhar 2007); and a prevailing influence of leaf water status on stomatal conductance (Brodribb and Holbrook 2003; Aasamaa and Sõber 2011a). Indeed, the concept of a passive hydraulic influence on stomatal conductance stems from a long history of experimentation demonstrating the link between stomatal aperture and guard cell

turgor (Heath 1938; DeMichele and Sharpe 1973; Wigger *et al.* 2002) and gas exchange in plants grown in the field (Oren *et al.* 1999; Salleo *et al.* 2000). However, there is evidence that simple models of leaf gas exchange based on passive stomatal responses do not provide an adequate explanation for the behaviour of angiosperm stomata (Buckley *et al.* 2003; Roelfsema and Hedrich 2005; Ache *et al.* 2010). Instead, it is necessary to incorporate an active regulation of guard cell turgor in the response to leaf water status (Buckley *et al.* 2003). The interconnection between stomatal aperture and both guard and epidermal cell turgor ensures that numerous models of stomatal behaviour include parameters that account for both the passive influence of leaf water status and the active regulation of guard cell turgor (Buckley *et al.* 2003; Damour *et al.* 2010).

Active control of aperture

As recognised by the earliest studies of stomatal physiology, guard cell turgor must change independently of leaf and epidermal cell turgor to induce stomatal movement, particularly to open stomata in the light (von Mohl 1856; Schwendener 1881; Darwin 1898). A number of studies, conducted soon after (Iljin 1914; Wiggans 1921), quantitatively demonstrated that in the morning, osmotic concentrations of the guard cells increased so that they reached levels higher than in epidermal cells. This change corresponded with stomatal opening. Furthermore, guard cell osmotic concentration decreased at night, at the same time that stomata closed. This research initiated investigation into active stomatal behaviour; the means by which plants exploit the simple control of aperture by guard cell turgor through actively regulating guard cell osmotic pressure by membrane ion transporters. The movement of ions into and out of guard cells has received concerted investigation, to the point where active regulation of guard cell turgor is considered the primary driver of stomatal responses (Schroeder *et al.* 2001; Roelfsema and Hedrich 2005; MacRobbie 2006; Kim *et al.* 2010). Studies have presented data that implicate active stomatal control in almost all guard cell responses to the environment, including drought (Tardieu and Davies 1992, 1993), vapour pressure deficit and humidity (Xie *et al.* 2006; Okamoto *et al.* 2009), leaf water status through ABA (Christmann *et al.* 2005; Ache *et al.* 2010), CO_2 concentration (Israelsson *et al.* 2006; Young *et al.* 2006; Kim *et al.* 2010), red light (Roelfsema *et al.* 2002; Wang *et al.* 2010), blue light (Inoue *et al.* 2010; Takemiya and Shimazaki 2010), photosynthesis (Roelfsema *et al.* 2006; Galvez-Valdivieso *et al.* 2009; Lawson 2009), reactive oxygen species (Kwak *et al.* 2006), pathogens (Desclos-Theveniau *et al.* 2012) and distant electrical signals (Hlaváčková *et al.* 2006).

Our understanding of the complex and abundant active mechanisms that regulate guard cell turgor and aperture stems largely from a molecular biological perspective; from studies in model angiosperm mutants defective in normal stomatal behaviour (Merlot *et al.* 2002; Mustilli *et al.* 2002), as well as the use of guard cells as models for understanding ion channels (Ward *et al.* 2009). These studies place a central role on the phytohormone abscisic acid (ABA) in regulating guard cell responses to leaf water status as well as most of the characterised guard cell ion signalling cascades (Tallman 2004; Geiger *et al.* 2009; Kim *et al.* 2010; Raghavendra *et al.* 2010; Geiger *et al.* 2011). ABA is synthesised by drought stressed plants (Wright and Hiron 1969). Increasing levels of ABA in the leaf actively lowers guard cell turgor even when leaves are hydrated, thereby closing stomata (Mittelheuser and Van Steveninck 1969; Jones and Mansfield 1970). The control of stomata by ABA has received a predominance of investigative attention because of two reasons: the presence of specific signalling mutants; and the allure of ABA actively closing stomata and reducing excessive water loss from plants in agricultural systems (Wilkinson and Davies 2002; Chaves and Oliveira 2004).

Evolution of stomatal control

The thorough examination of active mechanisms influencing guard cell turgor and aperture has ensured a comprehensive understanding of the way in which the stomata of a number of recently derived model angiosperm herbs, particularly *Arabidopsis thaliana*, respond to the environment (Clauss and Koch 2006; Nilson and Assmann 2007). Yet recent evidence suggests that stomata have not always behaved the same and that over their 400 million year history (Edwards *et al.* 1998) adaptive innovations in the control of stomata may have evolved with land plants, an idea that remains controversial.

The concept of a phylogenetic structuring of stomatal control stems from two studies investigating the responses of fern stomata to CO_2, red and blue light (Doi *et al.* 2006; Doi and Shimazaki 2008). In angiosperms blue light is an important driver of rapid active stomatal opening in the light and closure in the dark (Shimazaki *et al.* 2007). However, Doi *et al*. (2006) showed that in a number of leptosporangiate fern species there was no stomatal response to blue light despite the presence of the phototropin genes that regulate the response in angiosperms. They showed that the light response of fern stomata was limited to a red light response and did not accurately mirror a response to photosynthesis (Doi *et al.* 2006; Doi and Shimazaki 2008). In addition Doi and Shimazaki (2008), followed later by Brodribb *et al.*

(2009) demonstrated that fern and lycophyte stomata lack a response to CO_2. In angiosperms, low CO_2 induces stomatal opening while high CO_2 induces stomatal closure (Morison 1985). Doi and Shimazaki (2008) showed that in the dark, the stomata of the fern *Adiantum capillus-veneris* did not respond to any change in CO_2 unlike the stomata of the angiosperm *A. thaliana* showing a pronounced response to increases and decreases in CO_2. This lack of a stomatal response to CO_2 is ubiquitous to ferns and lycophytes and the sensitivity to CO_2 varies between the seed plant lineages, with marked sensitivity to high CO_2 evolving after the divergence of the gymnosperms (Brodribb *et al*. 2009). In addition to these differences in the active responses of stomata in different groups of vascular plants, Franks and Farquhar (2007) illustrated that the stomata of ferns and lycophytes are hydromechanically different to angiosperms, lacking a close ionic and mechanical connection to the subsidiary cells that results in the wrong-way hydropassive response of stomata commonly observed in angiosperms when exposed to rapid changes in water status. In spite of these studies some researchers do not find this evidence convincing and still hold to a concept that there has been no evolution in the stomatal control components in land plants (Beerling and Franks 2009).

Structure of this thesis

In this thesis I consider, using a phylogenetic approach, the possibility that stomatal control has evolved. This was examined by observing the responses of stomata from different lineages of vascular plants, including lycophytes, ferns, gymnosperms and angiosperms. Specifically I focus on key signals for stomatal regulation observed in angiosperms including the principal, active metabolic, guard cell signaller ABA, as well as, leaf water status, photosynthesis and CO_2 concentration. In addressing this aim the following research questions were investigated:

- Does the sensitivity of stomata to exogenously applied ABA vary across land plant phylogeny? (Chapter 2)
- How does this sensitivity affect the stomatal response to leaf water status in different land plant lineages? (Chapter 2)
- Does endogenously synthesised ABA during drought in ferns and lycophytes play a role in leaf gas exchange? (Chapter 3)
- Can the sensitivity of conifer stomata to CO_2 concentration be augmented by ABA? (Chapter 4)
- Are there differences in the way in which land plant stomata respond to photosynthesis and regulate water use? (Chapter 5)

- What are the ecological implications for ferns and lycophytes with stomata that are governed by a passive response to leaf water status in the light? (Chapter 6)

The five experimental chapters in this thesis are composed of self-contained units, presented in the style of scientific journal articles. Each of the experimental chapters contains an introduction to the literature providing an outline for the potential contribution of the investigation to the field; the results presented in each chapter are followed by a discussion of the findings and conclusions. In chapter 7, a concluding discussion of the major findings of the experimental chapters, their implications in relation to the relevant literature and directions for future research is presented.

CHAPTER 2

Ferns and lycophytes reveal a passive ancestral state of stomatal control in vascular plants

ABSTRACT

Stomata play a fundamental role in the regulation of terrestrial gas exchange, continually sensing and responding to the environment. Yet a complete understanding of the controls and evolution of stomatal behaviour remains elusive. Two metabolically governed stomatal control processes observed in angiosperms were examined in a diversity of ferns and lycophytes, (i) the response of stomata to the phytohormone abscisic acid (ABA) and (ii) wrong-way stomatal opening followed by hydroactive stomatal closure on leaf excision. Ferns and lycophytes lack active stomatal closure in response to ABA and also wrong-way stomatal opening following leaf excision. Yet stomatal conductance (g_s) in the basal lineages of vascular plants was highly sensitive to leaf dehydration and step changes in relative humidity. A dynamic model that predicts daytime stomatal aperture purely as a response to leaf water balance was then developed and tested in two ferns and a lycophyte species. Daytime g_s in a lycophyte and two fern species could be accurately predicted by the passive hydraulic model, indicating that the stomata of ferns and lycophytes act as passive hydraulic valves in the light. These results suggest a profound evolutionary change in the way plants regulate transpiration and respond to environmental conditions; with the development of complex active stomatal control mechanisms evolving in seed plants (gymnosperms and angiosperms) after the divergence of ferns.

INTRODUCTION

Two guard cells flanking the stomatal pores of land plants adjust stomatal aperture to accommodate both the uptake of carbon dioxide for photosynthesis and the limitation of plant water loss. The biological regulation of these two gases by guard cells has been immensely significant in atmospheric, ecological and evolutionary processes following their first appearance at least 400 million years ago (Edwards *et al.* 1998; Hetherington and Woodward 2003). An understanding of the function of stomata is crucial for predicting and modifying the responses of plants and ecosystems to changes in their environment (Berry *et al.* 2010).

Angiosperm stomata are active valves driven by the movement of ions across membranes, known to open in response to red and blue light, low carbon dioxide concentration, decreased evaporative demand; and close in response to darkness, high carbon dioxide, increased evaporative demand and radiation, phytohormones including ABA, reactive oxygen species and pathogens (Linsbauer 1917; Mittelheuser and Van Steveninck 1969; Buckley 2005; Acharya and Assmann 2009; Lawson 2009; Zeng *et al.* 2010). This common active regulation of stomatal aperture in angiosperms extends to the responses of stomata to changes in leaf hydration (Ache *et al.* 2010), with models predicting stomatal responses to leaf hydration incorporating metabolic signals (Buckley *et al.* 2003; Damour *et al.* 2010). In angiosperms there are two independent lines of evidence that support an active regulation of stomata in response to changes in leaf water status, one is the phytohormone ABA, driving the depolarisation of the guard cell membrane and a loss of guard cell turgor pressure regardless of leaf turgor (Geiger *et al.* 2011) and the other, hydroactive feedback on stomatal conductance following perturbations in leaf water status (Kaiser and Legner 2007).

It is well known that the hormone ABA actively reduces stomatal aperture when levels increase in the leaf (Mittelheuser and Van Steveninck 1969; Wright and Hiron 1969). The significance of ABA driving stomatal closure during drought and water stress cannot be understated (Wilkinson and Davies 2002). Investigations of guard cell membrane ion transporters would scarcely have progressed without single gene mutants identified in the ABA signalling and synthesis pathways (Leung and Giraudat 1998; Schroeder *et al.* 2001). Mutants of ABA synthesis and signalling have a characteristic wilty phenotype that is incapable of surviving water stress events, either increased evaporative demand or drought (Imber and Tal 1970; Mustilli *et al.* 2002; Wigger *et al.* 2002). The role of ABA in regulating

correct stomatal behaviour, especially the response of stomata to plant water status, places a high priority on ABA in governing plant survival (Tardieu and Davies 1992).

In angiosperms there is a close ionic and mechanical interaction between the guard cells and subsidiary cells that facilitates rapid stomatal movement and an increased stomatal aperture (Franks and Farquhar 2007; Kaiser and Legner 2007). However, when the leaves of angiosperms are exposed to perturbations in water status (e.g. following leaf excision (Powles *et al.* 2006), changes in humidity (Kappen *et al.* 1987), transpiration rate (*E*) (Kaiser and Grams 2006) or even heat induced electrical signals (Kaiser and Grams 2006)) instead of stomatal aperture responding passively to changes in leaf turgor, stomata experience hydropassive (or wrong-way) transient movement. This hydropassive stomatal behaviour occurs because of a prevailing influence of subsidiary cell turgor pressure on stomatal aperture (Franks and Farquhar 2007). In order for guard cells to re-establish a new steady-state leaf gas exchange, an active movement of ions between the guard cells and the subsidiary cells is required (Franks and Farquhar 2007). Both active stomatal closure at high levels of ABA and hydropassive stomatal responses ensure that the incorporation of an active movement of ions in the modelling of stomatal responses to changes in leaf water status is necessary (Buckley *et al.* 2003; Buckley 2005).

These active processes regulating stomatal aperture in response to changes in leaf hydration commonly observed in angiosperms raises the question: has this complexity evolved from a simple ancestral stomatal regulation in basal vascular land plants? One possibility is that the ancestral stomatal control in vascular plants is devoid of the numerous active ionic regulators of stomatal aperture relying simply on the passive turgor driven responses of stomata. The basal lineages of vascular plants, including ferns and lycophytes, are the ideal candidates in which to investigate the ancestral state of stomatal control in vascular plants. Both lineages diverged around 400 million years ago, before the emergence of the seed plants and have maintained ancestral foliar and stomatal morphologies (Rothwell 1996). Extant ferns and lycophytes have proved useful subjects in understanding the progression of plants on land (Boyce 2005; Boyce 2010), from the evolution of light perception genes (Wada 2007) to plant hydraulic traits (Brodribb *et al.* 2005). Ferns and lycophytes have a relatively simple epidermal anatomy lacking the highly differentiated stomatal complexes with abundant subsidiary cells found in angiosperms (Peterson *et al.* 2010), and are assumed to be largely free of the hydropassive stomatal behaviour characteristic of angiosperms (Franks and

Farquhar 2007). In addition, a number of studies suggest that the stomata of ferns and lycophytes are very sensitive to plant water supply (Lange *et al.* 1971; Hollinger 1987), with stomatal closure occurring before significant reductions in xylem hydraulic conductivity, in contrast to many angiosperms (Brodribb and Holbrook 2004; Lo Gullo *et al.* 2010). Yet fern and lycophyte stomata have weak or absent responses to a number of well characterised active regulators of stomatal aperture in angiosperms (see Chapter 1; Doi *et al.* 2006; Doi and Shimazaki 2008; Brodribb *et al.* 2009).

Here I examine the ancestral state vascular plant stomatal responses to changes in leaf water status. I observe the responses of stomata in a phylogenetically diverse selection of fern and lycophyte species to two key indicators of active regulation of stomatal conductance by leaf water status in angiosperms; hydroactive stomatal closure following hydropassive opening on leaf excision and the exogenous application of ABA through the transpiration stream. In the absence of an active regulation of stomatal conductance by leaf water status, a passive stomatal regulation by leaf hydration in the light in ferns and lycophytes is tested by comparing the observed stomatal conductance in a lycophyte and two fern species against a dynamic model that predicts stomatal aperture purely as a function of leaf water status.

MATERIALS AND METHODS

Plant Material

All species were represented by at least three individuals grown in pots in the glasshouses of the School of Plant Science, University of Tasmania or as individuals growing in the field (Table 2.1). Experiments were undertaken over autumn and winter (March to July, 2010). All glasshouse lycophyte, fern and gymnosperm species were grown under natural light conditions supplemented with 24 h heating maintaining the minimum night time temperature above 10ºC, all plants received 3 month applications of slow release fertiliser. Individuals of *H. annuus* were grown 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 in a growth cabinet (14 h days, 25°C/15°C day/night temperatures, receiving a maximum photosynthetic photon flux density (PPFD) at pot height of 500 µmol quanta m^{-2} s^{-1}, relative humidity maintained at 85%). All measurements on *H. annuus* individuals were carried out on plants no more than 4 weeks old, on at least the fourth fully expanded leaf.

Table 2.1 Experimental species including a description of native habitat and growth form.

Species	Family	Habitat
	LYCOPHYTES	
Lycopodium deuterodensum Herter[a]	Lycopodiaceae	Temperatue-subtropical perennial herb from open forests
Lycopodiella inundata (L.) Holub.	Lycopodiaceae	Circumpolar, circumboreal perennial herb from very wet habitats
Selaginella kraussiana (Kunze) A.Braun	Selaginellaceae	Ruderal perennial herb from very wet environments
	FERNS	
Todea barbara (L.) T.Moore[b]	Osmundaceae	Temperate wet forest understorey treefern
Cyathea australis (R.Br) Domin.[b]	Cyatheaceae	Temperate wet forest understorey treefern
Dicksonia antarctica Labill.[a]	Dicksoniaceae	Temperate wet forest understorey treefern
Hypolepis tenuifolia (G.Forst.) Bernh.	Dennstaedtiaceae	Tropical pioneer ground fern
Pteridium esculentum (G.Forst.) Cockayne[a]	Dennstaedtiaceae	Temperate-subtropical pioneer ground fern from open forest and grassland
Adiantum capillus-veneris L.	Adiantaceae	Warm temperate-tropical subcosmopolitan fern from moist rock faces
Nephrolepis exaltata (L.) Schott	Lomariopsidaceae	Temperate moist forest understory fern
Microsorum pustulatum (G.Forst.) Copel.[a]	Polypodiaceae	Temperate wet forest understorey epiphyte or ground cover
	GYMNOSPERMS	
Ginkgo biloba L.	Ginkgoaceae	Temperate deciduous tree
Callitris rhomboidea R.Br.[a]	Cupressaceae	Temperate dry forest tree
	ANGIOSPERM	
Helianthus annuus L. cv. Yellow Empress	Asteraceae	Domesticated temperate annual herb

[a] Leaf material collected from individuals growing in the field.
[b] Species not included in exogenous ABA feeding experiment

Quantifying hydropassive effects

Two leaves each from three individuals were used to record the dynamics of stomatal closure following leaf excision. Potted plants were transported and acclimated to laboratory conditions for at least 12 hours prior to experimentation, leaves collected in the field were excised in the morning either, cut underwater directly (*M. pustulatum*, *D. antarctica* and *L. deuterodensum*) or wrapped in damp cloth, double bagged and transported immediately to the laboratory (approximately 20 min) (*P. esculentum*) where the leaves were then enclosed in a sealable bag and recut with a razor blade under water allowing the leaf to fully rehydrate for at least 1 h.

Portable infrared gas analysers (Li-6400 and Li-6400XT, Li-Cor Biosciences, Lincoln, NE, USA) were used to measure g_s (mol m^{-2} s^{-1}) following leaf excision. Ambient conditions within the leaf chamber were maintained constant during the experiment (leaf temperature

was maintained at 22ºC, PPFD at 1000 µmol quanta m^{-2} s^{-1}, leaf chamber flow rate at 500 µmol s^{-1}, CO_2 concentration at 390 µmol mol^{-1} and vapour pressure difference (VPD) manually regulated between 1 and 1.3 kPa. In the case of *L. inundata* and *S. kraussiana* PPFD was set at 500 µmol quanta m^{-2} s^{-1} and the leaf chamber flow rate lowered to 300 µmol s^{-1} to maximise the sensitivity of the infrared gas analysers). During measurements g_s, assimilation (*A*) and other leaf environmental traits were logged every 60 s.

Intact leaves or leafy shoots were allowed to equilibrate in the chamber for 10 min while supplied with water, after which the leaf was excised in air and allowed to desiccate. Gas exchange data were recorded on the excised leaf until g_s had reached a minimum and stabilised (≤1% change in initial g_s over 3 minutes), the sample was then removed from the cuvette and leaf area measured.

Stomatal conductance and ABA feeding

To assess the response of g_s to ABA in nine lycophyte and fern species (Table 1) and the representative gymnosperms and angiosperm, single leaves or terminal segments of shoots from each species were excised and recut immediately in a dish containing 500 ml of filtered (0.22 µm membrane filter) and de-ionised (18 MΩ cm^{-1}) water (MilliQ, Millipore, Billerica, MA, USA). Leaf tissue was enclosed in the cuvette of a Li-6400 gas exchange system under conditions described above and g_s, *A* and leaf environmental parameters logged every 60 s.

To ensure that leaf water supply was not limited by xylem blockage or damage it was necessary to regularly recut submerged stems, thereby eliminating the accumulation of mucilage or other blocking agents at the cut end of the xylem. This was particularly important in the fern species (Schütze 1906) where a 2-4 mm segment was excised every 10 min by razor blade from the end of the rachis, rhizome or pinnae under the water

Leaves were allowed to equilibrate in the chamber for 10 min, after which, sections of leaf tissue near the chamber were removed for the quantification of initial leaf ABA levels (see below). An aliquot of a stock solution of unlabelled ABA, prepared by dissolving 90% crystalline ABA of mixed isomers (Sigma Chemical Co., St. Louis, MO, USA) in 1 ml of methanol then diluting up to 250 ml with filtered and de-ionised water, was added to the leaf water supply increasing the concentration of ABA in the water to 15000 ng ml^{-1} (equivalent to 56.7 µM in the xylem sap). In *D. antarctica* and *P. esculentum* three concentrations of ABA

in the water were used (7500 ng ml^{-1}, 15000 ng ml^{-1} and 30000 ng ml^{-1}). Following the addition of ABA to the leaf water supply, g_s continued to be recorded every 60 s for at least 90 minutes. In all nine lycophyte and fern species assessed the rachis was then cut in air, severing the leaf water supply to test whether stomata were responsive to leaf water status and to determine cuticular conductance. After approximately 5 minutes the leaf tissue in the chamber was removed, scanned for the quantification of leaf area, weighed and prepared for the physiocochemical gas chromatography-tandem mass spectrometry (GC-MS-MS) quantification of ABA with an added internal standard.

ABA extraction, purification and quantification

In all species approximately 1 g of leaf tissue was used in quantifying ABA level. In the fern and lycophyte species because leaf ABA level was undetectable, up to 3 g of leaf tissue was harvested. Harvested tissue was weighed then roughly chopped into cold (-20ºC) 80% (v/v) methanol in distilled water containing 250 mg l^{-1} of butylated hydroxytoluene and placed in a freezer for 24 h. Tissue was homogenised and ABA extracted for 24 h at 4ºC. The extract was filtered through Whatman no. 1 filter paper and an aliquot taken. [$^2H_6$]ABA (National Research Council of Canada, Saskatoon, Canada) was then added to the aliquot as an internal standard. Samples were reduced in volume to less than 1 ml under vacuum at 35ºC and taken up in 3 x 1 ml washes of 0.4% (v/v) acetic acid in distilled water. This volume was passed through a Sep-Pak® C18 cartridge (Waters, Milford, USA) (preconditioned with 15 ml of methanol followed by 15 ml of 0.4% (v/v) acetic acid in distilled water). The cartridge was washed with 15 ml of 20% (v/v) methanol in 0.4% (v/v) acetic acid and ABA eluted with 15 ml of 45% (v/v) methanol in 0.4% (v/v) acetic acid. The eluate was reduced to dryness under vacuum at 35ºC, taken up in 400 µl of methanol, methylated with 750 µl of a 1:10 dilution of (trimethylsilyl)diazomethane in diethyl ether for 30 min then dried under a nitrogen stream. Following methylation ABA was taken up in 2 x 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 or GC-SIM analysis. GC-MS-MS analysis was performed using a Varian 8400 Autosampler and a Varian 3800 GC coupled to a Varian 1200 triple quadrupole MS. An 1177 split/splitless injector was used to make a splitless injection of 1 µl onto a 30 m long x 0.25 mm inner diameter Varian FactorFour VF-5ms column with a 0.25 mm film thickness. Helium was used as the gas carrier at a constant flow rate of 1.4 ml min^{-1}. The initial temperature of the column oven was maintained at 50ºC for 2 min, increased to 230ºC at 30ºC min^{-1} and then to 270ºC at 10ºC min^{-1} and held for 5 min. The system was

operated in MS/MS selected reaction monitoring mode, the ions monitored were MS1 m/z 190, MS3 m/z 162 (endogenous ABA) and MS1 m/z 194, MS3 m/z 166 (internal standard [$^2H_4$]ABA). Samples of *P. esculentum* were quantified by GC-SIM using the fully comparable Hewlett-Packard 5890 gas chromatograph linked via an open split interface to a Kratos Concept ISQ mass spectrometer. One ml splitless injections were made at 250°C onto a 25-m x 0.32 mm i.d. Hewlett Packard HP1 column with a 0.17-mm film thickness. Helium was used as the carrier gas at a low rate of 2 ml min^{-1} under a pressure of 190 kPa. The temperature of the column oven was increased from 60 to 150°C at 30°C min^{-1} and then to 290°C at 10°C min^{-1}.

In all cases the ratio of endogenous ion intensity to internal standard ion intensity was calculated. The product of this ratio and the amount of internal standard added was divided by the fresh weight of the tissue sample and adjusted for aliquot volume to determine the level of ABA per gram fresh weight in the leaf.

Passive-hydraulic stomatal control model

To test whether the stomata in ferns and lycophytes behaved in a way consistent with a passive control of leaf hydration we formulated a passive-hydraulic stomatal control model that predicted g_s based on the assumption that guard cell hydration was in equilibrium with the bulk leaf tissue, and determined from the balance of hydraulic supply and transpirational loss of water. Modelled responses for each species were calculated in a stepwise fashion using measured parameters (Appendix 2.1).

Primarily, g_s was assumed to be a function of guard cell turgor pressure (P_g):

$$g_s = f(P_g) \quad (1)$$

Rather than measuring guard cell turgor pressure we assumed homogenous hydraulic and osmotic conditions existed within leaves and hence that P_g was equal to the average leaf cell turgor pressure. The function relating g_s and turgor pressure was determined empirically for each species by establishing the relationship between leaf water potential (Ψ_l) and g_s in slowly desiccating leaves (see below) and calculating turgor pressure by equation 2, from Ψ_l and leaf osmotic potential (Ψ_π) derived from pressure-volume analysis (see below):

$$\Psi_l = \Psi_\pi + turgor\ pressure \quad (2)$$

Following the estimation of g_s using equation 1, the evaporation of water from the leaf (E) could then be calculated as the product of g_s and the ambient leaf-to-air vapour pressure difference (VPD) divided by atmospheric pressure (P_{atm}):

$$E = g_s \times \frac{VPD}{P_{atm}} \quad (3)$$

Leaf boundary layer effects were not included because all measurements were made inside a cuvette with fan-forced mixing to reduce the boundary layer to insignificant dimensions. Initial conditions for each modelled response were set with specified VPD and specific leaf hydraulic parameters and allowed to come to an equilibrium g_s. Following a step change in VPD the immediate effect on E was first determined (equation 3). This immediate change in E resulted in a change in Ψ_l calculated from relaxation dynamics that treated the leaf as a capacitor/resistor series (Brodribb and Holbrook 2003). The model determined Ψ_l every second following the step transition in VPD (equation 4). Ψ_l was calculated by adding to the initial leaf water potential from the previous iteration of the model ($\Psi_{l,i}$) the calculated change in Ψ_l over one second (equation 4). The change Ψ_l was determined as the theoretical maximum change in Ψ_l (without the effects of stomatal closure or leaf capacitance) that would result from the instantaneous evaporative demand and hydraulic supply (K_{leaf}) of the leaf, less the change in Ψ_l as a result of leaf capacitance (C_{leaf}) and the instantaneous evaporative demand and hydraulic supply for a time (t) of one second:

$$-\Psi_l = \Psi_{l,i} + \left(\left(\frac{E}{K_{leaf}} - \Psi_{l,i} \right) - \left(\frac{E}{K_{leaf}} - \Psi_{l,i} \right) \times e^{-\frac{tK_{leaf}}{C_{leaf}}} \right) \quad (4)$$

In each case K_{leaf} was determined as a function of $\Psi_{l,i}$ derived empirically from leaf hydraulic vulnerability curves (see below):

$$K_{leaf} = f(\Psi_{l,i}) \quad (5)$$

Once Ψ_l was calculated by equation 4 a new transient g_s could then be determined by substituting Ψ_l into equation 2 yielding a new Ψ_p and g_s (equation 1). The new g_s then resulted in a change in E (equation 3) and subsequently a change in Ψ_l (equation 4). Cycling through equations 3-4,5-2-1-3 resulted in a predictive dynamic for g_s following stepwise transitions in VPD, as well as yielding steady state values for g_s if t=∞.

To predict the closing dynamics of g_s following leaf excision in air an alternative model based on E, C_{leaf} and t was used in place of equation 4, since K_{leaf} on excision is reduced to 0. Once E was determined by equation 3, a new Ψ_l was calculated using t=1s as the change from $\Psi_{l,i}$ simply as a result of E, t and C_{leaf}:

$$-\Psi_l = \Psi_{l,i} + \left(\frac{tE}{C_{leaf}}\right) \quad (6)$$

Once Ψ_l was calculated by equation 6 a new transient g_s was determined using equation 2 then equation 1. The cycling of these equations 3-6-2-1-3 established a model dynamic for g_s following leaf excision in air.

Establishing the parameters for the model

In this study two field collected fern species *D. antarctica* and *P. esculentum* and a lycophyte species *Lycopodium deuterodensum* were used to evaluate this hydraulic based model for stomatal conductance. Sporophytes of *D. antarctica* were sampled from eight individuals present in the understorey of a *Eucalyptus* spp. dominated wet forest, in a gully on the grounds of the University of Tasmania (Hobart, Australia: 147°19′E, 42°54′S). *Pteridium esculentum* sporophytes were sampled from two stands within 800 m of one another on cleared allotments in the Hobart suburb of Kingston (147°18′E, 42°58′S). *Lycopodium deuterodensum* sporophytes were collected from a single population in the understorey of a *Eucalyptus* spp. dominated dry forest on a ridge near Longley, Tasmania (147°11′E, 42°58′S).

Ψ_π and C_{leaf}

Pressure-volume (*PV*) analysis was performed on the foliage of the three species to determine C_{leaf} and Ψ_π (Tyree and Hammel 1972). Single foliage samples were collected for analysis from eight individuals of *D. antarctica*, three individuals from both stands of *P. esculentum* and five individuals of *L. deuterodensum*. Fronds (*P. esculentum*) or frond segments including whole pinnae (*D. antarctica*) were collected pre-dawn when leaf water potential (Ψ_l) was high (>-0.05 MPa), wrapped in damp cloth and double bagged for transport to the laboratory. Small terminal shoots or *L. deuterodensum* were excised from larger shoots cut underwater in the field and transported to the laboratory where the main stem was recut underwater and the shoot bagged overnight allowing rehydration to the conditions described above. In the fern species the apices of pinnae (c. 10 cm long) were selected for *PV* analysis for their lack of significant mucilage ducts, in *L. deuterodensum* small terminal shoots were selected. Selected tissue was excised and leaf weight (±0.0001 g, Mettler-Toledo MS204S, Switzerland) and Ψ_l (using a Scholander pressure chamber) periodically measured over gradual desiccation in the laboratory. *PV* curves were constructed by plotting $1/\Psi_l$ against relative water content (*RWC*) and turgor loss point determined by the inflection point of the graph. Slopes of the linear regression fitted through data before turgor loss point yielded C_{leaf}

in terms of *RWC*. The calculated C_{leaf} was then expressed in absolute terms and normalised by leaf area (Brodribb and Holbrook 2003):

$$C_{leaf} = \frac{\delta RWC}{\delta \Psi_l} \times \frac{DW}{LA} \times \frac{(WW/DW)}{M} \quad (7)$$

Where *DW* is leaf dry weight (g); *LA* is leaf area (m^2); *WW* is the mass of leaf water at 100% *RWC* (g); and *M* is the molar mass of water (g mol^{-1}).

The Ψ_π at full turgor was calculated as the intersection of the Ψ_l axis by a linear regression fitted through the *PV* data after turgor loss point from all *D. antarctica* and *L. deuterodensum* foliage and for each of the stands of *P. esculentum*.

Relationship between g_s and Ψ_p

The response of stomatal aperture to changes in Ψ_p of the guard cells was assessed by the simultaneous measurements of g_s and Ψ_l on at least six leaves or shoots from each of the three species, *P. esculentum* was represented by three leaves from each stand. Water potential equilibrium between tissues inside and outside the cuvette was important, so cuvette and ambient conditions were matched and declining Ψ_l was slowly induced by gradually blocking the leaf supply xylem. Pinnae of *D. antarctica* and *P. esculentum* were excised with a razor blade, from an already excised frond segment, under filtered and de-ionised water in the laboratory. An entire shoot of *L. deuterodensum* was selected with the main stem excised under filtered and de-ionised water. Pinnae or shoots were irradiated with a fibre-optic light source providing a leaf level PPFD of 300 µmol quanta m^{-2} s^{-1} and rested in the path of a gentle air stream proved by a small fan to strip away boundary layer effects equivalent to the conditions inside the leaf cuvette. Three pinnules of *D. antarctica* two thirds of the length from the base of the pinna, the centre of a pinnule of *P. esculentum* or a single terminal shoot approximately halfway up the stem of *L. deuterodensum* were enclosed in the leaf chamber of a portable infrared gas analyser (as described above). Conditions in the leaf chamber were programmed identically to the atmospheric conditions of the laboratory (leaf temperature was maintained at 22ºC, PPFD at 300 µmol quanta m^{-2} s^{-1}, leaf chamber flow rate at 500 µmol s^{-1}, the inlet air was supplied from a buffered container drawing from within the laboratory, CO_2 concentration was never more than 420 µmol mol^{-1} and VPD ranged between 1 and 1.3 kPa). The excised ends of the pinnae or shoots were initially regularly recut (as described above) to ensure maximum possible initial Ψ_l. After 10 min of adjustment simultaneous recordings of g_s and Ψ_l were made. For the quantification of Ψ_l hole pinnules of *D. antarctica*, the terminal 5 cm of pinnules of *P. esculentum* or separate individual shoots of *L. deuterodensum* outside

of the leaf chamber were excised from the pinna or shoot, immediately wrapped in damp paper towel and aluminium foil and pressure bombed to determine Ψ_l. Fortuitously the mucilage exuded from excised tissue of ferns gradually blocked the xylem at the terminus reducing hydraulic conductivity (K_{leaf}), such that after 15 min Ψ_l and g_s had begun to decline, the decline in K_{leaf} occurred over a very slow rate with disequilibrium in Ψ_l between pinnules minimal. In *L. deuterodensum* mucilage was absent so in order to stimulate a reduction in K_{leaf} and thereby a decrease in Ψ_l and g_s the terminus of the stem under water was briefly (<5 s) immersed in a slurry of equal parts flour and water. At least seven simultaneous measurements of g_s and Ψ_l were taken on each pinna or shoot, continuing until g_s reached a minimum and ceased to decline (at least 90 min), in some cases the terminus under water was recut and the relationship between Ψ_l and g_s assessed over rehydration. Stable minimum leaf conductances as leaves desiccated were assumed to represent cuticular conductance, and this residual value was subtracted from the measured conductance ensure the function $g_s = f(\Psi_l)$ passed through the origin. The relationship between g_s and Ψ_p was determined by rearranging a simplified equation based on the components of Ψ_l (Kramer *et al.* 1966) excluding matric potential and elastic modulus, using the Ψ_π determined from PV curves (in the case of *P. esculentum* the stand specific Ψ_π) (see equation 2).

Leaf hydraulic conductance

Leaf hydraulic conductance (K_{leaf}) was assessed over the water potential range equivalent to conditions experienced in the leaf cuvette (typically -0.2 to -1.5MPa) on 20-30 leaves or shoots from eight individuals of *D. antarctica* and *P. esculentum* collected from plants over the course of the day. In the lycophyte species *L. deuterodensum* maximum K_{leaf} was determined on entire shoots that had been excised under water in the field then bagged and rehydrated overnight in the laboratory. The apex (less than 20 cm) of the frond of *D. antarctica* was selected for measurements of K_{leaf} as it was found to be a region of the rachis with the least secreted mucilage following excision. The terminal portion of a pinna of *P. esculentum* was selected for the measurement of K_{leaf}, in both cases the lower pinnules were removed to reduce the water flux into the leaf and hence the rate of mucilage blocking.

The selected frond or shoot tissue was briefly enclosed in damp paper towel while a pinnule or side branch was excised, wrapped in damp paper towel and aluminium foil and bagged for assessment of Ψ_l in a pressure chamber. The frond or shoot was then unwrapped and immediately excised in filtered and de-ionised water under a light source (PPFD of 1000 µmol quanta m^{-2} s^{-1}) and temperature of 22°C, and connected to a high precision flowmeter

(Brodribb and Holbrook 2006). The assessment of K_{leaf} followed two protocols detailed by Brodribb and Cochard (2009), the first was based on the instantaneous flow rate into non-transpiring leaves (logged every 1 s) and the second method by allowing the leaf to reach an evaporational steady state (no more than 150 s). K_{leaf} was determined by the following equation:

$$K_{leaf} = \frac{I}{A_{leaf}\Psi_{leaf}} \quad (8)$$

Where, I is the instantaneous flow rate into the leaf (mmol s^{-1}) and A_{leaf} the projected leaf area.

Testing the model through a response to VPD and leaf excision

The response of steady state g_s to step-wise changes in VPD and following leaf excision in air was recorded in five leaves from three individual plants of *D. antarctica*, *P. esculentum* and *L. deuterodensum*. Pinnae or shoots were excised under water in the laboratory, with leaf tissue enclosed in a portable infrared gas analyser (as described above); the remainder of the pinna or shoot was prepared as previously described in the ABA feeding experiment. Leaf chamber VPD was regulated manually by adjusting the humidity of the inlet air, either by bubbling air through water to produce lower values of VPD (<1 kPa), by adjusting the amount of inlet air passing through a desiccant column containing calcium sulfate (1 to 2.5 kPa) or by increasing the leaf temperature to 30°C (>2.5 kPa). Increasing leaf temperature while regulating stable VPD had no effect on steady state g_s or when compared to expected g_s based on model calculations (data not shown). One to two step-wise transitions in VPD were undertaken on a single leaf with g_s data logged every 60 s and standardised against leaf area, transitions in VPD were not undertaken until g_s had stabilised, see above. Transitions in VPD encompassed a range of 0.5 to 3.5 kPa. For each leaf 3 VPD transitions were typically made (*e.g.* 1-2-1 kPa) after which the leaf was excised in air and declining g_s recorded as leaves desiccated. Residual leaf conductances after stabilization were assumed to represent cuticular water loss and this small conductance was subtracted from the measured conductance.

Response of g_s to darkness

The stomatal aperture response to darkness was recorded in all fern species as well as the angiosperm control. Fronds of species growing in the field (Table 2.1) were collected as described previously with leaf tissue enclosed in the leaf chamber of a portable infrared gas analyser under the conditions described in the ABA feeding experiment. Leaves were equilibrated to the leaf chamber conditions for 10 min after which the lights in the chamber

were switched off. Data were logged every 60 s until g_s had stabilised as described above, the leaf was then excised and data were standardised against the leaf area in the chamber.

Three individuals from each species grown in pots under glasshouse conditions were transported to a light insulated growth chamber in a dark room (temperature 18°C) at 15:00 h (leaf level PPFD of 150 µmol quanta m^{-2} s^{-1} provided by fluorescent incandescent lighting). The day/night light patterning was gradually reversed by initiating an initial 7 h 'night' starting at 17:00 h, followed by an 8 h 'day' starting at 01:00 h and concluding at 09:00 h the following morning. Instantaneous measurements of g_s were logged using a portable infrared gas analyser (leaf chamber conditions were maintained at PPFD 150 µmol quanta m^{-2} s^{-1}, leaf temperature at 18°C, leaf chamber flow rate at 500 µmol s^{-1}, with inlet air supplied through a buffer container drawing from the growth chamber) on the same location on a single leaf from each of the three individuals of each species. Once the leaf was enclosed in the leaf chamber 30 to 60 s was required before all parameters had stabilised and data could be logged. Data were collected twice before lights went out at 09:00 h then following the initiation of darkness every 15 min for the first 2 h then every hour after that until 14:00 h. Green fluorescent light (PPFD 0 µmol quanta m^{-2} s^{-1}) was provided by a fluorescent lamp during the measurements. Following data collection all leaves were excised in air and allowed time for sufficient stomatal closure to assess cuticular conductance. Data were standardised against the leaf area in the chamber.

RESULTS

Absent ABA induced stomatal closure

Stomatal conductance in ferns and lycophytes was insensitive to exogenous ABA taken up through the transpiration stream (Figs 2.1 and 2.12). The stomata of nine fern and lycophyte species did not close following at least 90 minutes of feeding 15000 ng ml^{-1} (or 56.7 µM in the xylem sap) ABA into the transpiration stream (Figs 1A; 2C and D). Stomata of the representative gymnosperms (*Ginkgo biloba* and *Callitris rhomboidea*) closed by 58% and 68% respectively while the stomata of the representative angiosperm (*Helianthus annuus*) closed by 90% following the addition of the same concentration of ABA in less than 90 minutes (Figs 2.1A; 2.2A; 2.2B and Appendix 2.2). Feeding 15000 ng ml^{-1} ABA into the transpiration stream increased foliar ABA levels in all fern and lycophyte species to levels above 2000 ng g^{-1} FW (Fig. 2.1B). Following stomatal closure foliar ABA level in the two gymnosperms (*G. biloba* and *C. rhomboidea*) had increased to 1989 ng g^{-1} and 1767 ng g^{-1} respectively and in

the angiosperm (*H. annuus*) to only 1722 ng g^{-1} (Fig. 2.1B). Despite the lack of stomatal closure following the feeding of ABA into the transpiration stream and the subsequent high levels accumulated in the leaf, leaf excision in the lycophyte *Lycopodium deuterodensum* and the fern *Pteridium esculentum* resulted in immediate and pronounced stomatal closure (Figs 2.2C and 2.2D).

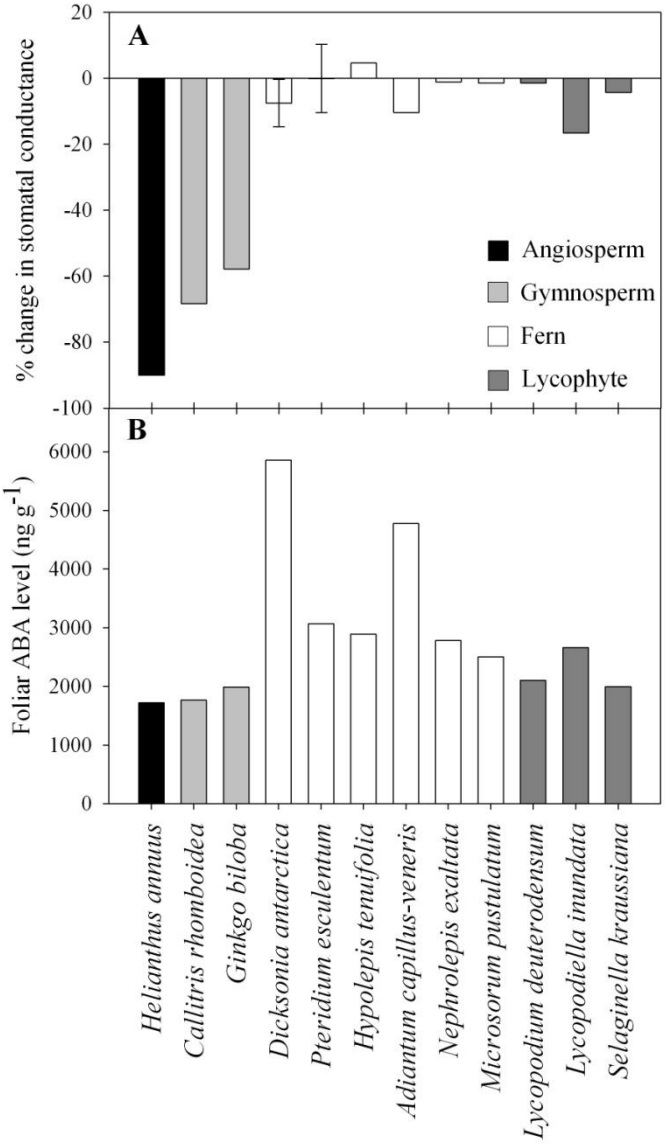

Fig. 2.1 Changes in stomatal conductance 90 min after the introduction of ABA into the transpiration stream at an equivalent xylem sap concentration of 5.67×10^4 mmol m^{-3}, in nine fern and lycophyte species and the representative angiosperm *Helianthus annuus* and gymnosperms *Ginkgo biloba* and *Callitris rhomboidea* (A). Values shown are percentages relative to an initial steadystate stomatal conductance established under optimal conditions. Means ±SE are shown for *Dicksonia antarctica* and *Pteridium esculentum* additionally fed both half and double the above-mentioned concentration. Foliar ABA level (ng g^{-1}) after 90 min of feeding an equivalent ABA xylem sap concentration of 56.7 µM into the transpiration stream (B).

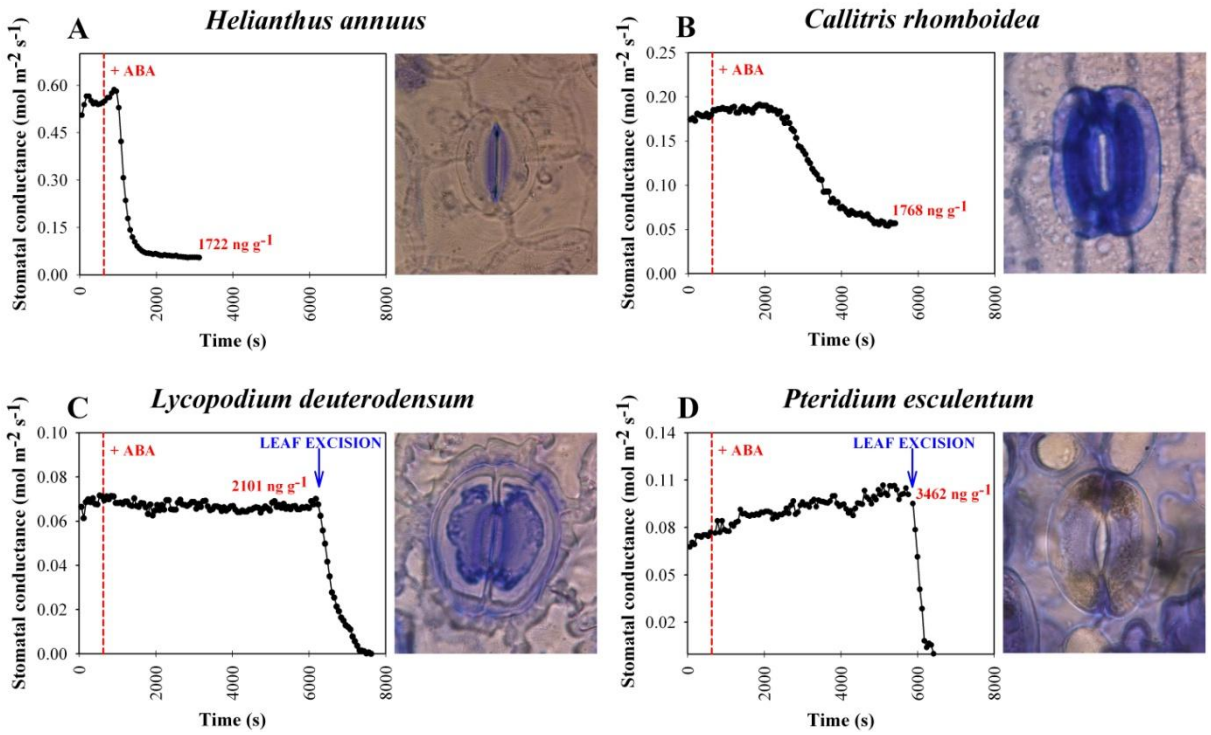

Fig. 2.2 Typical time courses of stomatal conductance (mol $m^{-2}s^{-1}$) after the introduction of a relatively concentrated 15000 ng ml^{-1} of ABA (or 56.7 µM in the xylem sap) into the transpiration stream a short distance from sample leaves. Dotted red lines show the moment of ABA feeding. The stomata of the angiosperm *Helianthus annuus* (A) and conifer *Callitris rhomboidea* (B) closed rapidly in response to ABA. In contrast neither the lycophyte *Lycopodium deuterodensum* (C) nor the fern species *Pteridium esculentum* (D) showed any response to high xylem ABA flux, despite the accumulation of high levels of ABA in the leaves of both species. Only leaf excision (arrowed) caused stomatal closure in these two ancestral lineages. Stomatal anatomy and dimensions for each species are shown (light microsocope images taken at 100x magnification; cuticles stained with crystal violet).

A range of ABA concentrations were fed into the transpiration stream of two fern species, the highest concentration resulting in foliar ABA levels in excess of 7000 ng g^{-1} FW, this range of ABA level fed into the transpiration stream likewise had no effect on g_s (Appendix 2.3). Not only were fern and lycophyte stomata insensitive to exogenous ABA but interestingly the ABA level in unstressed hydrated leaves was at least 2-fold lower compared to unstressed angiosperms and conifers (Appendices 2.4; 2.5 and 2.6).

Absent hydropassive effect

Wrong-way stomatal opening following leaf excision was absent in all 11 fern and lycophyte species examined (Fig. 2.3). In the representative angiosperm *H. annuus* immediately following leaf excision g_s increased by more than two-fold relative to initial g_s, after which stomata hydroactively closed (Fig. 2.3). In all fern and lycophyte species g_s declined immediately following leaf excision, this decline in g_s following excision occurred at species specific rates (Fig. 2.3).

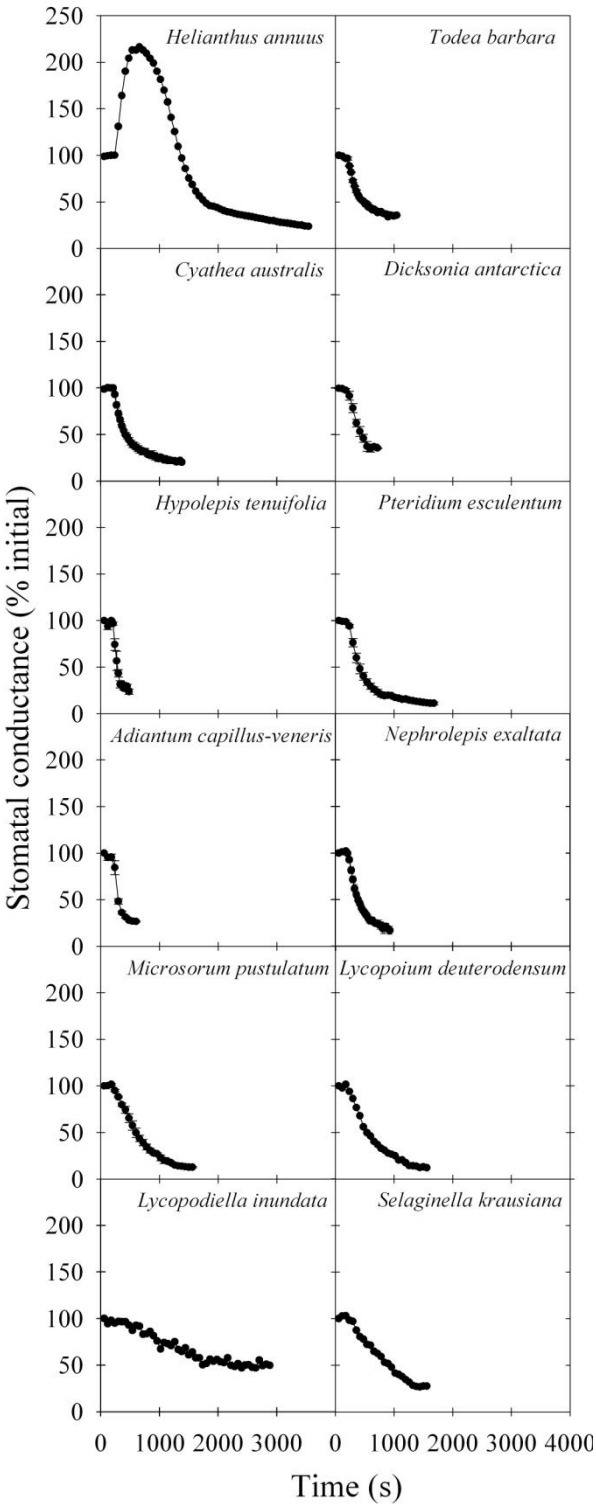

Fig. 2.3 The dynamics of stomatal conductance (represented as % of initial conductance) over time following leaf excision at 240 s in eleven species of fern and lycophyte and the angiosperms *Helianthus annuus*. Each species of fern is represented by the mean stomatal conductance of three individuals and standard errors, lycophyte species and *H. annuus* are each represented by a single individual.

Passive hydraulic model

The stomatal conductance of the lycophyte *L. deuterodensum* and two fern species *P. esculentum* and *Dicksonia antarctica*, could be consistently predicted by a purely passive hydraulic model for g_s in the light (Fig. 2.4). The model predicted that as VPD increases the water potential of the leaf declined which in turn directly decreased guard cell turgor pressure and thereby g_s and transpiration, and the opposite occurs when VPD decreases; this highly predictable behaviour was observed in the three species for which the model was parameterised and tested (Fig. 2.4). Observed g_s in the three species did not deviate from a 1:1 relationship with predicted conductance, in the steady state, transiently over steps of increasing or decreasing VPD or following rapid dehydration caused by leaf excision (Fig. 2.4). The dynamics of observed g_s following a series of changes in VPD and also leaf excision conformed to the predicted values of the passive hydraulic model in all species (Fig. 2.4). Consistent agreement between observed and predicted g_s occurred despite variability in the half-time of the stomatal kinetics between the three phylogenetically and functionally diverse species (Fig. 2.4), as well as variability in the hydraulic parameters used to formulate the model (Appendix 2.1).

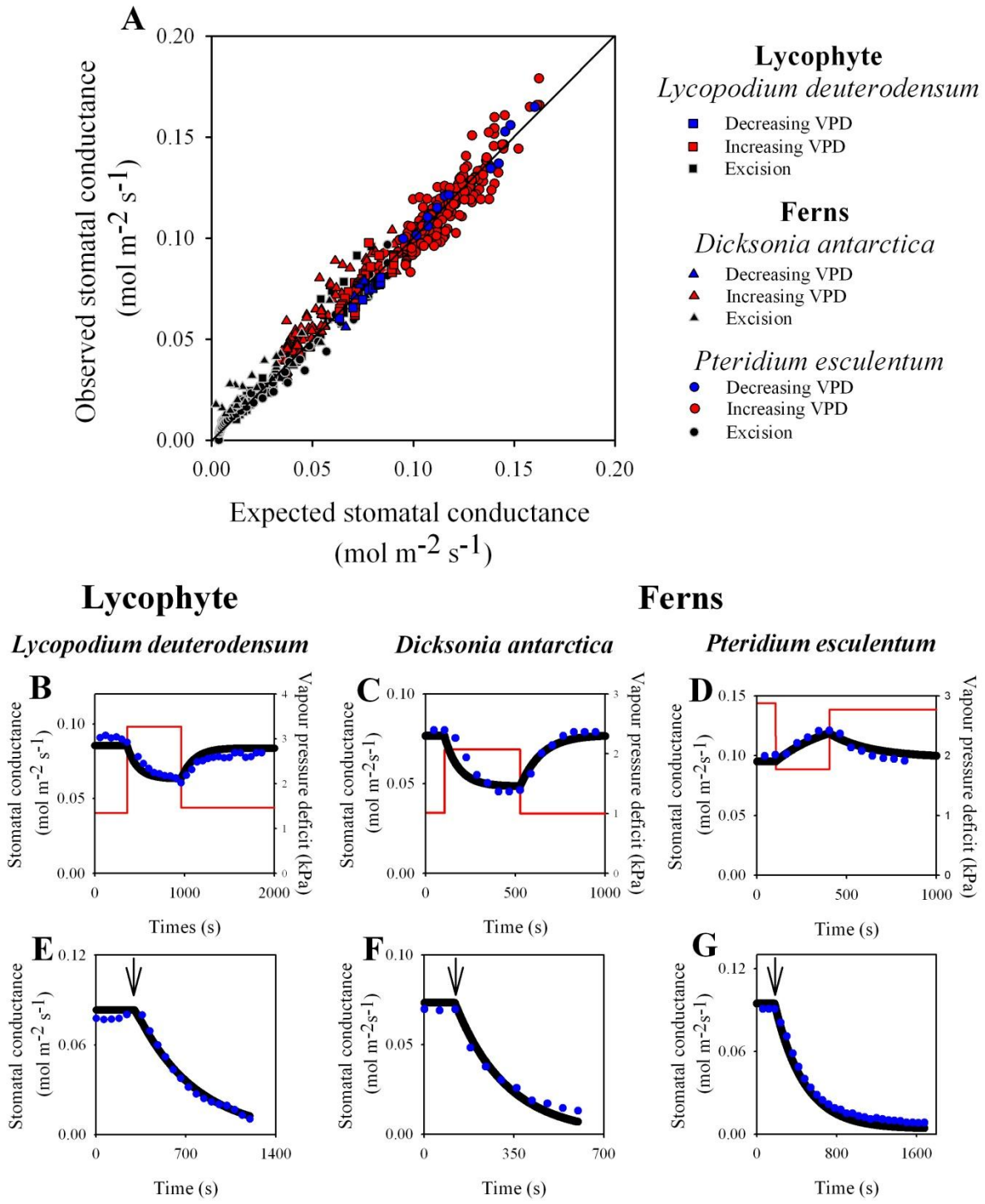

Fig. 2.4 The relationship between observed equilibrium and transient stomatal conductance following changes in vapour pressure deficit and following leaf excision, with predicted stomatal conductance from a passive hydraulic model in two fern species *Dicksonia antarctica* and *Pteridium esculentum* and a lycophyte *Lycopodium deuterodensum* (A). Representative dynamic responses of observed stomatal conductance (blue circles) and predicted stomatal conductance (thick black line) over step changes in vapour pressure deficit (red line) (B-D) and following leaf excision (marked by arrow) (E-G) are shown for each of the three species.

DISCUSSION

There are two stomatal processes that do not support a passive hydraulic stomatal response to leaf water status in angiosperms, (i) active closure of stomata by the exogenous application of the phytohormone ABA and (ii) a close ionic and mechanical connection of guard cells with the epidermis necessitating hydroactive stomatal responses following perturbations in leaf water status. Unlike angiosperms, ferns and lycophytes lack both active ABA induced stomatal closure (Figs 2.1 and 2.2) and wrong-way stomatal responses and associated hydroactive stomatal behaviour (Fig. 2.3). Yet the stomatal response to leaf water status in ferns and lycophytes, whether through altering VPD or following rapid leaf dehydration on excision, could be accurately predicted by a hydraulic model based solely on leaf water status in the light (Fig. 2.4). This suggests that the ancestral state of stomatal control in vascular plants was a simple passive response to leaf water status in the light, without many of the active regulators of stomatal aperture seen in angiosperms. The first vascular land plant stomata likely evolved to reduce transpiration when leaf water content declines and to open to facilitate photosynthesis when leaves are hydrated in the light.

Lack of active ABA-induced stomatal closure

Prior to this study the stomatal sensitivity to ABA in ferns and lycophytes had never been examined; despite ABA synthesis and signalling mutants being identified in the fern *Ceratopteris richardii* (Hickok 1985). Studies have reported an inconsistent response of stomata to ABA in the non-vascular bryophytes. Garner and Paolillo (1973) reported a brief (maximum 18 min) stomatal closure following the application of ABA to excised sporophyte paradermal sections of the moss *Funaria hygrometrica*, while in hornwort species the response to ABA is varied with some evidence for stomatal closure (Hartung *et al.* 1987) while others have reported response to ABA (Lucas and Renzaglia 2002). The definitive stomatal response of bryophytes to ABA remains largely unknown due to the technical limitation of observing aperture responses in less than 15 stomata. Here I show no physiological effect of ABA on the stomata of ferns and lycophytes.

It is unlikely that the exogenous level of ABA feed into the transpiration stream of ferns and lycophytes in this study was not sufficient to induce stomatal closure. The level of ABA in the leaves of the fern and lycophyte species fed exogenous solutions was higher (>2000 ng g^{-1} FW) than the endogenous levels of ABA encountered by angiosperms and conifers (rarely more than 500 ng g^{-1} FW) under the natural water stress conditions at which stomata close

(Appendices 2.5 and 2.6). The treatment of exogenous ABA to the leaves of the fern and lycophyte species examined in this study was sufficient to enable stomatal closure in two representative gymnosperms and an angiosperm herb (Fig. 2.2; Appendix 2.2) and based on the levels in the leaf should have been sufficient to close the stomata of a wider diversity of species (Appendix 2.5).

Stomatal closure in response to ABA in seed-plants is governed by Ψ_l (Tardieu and Davies 1992); a high Ψ_l reduces the responsiveness of stomata to ABA concentration (Tardieu and Davies 1993). Limited stomatal sensitivity to ABA at high Ψ_l has been reported at very low concentrations of ABA (500 μmol m^{-3}) (Tardieu and Davies 1993). In this study ABA concentrations fed into the xylem were 100-fold higher (56400 μmol m^{-3}) than this concentration. Based on an extrapolation of the relationship between g_s and xylem ABA concentration in hydrated leaves (Tardieu and Davies 1993) the levels employed in this study would result in at least 70% stomatal closure in angiosperms; this was observed in the representative angiosperm and gymnosperm species (Fig. 2.2; Appendix 2.2). Thus it is unlikely that the full hydration of fern and lycophyte leaves would have countered the ABA sensitivity of stomata.

In angiosperms the stomata of ABA-insensitive mutants, irresponsive to exogenous and endogenous ABA, have aberrant stomatal morphologies compared to wild-type individuals (such as asymmetrical guard cells (Wigger *et al.* 2002) and vastly increased pore sizes (Arend *et al.* 2009)). This however is not the case in ferns and lycophyte that are insensitive to ABA as well. Ferns and lycophytes have similar stomatal anatomies to seed plants (Zhang and Davies 1989), often with smaller pore sizes. Light microscopy images of the stomata of the lycopod *Lycopodium deuterodensum* and fern *Pteridium esculentum* and two representative seed plants illustrates these anatomical similarities (Fig. 2.2). While it is unlikely that stomatal morphology can explain a lack of stomatal sensitivity to exogenous ABA in ferns and lycophytes, the possibility that ferns and lycophytes have fewer or less effective guard cell membrane pumps compared to seed plants cannot be ruled out. This possibility could be resolved by monitoring guard cell ion fluxes and membrane depolarisation in ferns and lycophytes fed ABA.

Interestingly the absence of stomatal closure in response to exogenous ABA in ferns and lycophytes is likely not due to absent ABA signalling or synthesis genes given that most of

these genes are present in the sequenced genome of the moss, *Physcomitrella patens* (Rensing *et al.* 2008) and lycophyte *Selaginella moellendorffii* (Banks *et al*. 2011). The presence of these genes as well as the results of a number of physiological studies indicate that ABA in the basal lineages of land plants is physiologically active (Hsu *et al.* 2001; Liu *et al.* 2008; Khandelwal *et al.* 2010), even if it plays no role in regulating stomatal aperture (Fig. 2.2). ABA is synthesised in response to osmotic and abiotic stress and is vital for the initiation of desiccation-tolerance genetic pathways across a wide diversity of lineages of life (from bacteria (Karadeniz *et al.* 2006), basal animal clades such as the sponges (Zocchi *et al.* 2003), non-vascular plants including mosses (Khandelwal *et al.* 2010), and vascular plants such as desiccation-tolerant lycophytes in the genus *Selaginella* (Liu *et al.* 2008; Xie *et al.* 2008), ferns (Bagniewska-Zadworna *et al.* 2007) and angiosperms (Bartels 2005)). Increased ABA levels in ferns trigger anatomical changes in the leaves of the aquatic fern *Marsilea quadrifolia* from aquatic to aerial leaves (Hsu *et al.* 2001; Lin *et al.* 2005), as well as playing an important role in the sexual differentiation of gametophytes (Hickok 1983; Banks *et al.* 1993). With an ancient origin linking ABA with abiotic stress signalling, it seems a logical and advantageous evolutionary step for land plants to capitalise on this effective proxy for current and past water status by evolving an active regulation of stomatal aperture by ABA.

Lack of ionic connections to the epidermis

Stomatal closure in ferns and lycophytes following leaf excision was quite dissimilar to the hydroactive stomatal closure observed in angiosperms occurring after a transient wrong-way opening (Buckley *et al.* 2003; Powles *et al.* 2006; Kaiser and Legner 2007) (Fig. 2.3). It is likely that in angiosperms the development of highly patterned subsidiary cell complexes belonging to the same cell lineage as the guard cells (Peterson *et al.* 2010) greatly enhances the ionic connections between the guard cells and the epidermis, and may explain a strong metabolically active response of stomata to changes in leaf hydration. The lack of close ionic and mechanical connection between the guard cells and the subsidiary cells in ferns and lycophytes, and thereby a wrong-way stomatal response has been reported previously (Franks and Farquhar 2007).

Passive hydraulic stomatal control and vascular plant evolution

Daytime stomatal conductance of ferns and lycophytes could be predicted using a dynamic model based solely on leaf hydraulic parameters (Fig. 2.4). The strong correlation between predicted g_s based on a passive hydraulic model and observed g_s in the steady-state,

transiently over varying VPD and also following leaf excision was due to a lack of active stomatal control mechanisms in response to changes in leaf water status (Figs 2.2; 2.3 and 2.4). Interestingly the predictability of fern stomatal responses to changes in VPD has been recognised for over 40 years, however only in isolated epidermis (Lange *et al.* 1971) Whether or not there is any phylogenetic difference in the response of stomata from isolated epidermis to changes in VPD is yet to be explored. However angiosperm stomatal responses in isolated epidermes to changes in VPD appear to be as predictable as fern stomata (Shope *et al.* 2008).

The absence of a stomatal response to exogenous ABA suggests that one of the only active control mechanisms of g_s in the seedless vascular plant lineages is the response to red light, possibly through the induction of photosynthesis in the guard cells (Doi and Shimazaki 2008) (Appendix 2.7). The lack of active stomatal control processes in ferns and lycophytes does not mean these lineages have ineffective stomatal control (Figs 2.2; 2.2 and 2.4). The passive response of stomata to leaf water balance enables the stomata of ferns and lycophytes to regulate plant water loss depending on leaf hydration (Fig. 2.4). The passive response of stomata in the light to leaf water status in ferns and lycophytes is the simplest means of achieving the basic role of stomata, allowing optimal photosynthesis while preventing terminal water loss (Farquhar and Sharkey 1982), and appears to be the ancestral state for stomatal control in vascular plants.

Conclusion

The stomatal aperture of ferns and lycophytes is passively controlled by leaf water balance in the light (Fig. 2.4). The active process of closing stomata in response to ABA, instrumental in regulating the stomatal response to changes in leaf water status in angiosperms, is absent in the basal lineages of vascular plants (Figs 2.2 and 2.3). Ferns and lycophytes are insensitive to exogenously applied ABA with the turgor of the guard cells in the light in close hydraulic equilibrium with leaf water status (Fig. 2.4). This mechanism of stomatal control provides important insight into the evolution of metabolic processes which regulate the stomatal aperture of angiosperms. It seems that the active processes governing stomatal response to changes in leaf water status in angiosperms evolved after the divergence of ferns 400 million years ago, with the ancestral state of stomatal control in vascular plants being one of an active response to red light and a passive response to leaf water balance (Doi *et al.* 2006; Doi and Shimazaki 2008; Brodribb *et al.* 2009) (Fig. 2.4).

CHAPTER 3

Fern and lycophyte guard cells do not respond to endogenous abscisic acid

The text and results of this chapter are taken directly from the following publication:

McAdam, S.A.M. and Brodribb, T.J. (2012). Fern and lycophyte guard cells do not respond to endogenous abscisic acid. *Plant Cell* 24, 1510-1521.

ABSTRACT

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

INTRODUCTION

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

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

ABA mutants and the genes identified from these mutants have provided important information about the molecular and biochemical pathways regulating guard cell turgor (Pei

et al. 1997; Pei *et al.* 2000; Geiger *et al.* 2010; Geiger *et al.* 2011) and the role of ABA in these processes, with numerous comprehensive reviews on the topic (Schroeder *et al.* 2001; Outlaw Jr. 2003; Li *et al.* 2006; Kim *et al.* 2010). However there are many unresolved issues associated with the performance of ABA mutants that prevent this approach from providing a comprehensive understanding of the physiological controls of guard cells. Conflicting evidence from ABA mutants has been unable to convincingly resolve the control of stomatal responses to changes in humidity, particularly the role of ABA in regulating wild-type responses to these small changes in leaf hydration (Assmann *et al.* 2000; Xie *et al.* 2006). Adding to this ambiguity are data suggesting that the responses of wild-type stomata to changes in humidity can be accurately predicted without any influence of metabolic signals such as ABA (Peak and Mott 2011). Furthermore, the interconnectedness of many biochemical pathways responsible for stomatal control make it difficult to decipher particular guard cell metabolism and biochemistry responsible for stomatal responses to signals such as red light and photosynthesis (Lawson 2009). As a result there still remains little integration between the well documented guard cell membrane processes and the responses of stomata to changes in environmental conditions experienced in the field. In particular, data from hydraulic experiments raise uncertainty about the centrality of ABA in closing stomata during water stress in whole plants (Oren *et al.* 1999; Brodribb and Cochard 2009). This disparity between the membrane-level understanding of stomatal control and whole-plant behaviour widens considerably on consideration of current models predicting stomatal behaviour, many of which pay little heed to an appreciable role of ABA in regulating stomatal responses (Buckley *et al.* 2003; Buckley 2005; Damour *et al.* 2010). With the current array of guard cell signalling mutants unable to resolve many of these issues, prospects for alternative genetic approaches to understanding guard cell signalling are highly appealing. One such alternative is to investigate natural genetic variation observed across the phylogeny of land plants.

The phylogenetic approach to examining stomatal control is a recently emerging possibility that provides a means of identifying novel pathways associated with the evolution of components in the stomatal control network, and has the potential to bridge the gap between membrane-level processes and whole plant function. Several major discoveries have demonstrated the power of a phylogenentic approach in the investigation of stomatal physiology, including evidence for absent phototropin-mediated responses of guard cells to blue light in the basal lineages of vascular plants (Doi *et al.* 2006; Doi and Shimazaki 2008)

and more recently, evidence that the stomata of early-branching vascular plants are only equipped with very simple responses to light and water content (Brodribb *et al.* 2009; Brodribb and McAdam 2011b; Haworth *et al.* 2011). These studies suggest that over the 400 million years since the evolution of stomata (Edwards *et al.* 1998) the control of stomatal aperture has increased in complexity, and that this is reflected in the characteristic stomatal behaviours of the different clades of living vascular plants (McAdam and Brodribb 2012a). Central to the theory of gradualistic stomatal control evolution is evidence that regulation of transpiration by ABA is a derived character, evolving after the divergence of seed plants (Brodribb and McAdam 2011b). This idea has been recently challenged by linked reports of ABA sensitivity in the bryophyte genus Funariaceae (Chater *et al.* 2011) and the lycophyte species *Selaginella uncinata* (Ruszala *et al.* 2011). In their challenge to the gradualistic model of stomatal control evolution these authors conclude that stomata evolved 400 million years ago in possession of all of the signalling and control complexity seen in modern angiosperm stomata (Chater *et al.* 2011; Ruszala *et al.* 2011). Testing these diametrically opposed alternatives to the evolutionary history of stomatal function is imperative if we are to resolve the current impasses in our understanding of guard cell function in higher plants and the integration of guard cell membrane-specific processes with global models of transpiration.

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

MATERIALS AND METHODS

Responses of vascular plants to drought and the separation of the effects of ABA and water potential on stomatal closure in excised leaves

The response to drought was observed in six diverse vascular plant species including , two ferns *Dicksonia antarctica* (Dicksoniaceae) and *Pteridium esculentum* (Dennstaedtiaceae), a lycophyte *Selaginella kraussiana* (Selaginellaceae), two conifers *Callitris rhomboidea* (Cupressaceae) and *Pinus radiata* (Pinaceae) and the angiosperm *Pisum sativum* (Fabaceae) wild-type line derived from the cultivar Torsdag (Hobart L107). Six individuals of each species were grown in pots in the glasshouses of the School of Plant Science, University of Tasmania, Hobart, Australia under natural light, supplemented and extended to a 16 h

photoperiod by sodium vapour lamps ensuring a minimum 300 µmol quanta m^{-2} s^{-1} at the leaf surface throughout the day period, temperature was maintained at 25°C during the day and 15°C at night. In all species stomatal conductivity measurements were made at midday (1200 to 1300 h) using an infrared gas analyser (Li-6400; Li-Cor, Lincon, NE, USA) on healthy photosynthetic tissue (leaf cuvette conditions: leaf temperature 22°C, vapour pressure difference (VPD) between 1.1 and 1.2 kPa, 390 µmol mol^{-1} CO_2 concentration and light intensity at 1000 µmol quanta m^{-2} s^{-1}), until stomata had closed to less than 20% initial conductances (between 3 and 7 days depending on the species measured). All gas exchange data were adjusted for leaf area in the cuvette on each measurement. Concurrently with every measurement of gas exchange, tissue was taken for ABA quantification (see below) and leaf water potential measurement using a Scholander pressure chamber and microscope to precisely measure the xylem balance pressure. Measurements were made prior to drought when all individuals were well watered; drought was then initiated by withholding water.

To examine the effects of ABA and water potential on stomatal closure across the land plant phylogeny, three individuals from the six species listed above were selected. When the stomata of these individuals had closed to less than 20% initial conductances, plants were brought into the laboratory and the leaf, branch or pinnule, from which the initial rates of gas exchange prior to drought were measured from were enclosed in the chamber of the gas analyser and the majority of the leaves or pinnules on the same stem or rachis were excised; tissue again was taken for ABA quantification and water potential measurements, representing water potentials and ABA levels during peak water stress. Gas exchange and leaf environmental data were automatically logged every 1 min. After an acclimation period of at least 10 min in the cuvette the stem or rachis was excised under water containing ABA at a concentration of 1000 ng ml^{-1} (in the angiosperm *Pisum sativum* and conifer *C. rhomboidea* the experiment was repeated using water with no added ABA). The cut end of the stem was regularly recut to maximise water flow and avoid xylem blocking. After 10 to 20 min following recutting another branch or pinnule outside of the cuvette of the gas analyser was excised and immediately wrapped in damp paper towel for immediate measurement of water potential and quantification of ABA, representing hydrated tissue with high levels of ABA.

Fern responses to soil drying and rewetting

To examine the response of ABA level and transpiration to water stress and subsequent recovery by soil rewetting in ferns, two phylogenetically disparate species with contrasting

growth habit were selected. The understorey tree-fern from temperate rainforest, *Dicksonia antarctica* and the rhizomatous, cosmopolitan fern from relatively dry habitats in full sun, *Pteridium esculentum*. Each species was represented by three potted individuals acclimated to growth cabinet conditions under a 14 h photoperiod, with light supplied by mixed fluorescent and incandescent globes providing 600 µmol quanta m^{-2} s^{-1} at leaf height, 25°C/16°C day/night temperatures and 50% relative humidity (controlled by a dehumidifier with integrated humidity sensors (SeccoUltra 00563, Olimpia-Splendid, Italy). Temperature and humidity were logged for the duration of the experiment using a HOBO Pro-series data logger (Onset, MA, USA). Whole plant midday (1200 to 1300 h) gas exchange was monitored daily by weighing triple-bagged pots on a computer-interfaced balance to an accuracy of ± 0.01 g (Mettler-Toledo PG5002-S). Transpiration was calculated as the loss of weight between measurements divided by the total leaf area. Leaf area was measured by scanning leaves prior to and on conclusion of the experiment. In both species drought resulted in varying degrees of leaf senescence (up to 20%) apparent the day following rewatering and recovery; whole plant transpiration (expressed per m^2 of leaf area) was corrected for this change in leaf area. As vapour pressure difference remained constant through-out the experimental period, whole plant transpiration was proportional to stomatal conductance to water vapour.

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

Instantaneous regulation of stomatal aperture by water potential

In the three fern and lycophyte species, *Pteridium esculentum*, *D. antarctica* and *S. kraussiana,* as well as the angiosperm *Pisum sativum* and conifers *C. rhomboidea* and *Pinus radiata,* the role of leaf water potential in regulating stomatal aperture was investigated in non-droughted leaves with low levels of ABA. Branches or pinnules attached to the plant

were enclosed in the cuvette of a gas analyser under the conditions described above and data were automatically logged every 1 min. After an initial period, during which leaf gas exchange equilibrated to conditions in the cuvette, tissue on the same branch or leaf was excised, immediately wrapped in damp paper towel and bagged for water potential measurement. The branch or leaf bearing the tissue in the cuvette was excised, with approximately 30 cm of stem or rachis below the tissue in the cuvette and allowed to dehydrate. In all species gas exchange was monitored until stomata had closed by 50% of their initial conductance, following which tissue was collected from the same branch immediately wrapped in damp paper towel and bagged for water potential measurements (as above). The excised stem or rachis was recut under water and allowed to rehydrate. When gas exchange had reached stability following rehydration, tissue was again taken for water potential measurement. The cycle of dehydration to 50% stomatal closure then rehydration was repeated. Gas exchange measurements were adjusted for leaf area.

ABA and stomatal opening in the light

To test whether elevated levels of ABA in the leaf restricted stomatal opening in the morning in ferns, three species were selected spanning a spectrum of leaf gas exchange rate: (i) *Nephrolepis exaltata* (Lomariopsidaceae) with relatively low rates of gas exchange; (ii) *Pteridium esculentum* with moderate to high rates of gas exchange; and (iii) *Astrolepis sinuata* (Pteridaceae) with a high rate of gas exchange. Two angiosperms with similarly high rates of gas exchange were selected as controls: (i) *Lotus corniculatus* (Fabaceae); and (ii) *Oxalis corniculata* (Oxalidaceae). Leaves of the ferns and stems of the angiosperms were excised under water and, using marked fern pinnules or individual angiosperm leaves, steady-state stomatal conductivity to water vapour was recorded using an infrared gas analyser. Concurrently, with every measurement of leaf gas exchange, the level of foliar ABA was quantified (see below) from an adjacent pinnule or leaf. Once initial rates of gas exchange were recorded an aliquot of concentrated ABA was added to the water resulting in a concentration of 1000 ng ml^{-1} ABA entering the transpiration stream. The leaf or stem was then allowed to transpire under light for 20 min, with the end of the stem or rachis under water regularly re-cut allowing for continual hydration and flow of ABA into the leaf. After 20 min on the same pinnule or leaf, stomatal conductivity was recorded and an adjacent pinnule or leaf taken for ABA quantification. The leaf or stem, while still taking up ABA from the water, was covered with a black bag and left in the dark overnight. Prior to dawn (approximately 0600 h) the following morning the same pinnule or leaf was again enclosed in

the cuvette of the infrared gas analyser under the above described conditions and steady-state stomatal conductance was recorded prior to and following stomatal opening. At the same time an adjacent leaf was taken for ABA quantification. Leaf gas exchange measurements were adjusted for pinnule or leaf area in the cuvette.

ABA extraction, purification and quantification

In all species approximately 0.5 g of leaf tissue was used to quantify the foliar ABA level. ABA extraction and purification was performed according to the protocol of McAdam *et al*. (2011). Once the eluate was completely dried, ABA was taken up in 250 µl of 5% (v/v) methanol in 1% (v/v) acetic acid and centrifuged at 13 000 rpm for 3 min. Following centrifugation 100 µl of supernatant was taken for combined ultra-high performance liquid chromatography (UPLC) and multiple reaction monitoring (MRM) mass spectrometry/mass spectrometry (MS/MS) analysis using a Waters Acquity H-series UPLC coupled to a Waters Xevo triple quadrupole mass spectrometer. A Waters Acquity UPLC BEH C18 column (2.1 mm x 100 mm x 1.7 µm particles) was used. The solvents were 1% (v/v) acetic acid in water (Solvent A) and acetonitrile (Solvent B) at a flow rate of 0.35 ml min^{-1}, with a gradient of 80% A : 20% B to 5% A : 95% B at 5 minutes, followed by re-equilibration to starting conditions for 3 min. The column temperature was 45°C. A total of10µl of sample was injected. The mass spectrometer was operated in negative ion electrospray mode with a needle voltage of 2.7 kV; selected reaction monitoring was used to detect ABA and [$^2H_6$]ABA. The ion source temperature was 130°C. Desolvation gas was nitrogen at 950 l h^{-1}. Cone gas flow was nitrogen at 50 l h^{-1}. Desolvation temperature was 450°C. The MS/MS transitions monitored for ABA were *m/z* 263.2 to 153.1, 204.2 and 219.2; for [$^2H_6$]ABA the transitions were *m/z* 269.2 to 159.1, 207.2 (3 deuteriums were lost in this fragmentation) and 225.2. Cone voltage was 32 V in all cases. The collision energy was 18 V for the *m/z* 263.2 to 153.1 and 204.2 and corresponding deuterium labelled channels, and 16 V for the *m/z* 263.2 to 219.2 and corresponding deuterium labelled channel. Dwell time was 50 ms per channel. Data were analysed using Waters MassLynx and TargetLynx software. Quantitation was carried out using *m/z* 263.2 to 153.1 and the corresponding deuterium labelled channel. For all samples the ratio of endogenous ion intensity to internal standard ion intensity was calculated. The product of this ratio and the amount of internal standard added was divided by the fresh weight of the tissue sample and adjusted for aliquot volume to determine the level of ABA per gram fresh weight in the leaf.

RESULTS

Stomatal response to endogenous ABA during drought

In agreement with prevailing literature we found that our diverse sample of vascular land plants had identical physiological responses to water stress (Fig. 3.1). After the imposition of water stress all species closed their stomata concomitant with a decrease in leaf water potential and an increase in the level of ABA (Fig. 3.1). All species experienced a similar level of stress, with the decline in leaf water potential, augmentation of ABA and stomatal closure occurring over a period of 3 to 7 days. Leaf ABA levels rose markedly in all species, ranging from a 3-fold increase in conifers to a 20-fold increase in the fern *Dicksonia antarctica* (Fig. 3.1).

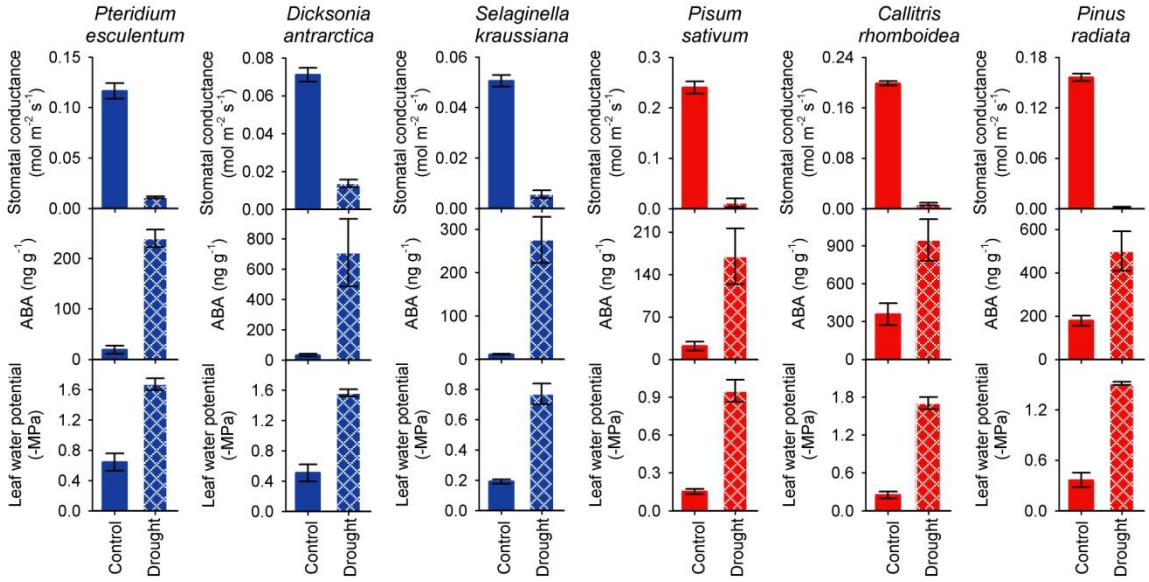

Fig. 3.1 The physiological responses of six diverse vascular plant species to drought stress.
In three seedless vascular plants, ferns and lycophytes (blue bars) and three seed-bearing vascular plants, angiosperms and conifers (red bars), stomatal conductivity (mol $m^{-2}s^{-1}$), foliar ABA level (ng g^{-1}) and leaf water potential (-MPa) was measured in pre-drought controls (solid bars) and following exposure to a water-stress that resulted in stomatal closure (hashed bars). Data represents means ± SE (n=6).

Rapid rehydration of excised droughted leaves allowed us to separate the simultaneous stomatal closing signals produced by leaf dehydration and ABA augmentation. In all species we found that after a period of minutes following excision under water, droughted leaves hydrated rapidly to water potentials equivalent to pre-drought levels while maintaining high endogenous ABA levels (Fig. 3.2). High levels of ABA in the leaf strongly reduced the reopening of droughted seed-plant stomata despite the restoration of pre-drought water potentials. Rehydrated stomatal conductances in our seed-plant sample (*Pisum sativum*, *Callitris rhomboidea* and *Pinus radiata*) were less than 20% that of pre-stressed leaves with low levels of ABA (Fig. 3.2). The suppression of stomatal reopening when droughted leaves

of seed-plants were instantaneously rehydrated was the same regardless of whether the leaf was rehydrated in a 1000 ng ml^{-1} concentrated solution of ABA or deionised water (Fig. 3.2).

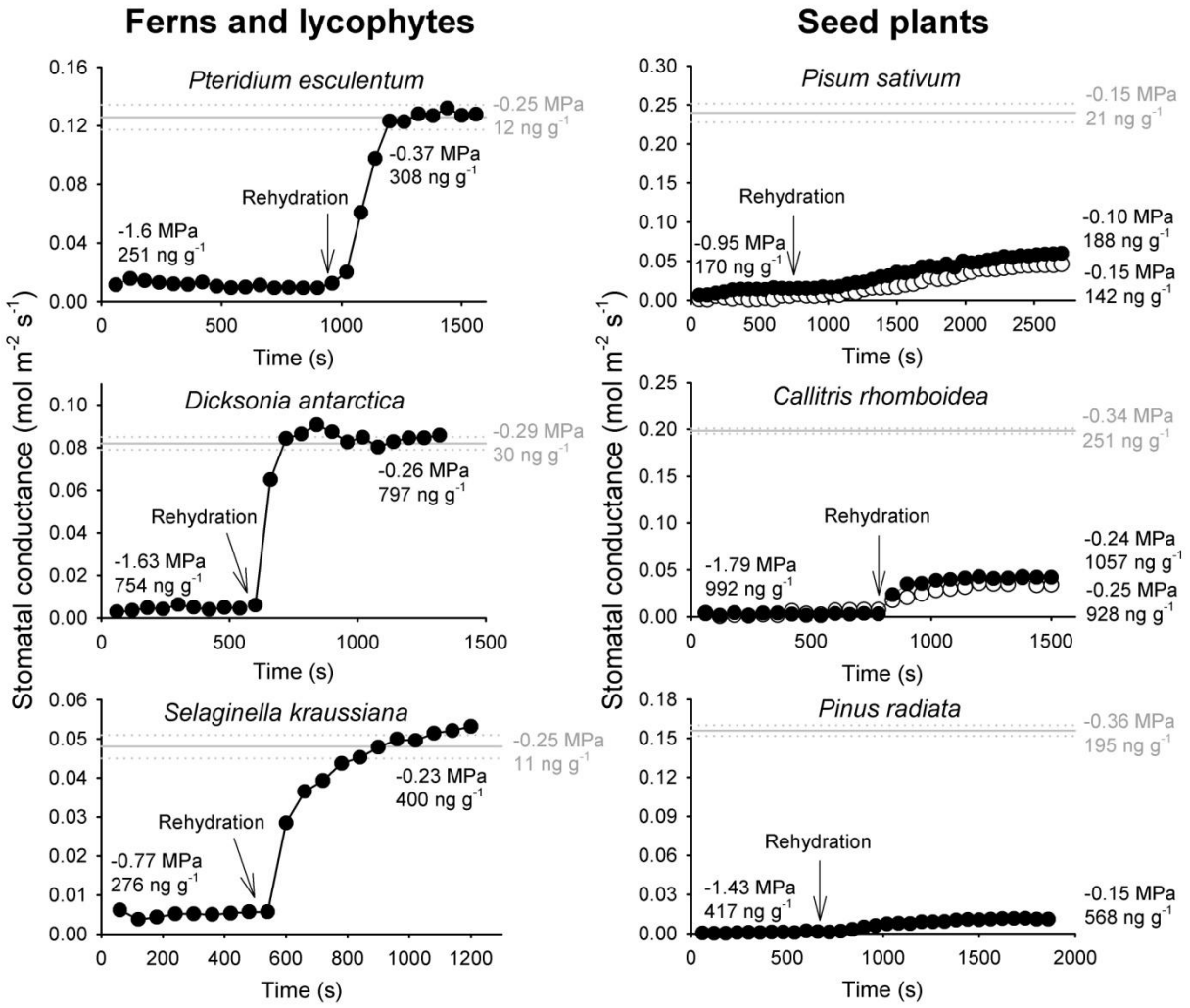

Fig. 3.2 Unlike seed-plants, instantaneous rehydration of droughted leaves allows the stomata of ferns and lycophytes to reopen despite high endogenous levels of ABA in the leaf.
Prior to drought, stomatal conductivity was measured on three leaves (mean represented by grey line; ± SE represented by dotted grey lines) in conjunction with leaf water potential (MPa) and foliar ABA concentration (ng g^{-1}) indicated in grey for each species. Plants were then droughted until stomata were closed and leaf water potential and foliar ABA concentration again measured. Stomatal conductivity was measured on a leaf that was instantaneously rehydrated in water containing 1000 ng ml^{-1} of ABA (black circles) and in two seed-plants in deionised water (white circles). Following rehydration, leaf water potential and foliar ABA concentration were again measured. Representative time courses of stomatal conductance are presented for each species.

In contrast to the stomata of seed-plants, the stomata of the representative ferns (*Pteridium esculentum* and *D. antarctica*) and lycophyte (*Selaginella kraussiana*) were found to be insensitive to the high endogenous levels of ABA produced by water stress, and were able to rapidly reopen to maximum apertures when the leaves were instantaneously rehydrated from a droughted state (Fig. 3.2). This instantaneous reopening of fern and lycophyte stomata occurred in leaves with high endogenous levels of ABA that were cut in water as well as leaves with endogenous ABA augmented by the 1000 ng ml^{-1} solution of ABA used to

rehydrate the leaves. The reopening of fern and lycophyte stomata during rehydration was rapid, occurring over less than 5 min, with stomatal conductances reaching pre-drought levels recorded in leaves from the same plants with very low levels of foliar ABA (Fig. 3.2).

Given the lack of ABA sensitivity in the stomata of water stressed ferns and lycophytes, we further investigated whether high levels of ABA augmented by water stress in ferns played any role in aiding plant recovery following water stress. We found that during water stress the stomata of two representative fern species (*Pteridium esculentum* and *D. antarctica*) were extremely sensitive to leaf water potential and not the concentration of ABA in the leaf (Fig. 3.3). During water stress, in these two fern species of contrasting growth habit and maximum transpiration rate, stomata closed predictably with the progressive dehydration of the plant (Fig. 3.3). Following recovery by rehydration of the soil, the stomata of both fern species reopened following a trajectory that reflected passive stomatal control by leaf water potential, as evidenced by minimal hysteresis in the leaf water potential stomatal conductance relationship during whole plant rehydration (Fig. 3.3). The recovery of leaf gas exchange to pre-droughted levels following rewatering was not dependent or influenced by the concentration of ABA in the leaf; ABA levels in the leaf increased during water stress but did not form any predictable relationship with stomatal aperture especially following rewatering and recovery from stress (Fig. 3.3).

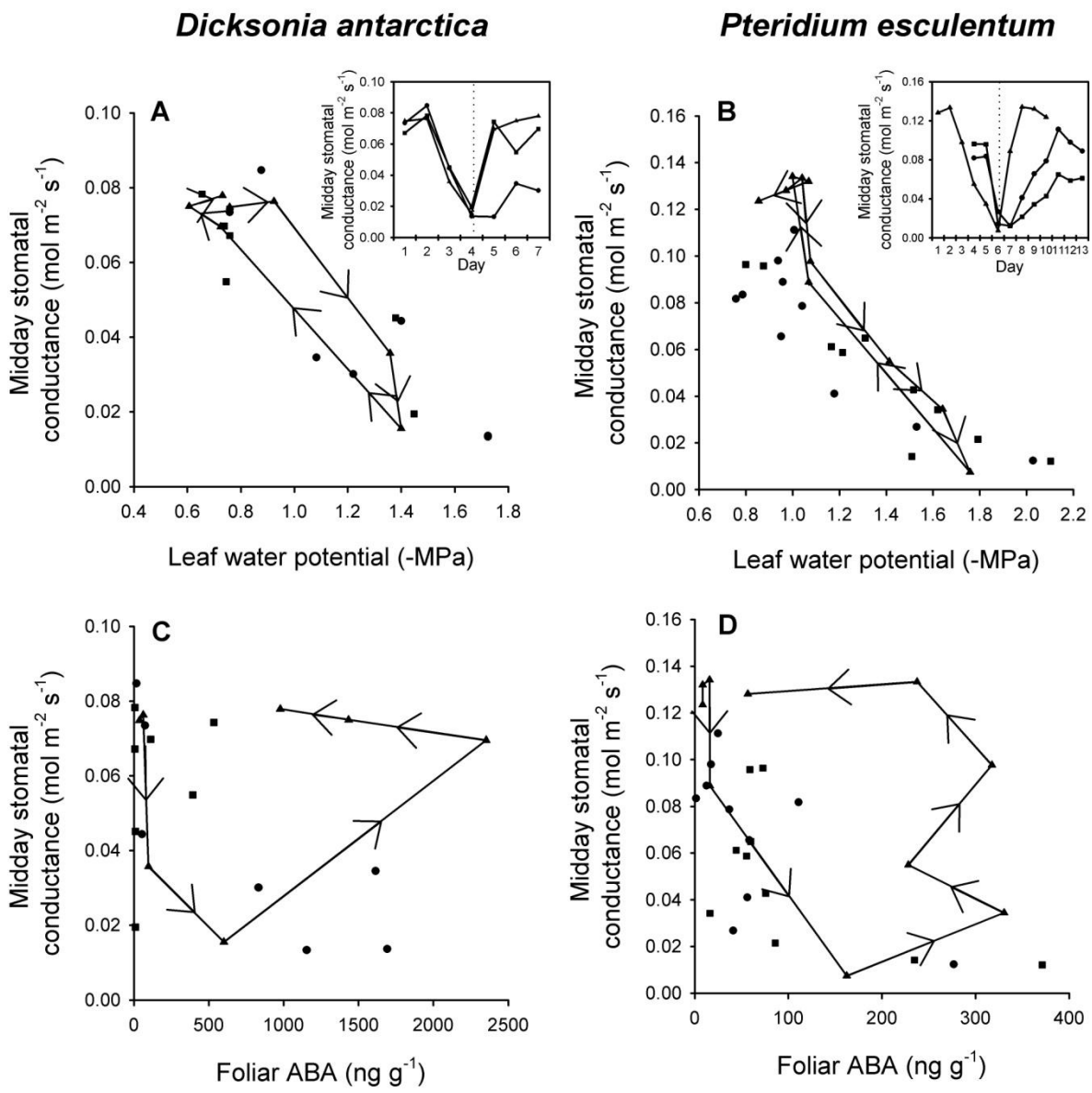

Fig. 3.3 During water-stress and recovery stomatal aperture in ferns is controlled by leaf water potential and not the concentration of ABA in the leaf.
In two fern species, *Dicksonia antarctica* and *Pteridium esculentum* midday stomatal conductance was measured in conjunction with leaf water potential (A and B) and foliar ABA concentration (C and D) in three individuals over a period of water stress and recovery by rewatering. Leaf water potential and foliar ABA concentration is shown for an individual of each species, with arrows indicating the progressive measurements during the drought and rehydration cycle; symbols correspond to the same individual of each species. The insert depicts midday stomatal conductance over drought and recovery in the three individuals (represented by different symbols) when water was withheld and the plants were stressed until stomata had closed; the dotted line represents the moment of rewatering and recovery from water stress.

Stomatal response to leaf water potential

Having demonstrated an insensitivity to water stress augmented ABA in fern and lycophyte stomata we examined the instantaneous effects of rapid fluctuations in leaf water potential on stomatal aperture in excised leaves to see if this responsiveness of fern and lycophyte stomata to leaf water potential occurred over short (<15 min) time scales. In two representative fern species (*Pteridium esculentum* and *D. antarctica*) and the lycophyte (*S. kraussiana*) leaf excision in the air resulted in immediate and progressive stomatal closure as leaves dehydrated (Fig. 3.4). The decline in stomatal aperture as fern and lycophyte leaves dehydrated could be arrested and reversed by rehydrating the leaf in water, with stomata rapidly reopening (Fig. 3.4). The response of fern and lycophyte stomata to leaf water potential was highly predictable over repeated cycling of leaf dehydration and rehydration (Fig. 3.4). In contrast, the stomatal responses of representative seed-plants (*Pisum sativum*, *C. rhomboidea* and *Pinus radiata*) to changes in leaf water potential were unpredictable (Fig. 3.4). In neither angiosperms nor conifers did stomatal aperture track changes in leaf water potential over cycles of leaf dehydration and rehydration (Fig. 3.4).

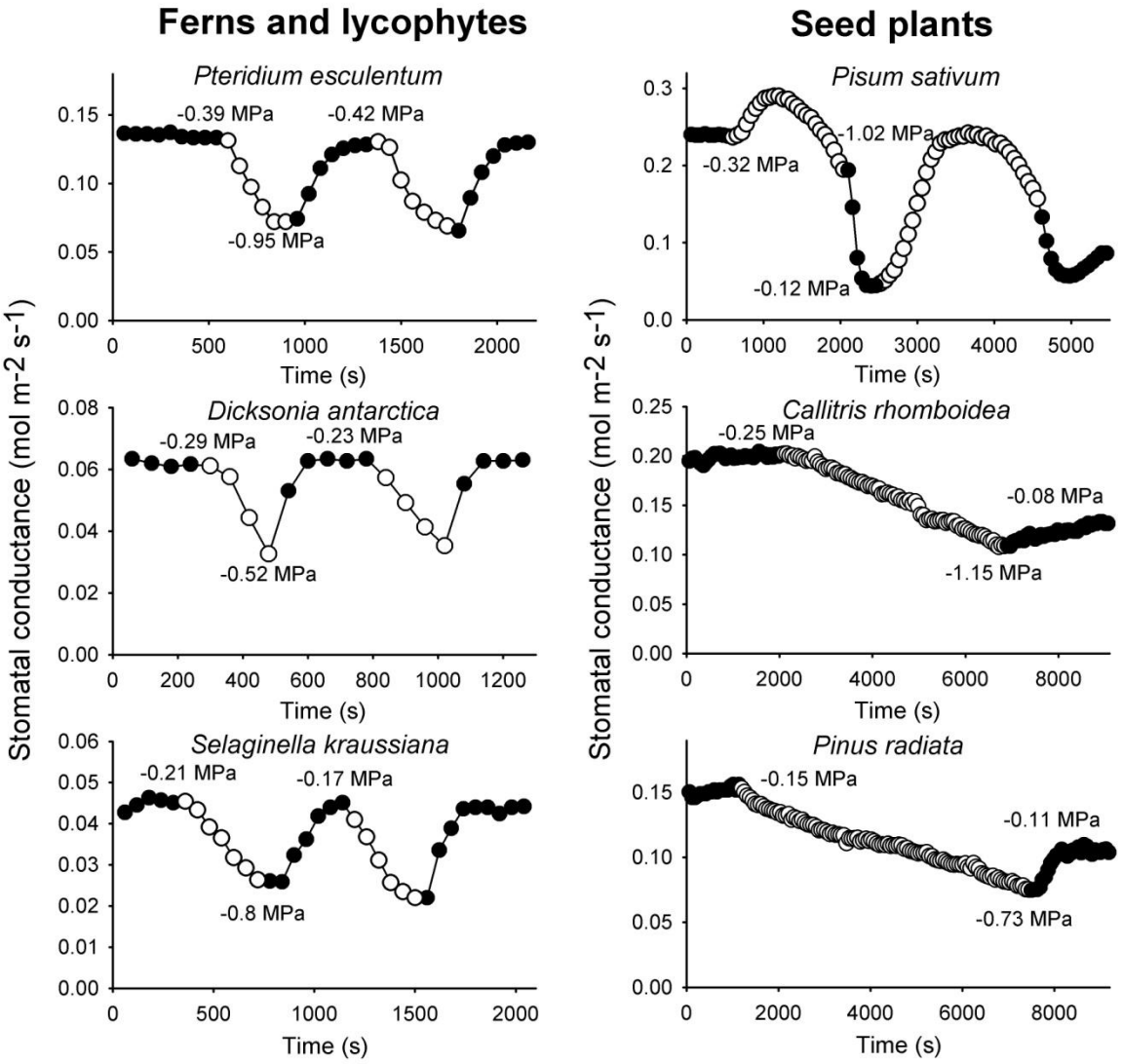

Fig. 3.4 Stomatal aperture responds directly to leaf water potential in excised leaves of ferns and lycophytes, but not in seed plants.
Leaf gas exchange was initially measured on the leaf of a fully hydrated plant. Leaf water potential was measured, then the leaf was excised and allowed to dehydrate (white circles); when stomata had closed by ~50% the leaf was recut under water and allowed to rehydrate (black circles) this cycle of dehydration and rehydration was repeated with leaf water potentials recorded prior to every transition.

Suppression of stomatal opening by ABA

In the absence of an ABA-mediated stomatal response to water stress in ferns and lycophytes we investigated whether exogenous ABA fed into the transpiration stream of representative ferns (*Astrolepis sinuata*, *Pteridium esculentum* and *Nephrolepis exaltata*) was able to supress light-activated stomatal opening in hydrated leaves after a normal nocturnal dark period during which the stomata of all species closed (Fig. 3.5). In two representative angiosperm species (*Oxalis corniculata* and *Lotus corniculatus*) exogenous ABA fed into the transpiration stream closed stomata and suppressed reopening in the light by more than 50% the following

morning, if the leaf was kept in the dark overnight (Fig. 3.5). In contrast, ABA was not able to supress light-activated stomatal opening in our photosynthetically diverse sample of fern species (Fig. 3.5). Regardless of maximum stomatal apertures, the stomata of ferns were not stimulated to close when exogenous ABA was fed into the transpiration stream and were able to reopen in the light in the presence of high leaf ABA levels after nocturnal closure (Fig. 3.5).

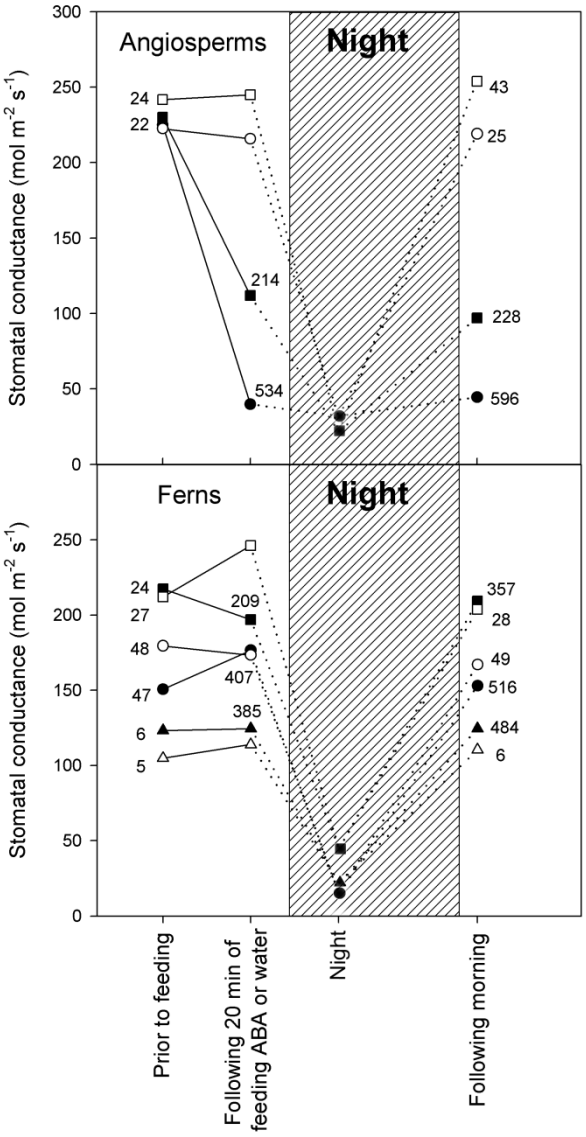

Fig. 3.5 ABA does not restrict metabolic stomatal opening in the morning when fed into the transpiration stream of ferns.
When ABA was fed into the transpiration stream (black symbols) of two angiosperm species, *Oxalis corniculata* (squares) and *Lotus corniculatus* (circles), stomata closed within 20 minutes and following 12 hours of darkness did not reopen to maximum apertures in the light the next day. Control leaves of angiosperms that were only fed water (white symbols) were able to open the following morning. When ABA was fed into the transpiration stream (black symbols) of three fern species, *Astrolepis sinuata* (squares), *Pteridium esculentum* (circles) and *Nephrolepis exaltata* (triangles), stomata did not close after 20 minutes; and the following morning were able to open in the light, just like control leaves fed only water. Foliar ABA levels (ng g^{-1}) are shown at each time point of gas exchange measurement (except during the night) in the ABA fed leaves and both morning measurements of the control leaves.

DISCUSSION

In agreement with the current paradigm of stomatal physiology we found that diverse lineages of vascular plants responded similarly to water stress, with stomata closing in parallel with increasing levels of ABA in the leaf and decreasing leaf water potential (Fig. 3.1). However, upon separating these two concurrent influences on stomatal closure we found profound differences between lineages. showing that fern and lycophyte stomata were insensitive to high endogenous levels of ABA, responding uniquely to the water potential of the leaf in a manner consistent with stomata acting as passive hydraulic valves (Brodribb and McAdam 2011b). Such passive stomatal responses in ferns and lycophytes contrast starkly with seed-plant stomata which actively regulate leaf hydration during water stress via ABA (Ache *et al.* 2010). These contrasting behaviours indicate a profoundly different process of turgor regulation occurring in the guard cells of ferns and lycophytes than that of seed-plants.

Contrasting stomatal responses to ABA across vascular plant phylogeny

Water stress appears to invoke stomatal closure in different ways depending on a species' position in the plant phylogeny. In seed-plants ABA regulates stomatal aperture especially during and after water stress, yet according to our results fern and lycophyte stomata respond passively to leaf water content and not ABA during changes in plant hydration (Figs. 3.2 and 3.4). The lack of stomatal regulation by ABA in ferns and lycophytes is surprising considering that all vascular land plants appear to synthesise ABA in response to water stress, including the ferns and lycophytes measured here (Fig. 3.1). However, rehydrating water stressed leaves with high levels of endogenous ABA provided a highly effective method for separating the influence of parallel changes in both leaf water content and ABA during drought stress on stomatal aperture. This technique revealed a dichotomy in stomatal control systems between ferns and lycophytes and seed-plants (Fig. 3.2) verifying the concept of passive stomatal control in ferns and lycophytes under natural conditions of water stress (Brodribb and McAdam 2011b).

Angiosperm stomata respond to water stress via a metabolic system of aperture control (Ache *et al.* 2010), and crucial to these responses is the presence of ABA in the leaf (Tardieu and Davies 1992). In the seed-plants sampled here, the effectiveness of endogneous ABA in reducing stomatal opening following the rehydration of droughted leaves supports extensive studies demonstrating the role of ABA in closing stomata during and after drought (Cornish and Zeevaart 1986; Christmann *et al.* 2005; Lovisolo *et al.* 2008). In contrast to seed-plants,

we found that the stomata of ferns and lycophytes were able to rapidly and fully reopen when rehydrated, despite the presence of high levels of endogenous ABA produced by water stress (Figs 3.1 and 3.2). Apparently, the only constraint for stomatal opening in the light in these early-branching clades of vascular plants is the hydration status of the leaf (Figs 3.2 and 3.5). The absence of an ABA-effect on the stomata of ferns and lycophytes leads to predictable stomatal responses to leaf water potential during short (Fig. 3.4) and long term (Fig. 3.3) water stress. The unpredictable responses of seed-plant stomata to instantaneous changes in leaf hydration are indicative of interactions between metabolic, hydraulic and mechanical effects on the guard cells as opposed to simple passive-hydraulic control of stomatal aperture suggested for ferns and lycophytes (Fig. 3.4; Brodribb and McAdam 2011b). *Pisum sativum* stomata epitomize the unpredictable nature of angiosperm stomata (Fig. 3.4). In this angiosperm the mechanical advantage of epidermal cells over guard cells produces "wrong-way" responses, (transient stomatal opening on dehydration and transient closure on rehydration) and these wrong-way transients are typically followed by right-way responses mediated by active changes in the osmotic balance between guard cells and epidermal cells (Fig. 3.4; Buckley 2005). Like ferns and lycophytes, the gymnospermous seed plants (*Callitris rhomboidea* and *Pinus radiata*) showed no evidence of wrong-way stomatal behaviour when their leaves were instantaneously dehydrated or rehydrated; however, the lack of correlation between leaf water potential and stomatal conductance in these species suggests a significant stomatal closing effect of ABA, even after short-term water stress (Fig. 3.4).

These data strongly support the theory that fern and lycophyte stomata are insensitive to ABA and highly responsive to the water potential of the leaf. Brodribb and McAdam (2011b) showed in nine species of ferns and lycophytes, that stomata were insensitive to ABA when fed exogenously into the transpiration stream. Similarly, Ruszala *et al*. (2011) showed that the stomata on live leaves of the lycophyte *Selaginella uncinata* were relatively insensitive to extremely high levels of exogenous ABA (exceeding 260 000 ng ml^{-1}) in the xylem, resulting in less than a 15% reduction in stomatal conductance. Very weak responses of stomatal aperture to very high levels of exogenous ABA have also been observed in the epiphytic fern *Platycerium bifurcatum* for which foliar spray applications of ABA again resulted in less than a 15% reduction in gas exchange (Rut *et al.* 2008).

Gradualistic evolution of stomatal control

The concept of gradualistic evolution of stomatal control components over the 400 million years since land plants evolved stomata (Brodribb and McAdam 2011b) is strongly supported by our observations of plants subjected to water stress. The contrasting stomatal behaviour of water stressed plants shown here provides compelling new evidence for ABA insensitivity in ferns and lycophytes adding to previous studies using exogenous ABA (Rut *et al.* 2008; Brodribb and McAdam 2011b; Ruszala *et al.* 2011). Experiments in this study were specifically designed to observe stomatal responses to endogenous levels of ABA simulated by the types of water stress conditions experienced in the field. By using living plants under water stress conditions, we were able to observe stomatal responses under realistic endogenous ABA levels, avoiding the potentially confounding exposure of stomata to unrealistically high levels of exogenously applied ABA. In simulating the water stress conditions under which guard cell closure is triggered, we found an absence of effective stomatal control by endogenous ABA in our sample of basal vascular plants, resulting in stomata that have contrasting environmental responses to seed plants (Fig. 3.2). Therefore we conclude that in the light, fern and lycophyte stomata open and close only in response to changes in leaf water potential (Fig. 3.4). Our results do not support the recent conclusions of Ruszala *et al.* (2011) and Chater *et al*. (2011) that stomatal sensitivity to ABA evolved before vascular plants. The reason for the disparity between these studies may rest in the different measurement techniques used to quantify stomatal behaviour. In this study we measured stomatal diffusive conductance to water vapour on live plants to quantify stomatal aperture, while Ruszala *et al.* (2011) and Chater *et al*. (2011) based their conclusions on the common practice of calculating a mean stomatal aperture from measurements of large numbers of stomata on excised epidermal strips and unbelievable standard errors on gas exchange data. We believe that directly measuring water loss from stomata provides a more sensitive and functionally meaningful index of stomatal opening than measurements of isolated stomata *in vitro*. Furthermore, a number of papers have indicated major limitations associated with direct stomatal observation on excised epidermes (Spence 1987; Mott 2009) as well as recently suggested modifications (Hubbard *et al.* 2012).

Our data supports an evolutionary progression of stomatal control components in land plants, with modern seed-plant stomata characterised by a number of metabolically controlled signals absent in the basal lineages of vascular plants including phototropin mediated blue light responses (Doi *et al.* 2006; Doi and Shimazaki 2008), effective regulation of water use

efficiency (McAdam and Brodribb 2012a), closure in response to high carbon dioxide (Brodribb *et al.* 2009), and closure in response to exogenous (Brodribb and McAdam 2011b) and endogenous ABA (Fig. 3.2). The gradualistic evolution of stomatal control components in land plants offers a great opportunity to further our understanding of guard-cell membrane-specific processes as well as global models of transpiration over geological time.

Evolution of stomatal control by ABA and relevant genes

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

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

seems highly unlikely that the earliest stomata evolved to regulate photosynthesis and plant hydration, and under such conditions the evolution of an ABA-associated stomatal closure mechanism is improbable.

Despite there being no compelling argument for a selective pressure driving the evolution of ABA regulated stomatal control in the earliest land-plant lineages, all of the relevant ABA signalling and synthesis genes as well as functioning proteins responsible for analogous seed-plant stomatal control are present across the phylogeny of land plants, including the earliest stomatal bearing lineages (Hanada *et al.* 2011). However, DNA sequence databases from lineages diverging prior to stomatal evolution, such as liverworts (Yamato *et al.* 2007), indicate the presence of all the major genes implicated by mutant analysis as essential for the regulation and sensitivity of seed-plant stomata to ABA (including, *OPEN STOMATA 1* (*OST1*), *ABA-INSENSITIVE 1* (*ABI1*) and *GROWTH CONTROLLED BY ABA 2* (*GCA2*)). In seed-plant systems, with ABA governing rapid stomatal responses to environmental signals, it is not surprising that mutants for the genes involved in ABA sensitivity and synthesis invariably have phenotypes characterised by open stomata and wilty leaves. However, underlying this obvious stomatal phenotype and rapid response to ABA is a wide range of essential roles played by ABA in plant growth, survival and reproduction; ranging from seed maturation and germination, phase change, stress tolerance (reviewed by, Finkelstein *et al.* 2002), and in ferns and lycophytes, the sexual differentiation of gametophytes (Banks *et al.* 1993). Even the genes known to play unique roles in regulating the specific guard cell anion channels responsible for a stomatal sensitivity to ABA in seed-plants, such as the *SUCROSE NON-FERMENTING-RELATED PROTEIN KINASE 2* (*SnRK2*) family, including *OST1*, play vital roles in the transduction of ABA signalling during seed dormancy and development (Nakashima *et al.* 2009), osmotic stress tolerance (Fujii *et al.* 2011), as well as other crucial non-ABA related metabolic roles (Zheng *et al.* 2010). Indeed on considering gene families associated with the metabolism and signalling of ABA across sequenced land-plant genomes, there is strong evidence for significant duplication events relating to morphological divergence (Hanada *et al.* 2011). Thus, it does not seem surprising that despite the presence of homologous gene families definitively responsible for stomatal sensitivity to ABA in seed-plants, fern and lycophyte stomata are insensitive to ABA (Fig. 3.2). Although speculative, it seems likely that through one of the many duplication events occurring over the evolutionary history of the orthologous gene families related to ABA metabolism and signalling, a

common ancestor of the modern seed plants co-opted the ancient role of ABA as a stress signal into the rapid regulator of guard-cell specific anion pumps.

CHAPTER 4

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

The text and results of this chapter are taken directly from the following publication:

McAdam, S.A.M., Brodribb, T.J., Ross, J.J. and Jordan, G.J. (2011). Augmentation of abscisic acid (ABA) levels by drought does not induce short-term stomatal sensitivity to CO_2 in two divergent conifer species. *J. Exp. Bot.* 62, 195-203.

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 we test two alternative explanations for the stomatal insensitivity of conifers to C_a: 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 pathway rather than insufficient ABA_f.

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 optimisation of water use during photosynthesis (Farquhar and Sharkey 1982). As a central component of water use optimisation, 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 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 been attributed to numerous physiological factors including abscisic acid (ABA) level (Raschke 1975b; 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 1975b). Raschke (1975) found 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 sensitised to changes in C_a. ABA-induced sensitivity of stomata to C_a and the resulting effect on transpiration, assimilation and water-use efficiency

was 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.* 1998a). Current consensus recognises 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 lycophyte) showed very weak or absent responses to changing C_a. A similar lack of instantaneous 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 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 experiment. *Pinus radiata* individuals (grown from commercially available seed of composite provenance) were 1 year of age at the time of experiment and grown 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 20ºC/15ºC 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, relative humidity and air circulation restricted vapour pressure deficit (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 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 was performed according to the methods of Jager *et al.* (2008) with the following modifications. After preconditioning, 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 eluted with 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 x 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 re-watering.

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 and 13:00 h and E (g s^{-1}) 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 re-watering, 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 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 standardised during measurements (leaf temperature was maintained at 20 ºC, 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 minimising 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 standardised 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 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 ABA_f (maximum 450 ng g^{-1}) occurred between 10:00 and 11:00 h on the day following rewatering, dropping to lower values of ~120 ng g^{-1} after 14:30 h (Fig. 4.1A). In contrast, 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. 4.1B). The pattern of ABA_f post-drought in the conifer *Pinus 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.

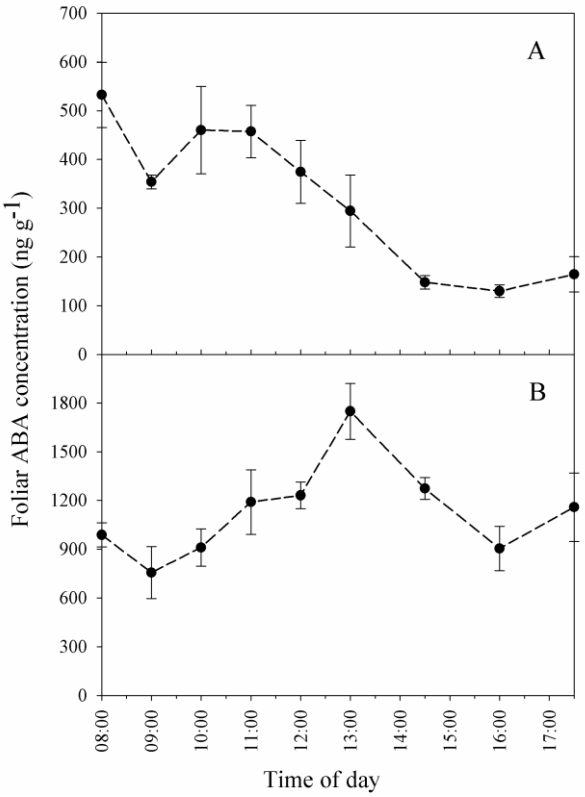

Fig. 4.1 Time course of foliar ABA concentration for *S. minimus* (A) and *C. rhomboidea* (B) on the day immediately following recovery from mild drought. Data points are means ±SE (n=3). Plants were re-watered at 05:00 h.

Trends of 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. 4.2). Drought caused stomatal closure in all species, reducing *E* to <25% of initial *E* over 3-5 d depending on the species (Fig. 4.2). Minimum Ψ_{leaf} at 25% initial *E* was different for each species, ranging from between -0.7 and -0.9 MPa in *S. minimus*, -1.9 and -2.1 MPa in *C. rhomboidea* and between -1.7 and -1.8 MPa in *P. radiata* (Fig. 4.2).

Following rewatering, a full recovery of Ψ_{leaf} to pre-drought levels took between one and two days in all species after which Ψ_{leaf} remained similar to or higher than pre-drought Ψ_{leaf} (Fig. 4.2). Following recovery from drought, *E* in *S. minimus* individuals increased to levels similar to pre-drought conditions by the fourth day (Fig. 4.2A). Transpiration in *C. rhomboidea* remained low for 2 d following drought recovery, after which *E* returned to pre-drought levels (Fig. 4.2B). However, in *P. radiata E* only recovered slightly, remaining low, between 0.002 and 0.003 g s^{-1} over the 4 d post-drought recovery (Fig. 4.2C).

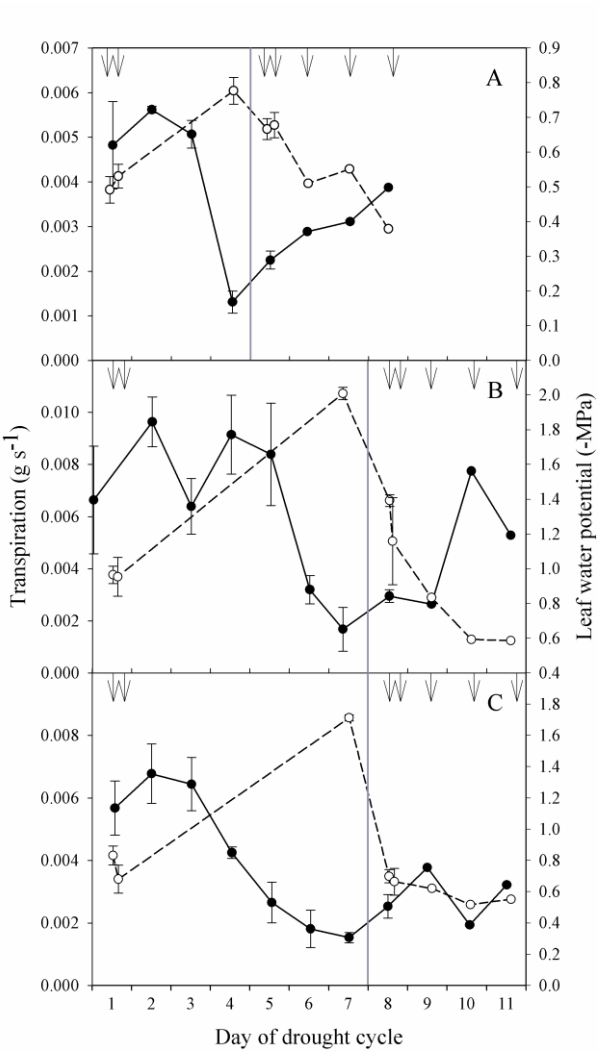

Fig. 4.2 Transpiration (solid line) and leaf water potential (Ψ_{leaf}) (dashed line) over the course of a mild drought cycle in *S. minimus* (A), *C. rhomboidea* (B) and *P. radiata* (C). Data points represent means ±SE (n=3) for all days except the final three when only one individual was represented. 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.

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. 4.3).

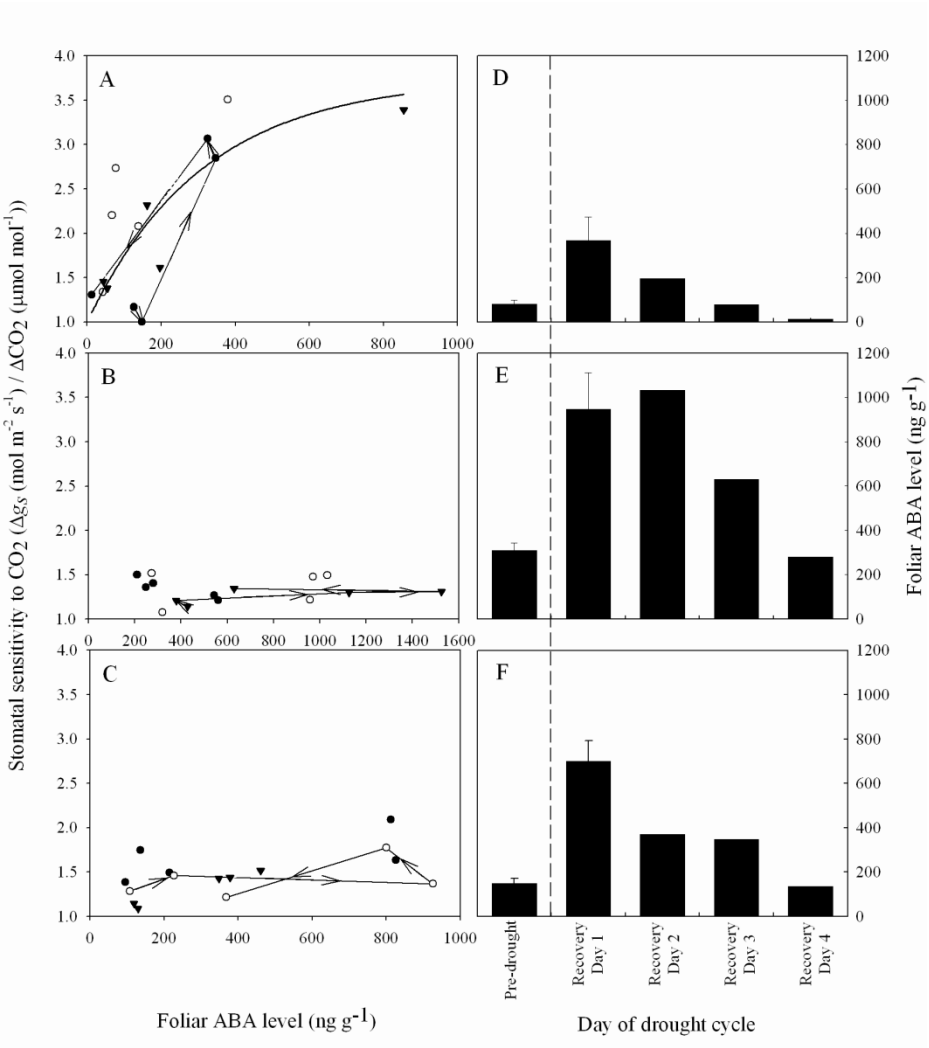

Fig. 4.3 Stomatal sensitivity to CO_2 relative to foliar ABA level in *S. minimus* (A), *C. rhomboidea* (B) and *P. radiata* (C). Three individuals are represented by different symbols, with a representative individual from each species linked with arrows indicating the transition from two initial well-watered states, two states on the first day following re-watering, 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 [$C_a = 2.745(1-e^{-0.0032ABA_f})$] as indicated by the solid line, although neither conifer species presented a significant relationship. Mean foliar ABA level and ±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.

Following rewatering, the highest recorded ABA levels in all species occurred on the first day following rewatering, after which levels gradually declined over the following 3 d (Figs. 4.3D, E and F). ABA_f increased on average 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 to the two conifers *C. rhomboidea* (320 ng g^{-1}) and *P. radiata* (190 ng g^{-1}) (Fig. 4.3).

In *S. minimus*, stomatal sensitivity to C_a was weak when ABA_f was comparatively low prior to drought stress (Fig. 4.3A). Stomatal sensitivity was highest in leaves immediately

recovered from mild drought, when ABA_f was ~4 times higher than pre-drought conditions (Fig. 4.3A). 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. 4.3A). 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. 4.3A). 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 (Figs 4.3B and C). In both conifer species the stomatal sensitivity to C_a was not significantly affected by increasing ABA_f and was no different to the stomatal sensitivity prior to drought ($P > 0.05$) (Figs 4.3A; B and C). The two conifer species displayed a similar lack of stomatal response to C_a prior to and following drought (Fig. 4.4). Only 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. 4.4).

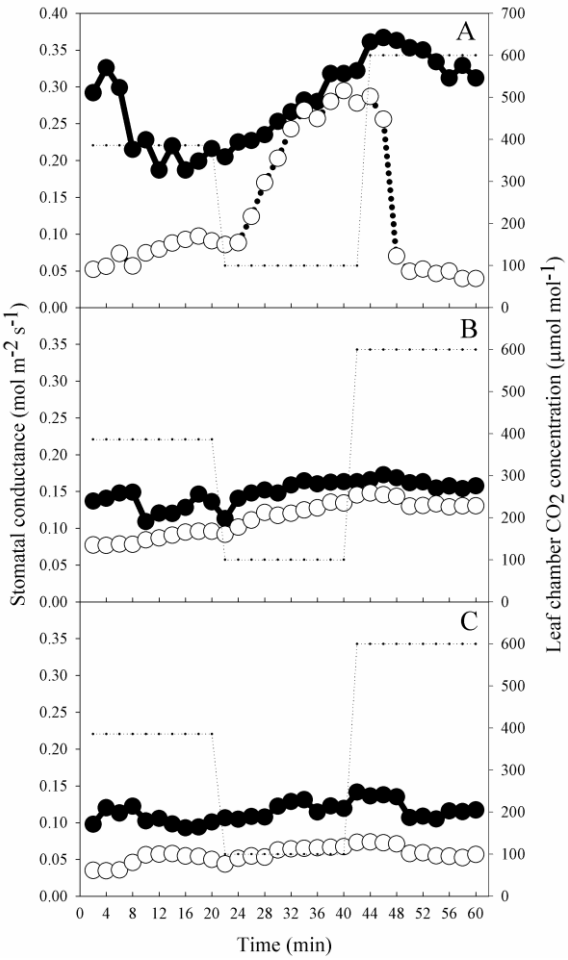

Fig. 4.4 Time-courses showing the responses of stomatal conductance (g_s) to step changes in CO_2 concentration (small dotted line) in a single representative individual of *S. minimus* (A), *C. rhomboidea* (B) and *P. radiata* (C) in the morning prior to the commencement of drought (filled circles) and the morning immediately following recovery from mild drought stress (open circles).

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. 4.5). 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. 4.5). 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. 4.5).

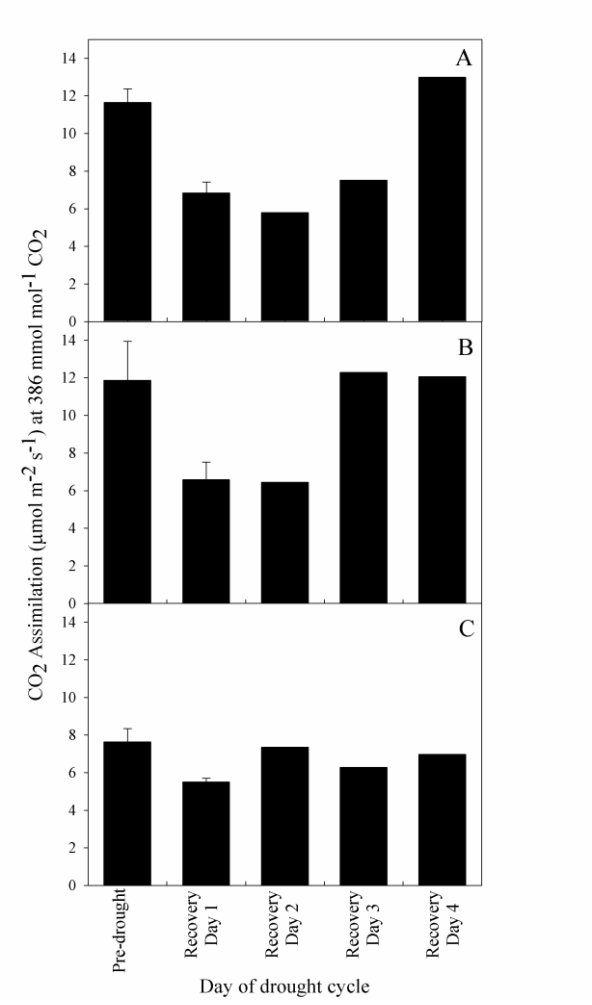

Fig. 4.5 Mean assimilation of CO_2 at ambient (386 mmol mol^{-1}) CO_2 and ±SE (n = 3) over the mild drought cycle including prior to the drought, and the 4 d post-drought recovery in *S. minimus* (A), *C. rhomboidea* (B) and *P. radiata* (C), (n=3 for pre-drought and recovery day 1, n=1 for the remaining days following drought recovery).

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. 4.3). 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. 4.3). 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 1975b; Dubbe *et al.* 1978; Raschke and Hedrich 1985; Ridolfi *et al.* 1996; Bunce 1998; Leymarie *et al.* 1998a).

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 to 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 to the angiosperm species (Fig. 4.3). 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. 4.5). The mesophyll plays a significant role in regulating the stomatal sensitivity to C_a in angiosperms (Mott *et al.* 2008) with photosynthesis likely 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. 4.5). The stomata of *S. minimus* were most sensitive to C_a on the day immediately following drought recovery (Figs 4.3A and D) when A in this species was 41% lower than on the day before the initiation of drought (Fig. 4.5). 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. 4.5).

The third explanation that the pathway responsible for the C_a response is entirely absent in conifers and hence the sensitisation 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 (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 the angiosperm-like regulation of stomatal control by ABA is lacking in the two coniferous species.

A diminished role of 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 conifers 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 (Murphy and Ferrell 1982; Kraus and Ziegler 1993; Hoffman *et al.* 1999; Kong *et al.* 2009). These studies all indicate that ABA_f in conifers, including Pinaceae (Murphy and Ferrell 1982; 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 190 ng g^{-1} for *P. radiata* compared with mean levels of 75 ng g^{-1} in *S. minimus* (Figs 4.3D; E and 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 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 (Figs 4.2A; B and C) and g_s (Figs 4A; B and C) prior to drought were similarly high in the angiosperm and two conifer species despite the relatively high ABA_f in the two conifers (Figs 4.3D; E and F). Additionally, by the fourth day following drought recovery ABA_f had returned to pre-drought levels in all species (Figs 4.3D; E and F) but this recovery did not correspond to a full recovery in E (Fig. 6.2). 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 of 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 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 similar 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-evaluating generalisations about the role of ABA in plants.

CHAPTER 5

Stomatal innovation and the rise of seed plants

The text and results of this chapter are taken directly from the following publication:

McAdam, S.A.M. and Brodribb, T.J. (2012). Stomatal innovation and the rise of seed plants. *Ecol. Lett.* 15, 1-8.

ABSTRACT

Stomatal valves on the leaves of terrestrial plants close to prevent desiccation, but also dynamically regulate transpiration to maintain efficient daytime water use during photosynthesis. The absence of core active stomatal control mechanisms in early-branching vascular plants raises the possibility that evolutionary change in stomatal function has fundamentally impacted how different plant lineages managed water through geological time. To test this hypothesis, we compared 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. Observations of excised stomata suggest that ferns and lycophytes lack a feedback signal from photosynthetic tissue to the guard cells that is linked to the efficient use of water in seed plants. 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.

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 and Woodward 2003). But 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 and Handley 1987; Tyree and Sperry 1989; Raven and 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 minimize 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 to satisfy the dual requirements of enhancing CO_2 uptake for photosynthesis while preventing excessive loss or wastage of water from the leaf (Cowan 1977; Cowan and Farquhar 1977; Farquhar and 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 and 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 and 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 and Shimazaki 2008; Brodribb *et al.* 2009; Brodribb and McAdam 2011b; 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 and 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 absent active metabolic responses to increased CO_2 concentration in both the light and dark (Doi and Shimazaki 2008; Brodribb *et al.* 2009; Ruszala *et al.* 2011), the phytohormone ABA (Brodribb and McAdam 2011b; 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 and McAdam 2011b). 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 outcomes between major plant lineages during land plant evolution, and for modelling atmosphere-plant canopy interactions through geologic time (Hetherington and Woodward 2003; Berry *et al.* 2010). Investigating such a possibility, here 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 seven angiosperms, five gymnosperms, 11 ferns and two lycophytes (Table 5.1). 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 5.1). 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, 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 three month applications of slow release fertilizer. In all species the most recent fully expanded leaf, leaves, scale-leaved branches or non-reproductive pinnules were chosen for experiments.

Table 5.1. The species used to examine the optimisation of water use and stomatal response to light intensity including a description of habitat, growth habit and the symbol used to represent each species in Fig. 5.2.

Species	Family	Habitat and growth habit	Symbol
		ANGIOSPERMS	
Amborella trichopoda Baill.	Amborellaceae	Tropical rainforest understorey to sub-canopy shrub	⬢
Atherosperma moschatum Labill.	Atherospermataceae	Temperate rainforest canopy tree	◆
Trimenia moorei (Oliv.) Philipson*,†	Trimeniaceae	Sub-tropical closed rainforest liana	+
Lotus corniculatus L.*,†	Fabaceae	Temperate grassland ruderal herb	♀
Nothofagus cunninghamii (Hook.) Oerst.‡	Nothofagaceae	Temperate rainforest canopy tree	■
Senecio minimus Poir.	Asteraceae	Temperate moist forest ruderal herb	●
Tradescantia virginiana L.	Commelinaceae	Temperate open forest and prairie herb	▲
		GYMNOSPERMS	
Agathis robusta (C.Moore ex. F.Muell) Bailey	Araucariaceae	Tropical rainforest canopy tree	✚
Callitris rhomboidea R.Br.	Cupressaceae	Temperate dry forest tree	✖
Ginkgo biloba L.*,†	Ginkgoaceae	Temperate deciduous tree	♂
Phyllocladus aspleniifolius (Labill.) Hook.f.	Podocarpaceae	Temperate rainforest canopy tree	▼
Pinus radiata D.Don	Pinaceae	Temperate dry forest pioneer tree	★
		FERNS	
Astrolepis sinuata (Lagasca ex. Swartz) Bentham and Windam†,‡	Pteridaceae	Temperate-sub-tropical full sun and desiccation tolerant from rocky semi-desert	⬢
Cyathea australis (R.Br.) Domin.	Cyatheaceae	Temperate wet forest treefern	●
Dicksonia antarctica Labill.†,‡	Dicksoniaceae	Temperate wet forest treefern	▼
Dryopteris cycadina (Franch. et Sav.) C.Chr.*,†,‡	Dryopteridaceae	Cool-temperate riparian mid-altitude closed forest	✖
Gleichenia microphylla R.Br.†,‡	Gleicheniaceae	Temperate-subtropical open forest in wet peaty sites	★
Hypolepis tenuifolia (G. Forst.) Bernh.	Dennstaedtiaceae	Tropical pioneer groundfern	▲
Marsilea hirsuta R.Br.	Marsileaceae	Aquatic-amphibious perennial herb	◖
Microsorum pustulatum (G.Forst.) Copel.	Polypodiaceae	Temperate wet forest understorey epiphyte or ground cover	+
Nephrolepis exaltata (L.) Schott†,‡	Lomariopsidaceae	Sub-tropical moist forest understorey	♂
Pteridium esculentum (G. Forst.) Cockayne†,‡	Dennstaedtiaceae	Temperate-subtropical pioneer groundfern from open forest and grassland	✚
Todea barbara (L.) T.Moore	Osmundaceae	Temperate wet understorey treefern	■
		LYCOPHYTES	
Huperzia varia (R.Br.) Trevis.	Lycopodiaceae	Temperate epiphyte from treeferns or rocks	◆
Selaginella kraussiana (Kuntze) A. Braun*,†,‡	Selaginellaceae	Ruderal perennial herb from very wet environments	♀

***** Species used in the observations of stomatal aperture over transitions in light intensity
† Species with 3 replicates of the single decreasing transition in light intensity
‡ Species that are included in the long-time course series transitions in light intensity

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 one hour after light transitions between dark, low, high and low light intensity (Table 5.1). 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 minutes was sufficient time to achieve equilibrium stomatal conductance (<1% change over three minutes) and hence we examined a second, larger group of seven angiosperms, five gymnosperms, 11 ferns and 2 lycophytes allowing 30 minutes equilibration between transitions from dark to high to low light. Leaf level gas exchange was measured with an infrared gas analyser (Li-6400; Li-Cor, Lincon, NE, USA) with leaves enclosed in a cuvette maintained at a constant 22°C and vapour pressure difference (VPD), a between 1.2 and 1.4 kPa and CO_2 concentration in the air was maintained 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 for the duration of the experiment and damp by misting with water from a hand-held spray bottle.

Stomatal aperture observation over light transitions

Light microscopy was used to examine the response of stomatal aperture of live leaves, isolated epidermes and xenografts to light intensity in five species selected to span the phylogeny of vascular plants (Table 5.1). 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 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, 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 four hours (Mott *et al.* 2008; Sibbernsen and Mott 2010). Epidermes were then mounted on a bridge of damp blotting paper standing 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, 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 by 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) 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, 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.

Xenografts were prepared by placing isolated epidermis of the eudicot angiosperm *Lotus corniculatus* on exposed mesophyll of the fern *Dryopteris cycadina*. A 50 mm diameter disk of leaf tissue was taken from *D. cycadina* at least 12 h before light transitions, under a disecting microscope the abaxial epidermis was removed using razor blade and fine forceps with care taken not to damage the mesophyll cells. The disk of leaf tissue was then placed in a similarly sized hole-punched hole in a damp blotting paper bridge standing in a petri dish containing the ionic solution described in the materials and methods, the close connection of leaf tissue with the damp blotting paper ensured the leaf tissue did not dehydrate. Isolated epidermis of *L. corniculatus* was then prepared as described in the materials and methods and placed over the exposed mesophyll entriely covering the exposed tissue. The petri dish was then covered and sealed with parafilm and placed in the dark for at least 12 h. The blotting paper bridge was then taken and enclosed in the cuvette and measurements of stomatal aperture over transitions in light intensity were undertaken as described above.

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 x20 long-working distance objective (LD Epiplan x20/0.4,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 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 be shown to offer the least variability in measurements of stomatal aperture from isolated epidermis (Gorton *et al.* 1989). 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 x2.5 magnification tube, aperture measurement of the four stomates took less than 1 min. The treatments for each species were repeated so that data represent 8 individual live and viable stomata.

Gas exchange observations over the same transitions in light intensity

A portable infrared gas analyser (Li-6400; Li-Cor, Lincon, NE, USA) was used to record the response of g_s and A to the same series of transitions in light intensity in the five species for which the stomatal aperture was observed (Table 5.1). All variables in the leaf cuvette of the Li-6400 were maintained in as similar a condition to the variables in the controlled environment chamber under the microscope as practical. Cuvette block temperature was maintained at 22°C, VPD was kept below 1.1 kPa by manually tuning the proportion of inlet air directed through a desiccant column, the flow rate was set at 300 ml min^{-1} and CO_2 concentration in the air maintained at ambient concentrations (390 µmol mol^{-1}). A single leaf from three individuals was sampled from each species. Individual plants were brought into the laboratory the night before and the selected leaf or branch bagged as described above. All remaining leaves on the stem of the selected leaf were excised and the remainder of the plant was covered in a black plastic bag while experiments took place. A series of transitions in light intensity were undertaken on each leaf with g_s, A and leaf environmental conditions logged every 1 min. Initially the leaf was exposed to 30 min of darkness in the leaf chamber, followed by a 45 min period at 1000 µmol quanta m^{-2} s^{-1} PPFD, then 30 min at 100 µmol

quanta m^{-2} s^{-1} and finally 30 min in darkness. Following gas exchange measurement, all logged data were standardised against the leaf area in the cuvette.

Cuticular conductance

Three 10 cm long terminal segments of leaves or branches each from different individuals of the eleven fern and lycophte species (Table 5.1) were excised and allowed to dehydrate on the laboratory bench. After approximately 3 h the leaves or microphylls were secured in the leaf cuvette of a portable infrared gas analyser (Li-6400; Li-Cor, Lincon, NE, USA) and allowed to equilibrate for 10 min. Conditions in the cuvette were maintained as described above with the exception of light intensity which remained at 0. Following equilibration g_s was manually logged three times over approximately 3 minutes. Leaf area in the cuvette was marked on to the leaf segment or branch, for standardisation of the gas exchange data, and the water potential of the leaf segment or branch was taken using a Scholander pressure chamber.

Dark conductance

Three individuals of each fern and lycophyte species (Table 5.1) were kept in the laboratory in the dark overnight. Prior to dawn (06:00 h) a single leaf segment or microphyll bearing branch from each individual was secured in the leaf cuvette of a portable infrared gas analyser (Li-6400; Li-Cor, Lincon, NE, USA), variables in the cuvette were maintained at 22°C and VPD was kept between 1.1 and 1.2 kPa. Gas exchange parameters were monitored until stability of g_s was reached (approximately 5-10 minutes), data were then logged and standardised against the leaf area in the cuvette.

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 ± standard error, 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 5.1; 5.2 and Appendix 5.1). 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) ± standard error, seed plants: 56.68% ± 2.8%;

ferns and lycophytes: 11.9% ± 4.2%, P<0.001, single factor ANOVA, Figs 5.1; 5.2 and Appendix 5.1). 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) (Appendix 5.2). 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;* Appendix 5.2). 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 (Appendix 5.3). Differences in stomatal behaviour between the groups of vascular plants were not associated with incompetent stomatal closure or high rates of cuticular water loss (Appendix 5.4) as demonstrated by the fact that mild 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*) (Appendix 5.4). However, hydrated leaves of ferns and lycophytes produced significantly higher stomatal conductances than seed plants in the dark (P<0.001, single factor ANOVA, Appendix 5.1).

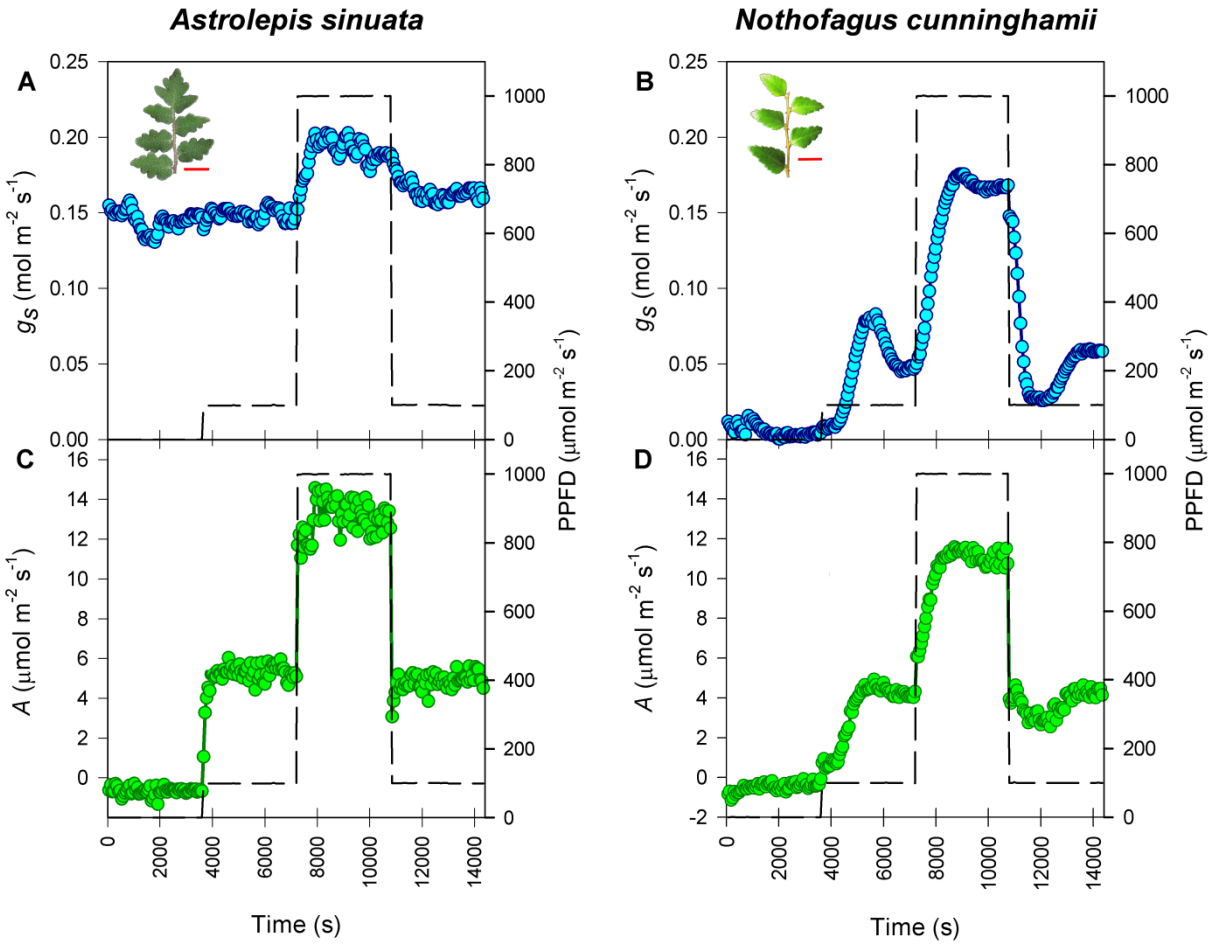

Fig. 5.1 Ferns display a reduced stomatal response to transitions in light intensity (PPFD) without evidence of a feedback between assimilation and stomatal conductance commonly observed in seed plants. Leaves of the representative fern *Astrolepis sinuata* (A and C) and angiosperm *Nothofagus cunninghamii* (B and D) were 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).

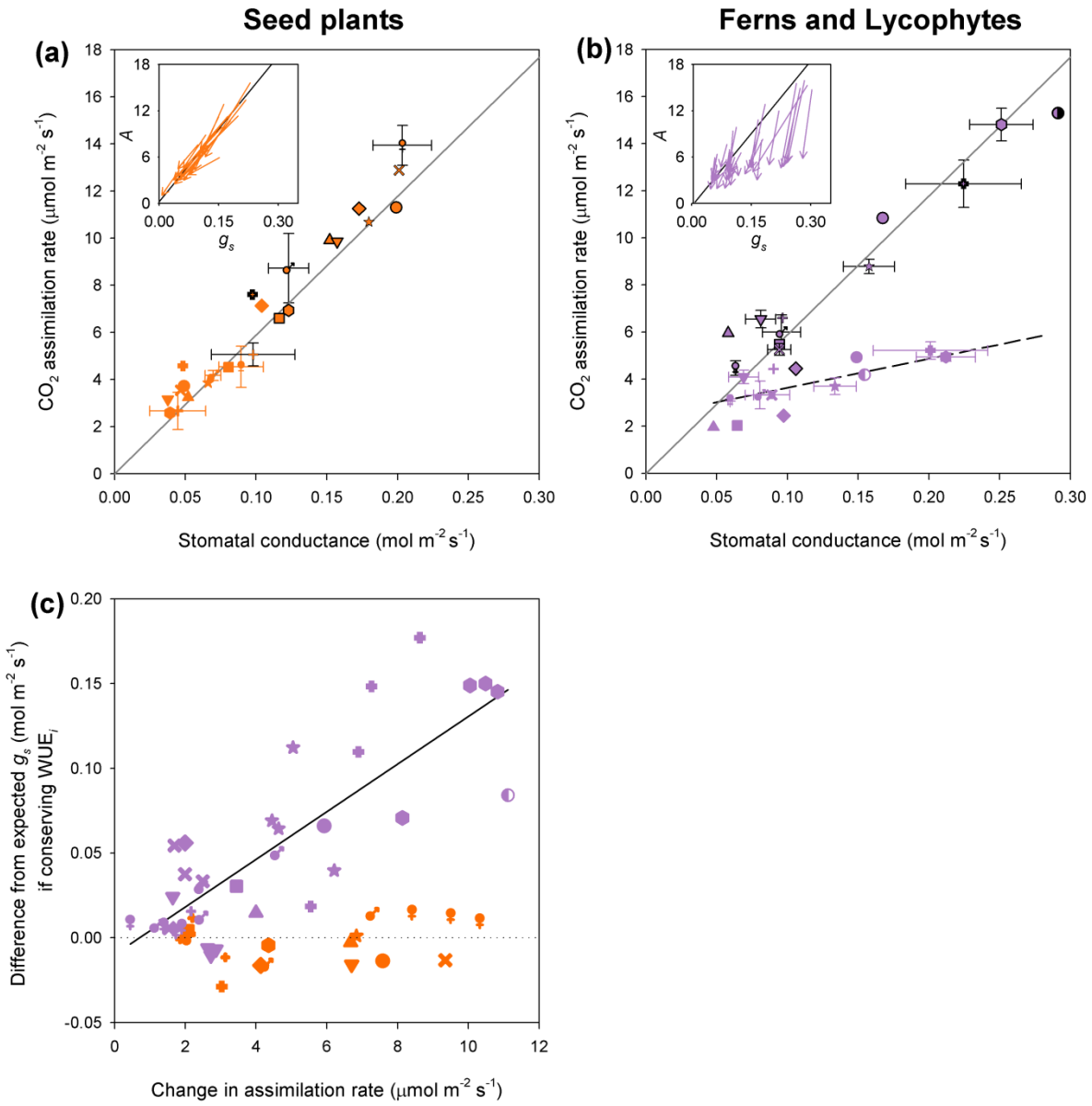

Fig. 5.2. Reduced stomatal response to decreasing light intensity in ferns and lycophytes (purple symbols) results in water wastage relative to seed plants (orange symbols). Under saturating light (1000 µmol quanta m^{-2} s^{-1}; all black-bordered symbols) the ratio of assimilation (*A*):stomatal conductance (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). By 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, while 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 Figs 5.2A and B) in fern and lycophyte species was correlated with the change *A* during the transition from high to low light (see Table 5.1 for a key to the symbols and corresponding species).

Regulation of water use efficiency

Reduced responsiveness of fern and lycophyte stomata to light when compared to seed plants markedly affected the ability of these plants to maintain high water use efficiency at non-saturating light intensity (Fig. 5.2). 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 (Figs 5.2A and B). By 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. 5.2B). 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. 5.2A), 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. 5.2B). Reduced WUE_i in ferns and lycophytes was the greatest for species that displayed the largest changes in assimilation rate following the transition from high to low light intensity (Fig. 5.2C). 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}, while 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. 5.2C). Very similar results were obtained when light intensities were increased from dark to 100 μmol quanta m^{-2} s^{-1} (Appendix 5.3), 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 (Appendix 5.3).

Mesophyll-stomatal feedback

When stomata were isolated from the leaf mesophyll the behaviour of seed plant stomata changed, while the representative fern and lycophyte species showed little impediment of function (Fig. 5.3). When examined *in situ* on the leaf the stomata of the seed plants examined changed by > 4μm during transitions between high and low light (Fig. 5.3), with a similar dynamic to that observed in g_s (Appendix 5.5). 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 (Fig. 5.3). 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 (Appendix 5.6).

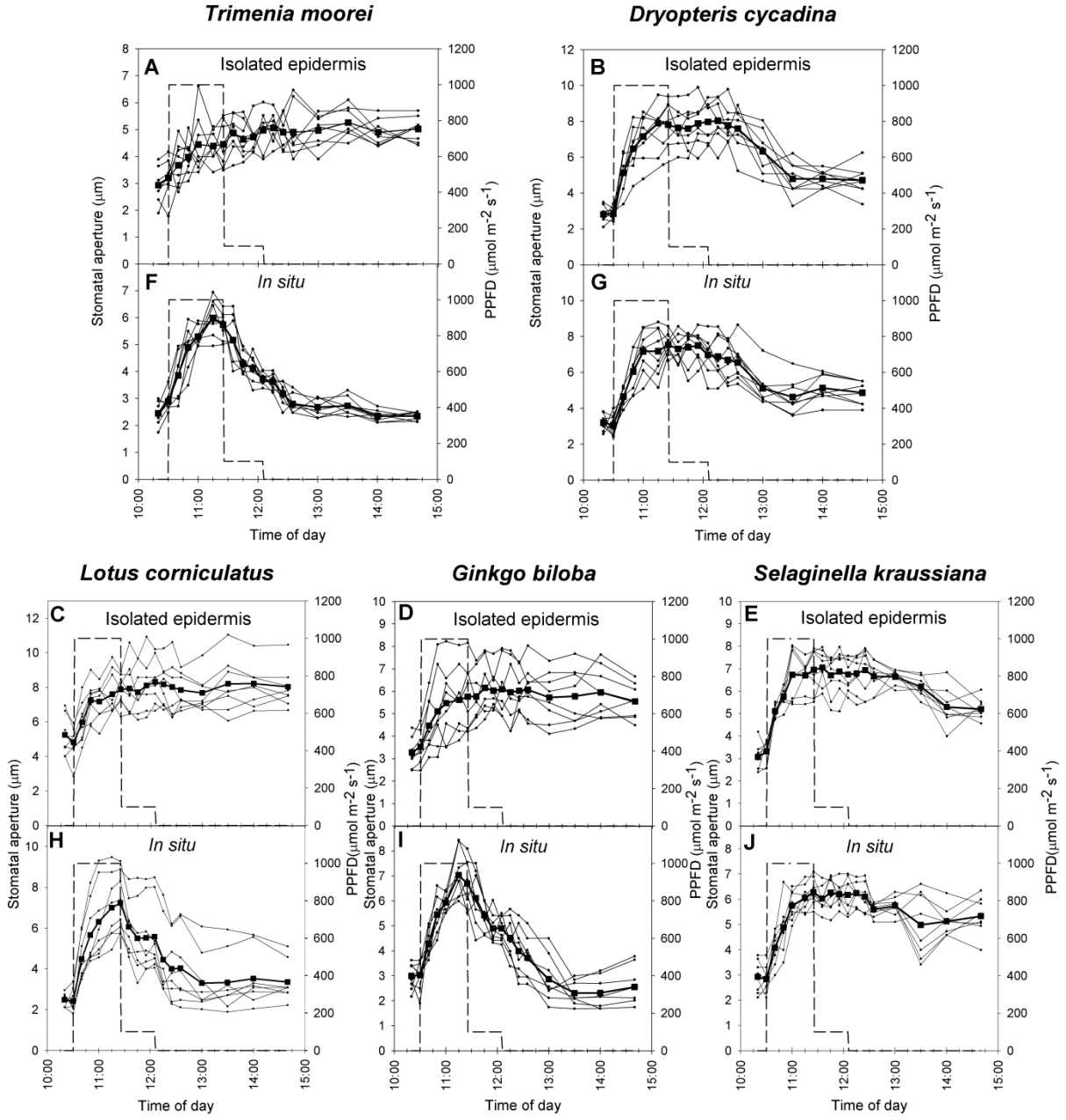

Fig. 5.3 The response of stomatal aperture to a sequence of light intensities (dashed line) on isolated epidermes (A-E) and intact leaves (F-J) of the basal angiosperm *Trimenia moorei* (A and F), fern *Dryopteris cycadina* (B and G), eudicot angiosperm *Lotus corniculatus* (C and H), gymnosperm *Ginkgo biloba* (D and I) and lycophyte *Selaginella kraussiana* (E and J). Small circles on thin lines represent individual stomata, large squares on the thick line represents the average (N=8). The stomata of seed plants became insensitive to reductions in light intensity once isolated from the leaf, while fern and lycophyte stomata behaved identically whether they were connected or excised from leaves.

Unlike seed plants, ferns 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; Fig. 5.3). *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 μm to > 6 μm in both species (Fig. 5.3). Following the transition from high to low light stomatal aperture from both isolated epidermis and live leaves did not decrease significantly over 30 min (Fig. 5.3), but the transition to dark caused a similar closure dynamic to that observed from g_s measurements (Fig. 5.3 and Appendix 5.5).

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. 5.2). Amongst our sample of two lycophyte clades and early and late branching fern clades (including sun and shade dwelling species) (Table 5.1), 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 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 and Franks 2009; Chater *et al.* 2011).

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 and 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 (Appendix 5.2), yet during both stomatal opening and closure we found that fern stomata were more wasteful of water compared with seed plants (Fig. 5.2 and Appendix 5.3). 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 (Brodribb & McAdam 2011) fern and lycophyte species have high stomatal sensitivity to changes in guard cell turgor and very effective closure in response to desiccation (Appendix 5.4). 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 and Bowling 1992, 1995; Sibbernsen and 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, while in seed plants we found excised stomata lost the ability to respond optimally to light (Fig. 5.3; 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. 5.4). 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. 5.4). 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 and Pearce 1957; Lucas and Renzaglia 2002; Duckett *et al.* 2009), and are often not involved in water conservation (Garner and Paolillo 1973; Hartung *et al.* 1987; Lucas and 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 and Paolillo 1973; Chater *et al.* 2011) but contradictory data have been shown for mosses and hornworts (Paton and Pearce 1957; Lucas and Renzaglia 2002).

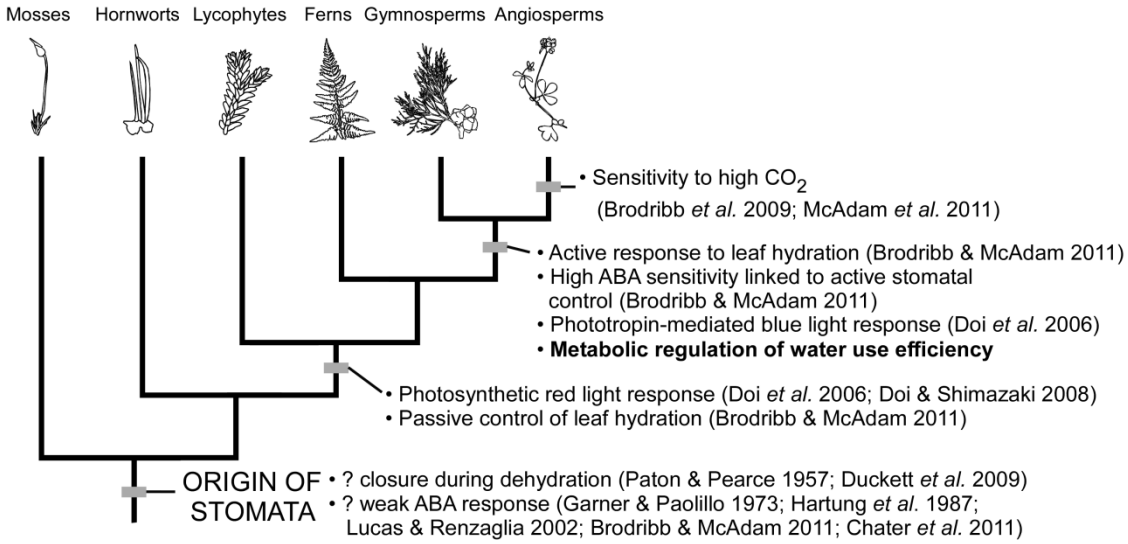

Fig. 5.4 Reconstructed evolution of stomatal control processes based on extant representatives of major plant clades. Evolution of metabolic regulation of high water use efficiency occurs coincidently with other key functional metabolic innovations, suggesting a major evolutionary transition of stomatal physiology following the divergence of seed plants.

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 and Renzaglia 2002; Raven 2002; Duckett *et al.* 2009; Brodribb and McAdam 2011b). Stomata of the basal lineages of vascular plants, the lycophytes and ferns, are characterised by an opening response to photosynthetically active red light (Doi and Shimazaki 2008; Lawson 2009) (Fig. 5.3), but transpiration rates are insensitive to physiologically relevant concentrations of ABA (Brodribb and McAdam 2011b; Ruszala *et al.* 2011), with stomata controlled passively by leaf water content during the day (Brodribb and McAdam 2011b). 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. 5.4). 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 (Brodribb and McAdam 2011b). 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. 5.2). 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 and 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. 5.4).

Conclusion

The major transition in stomatal physiology reconstructed here as occurring with the evolution of seed plants during the Paleozoic (Fig. 5.4), 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. 5.2), while 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. 5.2) 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 and Feild 2010). Additionally 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.* 2007b)) to account for the ecological limitations of ferns and the rise of seed plants.

CHAPTER 6

Passive hydraulic stomatal control results in a canalisation of drought response in ferns and lycophytes

The text and results of this chapter are taken directly from the provisionally accepted manuscript:

McAdam, S.A.M. and Brodribb, T.J. (2012). Ancestral stomatal control results in a canalisation of fern and lycophyte adaptation to drought. *New Phytol.*

ABSTRACT

- Little is known about how a predominantly passive hydraulic stomatal control in ferns and lycophytes might impact water use under stress. Ferns and lycophytes occupy a diverse array of habitats from deserts to rainforest canopies; raising the question of whether stomatal behaviour is the same under all ecological strategies and poses ecological or functional constraints on ferns and lycophytes.
- We examine the stomatal response of a diverse sample of fern and lycophyte species to both soil and atmospheric water stress. Assessing the foliar levels of the hormone abscisic acid (ABA) over drought and recovery and the critical leaf water potential (Ψ_l) at which photosynthesis in droughted leaves failed to recover.
- The stomata of all ferns and lycophytes showed very predictable responses to soil and atmospheric water deficit via Ψ_l, while stomatal closure was poorly correlated with changes in ABA. We found all ferns closed stomata at very low levels of water stress and their survival afterwards was limited only by their capacitance and desiccation tolerance.
- Ferns and lycophytes have constrained stomatal responses to soil and atmospheric water deficit due to a lack of stomatal regulation by ABA. The result is a monotypic strategy of ferns and lycophytes under water stress.

INTRODUCTION

Despite a relative stasis in stomatal morphology over more than 400 million years, the vegetative tissue bearing stomata has evolved immense diversity and complexity in form and function, from the small bifurcating axes of the oldest fossilised stomatal bearing plants (Bateman *et al.* 1998; Edwards *et al.* 1998) to highly productive modern angiosperm leaves (Brodribb *et al.* 2009). In conjunction with this morphological evolution, the functional behaviour of stomatal opening and closure has similarly radiated in complexity, with significant implications for productivity and water use (Doi *et al.* 2006; Doi and Shimazaki 2008; Brodribb *et al.* 2009; Brodribb and McAdam 2011b; Haworth *et al.* 2011; Haworth *et al.* 2012; McAdam and Brodribb 2012a, b).

Extensive research has investigated the controls of stomatal aperture in angiosperms (Raschke 1975a; Cowan and Farquhar 1977; Damour *et al.* 2010), with two processes responsible for regulating stomatal aperture, the passive control of guard cell turgor by leaf water status (Buckley and Mott 2002; Buckley 2005) and the active control of guard cell osmotic potential by the transport of ions across cell membranes (Schroeder *et al.* 2001; Shimazaki *et al.* 2007; Lawson 2009). While much work has focused on the stomatal behaviour of angiosperms our current understanding of the evolution of stomatal control comes from relatively recent investigation into both functional stomatal behaviour in modern representatives of extant lineages of vascular plants (Brodribb and McAdam 2011b; McAdam and Brodribb 2012a) as well as molecular investigations into the function of key genetic components essential for stomatal signalling (Chater *et al.* 2011; Ruszala *et al.* 2011). Particular focus in the investigation of the evolution of stomatal control has been placed on comparing the stomata of the well-researched seed plants with non-seed plant groups. While complex metabolically driven stomatal control predominates in the ecologically dominant and successful seed plants (Ache *et al.* 2010) fern and lycophyte stomata appear to be overwhelmingly regulated by a passive response to leaf water status in the light (Brodribb and McAdam 2011b; McAdam and Brodribb 2012b). The predominance of a passive response of fern and lycophyte stomata occurs despite the presence and function of key genetic components in these lineages that are essential for effective metabolic stomatal signalling (Chater *et al.* 2011; Ruszala *et al.* 2011). While these two lines of investigation appear contradictory it seems most likely that while the basal lineages of land plants are in possession of an operational genetic framework to illicit metabolic stomatal responses, the passive control of stomata by water balance predominates in the regulation of stomatal behaviour. Fern and lycophyte stomata have only a limited

functional metabolic control, namely a stomatal response to red light that originates in the guard cells (Doi and Shimazaki 2008; McAdam and Brodribb 2012a). The behaviour of fern and lycophyte stomata in the light is highly predictable, with guard cells passively linked to changes in the turgor pressure of the leaf (Brodribb and McAdam 2011b; Brodersen *et al.* 2012; McAdam and Brodribb 2012b). It has been suggested (McAdam and Brodribb 2012a) that an evolutionary transition in stomatal control towards a predominantly metabolic regulation of stomatal aperture occurred during the radiations of seed plant lineages in drier late-Palaeozoic environments (DiMichele and Aronson 1992) and was instrumental in ensuring the competitive success and ecological dominance of seed plants and the demise of fern and lycophyte dominated forests into the Mesozoic. Indeed during the Palaeozoic, lycophytes and ferns enjoyed a rich period of ecological dominance (Phillips *et al.* 1985) and over their evolutionary history have included morphological representatives of all terrestrial life forms currently represented by extant seed plants (Rothwell 1996).

Despite a reputation as mesic relicts, modern ferns and lycophytes are certainly not restricted to ever-wet environments. Evidence from the fossil record and extant taxa indicate that ferns and lycophytes have repeatedly radiated into xeric, cold and variable habitats (DiMichele and Phillips 2002; Hietz 2010), with the ability to survive desiccation widely represented (Hietz 2010). The epiphytic growth habit which exposes individuals to fluctuating water availabilities (Hietz and Briones 1998; Watkins *et al.* 2007a; Watkins and Cardelús 2009) is an ecological diversification widely represented in ferns (Schneider *et al.* 2004; Schuettpelz and Pryer 2009) and the lycophyte genus *Huperzia* (Wikström and Kenrick 2000; Wikström 2001), concurrent with the rise of angiosperm dominated forests. A number of extant fern genera are highly competitive and invasive, successfully out-competing seed plants and dominating landscapes and vegetation types both in modern forests (e.g. *Pteridium* (Marrs and Watt 2006) and *Lygodium* (Pemberton and Ferriter 1998)) as well as throughout the Mesozoic (Wing *et al.* 1993). Associated with this ecological diversity within the ferns and lycophytes is a substantial variability in maximum photosynthetic rates (A) and stomatal conductances (g_s) (McAdam and Brodribb 2012a) as well as a recent suggestion of highly variable maximum xylem hydraulic resistances and vulnerability in the stipe of different species (Pittermann *et al.* 2011; Brodersen *et al.* 2012). Ecological diversity, geological persistence and the capacity for physiological and morphological variability observed in ferns and lycophytes raises the question: do all ferns and lycophtyes have the same predominance of passive control of leaf hydration by stomata and if so what are the adaptations adopted by

ferns and lycophytes that allow them to survive with this stomatal control mechanism (Watkins and Cardelús 2012)?

To answer these questions we examined the physiological responses to water stress of a diversity of fern and lycophyte species including mesophytic terrestrial species, epiphytes and a desiccation-tolerant species. In particular we quantified the sensitivity of stomata to soil drought, critical water potentials at which plants died, and the role of ABA in the response to drought. Our results provide a standardised comparison of the diversity of water management strategies in ferns and lycophytes.

MATERIALS AND METHODS

Species examined

To observe the diversity of physiological responses of ferns and lycophytes to water deficit six fern species and a lycophyte were specifically selected to span a wide ecological range of spore-bearing vascular plants, including mesophytic, drought deciduous, terrestrial species, epiphytes and a desiccation-tolerant species (Table 6.1). In addition a mesophytic angiosperm herb was selected to act as a seed plant comparison for the fern/lycophytes group. One characteristic of seed plant stomatal responses is their variability, and hence this single angiosperm species was included as a random sample from a successful genus at the core of the eudicot clade.

Table 6.1. Species used to assess the physiological diversity of ferns and lycophytes to water deficit, their distinctive morphological features and ecologies, see Appendix 6.1 for images of each species.

Species	Family	Morphology	Ecology
		FERNS	
Adiantum capillus-veneris L.	Pteridaceae	Rhizomatous terrestrial, mesophytic	Temperate to tropical moist, shaded habitats
Cheilanthes myriophylla Desv.	Pteridaceae	Rhizomatous terrestrial, mesophytic	Desiccation-tolerant from rock crevices
Dicksonia antarctica Labill.	Dicksoniaceae	Tree fern, mesophytic	Temperate rainforest understorey
Pteridium esculentum (G. Forst.) Cockayne	Dennstaedtiaceae	Subterranean rhizome, drought deciduous	Cosmopolitan, full sun to open canopied habitats
Pyrrosia lingua (Thunb.) Farw.	Polypodiaceae	Epiphytic, rhizomatous	Sub-tropical canopy dwelling
Rumohra adiantiformis (G. Forst.) Ching	Dryopteridaceae	Epiphytic/lithophytic, rhizomatous	Temperate closed or open canopy
		LYCOPHTE	
Selaginella kraussiana (Kuntze) A. Braun	Selaginellaceae	Terrestrial	Sub-tropical, ever wet habitats
		ANGIOSPERM	
Viola x wittrockiana Gams. cv. Giant Butterfly	Violaceae	Terrestrial herb	Cultivated temperate herb

All species examined were represented by three, identical-aged individuals either grown from spores or from rhizomes collected in the field. Plants were grown 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, housed in the glasshouses of the School of Plant Science, University of Tasmania, Hobart, Australia. No individual had experienced prior drought stress. Plants were grown under natural light conditions supplemented and extended to a 16-h photoperiod by sodium vapour lamps, ensuring a minimum 300 µmol quanta m^{-2} s^{-1} at the leaf surface throughout the day period (maximum light intensity on cloudless days did not exceed 1100 µmol quanta m^{-2} s^{-1} at leaf level). Temperatures in the glasshouse were maintained at 22°C during the day and 15°C at night. All plants were watered daily and fertilised with liquid nutrients weekly (unless undergoing drought).

Drought, leaf gas exchange, water potential and foliar ABA level

Drought was initiated in three individuals per species by withholding water. Over the course of the drought, once a day (reducing to once a week after 10 days in more drought tolerant species), between 1200 h and 1300 h plants were transported to the laboratory where leaf gas exchange, leaf water potential (Ψ_l) and foliar ABA level were quantified on each individual.

Leaf gas exchange was measured using an infrared gas analyser (Li-6400; Licor) on photosynthetic tissue from a single leaf or stem (cuvette conditions: leaf temperature 22°C, vapour pressure difference (VPD) maintained between 1.1 and 1.2 kPa, 390 µmol mol^{-1} CO_2, light intensity 1000 µmol quanta m^{-2} s^{-1}, flow rate 500 ml min^{-1}), tissue was allowed to equilibrate to chamber conditions until stability was reached (approximately 20 minutes). On the same leaf or stem, Ψ_l was quantified on tissue excised and immediately wrapped in damp paper towel using a Scholander pressure chamber and microscope to precisely measure the xylem balance pressure without the loss of any water from the leaf tissue. Foliar ABA level was extracted, purified and physicochemically quantified using an UPLC (ultra-high performance liquid chromatography tandem mass spectrometry) with an added internal standard, according to the methods of (McAdam and Brodribb 2012b) , with the following modifications for improving sample purification for UPLC analysis. Following extraction and reduction under vacuum at 35°C to <1 ml the sample was taken up in 3 x 1 ml washes of weak aqueous sodium hydroxide (pH 8). These washes were loaded on to a solid-phase extraction 600 mg SAX cartridge (Maxi-Clean™, Grace Davison Discovery Sciences), preconditioned with 10 ml of weak aqueous sodium hydroxide (pH 8). The loaded sample on the cartridge was washed with 10 ml of methanol and ABA was eluted with 15 ml of 2% acetic acid in methanol (v/v).

Assessing recovery using rehydrated leaves and plants

Following leaf gas exchange measurements a pinnule, leaf or stem in closest proximity to the tissue sampled for gas exchange was excised in water and bagged. Over the course of the imposed drought no individual suffered defoliation in excess of 20% of the original leaf area. The excised tissue was left overnight rehydrating through the cut end of the rachis, petiole or stem to determine the viability of photosynthesis upon rehydration. Rehydrating individual fronds or leaves simulated the effect of soil rewetting without having to rewater whole plants, thereby allowing the three individuals to be tracked through an entire drought cycle. The following morning leaf gas exchange was measured, as described above, on the rehydrated tissue, while the cut end remained under water, this was performed to determine the Ψ_l at which different species sustained photosynthetic damage from water stress. Leaf recovery was defined as the recovery of photosynthesis following rehydration of tissue overnight, leaf death was defined as the Ψ_l at which photosynthesis failed to recover following overnight rehydration (above 0.5 µmol m^{-2} s^{-1}; A_0). Once photosynthesis in rehydrated leaves failed to recover in individual plants rehydration of leaves did not continue, in *Adiantum* only, drought

continued until plants reached -6 MPa. Following leaf gas exchange measurement the leaf area in the chamber was marked and the leaf or branch was taken for immediate Ψ_l measurement to assess effective rehydration of the tissue (>-0.2 MPa). In the desiccation-tolerant species *Cheilanthes myriophylla*, *A* recovered in rehydrated leaves when the plants were droughted beyond the limit of the pressure chamber (-10 MPa), at this point all three individuals were rewatered and leaf gas exchange, Ψ_l and ABA level were quantified daily until g_s had fully recovered to predrought levels. In the two epiphytic fern species, *Rumohra adiantiformis* and *Pyrrosia lingua*, the three droughted individuals were rewatered at different stages to observe the response of g_s, *A*, Ψ_l and ABA level over recovery. The first individual was rewatered on the first day that stomata closed (stomatal closure was defined as stomatal conductance <20% that of initial fully hydrated stomatal conductance), the second and third individuals were rewatered over the extended period that followed when leaves were losing water but not declining in Ψ_l or showing signs of leaf death. Individuals of the angiosperm herb *Viola x wittrockiana* were droughted as above and rewatered sequentially immediately upon stomatal closure, 24 hours later and 48 hours following complete stomatal closure to observe the effect of elevated ABA levels augmented by drought on g_s following recovery.

Turgor loss point and volume of available water until leaf death

Pressure-volume (PV) analysis was performed on at least 5 foliage samples, each from different plants, for each species to determine turgor loss point (Ψ_{tlp}) (Tyree and Hammel 1972) as well as the volume of water in the leaf that was available until plants reached lethal relative water content (RWC). The night prior to measurements foliage was bagged and excised under water to ensure Ψ_l was high (>-0.05 MPa). First leaf weight (±0.0001 g, Mettler-Toledo) followed immediately by Ψ_l were periodically measured over gradual desiccation in the laboratory, care was taken to ensure no water was lost from the petiole of stem during Ψ_l assessment. PV curves were constructed by plotting Ψ_l against RWC of an aggregation of points from each of the five leaves and Ψ_{tlp} determined by the inflection point of the relationship (Appendix 6.2), RWC was determined according to the following equation:

$$\mathrm{RWC} = \frac{(FW - DW)}{(TW - DW)}$$

Where *FW* is the fresh mass of tissue, *DW* is the dry mass of tissue and *TW* is the mass of fully hydrated, turgid tissue.

In all species the volume of water available, as capacitance, between stomatal closure and leaf death was calculated by extrapolating from the PV relationship the RWC at which leaves died by using the Ψ_l at which *A* in rehydrated leaves failed to recover. In the desiccation-tolerant *C. myriophylla* three additional individuals were droughted to determine an accurate RWC and time until *A* in rehydrated leaves failed to recover or died. The fresh weight of leaves at this lethal RWC was then used to calculate the volume of water available until leaf death according to the following formula:

$$\text{Available water (mmol/m}^2) = (WW - (FW_{death} - DW)) \times M/LA$$

Where *WW* is the mass of leaf water at 100% RWC (g); FW_{death} is the fresh leaf mass at the lowest recoverable RWC of leaves (g); *DW* is dry leaf mass (g); *M* is the molar mass of water (g mol^{-1}); and *LA* is leaf area (m^2).

Due to slow equilibration of Ψ_l in droughted leaves of the two epiphytic fern species (*R. adiantiformis* and *P. lingua*) PV curves were constructed using a modified method to observe the relationship between RWC and Ψ_l over the extended period of desiccation. Following the inflection point of the relationship between RWC and Ψ_l leaves were sealed in a bag containing damp paper towel to maintain high humidity and allowed to equilibrate overnight. The following morning leaf weight and Ψ_l were concurrently measured and the leaves were then allowed to desiccate on the bench for approximately 10 hours before leaf weight and Ψ_l were again measured. The leaf was then bagged and allowed to equilibrate overnight. This cycle was continued for five days or until Ψ_l in equilibrated leaves failed to recover.

Stomatal response to vapour pressure difference

The response of stomata to atmospheric water stress (as opposed to soil water stress) was investigated by examining the response of g_s to step-wise transitions in VPD in well watered individuals. Three individuals of each species were acclimated to laboratory conditions overnight and the following day the response of g_s to a sequence of VPD transitions (1-2-1 kPa) was measured on photosynthetic tissue using an infrared gas analyser (Li-6400; Licor), leaf cuvette conditions were maintained at 22°C, vapour pressure difference was regulated at the required kPa by adjusting the humidity in the inlet air by bubbling the incoming air through water and adjusting the amount passing through a desiccant column containing calcium sulphate, 390 µmol mol^{-1} CO_2, light intensity 1000 µmol quanta m^{-2} s^{-1} and flow rate

500 ml min^{-1}. All conditions in the chamber including A and g_s were automatically logged every minute. Leaves were acclimated for 20 minutes following each VPD step.

RESULTS

Stomatal response to leaf water potential

The stomata of a diverse sample of fern and lycophyte species closed over a relatively small window of Ψ_l between -0.6 and -2.1 MPa when droughted (Fig. 6.1). A significant linear relationship between Ψ_l at stomatal closure and Ψ_{tlp} was observed when all species, including the angiosperm were compared together ($P<0.05$; $R^2=0.616$). During the imposition of water stress and recovery from stress, g_s of all fern and lycophyte species was strongly influenced by Ψ_l, with no hysteresis in g_s observed in individuals that were rehydrated (Fig. 6.1). This pattern was very different in droughted individuals of the angiosperm *V. x wittrockiana* which showed strong hysteresis in stomatal recovery after rewatering (Fig. 6.1).

A small margin between stomatal closure and leaf death in ferns and lycophytes

Terrestrial fern and lycophyte species typically had a small Ψ_l margin between stomatal closure and leaf death from water stress, except for the dessication-tolerant species *Cheilanthes myriophylla* which could survive extremely low Ψ_l (Figs 6.1 & 6.2). In the mesophytic lycophyte *Selaginella kraussiana* the Ψ_l margin between stomatal closure and leaf death was only 0.01 MPa while in the terrestrial fern species this margin ranged from 0.19 MPa in *Adiantum capillus-veneris* to 0.4 MPa in *Pteridum esculentum* (Fig. 6.2). The two epiphytic fern species had slightly larger safety margins between stomatal closure and irreversible photosynthetic damage with 0.42 MPa and 0.92 MPa for *P. lingua* and *R. adiantiformis* respectively (Fig. 6.1).

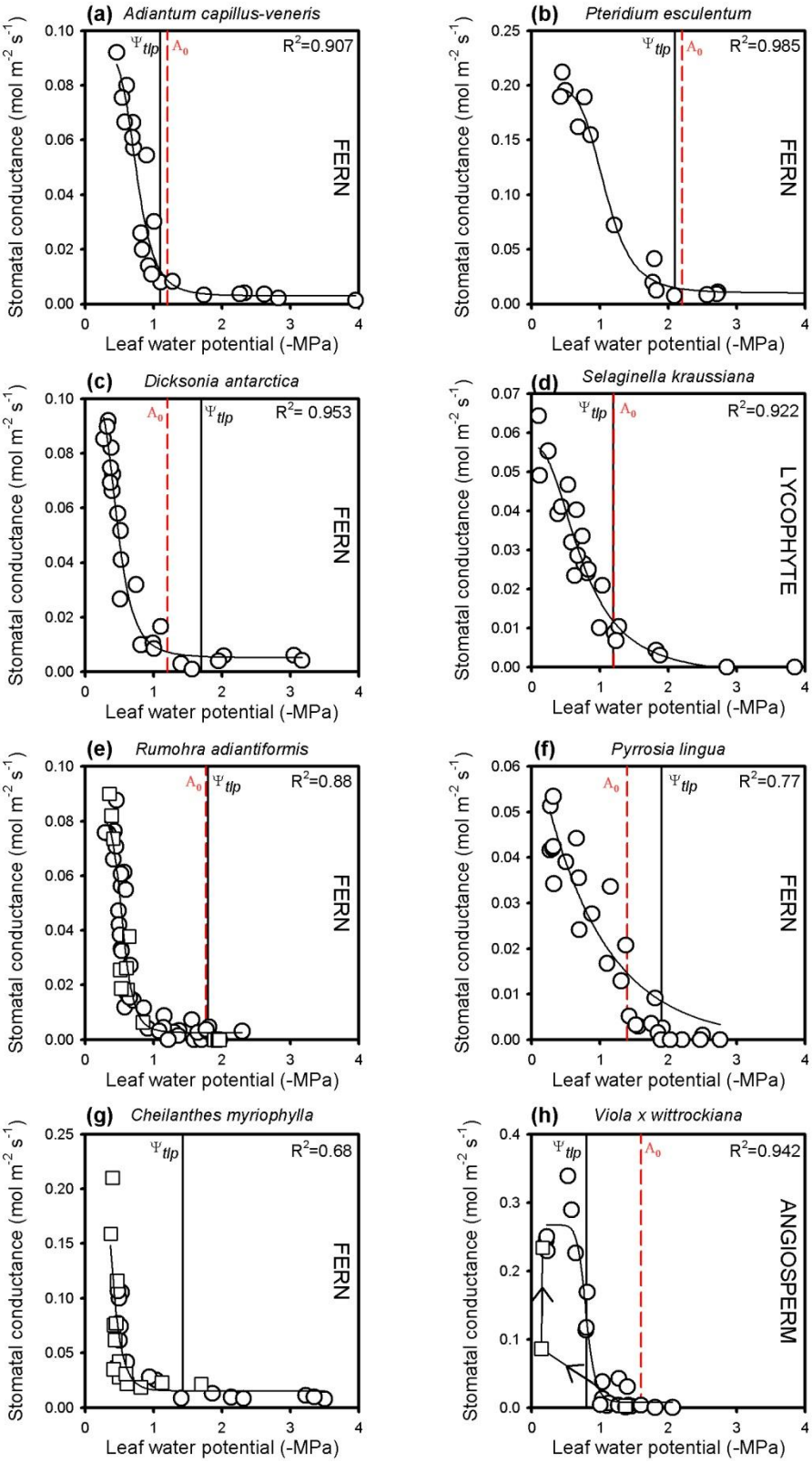

Figure 6.1. The relationship between stomatal conductance and leaf water potential pooled from three individuals of three mesophytic terrestrial fern species (a-c) and a lycophyte (d), two epiphytic fern species (e and f), a desiccation-tolerant fern species (g) and an angiosperm (h) when droughted by withholding water (open circles). Solid vertical lines represent Ψ_{tlp} (see Appendix 6.2) and dashed red vertical lines represent Ψ_l at which assimilation rate did not recover in rehydrated leaves (A_0). Square symbols represent measurements made on individuals that were rehydrated, the arrowed line tracks the recovery of stomatal conductance in an individual of the angiosperm *Viola x wittrokiana* following rehydration.

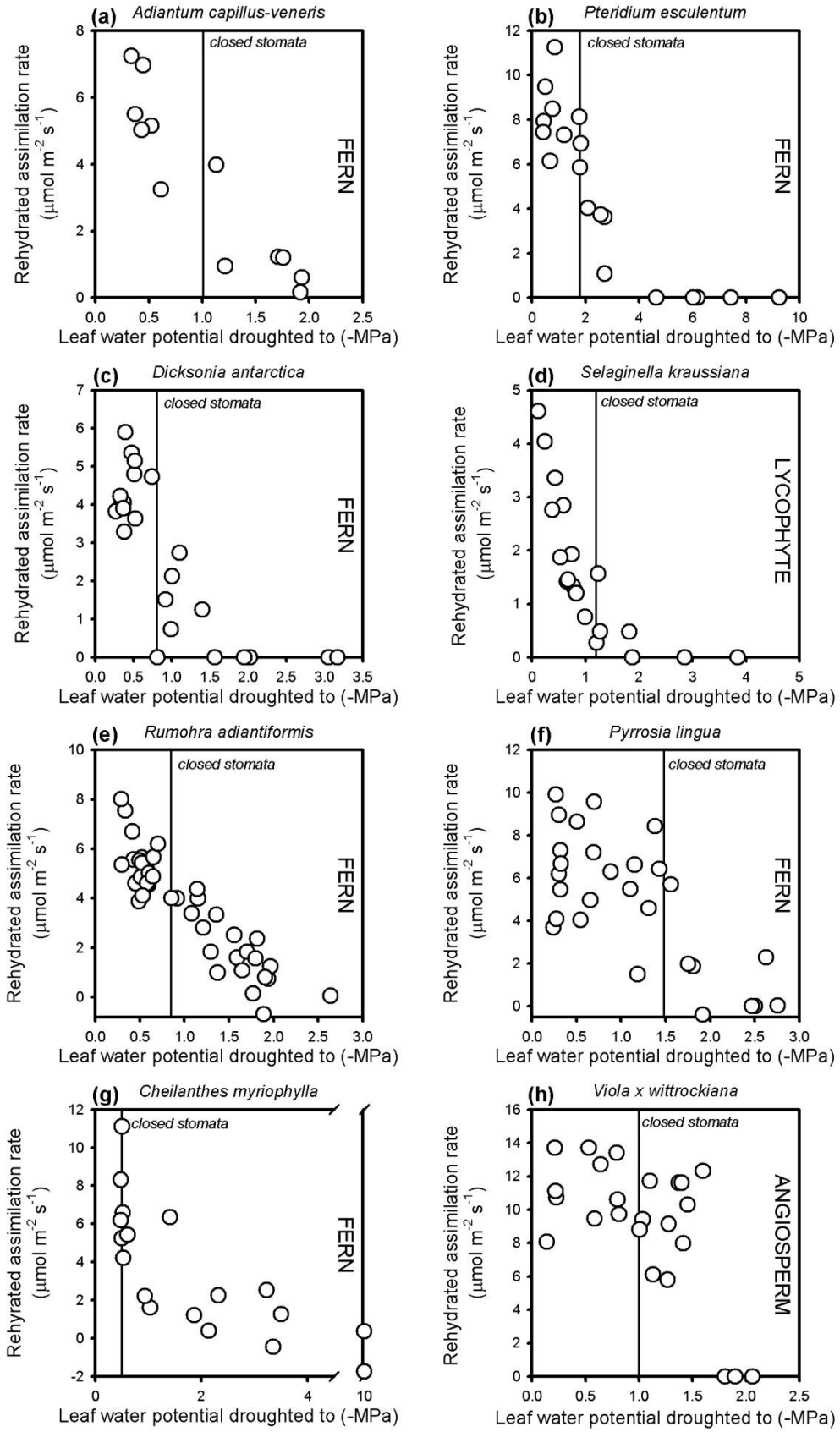

Figure 6.2. The assimilation rate of excised leaves rehydrated overnight to full hydration (>-0.2 MPa) from known droughted leaf water potentials, in three mesophytic terrestrial drought deciduous ferns (a-c), a lycophyte (d), two epiphytic ferns (e and f), a desiccation-tolerant fern (g) and an angiosperm (h). Solid vertical lines represent the leaf water potential at which stomata close (defined as <20% of initial stomatal conductance).

There was very little difference between the Ψ_l when drought stressed leaves died in all fern species (around 0.8 MPa lower in the epiphytic species; Fig. 6.1). In terrestrial fern and lycophyte species >80% of leaves died as a result of drought between at a Ψ_l ranging from -1.2 MPa (*A. capillus-veneris* and *S. kraussiana*) and -2.2 MPa (*P. esculentum*), while the leaves of the epiphyte *R. adiantiformis* perished when droughted to -2.2 MPa and >50% of leaves of *P. lingua* when droughted to -3 MPa (Figs 6.1 & 6.2). Importantly there was no evidence of a feedback in stomatal control between *A* and g_s, whereby reduced or damaged *A* as a result of drought caused stomata to remain closed on rehydration, in recovered leaves with depressed photosynthesis g_s remained relatively high (Appendix 6.3). This resulted in highly inefficient loss of water in plants recovering from drought damage.

The response of ferns and lycophytes to drought is not mediated by ABA

In all species ABA levels were augmented in response to water deficit, but in most species this augmentation did not correlate with changes in g_s (Fig. 6.3), instead occurring after stomatal closure and Ψ_{tlp}, following Ψ_{tlp} the levels of ABA in the leaves of the terrestrial and desiccation-tolerant species increased to levels exceeding 1000 ng g^{-1} FW (Fig. 6.4), this was the case when ABA level was quantified as a proportion of leaf FW, DW or water content (data not shown). Only two species showed overlap between the phase of ABA rise and stomatal closure. In *P. lingua* this relationship occurred over extremely low foliar ABA levels (<20 ng g^{-1} FW) (Fig. 6.3) and in *R. adiantiformis* the recovery of g_s when droughted plants were rewatered was not significantly influenced by the ABA level in the leaf (Fig. 6.1). By contrast, in the angiosperm *V. x wittrockiana* there was a significant linear relationship ($P<0.001$; $R^2=0.62$) between foliar ABA level and g_s as the plants were droughted and recovered from stress (Fig. 6.3).

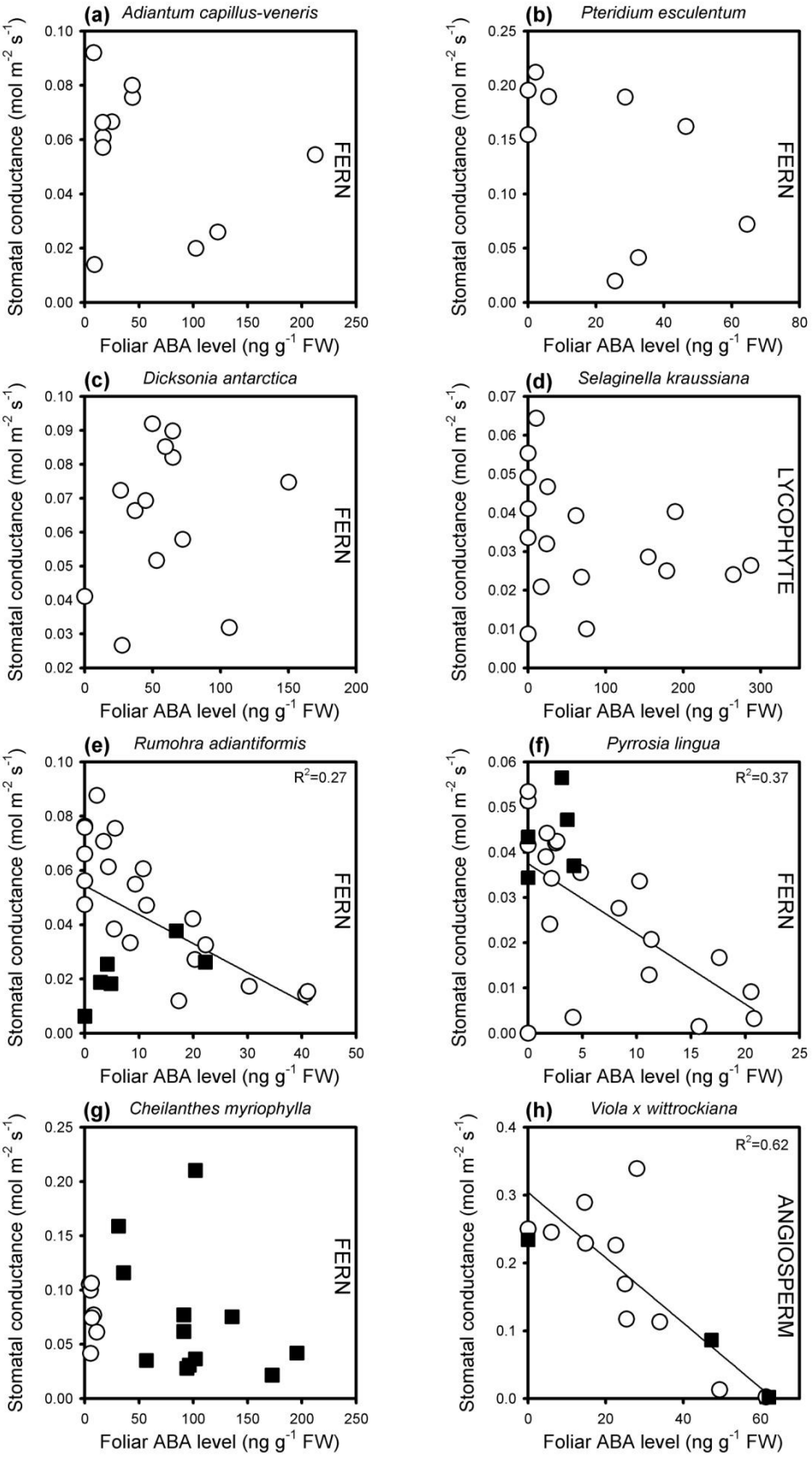

Figure 6.3. The relationship between foliar ABA level (ng g^{-1} fresh weight) and stomatal conductance prior to stomatal closure in three mesophytic terrestrial fern species (a-c) and a lycophyte (d), two epiphytic fern species (e and f), a desiccation-tolerant fern species (g) and an angiosperm (h) when droughted (white circles) and recovered (black squares). Significant regressions (solid lines) and R^2 are shown where present.

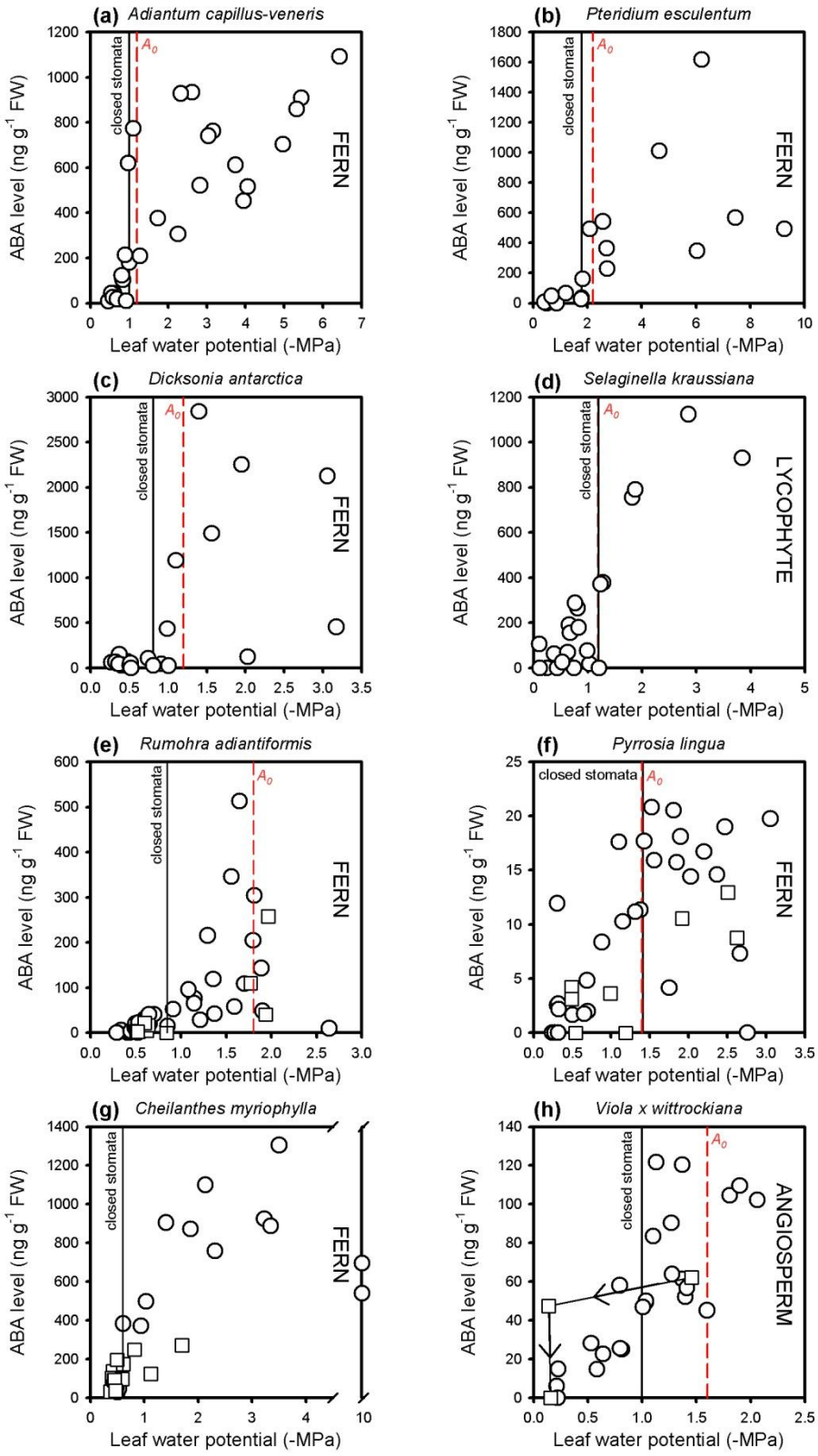

Figure 6.4. The relationship between foliar ABA level (ng g^{-1} fresh weight) and leaf water potential (-MPa) showing a clear relationship between water stress and ABA levels, pooled from three individuals of three mesophytic terrestrial fern species (a-c) and a lycophyte (d), two epiphytic fern species (e and f), a desiccation-tolerant fern species (g) and an angiosperm (h) when droughted by withholding water (open circles). Solid vertical lines represent Ψ_l when stomata closed (see Fig. 6.1) and dashed red vertical lines represent Ψ_l at which assimilation rate did not recover in rehydrated leaves (A_0). Closed symbols each represent measurements made on individuals that were rehydrated, a line tracks the recovery of ABA in an individual of *Viola x wittrokiana* following rehydration.

Very predictable responses of fern and lycophyte stomata to atmospheric humidity

All fern and lycophyte stomata were highly sensitive to atmospheric humidity (Fig. 6.5). Following an increase in VPD from 1 to 2 kPa the fern and lycophyte stomata closed following a single exponential decay function reaching a new steady-state g_s in less than 20 minutes, with no hydropassive, wrong-way response (Fig. 6.5). The degree of stomatal closure in response to this increase in VPD varied between species, with the stomata of *C. myriophylla* closing by only 30.95 ±3.65% while the stomata of *S. kraussiana* closed by 66.26 ±4.37% (Table 6.2). Stomatal responses to a subsequent decrease in VPD from 2 to 1 kPa resulted in stomata reopening that followed the same exponential trajectory with no evidence of hydropassive closure (Fig. 6.5 and Table 6.2). In contrast to the predictable and highly sensitive stomata of ferns and lycophytes to atmospheric water stress, the stomata of the angiosperm *V. x wittrockiana* were relatively insensitive to an increase in VPD from 1 to 2 kPa or decrease (Fig. 6.5 and Table 6.2).

Table 6.2. The mean percentage reduction in stomatal conductance (g_s) following an increase in vapour pressure difference from 1 to 2 kPa and the mean g_s (as a percentage of an initial g_s at 1 kPa) after a decrease in VPD from 2 to 1 kPa in seven fern and lycophyte species and an angiosperm (n=3).

Species	% stomatal closure (1-2 kPa)	±se	% g_s of initial (2-1 kPa)	±se
Adiantum capillus-veneris	47.50	4.68	105.45	3.81
Cheilanthes myriophylla	30.95	3.65	99.85	0.63
Dicksonia antarctica	33.53	3.82	100.30	3.26
Pteridium esculentum	35.37	5.31	102.65	2.60
Pyrrosia lingua	32.89	5.70	98.04	1.02
Rumohra adiantiformis	42.60	9.87	106.75	1.53
Selaginella kraussiana	66.26	4.37	100.23	1.85
Viola x wittrockiana	-3.23	3.36	113.98	12.29

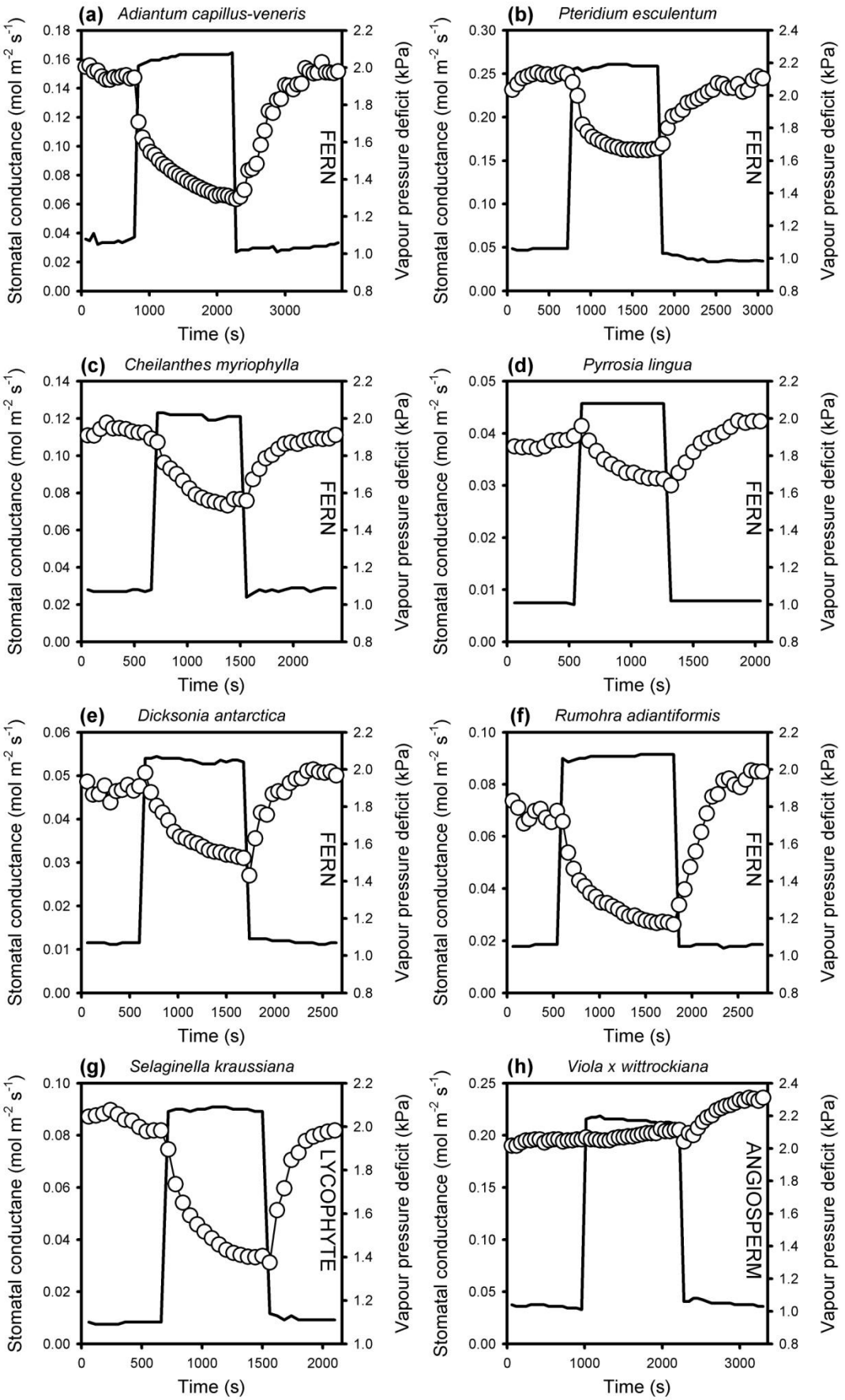

Figure 5. Representative responses of stomatal conductance (mol m^{-2} s^{-1}) to step-wise transitions in vapour pressure deficit (kPa) in six fern species (a-f), a lycophyte (g) and an angiosperm (h).

A limited diversity of fern and lycophyte strategy to water stress

There was a large variability in the minimum RWC that the leaves of fern and lycophyte species could survive (Fig. 6.6). An increase in desiccation tolerance increased the volume of internal leaf water available to the plant during drought (Fig. 6.6). The leaves of terrestrial fern and lycophyte species were unable to survive desiccation to a RWC <85% and had only a minimal volume of water available before leaf death, ranging from 46.4 mmol H_2O m^{-2} in *A. capillus-veneris* to 492.1 mmol H_2O m^{-2} in *S. kraussiana* (Fig. 6.6). The leaves of the least resistant species, *D. antarctica* were unable to survive a RWC below 97.2%, while the leaves of the lycophyte *S. kraussiana* survived to a RWC of only 87.3% (Fig. 6.6). The limited tolerance of leaf desiccation was a trait characteristic of the mesophytic terrestrial species only (Fig. 6.6). The leaves of the two epiphytic fern species were characterised by a tolerance of low RWC, with leaves of *R. adiantiformis* surviving to a RWC of 41.7% and *P. lingua* to 26.7%, this tolerance of low RWC resulted in a large volume of available water in the leaf before death as a result of drought (Fig. 6.6). The desiccation-tolerant fern species *C. myriophylla*, was able to survive extremely low RWCs (around 10%) and like the two epiphytic species had a large volume of internal leaf water (3824.8 mmol H_2O m^{-2} ± 44.07) available before leaf death (Fig. 6.6). The size of the internal leaf water buffer was a product of leaf volume and desiccation tolerance and was highly correlated with the duration of drought that could be tolerated ($P=0.001$, $R^2=0.902$, Fig. 6.6).

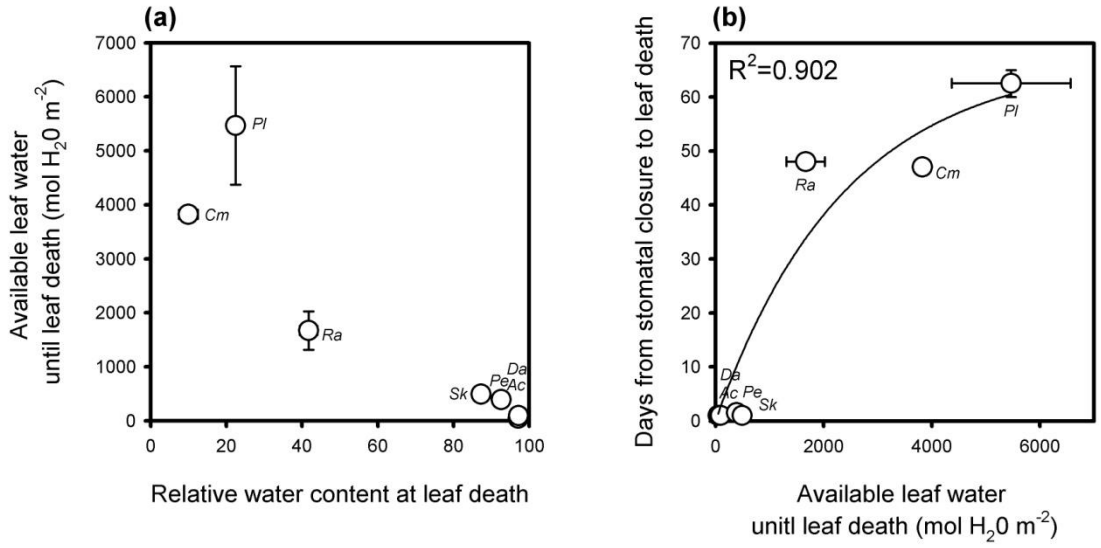

Figure 6. In a wide diversity of fern and lycophyte species the tolerance of leaves to minimal relative water contents influenced the volume of available leaf-water each species could use before leaf death (a). There was a significant relationship between available leaf water until leaf death and the number of days between stomatal closure and leaf death in ferns and lycophytes (P=0.001, $\text{Days to leaf death} = 67.34 \times \left(1 - e^{(-0.0004 \times \text{ available leaf water})}\right)$, R^2=0.902 n=7 species) (b). Each symbol represents the mean ± se (n=5) for an individual species (notation for symbols: *Ac*, *Adiantum capillus-veneris*; *Cm*, *Cheilanthes myriophylla*; *Da*, *Dicksonia antarctica*; *Pe*, *Pteridium esculentum*; *Pl*, *Pyrrosia lingua*; *Ra*, *Rumohra adiantiformis*; *Sk*, *Selaginella kraussiana*).

DISCUSSION

Passive stomatal control in the light is ubiquitous to ferns and lycophytes

The stomatal behaviour among fern and lycophyte species with diverse growth habits and ecologies was found to be remarkably conservative, with the stomata of all species predominantly controlled by a passive response to leaf water status (Brodribb and McAdam 2011b). Such uncomplicated regulation of plant hydration appears to result in a very simple drought adaptation strategy whereby ferns and lycophytes modify water storage and desiccation tolerance as a means of extending drought survival time (Fig. 6.6). This canalised adaptive pathway in response to drought must have significant ramifications for the ability of ferns and lycophytes to compete with seed plants which have complex stomatal control and diverse water management strategies (Tardieu and Simonneau 1998a; Tardieu and Simonneau 1998b). The complexity of a largely metabolic regulation of stomatal aperture in seed plants can be recognised in the variability of seed plant responses to changes in VPD, such as that observed in *Viola x wittrockiana* in this study (Fig. 6.5). This variability in response to VPD in seed plants ranges from highly sensitive plants that illicit 'feed-forward' responses to increases in VPD, to plants that are relatively insensitive (Farquhar 1978; Franks and Farquhar 1999; Mott and Peak 2012). This diversity in response is unlike the predictable

responses of fern and lycophyte stomata to changes in VPD which are largely regulated by a passive response to leaf water status (Fig. 6.5) (Brodribb and McAdam 2011b; McAdam and Brodribb 2012b).

Physiological and morphological adaptations alone are essential for ferns and lycophytes to survive dry environments

Many epiphytic fern and lycophyte species are exposed to dry and/or fluctuating environments, which raises the question of how these plants compete with seed plants when their stomata are predominantly regulated passively by leaf water status (Watkins and Cardelús 2012). Only a small amount of information is known about the physiological adaptations of epiphytic fern species. These include a lower leaf hydraulic conductance compared to terrestrial ferns (Watkins *et al.* 2010), desiccation-tolerant gametophytes (Watkins *et al.* 2010) and occasionally Crassulacean acid metabolism (CAM) (Ong *et al.* 1986; Holtum and Winter 1999). Interestingly the adaptation of CAM in tropical epiphytic ferns is not associated with an enhanced tolerance to long periods of drought as seen in angiosperm CAM species; rather improving carbon and water balance over short (diurnal) periods of water stress (Ong *et al.* 1986).

While we found the function of stomatal in a diversity of fern and lycophyte species was the same, species from drier environments (epiphytes and desiccation-tolerant species) were able to survive for a long period of time with stomata closed before leaves died unlike the mesophytic terrestrial species (Fig. 6.6). This increased tolerance of epiphytic fern species to drought stress was due to the combination of both a physiological tolerance of low RWC and anatomical modification of leaves to increase the amount of water available before leaf death during drought (Fig. 6.6). Epiphytic ferns are capable of developmentally adapting a reliance on either of these two strategies to survive water-stress, with *Asplenium auritum* being desiccation-tolerant as a young sporophyte and maturing into a drought-avoiding, high-capacitance plant as size increases (Testo and Watkins 2012). Epiphytic ferns are known to have large water storage capabilities in either leaves (as in the genus *Pyrrosia* with specialised hydrenchyma cells in the leaf (Ong *et al.* 1992)) as well as water storing rhizomes and stems (Dubuisson *et al.* 2009). Available water as defined here by the leaf capacitance multiplied by the viable range of leaf water content after stomatal closure, gives a volume of water that can supply the slow leakage of water from plants with closed stomata assuming hydraulic isolation from the soil (Linton and Nobel 1999). The significant relationship we found

between the time to leaf death and leaf available water highlights the fundamental importance of the interaction between the storage and partitioning of leaf water from the stomata and the time to leaf death as a result of drought. Epiphytic ferns are known to maintain a Ψ_l low enough to close stomata for an extended period of time before leaves die (Ong *et al.* 1992). The combination of these two strategies, a physiological tolerance of low RWC and partitioning and isolation of large volumes of water from the stomata is a common feature of epiphytic ferns and lycophytes, which are largely characterised by succulent leaves or rhizomes and can survive in environments with highly sporadic water availability (Watkins and Cardelús 2012). This apparent dependence on water storage and desiccation tolerance contrasts with seed plants that, with a predominantly metabolic regulation of stomatal control, have the opportunity of modifying stomatal behaviour through hormones such as ABA during drought stress (Wilkinson and Davies 2002).

High physiological tolerance of leaves to low RWC is common in (fern) epiphytes and exemplified by desiccation-tolerant and poikilohydric species (Hietz 2010). Within ferns desiccation-tolerance is widely represented, occurring in most major lineages, with estimates ranging from 5 to 10% of fern species being desiccation-tolerant or poikilohydric, while in the lycophytes two of the three extant families have desiccation-tolerant species (Hietz 2010). The over-representation of desiccation-tolerance in ferns and lycophytes relative to seed plant lineages (Oliver *et al.* 2000) is likely a reflection of the limited options of water strategy provided by a predominantly passive stomatal control. The stomatal responses of desiccation-tolerant and epiphytic ferns is the same as mesophytic terrestrial ferns.

Implications for the evolution of land plants

In ferns and lycophytes, the less complex predominance of a passive stomatal control of leaf hydration still provides an efficient means of regulating leaf hydration in response to changes in water stress (Brodribb and McAdam 2011b) (Fig 6.5). Yet this mechanism of stomatal control in ferns and lycophytes does not offer plants the ability to entertain a diversity of responses to soil water content through changes in metabolic regulation of stomata like seed plants (Tardieu and Simonneau 1998a). It is possible that the evolution of an increased regulation of stomata by ABA in seed plants may have been driven by the selective pressure for a stomatal strategy that could enhance the survival of plants over both short and long term periods of soil water stress, this would offer an advantage over the changes in morphology or desiccation tolerance required by ferns and lycophytes (Fig. 6.6). This might have been

particularly advantageous during the drying climate of the early Permian (DiMichele and Aronson 1992). A heightened sensitivity of stomata to increased ABA levels, augmented by drought, offers seed plants a dynamic, stomatal-mediated response to episodes of soil drought that occurs over relatively brief periods (hours to days). The predominance of a metabolic regulation of stomata particularly by ABA in seed plants additionally allows the leaves of species that do not have physiological and morphological adaptations to dry environments to survive periods of soil drought by modifying the sensitivity of stomata to small changes in Ψ_l (Umezawa *et al.* 2004; Fujita *et al.* 2005) unlike the mesophytic terrestrial ferns and lycophytes (Fig. 6.1). Adopting the strategy of ABA-regulated stomatal control in seed plants is also likely to be important both for optimising water use during fluctuating soil water availability (Chaves *et al.* 2003) and during the recovery from stress in facilitating the repair of xylem tissue after drought (Lovisolo *et al.* 2008).

The response of fern and lycophyte stomata to drought stress suggests that stomatal closure occurs with a relatively small Ψ_l margin before leaf death in these species (Fig. 6.1). Interestingly ferns and lycophytes close their stomata with a similar Ψ_l margin before leaves lose hydraulic conductivity (Brodribb and Holbrook 2004) suggesting that cavitation-induced losses in hydraulic conductivity probably lead to plant death during drought. Further investigation is required to develop a more comprehensive understanding of the role of xylem and leaf hydraulics in influencing the survival and ecology of ferns and lycophytes.

Suggested roles for ABA in droughted ferns and lycophytes

While the augmentation of ABA levels occurs in fern and lycophyte leaves during drought (Fig. 6.4), this increase is not typically associated with the closing of stomata (Fig. 6.3), which is traditionally held to be the primary function of this phytohormone (Wilkinson and Davies 2002). Instead ABA levels in ferns and lycophytes increase during drought largely after plants has been stressed beyond Ψ_{tlp} (Fig. 6.4), this could be due to ABA in the leaf being unbound from a fettered state when the leaf loses turgor, this free ABA then drives the release of much higher concentrations of ABA in the leaf (Georgopoulou and Milborrow 2012). There are a number of functional roles for ABA beyond active stomatal control that have been suggested in ferns and lycophytes, not all associated with the sporophyte, such as the regulation of sex expression in the gametophyte (Banks *et al.* 1993). In most land plants, including ferns and lycophytes, ABA enhances the survival of tissues grown *in vitro* and is instrumental in initiating tissue desiccation-tolerance (Bagniewska-Zadworna *et al.* 2007). In

the diversity of droughted fern and lycophyte species we examined, the level of ABA in the leaf dramatically increased when leaves were dehydrated beyond the critical Ψ_l at which point photosynthesis was unable to recover and leaves died (Fig. 6.4). It is possible that the augmentation of ABA in ferns and lycophyte occurring beyond Ψ_{tlp} and the recovery of photosynthesis reflects a role of ABA in initiating leaf senescence. High levels of ABA in seed-plant leaves have been shown to initiate leaf senescence pathways following stress (Lee *et al.* 2011). Senescence of droughted leaves of ferns and lycophytes would be particularly important because the stomata of ferns and lycophytes reopen on leaf hydration regardless of photosynthetic damage (Appendix 6.3) constituting a substantial transpiration cost without photosynthetic benefit (McAdam and Brodribb 2012a). It would be highly advantageous to initiate leaf senescence and shed leaves that have incurred photosynthetic damage following drought stress, through an increase in ABA level. Leaf senescence triggered by ABA in droughted ferns and lycophytes would additionally prevent hydraulic damage to the rhizome in recovering plants. This role for ABA may explain the prevalence of drought deciduousness and the ability of many terrestrial, mesophytic and epiphytic fern species to resprout from a protected meristem following drought (Hietz 2010) .

Conclusion

The stomata of all ferns and lycophytes studied showed a predominantly passive regulation of stomata by leaf hydration, regardless of ecology or morphology. While this mechanism of stomatal control means that ferns and lycophytes have an efficient regulation of water loss under fluctuations in vapour pressure difference and also close in response to soil drought, it results in canalised adaptation when it comes to surviving extended periods of water stress. While Ψ_l is sufficient to close the stomata of ferns and lycophytes when water-stressed, ferns and lycophytes must make significant morphological and physiological adaptations over generational time scales in order to adapt and survive for extended periods of drought or in water limiting environments.

CHAPTER 7

Concluding Discussion

Evolution of stomatal physiology across land plant lineages is a relatively recent field of research that has generated some controversy. The idea that stomatal responses might be different across land plant lineages has been raised for nearly 45 years (Mansfield and Willmer 1969). For most of this time technical limitations, such as those involved in measuring aperture changes in isolated epidermis, ensured that differences in stomatal responses across land plant lineages remained difficult to identify. In an early study directly observing stomata on isolated paradermal sections, Mansfield and Willmer (1969) concluded that the stomata of the fern *Asplenium* (*Phyllitis*) *scolopendrium* showed similar responses to CO_2 concentration as angiosperms. However, measurements using infrared gas analysers have subsequently shown no stomatal response to CO_2 in ferns and lycophytes (Doi and Shimazaki 2008; Brodribb *et al.* 2009; Ruszala *et al.* 2011; Haworth *et al.* 2012). The widespread use of leaf gas exchange measurements led Hollinger (1987) to suggest ferns lacked the complexity of stomatal control observed in seed plants. Yet it was not until the mid-2000's that the earliest physiological differences in stomatal control were identified, with two papers illustrating absent stomatal responses to blue light and CO_2 in ferns (Doi *et al.* 2006; Doi and Shimazaki 2008). In this thesis I have extended this evidence for evolution in stomatal control in vascular plants using both measurements of leaf gas exchange and stomatal apertures. I consider the ancestral state of stomatal control in vascular plants and offer speculation on the function of the very first land plant stomata. Recent controversy surrounding stomatal evolution stems largely from approaching the question of stomatal control from a molecular biological understanding of guard cell behaviour (Chater *et al.* 2011; Ruszala *et al.* 2011) as opposed investigating stomatal function on live leaves (Doi *et al.* 2006; Doi and Shimazaki 2008; Brodribb and McAdam 2011b; McAdam and Brodribb 2012a). I conclude with a discussion on the ecological and evolutionary implications of evolution in stomatal control in land plants.

Reconstructing the ancestral state of stomatal control in vascular land plants

The extant vascular land plants are partitioned into four separate lineages based on their distinct evolutionary histories, the lycophytes, ferns, gymnosperms and angiosperms.

Lycophytes are small group of spore-bearing plants that diverged shortly after the evolution of vascular tissues over 410 million years ago (Rickards 2000; Gensel and Berry 2001; Banks 2009; Banks *et al.* 2011). This group of plants came to dominate equatorial swamps as trees of determinate growth throughout the Carboniferous (Phillips *et al.* 1985). Only a few studies have investigated the stomatal behaviour of lycophytes. These studies show no epidermal mechanical advantage over the guard cells identified (Franks and Farquhar 2007) and no response to changes in CO_2 (Brodribb *et al.* 2009). In this thesis I have presented data that show lycophyte stomata lack a number of active regulators of stomatal aperture observed in seed plants. In particular, these stomata show no response to either exogenous (Figs 2.1 and 2.2) or endogenous (Fig. 3.2) ABA. Subsequent studies have supported these findings, with the stomata of *Selaginella bryopteris* under natural vapour pressure deficits (1 kPa) being insensitive to ABA (Soni *et al.* 2012), while the stomata of *S. uncinata* when fed astronomically high levels of ABA in the transpiration stream did not show any functionally significant reduction in stomatal conductance (Ruszala *et al.* 2011). Lycophyte stomata are also unable to efficiently regulate water use following changes in light intensity (Fig. 4.2) due to an ineffective photosynthetic signal from the mesophyll influencing stomatal aperture (Fig. 4.3). In place of these metabolic regulators of stomatal aperture, in the light, lycophyte stomata are passively controlled by leaf water status, with highly predictable responses to changes in leaf hydration (Figs 2.3; 2.4; 3.4 and 6.1).

The ferns were the next discreet lineage of vascular plants to evolve after the lycophytes. Ferns are a diverse clade of spore-bearing plants including one large lineage (the leptosporangiate ferns) that diversified in the Cretaceous (Schneider *et al.* 2004) and a number of smaller, relictual lineages (Pryer *et al.* 2004). While extant ferns are more successful than lycophytes, my studies showed the same mechanisms of stomatal control in both groups: no response to either exogenous or endogenous ABA (Figs 2.1; 2.2 and 3.2) and an inability of leaves to efficiently regulate water use (Figs 4.2 and 4.3), resulting in a highly predictable stomatal behaviour in the light with stomata responding as passive hydraulic valves (Figs 2.3; 2.4; 3.4 and 6.5). Fern stomata also lack responses to blue light (Doi *et al.* 2006) and CO_2 (Doi and Shimazaki 2008; Brodribb *et al.* 2009; Haworth *et al.* 2012). Based on the similar responses of lycophyte and fern stomata to changes in light intensity it seems likely that lycophytes also do not have a stomatal response to blue light; however this remains to be tested.

Angiosperm stomata are physiologically controlled by a diverse array of metabolic signals (Schroeder *et al.* 2001; Ache *et al.* 2010). Although angiosperm stomata are characterised as actively regulating leaf turgor (Ache *et al.* 2010) rather than the ancestral condition of a passive regulation of stomatal aperture by leaf hydration (Chapter 1), a stomatal response to leaf hydration is widely recognised in seed plants (Brodribb and Holbrook 2003; Brodribb and Cochard 2009; Aasamaa and Sõber 2011b, a; Peak and Mott 2011). There is a diversity of stomatal control mechanisms in the extant seed plant lineages (gymnosperms and angiosperms); with the stomata of a number of conifers insensitive to increasing CO_2 concentration (Fig. 6.4) and unlike angiosperms which are known to lose sensitivity to CO_2 when ABA levels in the leaf are low, the stomatal sensitivity of conifer species to CO_2 cannot be recovered by increasing the levels of ABA in the leaf (Fig. 6.3). This absence of a stomatal response to high CO_2 in gymnosperms suggests that the ancestral state of angiosperms was a lack of sensitivity to CO_2, which may be due to a component of the shared guard cell signalling pathway between ABA and CO_2 (Young *et al.* 2006) being co-opted into a stomatal function in the earliest angiosperms.

The similarity of stomatal control mechanisms in the two basal vascular land plant lineages, the ferns and lycophtyes, in spite of more than 400 million years of independent evolution, suggests that the ancestral condition of the first vascular plant stomata was a passive hydraulic control of leaf hydration in the light (Fig. 4.4). The evolution of the complex active mechanism of stomatal regulation seen in angiosperms may have involved multiple steps, but the fact that the stomata of extant gymnosperms show most of the important components suggest that much of the evolution occurred during the diversification of the earliest seed plant lineages (Doyle and Donoghue 1992).

The responses of the very first stomata

While the evolution of stomatal physiology across vascular land plants appears a simple story of increasing complexity from an ancestral passive hydraulic control of stomata in the light, a question remains about the responses of the very first stomata in land plants. Two lineages that diverged before the vascular plants, mosses and hornworts, have small numbers of stomata on their sporophytes (Paton and Pearce 1957; Abella *et al.* 1999; Lucas and Renzaglia 2002). However, it is very unclear whether these stomata function in an analogous way to those of vascular plants (Paton and Pearce 1957; Garner and Paolillo 1973; Hartung *et al.* 1987; Lucas and Renzaglia 2002; Duckett *et al.* 2009; Chater *et al.* 2011). Current opinion favours a role of these stomata in nutrient delivery to the developing sporophyte by the

maintenance of a transpiration stream as well as providing a means of desiccating sporophytic tissue in aid of spore release, similar to that documented in the moss genus *Sphagnum* (Duckett *et al.* 2009; Ligrone *et al.* 2012). Developmentally controlled stomatal behaviour has been reported in hornworts in a brief communication (Lucas and Renzaglia 2002) as well as in a recent review on sporophyte evolution (Ligrone *et al*. 2012a). In hornworts stomata are initially closed in the young sporophyte then open to allow mucilage in the intercellular spaces to dry, following which they never close (Lucas and Renzaglia 2002). If further investigation into the physiology of bryophyte stomata validates this behaviour, an inversion in stomatal function from facilitating water loss, to maintaining homoiohydry during photosynthesis would have occurred in the ancestors of the earliest vascular land plants. Although speculative, this major change of stomatal function would have required two crucial innovations in guard cell behaviour. The first would be the evolution of guard cell anatomy that ensures closure of the pore when cell turgor pressure declines. If the resting state of the stomata was closed the second requirement would be for an ionic mechanism to increase guard cell turgor pressure in the light, such as an H^+-ATPase and associated inward potassium channels that are active in the guard cells of most vascular plants (Willmer and Pallas 1973).

The evolution of stomatal control: a molecular perspective

The concept of evolution in stomatal control has generated heated debate between researchers approaching the question from a molecular biological perspective (Bowman 2011; Chater *et al.* 2011; Ruszala *et al.* 2011) as opposed to a functional perspective (Doi *et al.* 2006; Doi and Shimazaki 2008; Brodribb and McAdam 2011b; Brodribb and McAdam 2011a; Haworth *et al.* 2011; McAdam and Brodribb 2012b). The linked papers of Ruszala *et al*. (2011) and Chater *et al*. (2011) claim that the earliest stomata had the entire suite of complex stomatal control now observed in seed plants. Their conclusions are based on three major limitations: (1) the reliance on an experimental method designed to observe large aperture changes in angiosperms; (2) cross-species gene transplantation and (3) a singular focus on the role of ABA in closing stomata. In this thesis (Chapter 3), I focussed on the validity of the conclusions reached by Ruszala *et al*. (2011), which pivoted on the examination of *S. uncinata* stomata responding to ABA in isolated epidermis, as well as the interpretation of physiologically irrelevant (<3 mmol m^{-2} s^{-1}) changes in stomatal conductance following the feeding of unrealistically high levels of ABA.

Evidence underpinning the conclusions of Ruszala *et al*. (2011) and Chater *et al*. (2011) was that cross-species gene transplantation of both the moss and lycophyte OST1 homologues

restored the sensitivity of transformed *Arabidopsis thaliana* ABA-insensitive *ost1* mutants. While this evidence supports a conserved role of OST1 interacting with the SLAC1 anion channel in land plants it does not address the crucial issue of whether this pathway is active in guard cells. There are several examples in which the transplantation of genes from basal land plants into *A. thaliana* mutants have restored wild-type phenotypes even though the genes do not elicit the same function in the basal species (Yasumura *et al.* 2007; Liu *et al.* 2009). A particularly relevant example is the interaction between gibberellin receptor proteins and the growth preventing DELLA proteins; the two proteins bind under the influence of gibberellin, which deactivates the DELLA proteins and thereby promotes growth in vascular plants (Weston *et al.* 2008). In the moss *Physcomitrella patens* DELLA proteins do not supress growth, yet when transplanted into *A. thaliana* mutants, *P. patens* DELLA proteins can supress growth and restore a wild-type phenotype (Yasumura *et al.* 2007). As has been additionally demonstrated in studies investigating stomatal patterning genes, caution must be applied to the interpretation of cross-species gene transplantation experiments without an accurate phenotypic characterisation of function in both species (Liu *et al.* 2009; Vatén and Bergmann 2012).

Given that all of the genes currently known to be involved in guard cell ABA signalling are present in all lineages of land plants, including liverworts, which lack stomata (Yamato *et al.* 2007; Tougane *et al.* 2010), it is hardly surprising that cross-species transplantation restored mutant stomatal phenotypes in *A. thaliana*. Instead, it is important to consider the alternative roles for ABA signalling genes. The same suite of ABA signalling genes identified in angiosperm guard cells operate in many major lineages of organisms, including bacteria and animals (Zocchi *et al.* 2003; Nakashima *et al.* 2009; Li *et al.* 2011). However, in these lineages ABA can be involved in the regulation of cellular osmotic concentration under stress (Karadeniz *et al.* 2006; Hartung 2010), initiation of desiccation-tolerance pathways (Khandelwal *et al.* 2010), regulation of growth (Lin *et al.* 2005; Sakata *et al.* 2009; Takezawa *et al.* 2011) and the control of sex expression (Banks *et al.* 1993; Banks 1994). Thus, the simplest interpretation of the origin of a role for ABA in stomatal control is that this ancient interactive pathway triggered by environmental stress was gradually integrated into stomatal control over the course of land plant evolution. The alternative view that the entire suite of complex active stomatal control mechanisms (see Geiger *et al*. 2010) evolved with the first stomata and has not changed in 400 million years as argued by both Ruszala *et al*. (2011) and Chater *et al*. (2011) leads to a number of overstated conclusions. These include the evolution

of at least five discreet molecular interactions from the perception of ABA with three receptors through to the activation of a potassium membrane channel (Geiger *et al.* 2010) for the specific purpose of closing stomata (Chater *et al.* 2011; Ruszala *et al.* 2011). The no-evolution model also implies stomatal responses are largely irrelevant to the major ecological differences among land plant lineages.

Further molecular investigations

While the data in this thesis elucidate functional stomatal control, further study is required to clarify several molecular, biochemical and guard cell signalling facets of stomatal behaviour. Further studies into the evolution of stomatal control could include a thorough investigation of ABA action at a guard cell membrane level in bryophytes, lycophytes and ferns. The ancient origin of the genes involved in ABA signalling and synthesis begs the question of, whether the anion channels vital to angiosperm guard cell responses are present in the guard cells of ferns and lycophytes? If all of the relevant ion channels and signalling apparatus are present then guard cell pressure probe and membrane depolarisation studies would be instrumental in understanding their functional role in influencing guard cell turgor. ABA signalling mutants in angiosperms have been crucial in cementing the essential role of ABA in the normal stomatal responses of seed plants, if the stomata of ferns and lycophytes are functionally controlled by ABA then ABA signalling mutants should have the similar and severe, wilty phenotype of angiosperms. Thus the identification of ABA signalling mutants in lycophytes and ferns would prove pivotal in resolving the functional relevance of ABA in stomatal behaviour and survival in these lineages. There is the possibility that even though ferns and lycophytes have all of the molecular signalling components for an ABA response of stomata, these plants may lack the complex web of signalling interactions in seed plant guard cells. Examining the interactions between discreet ABA signalling components between a diversity of organisms would be highly informative in revealing the evolutionary origins of the genetic signalling interactions.

The ecological implications of innovation in stomatal control

The phylogenetic constraints of stomatal control have played an instrumental role in governing the ecological strategies employed by different lineages of land plants (Chapter 5). In seed plants, the active metabolic regulation of water use is extremely dynamic and intimately controlled by current and past experiences. It can also modify future stomatal behaviour. This active regulation of water use offers seed plants a diversity of ecological

roles, from water-wasting herbs to slow-growing trees in dry environments, as well as diversity in stomatal behaviours ontogenetically from understorey shrub to canopy dominant (Kolb *et al.* 1997; Van Wittenberghe *et al.* 2012). By contrast, in fern and lycophytes a passive response of stomata in the light to changes in leaf hydration offers an efficient regulation of water use only when water is limiting. This ancestral regulation of stomatal control appears to have been effective in allowing ferns and lycophytes to dominate both wet and drier ecosystems prior to the diversification of seed plants in the Permian (DiMichele and Phillips 2002). During this time terrestrial fluxes in carbon and water from vegetation would be decidedly simple to model provided estimations of the hydraulic properties of the dominant species (Chapter 2). However, this ancestral regulation of stomata places substantial limitations on the adaptability of individual leaves and whole plants to dynamic changes in the environment (Chapter 5) and may explain the relative conservatism in the leaf morphologies adopted by lycophytes and ferns over geological time (Boyce 2005).

In order to survive water limiting environments with passive hydraulic stomatal control ferns and lycophytes have made anatomical and physiological modifications that influence leaf water relations (Fig 6.6). This strategy is exemplified by epiphytic ferns which have an enhanced tolerance of low relative water contents and, combined with high leaf water content, thereby have a greater amount of available water available in the leaf extending the period which plants can remain with stomata closed (Fig. 6.6). These morphological adaptations required by ferns and lycophytes occur at a taxonomic level, with minimal functional plasticity offered by stomatal behaviour. The slow (generational) response of fern and lycophyte species to changes in the environment through morphological adaptations would ensure the rapid replacement of ecosystems dominated by ferns and lycophytes by seed plants in possession of an active metabolic regulation of stomatal aperture. The active metabolic regulation of stomata allows individual leaves to instantaneously respond to a diverse array of environmental conditions, such as soil drought, fluctuating light intensities and changes in photosynthetic rate. Seed plants are characterised by a wide diversity of stomatal function and leaf morphological forms, the two of which are not always associated. Examples of this include the deciduous angiosperm tree *Populus trichocarpa* which lacks a functional SLAC1 guard cell anion channel yet is morphologically analogous to other northern hemisphere deciduous tree species which have functional SLAC1 channels (Ehonen pers. comm.). Another example of diverse stomatal behaviour in angiosperms is the large leaved species *Gunnera tinctoria* which has stomata that behave similarly to ferns and lycophytes, with a

suboptimal response to light intensity and insensitivity to changes in CO_2 (Osborne *et al.* 1991). These examples highlight only a fraction of the diversity in water use strategy offered to seed plants by active stomatal control mechanisms; a result of which includes the dominance of these lineages in most terrestrial ecosystems as well as an opportunity for plants with diverse water use strategies to co-occur in the same environment.

The evolutionary implications of innovation in stomatal control

Classical explanations for the ecological and evolutionary success of the seed plants, particularly the angiosperms, have focussed on reproductive innovations (Regal 1977; Stebbins 1981). While reproductive traits are easy to recognise in the fossil record, evidence for evolutionary innovations in physiological capacity are decidedly harder to reconstruct. However, a detailed examination of the physiology of extant species has initiated the investigation of physiological evolution in land plants (Bond 1989; Boyce *et al.* 2009; Brodribb and Feild 2010). The abrupt increase in vein density during the Cretaceous in the angiosperms is a key example, allowing substantially higher hydraulic conductivities of seed plants compared to ferns and lycophytes (Boyce *et al.* 2009; Brodribb 2009; Brodribb and Feild 2010) producing a rise in maximum rates of photosynthesis (Boyce *et al.* 2009; Brodribb and Feild 2010). The evidence for evolution of stomatal control in vascular plants adds to the concept that physiological evolution is a primary driver of lineage success over geological time. Crucial further investigation of this hypothesis involves the linking of stomatal control with anatomical traits that can be observed in the fossil record. A potential candidate for this investigation might lie in the anatomy of the guard cell and subsidiary cell interface, stemming from the prominent association of the ancestral passive regulation of stomatal control in the light with the absence of a mechanical advantage of the epidermis over the guard cells (Franks and Farquhar 2007).

Conclusion

In this thesis I have demonstrated that stomatal control has evolved in vascular land plants from an ancestral passive response to changes in leaf hydration in the light to a complex metabolically driven active regulation of stomatal aperture in seed plants. Further investigations into this emerging field are crucial in offering insights into the evolution of terrestrial vegetation as well as a deeper understanding of the evolution and function of molecular and biochemical mechanisms responsible for stomatal control in angiosperms.

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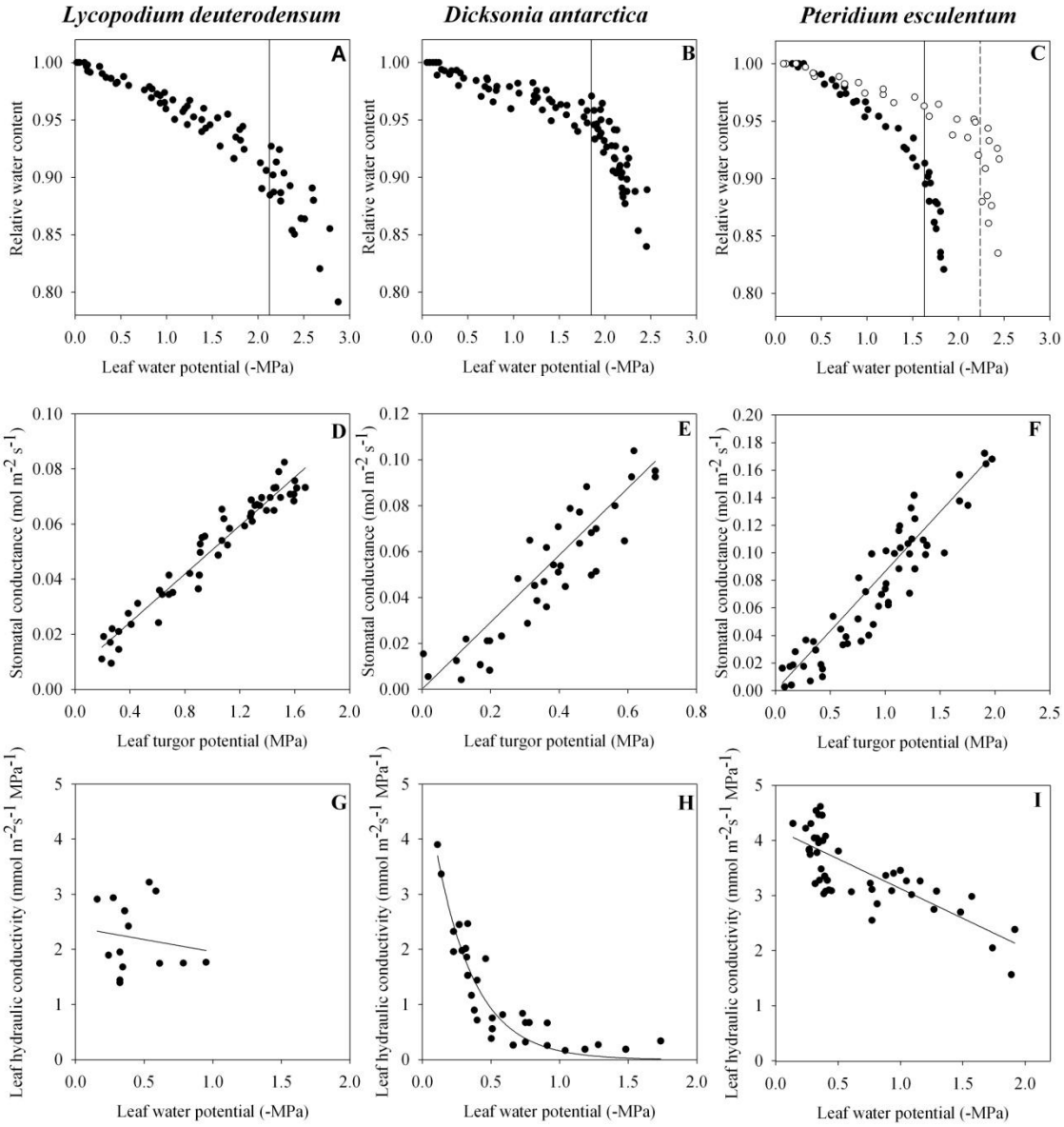
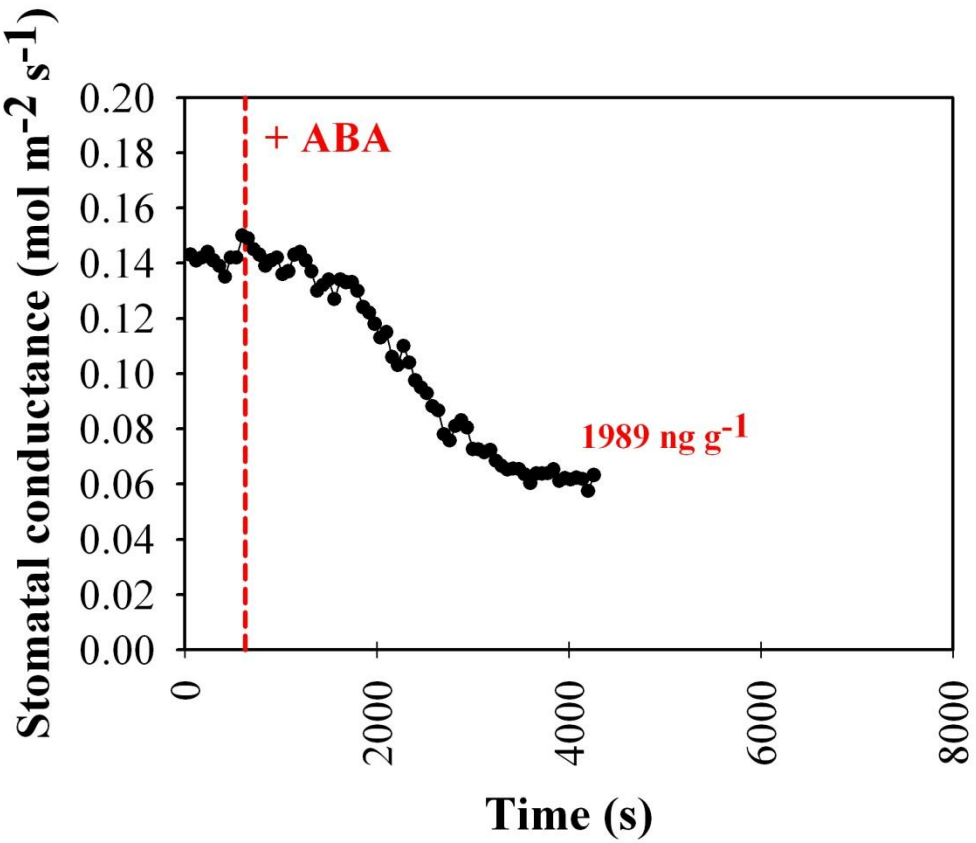
APPENDIX 2.1 Parameters used to formulate the hydraulic based model for stomatal conductance in two ferns and a lycophyte species.



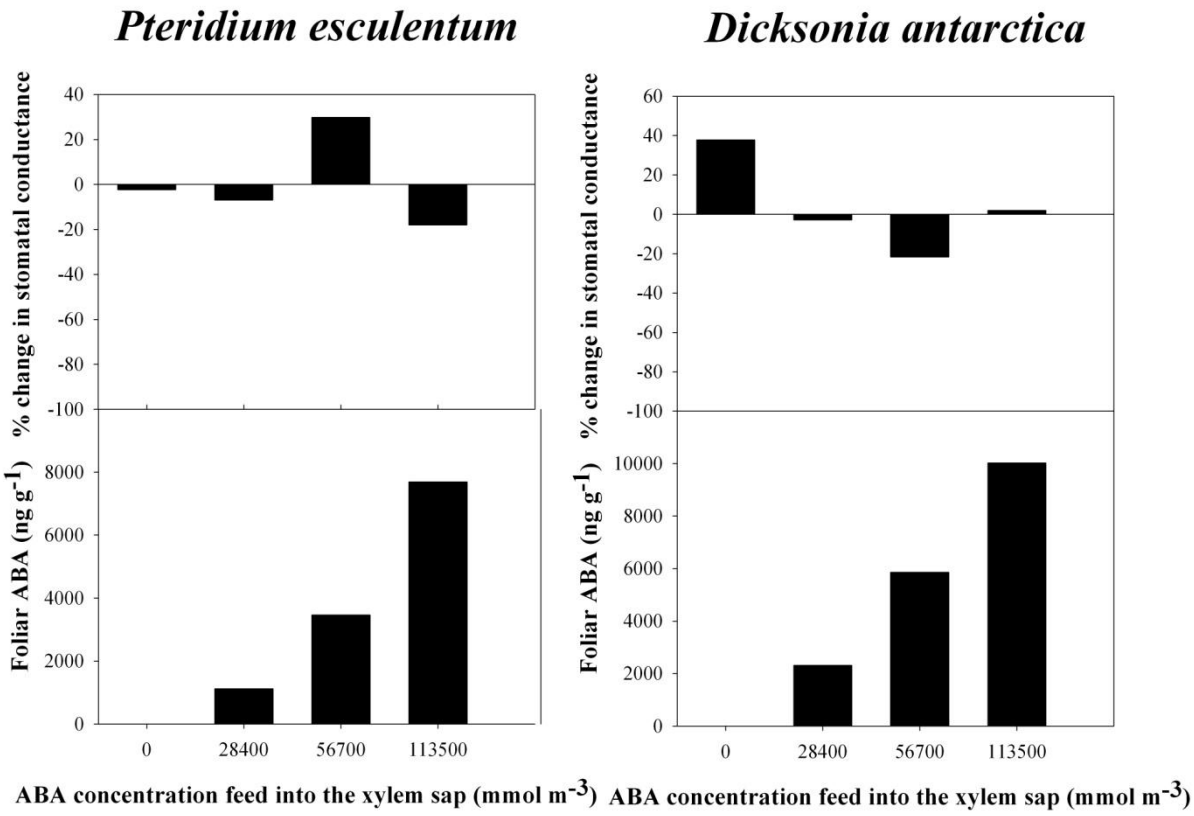
Appendix 2.1 Relationships used to formulate the hydraulic based model for stomatal conductance in the two fern species *Pteridium esculentum* and *Dicksonia antarctica* and the lycophyte *Lycopodium deuterodensum*. (A-C) Pressure-volume relationships for pooled data of *L. deuterodensum* (A), *D. antarctica* (B) and *P. esculentum* (C) with vertical lines representing turgor loss point. Separate populations of *P. esculentum* are represented by open and closed circles. Leaf capacitance and osmotic potentials were determined from data either side of turgor loss. (D-F) The relationship between stomatal conductance and leaf turgor in *L. deuterodensum* (D), *D. antarctica* (E) and *P. esculentum* (F), with linear functions fitted to the data through zero (R^2 = 0.94, 0.81 and 0.81 respectively). (G-I) The decline in leaf hydraulic conductivity over decreasing leaf water potential was determined from pooled data for *L. deuterodensum* (G), *D. antarctica* (H) and *P. esculentum* (I) and represented by a linear relationship for *L. deuterodensum* (R^2=0.028) and *P. esculentum* (R^2=0.61) and an exponential decay for *D. antarctica* (R^2=0.93).

APPENDIX 2.2 The response of stomatal conductance in *Ginkgo biloba* to ABA fed into the transpiration stream



Appendix 2.2 The response of stomatal conductance to the exogenous application of 15000 ng ml^{-1} ABA to the transpiration stream of the basal gymnosperm *Ginkgo biloba*.

APPENDIX 2.3 The percentage change in stomatal conductance in two fern species over a range of increasing ABA concentrations fed into the transpiration stream



Appendix 2.3 The percentage change in stomatal conductance (mol $m^{-2}s^{-1}$) following the feeding of three increasing concentrations of ABA into the transpiration stream and a control in the two fern species *Dicksonia antarctica* and *Pteridium esculentum*.

APPENDIX 2.4 The level of ABA under unstressed conditions in an angiosperm, two gymnosperms and nine fern and lycophyte species

Appendix 2.4 Concentration of ABA in unstressed leaves of nine fern and lycophyte species and the two representative gymnosperms *Ginkgo biloba* and *Callitris rhomboidea* and angiosperm *Helianthus annuus*.

Species	Natural unstressed foliar ABA level ($ng\ g^{-1}$)
Helianthus annuus	65
Callitris rhomboidea	90
Ginkgo biloba	272
Dicksonia antarctica	<10
Pteridium esculentum	12
Hypolepis tenuifolia	<10
Nephrolepis exaltata	<10
Adiantum capillus-veneris	<10
Microsorum pustulatum	<10
Lycopodium deuterodensum	<10
Lycopodiella inundata	<10
Selaginella kraussiana	<10

APPENDIX 2.5 Summary of the published literature of endogenous foliar ABA level under unstressed and drought stressed conditions

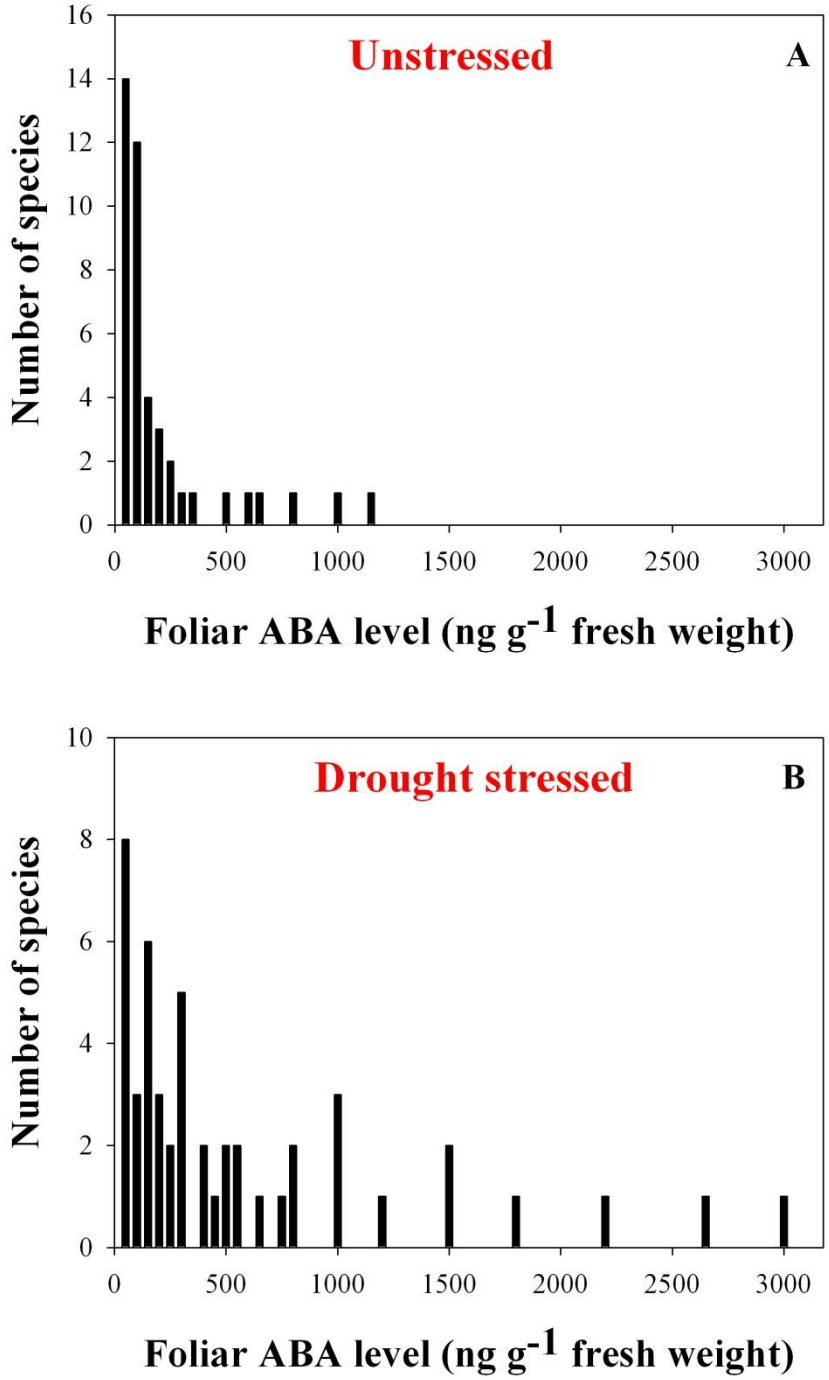
Appendix 2.5 The endogenous foliar ABA level (ng g^{-1} fresh weight) under normal unstressed conditions and at stomatal closure including the degree of drought stress imposed and the ABA quantification method from a range of species.

Species	Family	Minimum ABA (ng g^{-1}) FW	Minimum ABA level at stomatal closure (ng g^{-1}) FW	Degree of drought stress	Reference	ABA quantification method
			CONIFERS			
Callitris rhomboidea	Cupressaceae	320	1000	-1.8 MPa	(McAdam *et al.* 2011)	Physicochemical
Picea asperata	Pinaceae	800	1200	72% RWC	(Duan *et al.* 2009)	Immunoassay
Pinus radiata	Pinaceae	190	750	-2 MPa	(McAdam *et al.* 2011)	Physicochemical
Pseudotsuga menziesii	Pinaceae	24.5-89.0*	243.5*	-18.5 MPa	(Newville and Ferrell 1980)	Physicochemical
			ANGIOSPERMS			
			Monocots			
Allium cepa	Alliaceae	75-106	123-392	62.2% RWC	(Upreti and Murti 2004)	Immunoassay
Cocos nucifera var. *nana*	Arecaceae	132	264	-0.25 MPa	(Gomes *et al.* 2009)	Immunoassay
Dimerandra emarginata	Orchidaceae	13.26	105.72	-2 MPa	(Zotz *et al.* 2001)	Immunoassay
Hordeum vulgare	Poaceae	-	28.96*	50% RWC	(Sanguineti *et al.* 1994)	Immunoassay
Miscanthus x giganteus Greef and Deuter ex Hodkinson and *	Poaceae	2.0*	12.5*	-1.5 MPa	(Clifton-Brown *et al.* 2002)	Immunoassay
Miscanthus sacchariflorus	Poaceae	2.0*	12.5*	-1.5 MPa	(Clifton-Brown *et al.* 2002)	Immunoassay
Miscanthus sinensis hybrid (triploid)	Poaceae	1.0*	5.0*	-1.5 MPa	(Clifton-Brown *et al.* 2002)	Immunoassay
Pennisetum americanum	Poaceae	2-5	100-500	7% RWC	(Henson 1984)	Physicochemical
Poa pratensis	Poaceae	2.64	26.43-265.11	-4 MPa	(Wang and Huang 2003)	Immunoassay
Triticum aestivem cv. Gamenya and Warigal	Poaceae	100	250	-1.6 MPa	(Henson *et al.* 1989)	Immunoassay
Zea mays	Poaceae	26.43*	462.56*	50% RWC	(Jiang *et al.* 2007)	Immunoassay
			Eudicots			
Mangifera indica cv. Chokanan	Anacardiaceae	1000-2000*	3000*	-2.5 MPa	(Zaharah and Razi 2009)	Immunoassay
Gerbera jamesonii cv. Bolus	Asteraceae	5.2886	23.789	-2 MPa	(Olivella *et al.* 1998)	Immunoassay
Helianthus annuus	Asteraceae	6.068-13.2	39-59		(Rauf and Sadaqat 2007)	Immunoassay
Senecio minimus	Asteraceae	75	400	-0.8 MPa	(McAdam *et al.* 2011)	Physicochemical
Aptenia cordifolia	Azioaceae	65*	110*		(Cela *et al.* 2009)	Physicochemical

Arabidopsis thaliana	Brassicaceae	5*	45*		(Huang *et al.* 2008)	Physicochemical
Carica papaya cv. Baixinho de Santa Amalia	Caricaceae	3.5-12.5*	20.0-24.0*		(Mahouachi *et al.* 2007)	Physicochemical
Cucumis sativus cv. suyo	Cucurbitaceae	50	300	-1 MPa	(Nada *et al.* 2003)	Physicochemical
Hippophae rhamnoides	Elaegnaceae	260-380	535-663		(Xu *et al.* 2008)	Physicochemical
Cicer arietinum	Fabaceae	10	50	-2.48 MPa	(Nayyar *et al.* 2005)	Physicochemical
Glycine max cv. Hwangkeumkong.	Fabaceae	60*	100*		(Hamayun *et al.* 2010)	Physicochemical
Lupinus cosentinii cv. Eregulla	Fabaceae	200	800	-0.9 MPa	(Henson *et al.* 1989)	Immunoassay
Medicago sativa cv. Aragòn	Fabaceae	206.1*	283.0*	-2.70 MPa	(Goicoechea *et al.* 1997)	Physicochemical
Pisum sativum	Fabaceae	21±0.2	440±10		(Jager *et al.* 2008)	Physicochemical
Pisum sativum mutant argenteum	Fabaceae	94*	103*	88% RWC	(Cornish and Zeevaart 1986)	Physicochemical
Vicia faba	Fabaceae	200*	620*	10% RWC	(Harris *et al.* 1988)	Immunoassay
Vicia faba	Fabaceae	78.77 ±411.37	528.64	-0.87 MPa	(Zhang and Outlaw 2001)	Immunoassay
Vicia faba cv. Long Pod	Fabaceae	39±2*	116±23*	88% RWC	(Cornish and Zeevaart 1986)	Physicochemical
Vigna unguiculata	Fabaceae	100	1500	55% RWC	(Iuchi *et al.* 2000)	Physicochemical
Fagus sylvatica	Fagaceae	13.22*	52.8-132.2*		(Peuke *et al.* 2002)	Immunoassay
Gossypium hirsutum cv. Tamcot	Malvaceae	1140 ±270	1800±420		(Ackerson and Radin 1983)	Physicochemical
Eucalyptus microtheca	Myrtaceae	648-678	2628-3836	-2.37 MPa	(Li and Wang 2003)	Physicochemical
Eucalyptus tetrodonta	Myrtaceae	37.0*	113.66*		(Thomas and Eamus 1999)	Physicochemical
Olea europaea cv. Chemlali and Chetoui	Oleaceae	43.08	198	-3.75 MPa	(Guerfel *et al.* 2009)	Immunoassay
Olea europaea cv. Koroneiki	Oleaceae	20	50-80	-2 MPa	(Kitsaki and Drossopoulos 2005)	Physicochemical
Malus domestica cv. Imperial gala	Rosaceae	5.9-9.8	145-193	-1 MPa	(Thomas Fernandez *et al.* 1997)	Immunoassay
Prunus salicina cv. Santa Rosa	Rosaceae	7.1-19.6*	173.2*	57% RWC	(Pustovoitova *et al.* 1996)	Immunoassay
Citrus sinensis cv. Pêra	Rutaceae	100*	300*	-3 MPa	(De Assis Gomes *et al.* 2004)	Immunoassay
Populus davidiana	Salicaceae	600	1500-2750		(Zhang *et al.* 2004)	Immunoassay
Populus yunnanensis	Salicaceae	250	800	-1.69 MPa	(Duan *et al.* 2008)	Physicochemical
Lycopersicon pennellii	Solanaceae	500	2200	-2 MPa	(Kahn *et al.* 1993)	Immunoassay
Vitis labruscana	Vitaceae	140-330	1000-2500	-1.6 MPa	(Liu *et al.* 1978)	Physicochemical
Vitis vinifera cv. Sultana	Vitaceae	198.24	991.2	-1.02 MPA	(Stoll *et al.* 2000)	Physicochemical

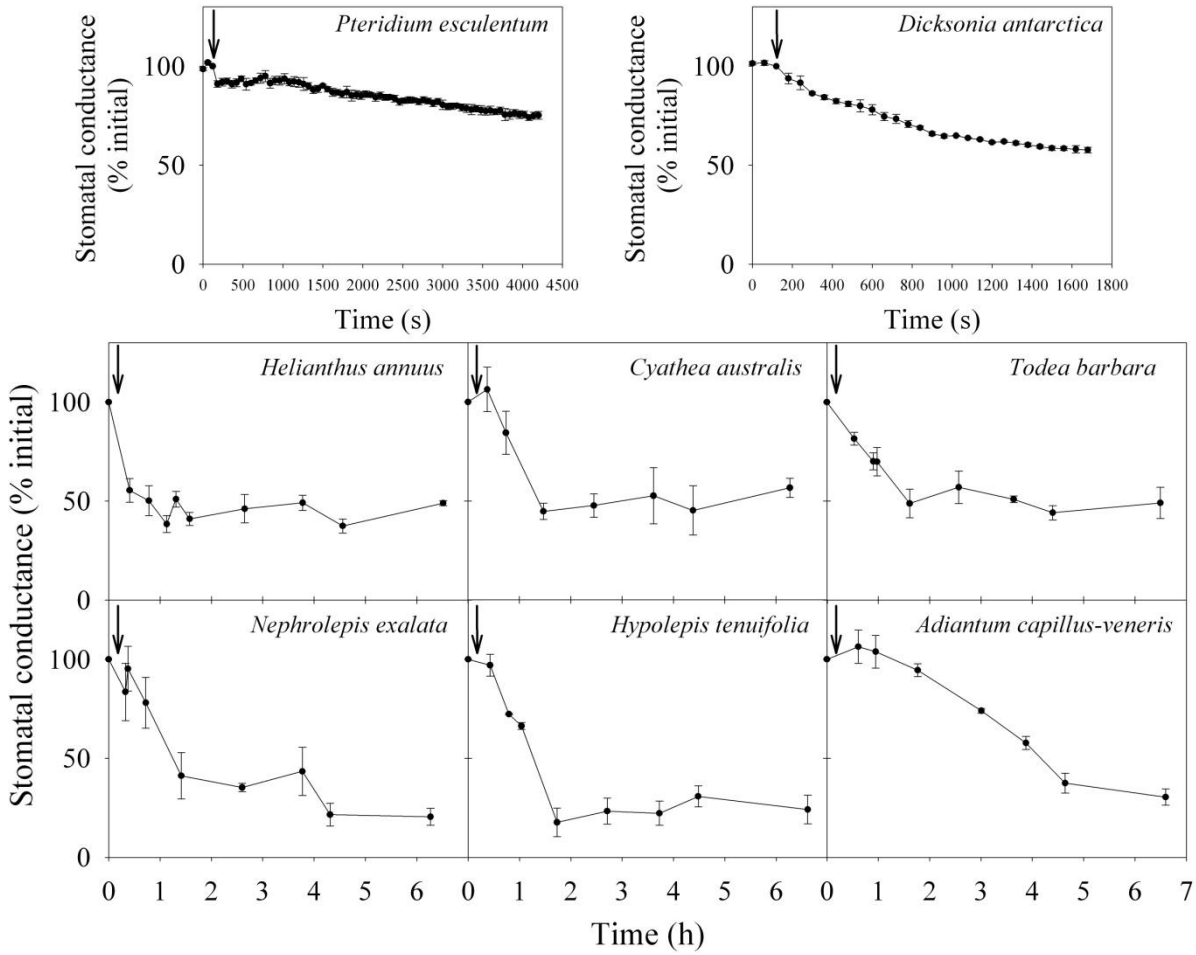
* indicates ABA level adjusted from values calculated as dry weight by assuming dry weight represented 10% of initial fresh weight of sample.

APPENDIX 2.6 Number of species corresponding to unstressed and drought stressed foliar ABA level



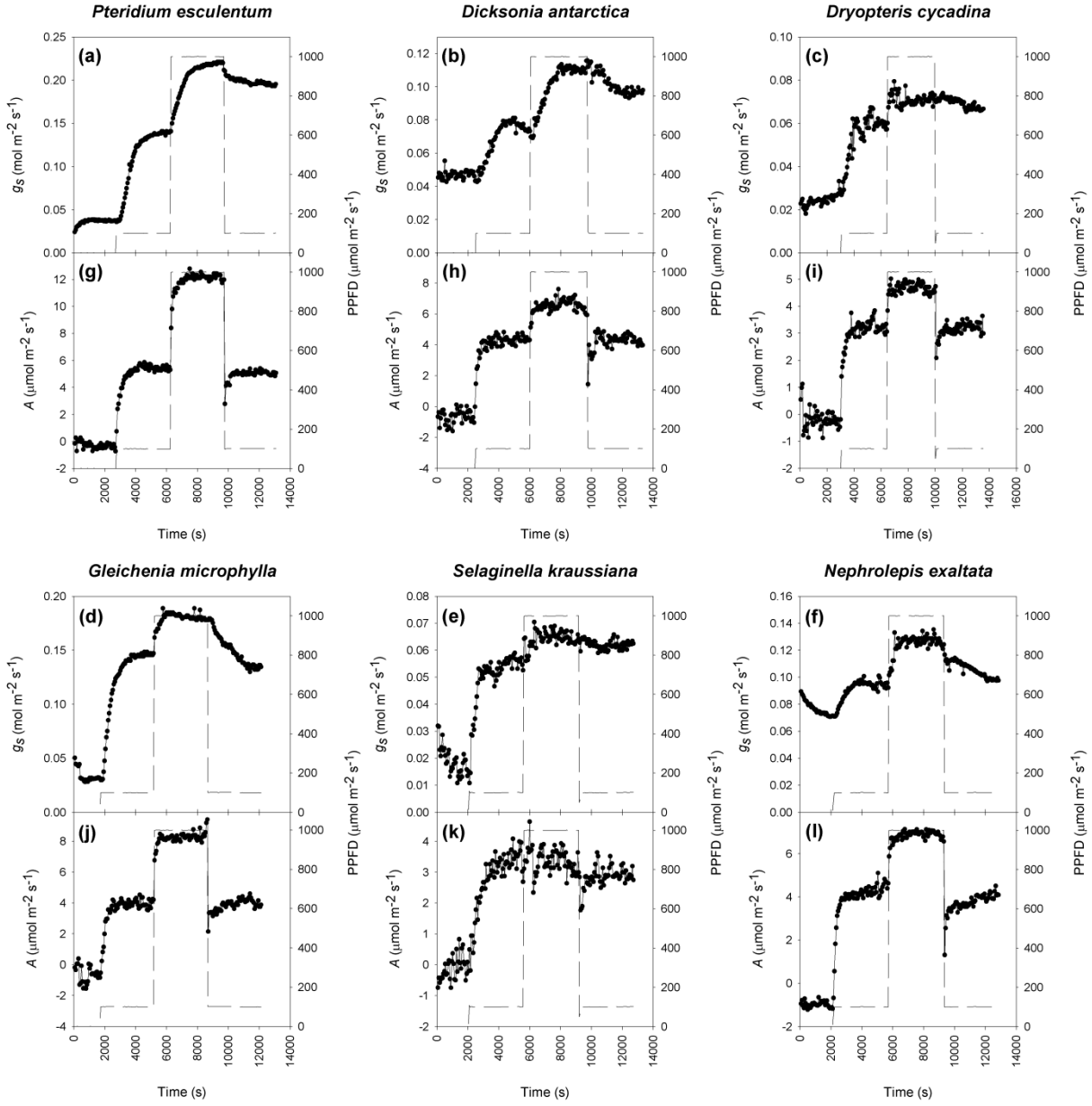
Appendix 2.6 Frequency distribution of reported foliar ABA level (ng g^{-1} FW) in unstressed conditions (A) and when stomata were closed as a result of drought stress (B) using data from Appendix 2.5.

APPENDIX 2.7 Stomatal closure in the dark in an angiosperm and seven fern species



Appendix 2.7. The dynamics of stomatal closure in darkness. Stomatal conductance (represented as a percentage of initial conductance) in three leaves following the initiation of darkness represented by an arrow in seven fern species and the angiosperm *Helianthus annuus*.

APPENDIX 5.1 Response of stomatal conductance and assimilation to changes in light intensity in representative fern and lycophyte species



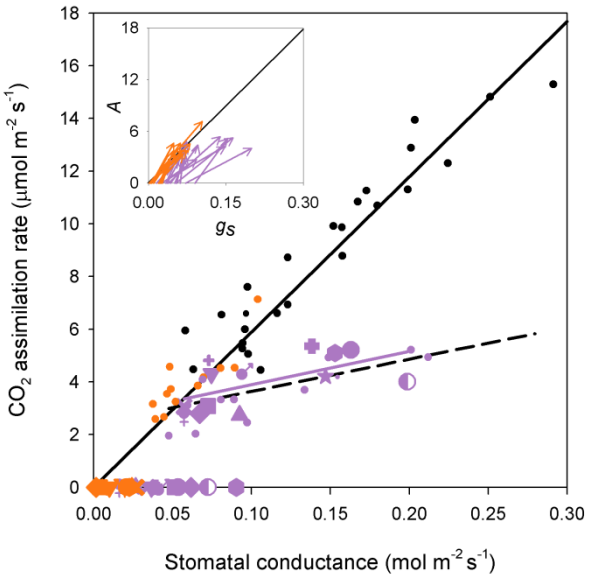
Appendix 5.1 The response of stomatal conductance (g_s) (A-F) and CO_2 assimilation rate (A) (G-L) in a wide phylogenetic and ecological selection of six fern and lycophyte species for 60 minutes after each of three transitions in light intensity (PPFD) (dashed line).

APPENDIX 5.2 Rates of stomatal movement in representative vascular plants

Appendix 5.2 The rate of change in stomata conductance (as percentage change in g_s per s or absolute rate, both calculated over the initial period of stomata movement, at least 300 s) during stomatal opening caused by increasing PPFD (100 to 1000 μmol m^{-2} s^{-1}) and during closure caused by decreasing PPFD (1000 to 100 μmol m^{-2} s^{-1}) in 11 ferns, 2 lycophytes, 3 angiosperms and a gymnosperm. No statistical difference in the rate of stomatal opening represented as either a percentage or as absolute values was observed between seed-bearing vascular plants and seedless vascular plants (P>0.5, single factor ANOVA).

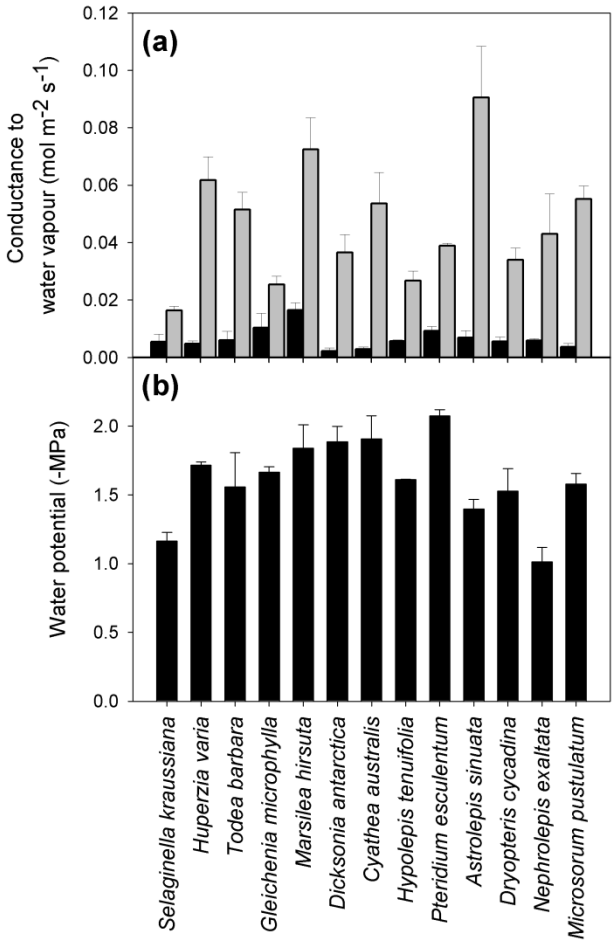
Species	**Type of vascular plant**	**% rate stomatal opening (%g_s/s)**	**Absolute rate of stomatal opening (g_s/s)**	**rate stomatal closure (%g_s/s)**	**Absolute rate of stomatal closure (g_s/s)**
Nothofagus cunninghamii	Angiosperm	0.203	9.30×10^{-5}	0.127	2.14×10^{-4}
Lotus corniculatus	Angiosperm	0.391	3.50×10^{-4}	0.1	2.03×10^{-4}
Trimenia moorei	Angiosperm	0.654	2.92×10^{-4}	0.05	4.90×10^{-5}
Ginkgo biloba	Gymnosperm	0.412	2.86×10^{-4}	0.045	5.54×10^{-5}
Astrolepis sinuata	Fern	0.051	1.08×10^{-4}	0.031	7.79×10^{-5}
Cyathea australis	Fern	0.607	9.03×10^{-4}	0.048	8.03×10^{-5}
Dicksonia antarctica	Fern	0.058	4.01×10^{-5}	0.012	9.73×10^{-6}
Dryopteris cycadina	Fern	0.146	1.30×10^{-4}	0.01	9.43×10^{-6}
Hypolepis tenuifolia	Fern	0.164	7.81×10^{-5}	0.047	2.73×10^{-5}
Gleichenia microphylla	Fern	0.39	5.21×10^{-4}	0.017	2.68×10^{-5}
Nephrolepis exaltata	Fern	0.032	2.58×10^{-5}	0.027	2.59×10^{-5}
Marsilea hirsuta	Fern	0.708	1.09×10^{-3}	0.091	2.65×10^{-4}
Microsorum pustulatum	Fern	0.725	6.55×10^{-4}	0.065	6.28×10^{-5}
Pteridium esculentum	Fern	0.191	3.84×10^{-4}	0.029	6.51×10^{-5}
Todea barbara	Fern	0.213	1.38×10^{-4}	0.039	3.68×10^{-5}
Huperzia varia	Lycophyte	0.531	5.17×10^{-4}	0.007	7.41×10^{-6}
Selaginella kraussiana	Lycophyte	0.635	3.77×10^{-4}	0.008	5.06×10^{-6}

APPENDIX 5.3 Sub-optimal stomatal responses during stomatal opening in ferns and lycophytes



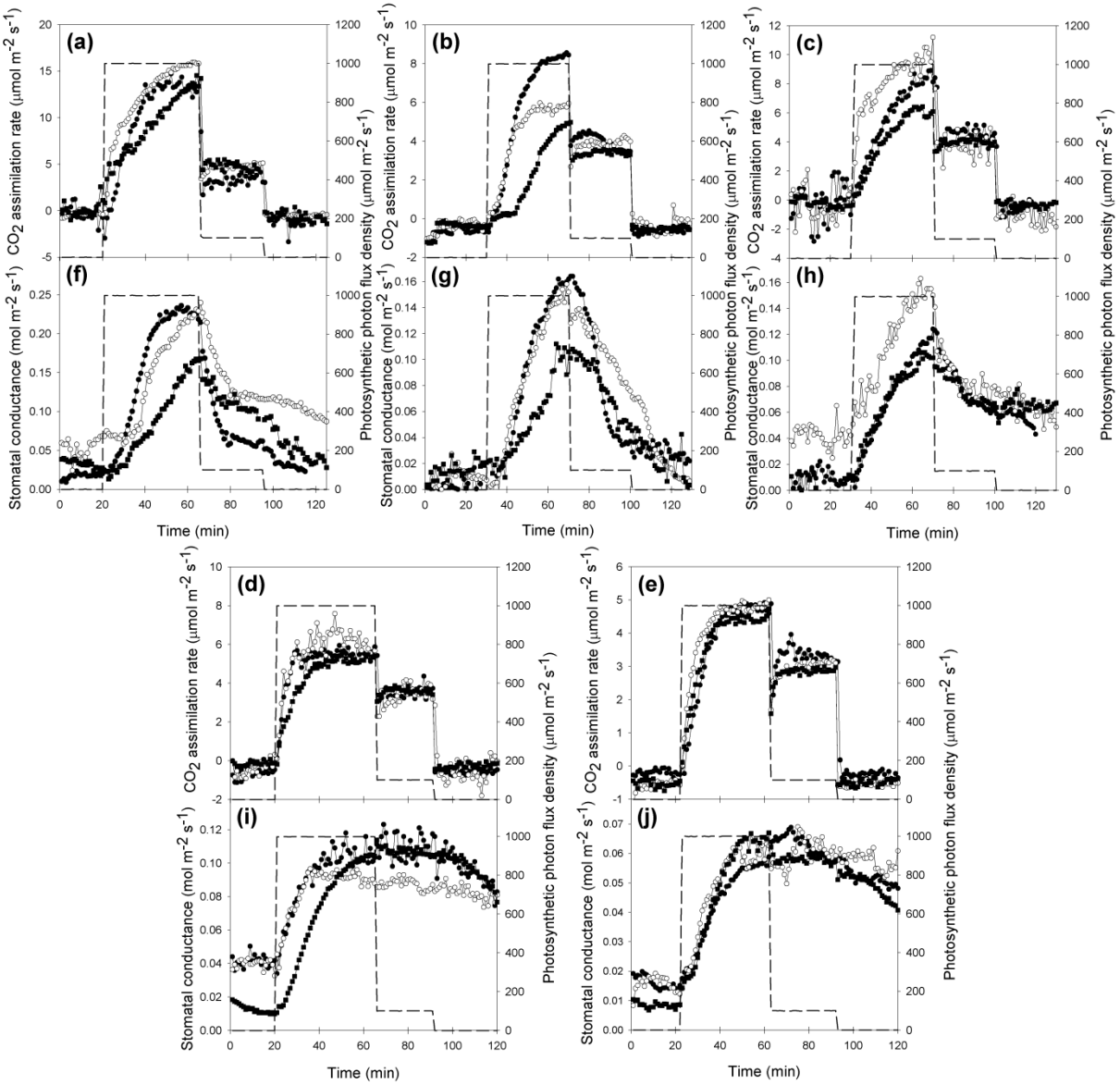
Appendix 5.3 As well as a reduced stomatal response to decreases in light intensity ferns and lycophytes (purple symbols) have an identical reduced stomatal response to increasing light intensity (from darkness to 100 µmol m^{-2} s^{-1}), resulting in water wastage relative to seed plants (orange symbols). At saturating light intensity (1000 µmol m^{-2} s^{-1}) the ratio of assimilation/stomatal conductance was similar in all species including seed plants and ferns and lycophytes (black circles, regression represented by the solid black line reflects a conservation of intrinsic water use efficiency (WUE_i)). Following a decrease in light intensity seed plants (orange circles) maintain high WUE_i, whereas ferns and lycophytes (purple circles) waste water. This lack of maintenance of high WUE_i in ferns and lycophytes to low light intensity is the same regardless of whether light intensity is decreasing from a saturating intensity or increasing from darkness (large purple symbols). Stomatal conductivity of individual species during darkness are represented by symbols at an assimilation rate of 0 µmol m^{-2} s^{-1} despite negative values being recorded due to respiration. Arrowed lines in the insert depict the trajectory of stomatal opening for each species (orange lines represent seed plants; purple lines represent ferns and lycophytes). See Table 5.1 for a key to the symbols and corresponding species.

APPENDIX 5.4 Cuticular conductance in representative ferns and lycophytes



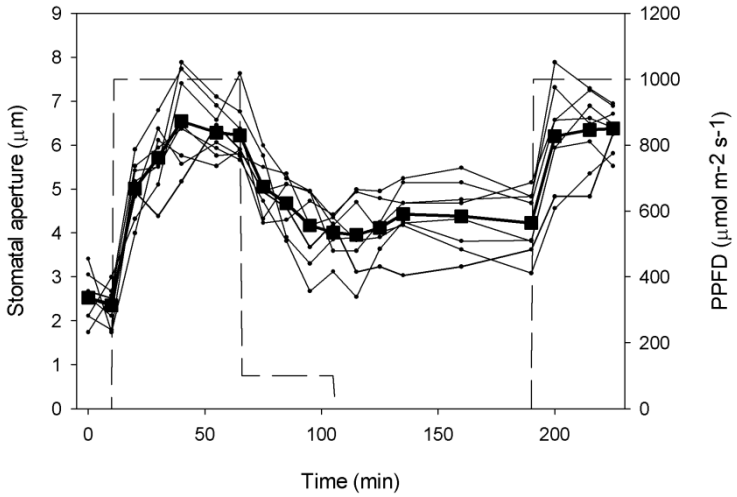
Appendix 5.4 (a) Cuticular conductance (black bars) due to mild water stress following leaf excision and dehydration and conductivity to water vapour in the dark (grey bars) measured prior to dawn in thirteen species of ferns and lycophytes. (b) Mean water potentials of each species during cuticular conductance measurements, bars represent standard error (N=3).

APPENDIX 5.5 Corresponding gas-exchange data for species over the same variations in light intensity used to observe stomatal aperture in Chapter 5



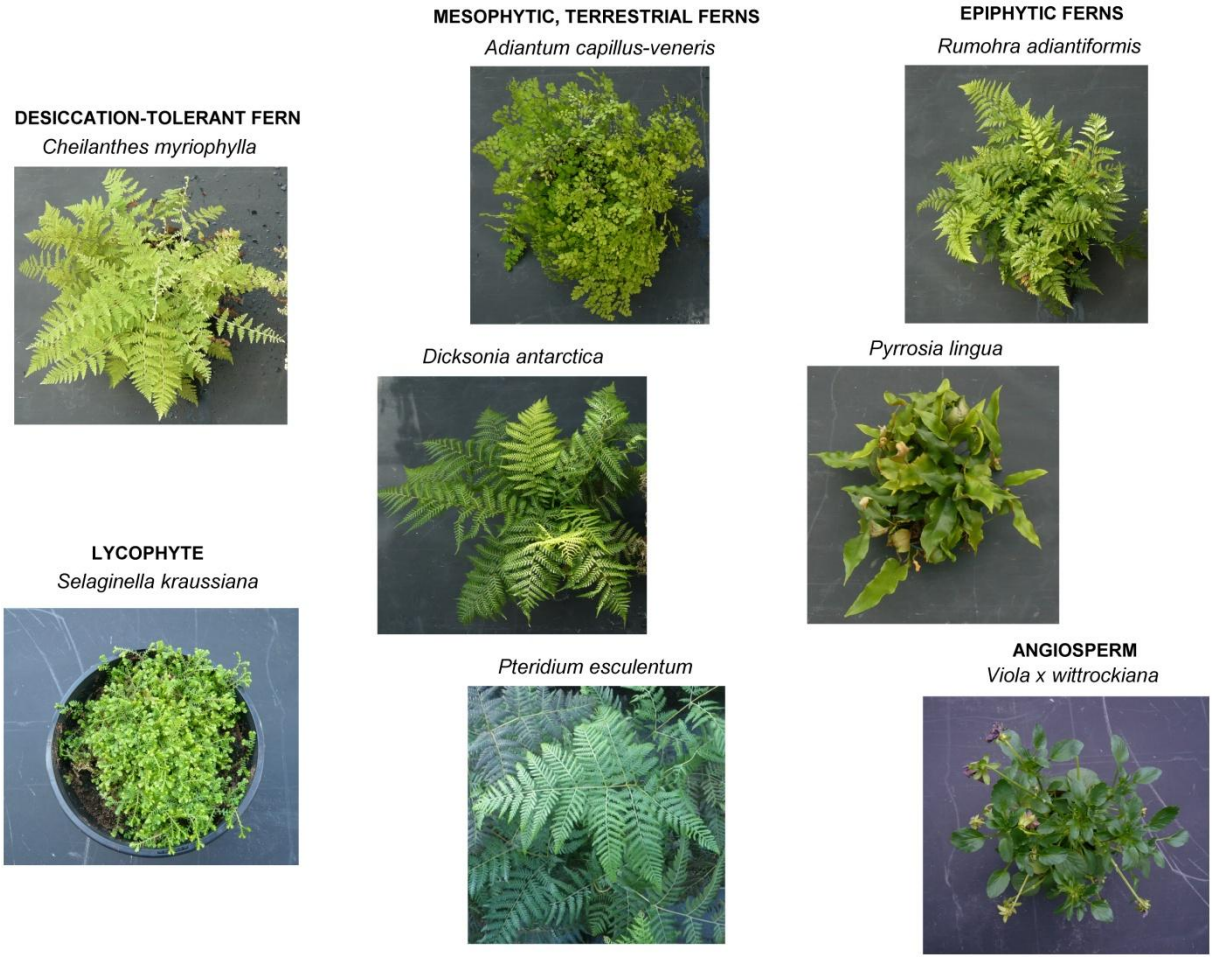
Appendix 5.5 Gas exchange measurements of CO_2 assimilation rate (A-E) and stomatal conductance (F-J) over the light intensity series; darkness to 1000 to 100 µmol m^{-2} s^{-1} (dashed line) in five species, the eudicot angiosperm *Lotus corniculatus* (A and F), basal angiosperm *Trimenia moorei* (B and G), gymnosperm *Ginkgo biloba* (C and H), fern *Dryopteris cycadina* (D and I) and lycophyte *Selaginella kraussiana* (E and J). Each species is represented by three individuals (different symbols).

APPENDIX 5.6 The stomatal response of angiosperm epidermis xenografted onto fern mesophyll



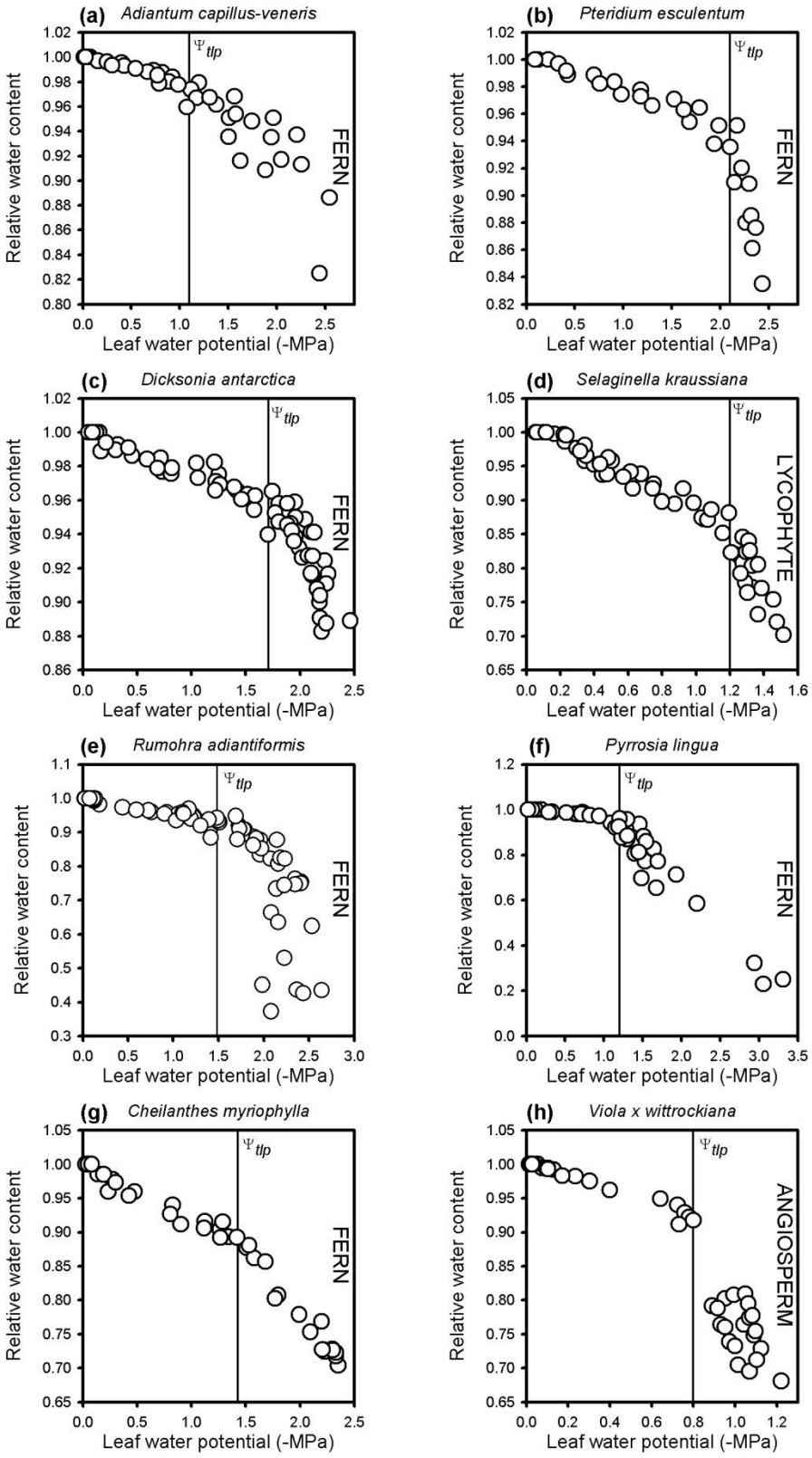
Appendix 5.6 The response of stomatal aperture to a sequence of light intensities (dashed line) in an epidermal xenograft from the angiosperm *Lotus corniculatus* on the mesophyll of the fern *Dryopteris cycadina*. Small circles on thin lines represent individual stomata, large squares on the thick line represents the average (N=8).

APPENDIX 6.1 Images of species used in the experiments of Chapter 6



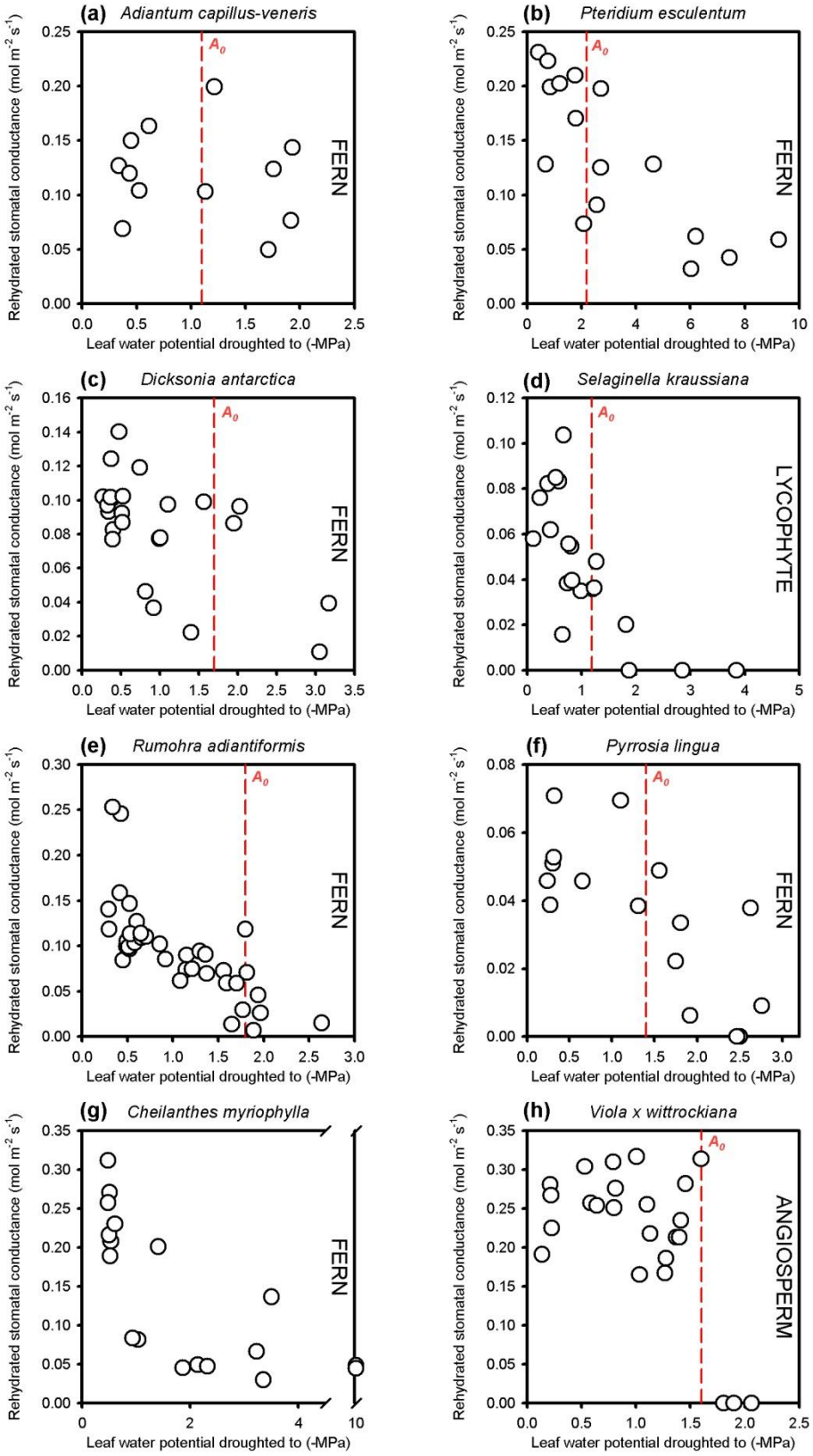
Appendix 6.1. Images of a representative individual of each species including a single angiosperm, lycophyte and desiccation-tolerant fern, two epiphytic ferns and three mesophytic, terrestrial fern species.

APPENDIX 6.2 Pressure-volume curves from six representative ferns, a lycophyte and an angiosperm



Appendix 6.2 Pressure-volume relationships for three mesophytic terrestrial fern species (a-c), a lycophyte (d), two epiphytic fern species (e and f), a desiccation-tolerant fern (g) and an angiosperm (f) with vertical lines representing turgor loss point (data points represent the combined data from 5 leaves).

APPENDIX 6.3 The stomatal conductance of droughted leaves that were rehydrated over night



Appendix 6.3 Stomatal conductivity of leaves rehydrated overnight from known droughted leaf water potentials, in three mesophytic terrestrial ferns (a-c), a lycophyte (d), two epiphytic ferns (e and f), a desiccation-tolerant fern (g) and an angiosperm (h). Dashed red vertical lines represent the leaf water potential at which assimilation rate does not recover (A_0) (absent from the desiccation-tolerant fern *Cheilanthes myriophylla*).