New Phytologist

Research

Differential responses of stomatal kinetics and steady-state conductance to abscisic acid in a fern: comparison with a gymnosperm and an angiosperm

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Received: *28 September 2018*
Accepted: *4 February 2019*

New Phytologist (2019) **222:** 1883–1892
doi: 10.1111/nph.15736

Key words: abscisic acid (ABA), asymmetric stomatal response, dynamic irradiance, ferns, light flecks, stomata, stomatal evolution, water deficit.

Summary

• Origins of abscisic acid (ABA)-mediated metabolic control of stomatal conductance have been suggested to be recent, based on a gradualistic model of stomatal evolution. In ferns, steady-state stomatal conductance (g_s) was unresponsive to ABA in some studies, supporting this model. Stomatal kinetic responses to ABA have not been considered.

• We used dynamic gas exchange methods to characterise half times of stomatal opening and closing in response to step changes in light, across a range of ABA exposures in three diverse taxa.

• All taxa had asymmetric kinetics, with closure slower than opening in fern and cedar, but faster than opening in soybean. Closing was fastest in soybean but opening was slowest. Stomatal kinetics, particularly for closure, responded to ABA in all three taxa. Steady-state g_s did not respond significantly to ABA in fern or cedar but responded strongly in soybean. Stomatal kinetics were responsive to ABA in fern. This finding supports a contrasting, single origin model, with ABA-mediated regulation of stomata arising early, in conjunction with stomata themselves.

• Stomatal kinetics are underutilised. Differential responses of opening and closing rates to environmental and hormonal stimuli may provide insights into phylogeny and stomatal regulatory strategies with potential application to selection for crop improvement.

Introduction

Angiosperms regulate plant water status actively, with stomatal responses mediated by the ancient phytohormone, abscisic acid (ABA). A gradualistic model (Brodribb & McAdam, 2011a; Sussmilch *et al.*, 2017) suggests that this metabolic (hydroactive) stomatal response to tissue water deficit evolved recently, after the divergence of spermatophytes (seed plants) from Monilophyta and Lycophyta (formerly ferns and fern allies) *c.* 400 million yr ago (Ma). ABA-mediated stomatal responses to humidity and CO_2 in this model are considered to have developed even later (Brodribb *et al.*, 2009; Brodribb & McAdam, 2013; McAdam & Brodribb, 2014), after divergence of angiosperms from gymnosperms (*c.* 360 Ma; Pryer *et al.*, 2004).

A contrasting single origin model suggests a coordinated emergence of stomata and their metabolic control by ABA in the earliest land plants *c.* 475 Ma (Cai *et al.*, 2017; Chater *et al.*, 2011; Ruszala *et al.*, 2011; Tougane *et al.*, 2010). The gradualistic and single origin models differ significantly in the predicted ABA-response characteristics of ferns, which we address in the current study.

Neither model is wholly supported. Recent phylogenetic analyses (PPG, 2016; Puttick *et al.*, 2018), suggested monophylly of the bryophytes, close association of liverworts and mosses, and the probable evolution of stomata in a common ancestor of Bryophytes and Tracheophytes. Both models, and other statistically viable phylogenetic scenarios, required a clade-specific evolutionary loss or gain of stomata (Rensing, 2018).

Stomata of early taxa respond to photosynthetically active radiation, mediated by ATPase-driven membrane polarisation. This evolved before the origin of vascular plants (Doi *et al.*, 2015; Sussmilch *et al.*, 2017). A physiological refinement to stomatal kinetics is response to low fluence blue light with phototropins as alternative photoreceptors (Doi *et al.*, 2006). This blue light response is present in both Lycophyta and early Monilophyta of the Equisetaceae, Psilotaceae, and Marattiaceae (Doi *et al.*, 2015). It was subsequently lost during or after divergence of the Polypodiidae (leptosporangiate ferns) and is not expressed in the more recently evolved fern families, Pteridaceae, Aspleneiaceae and Lomariopsidaceae (Doi *et al.*, 2006). These light-mediated responses are not directly linked to ABA but they suggest that a similarly complex evolutionary scenario may apply to other aspects of stomatal regulation.

The endpoint of stomatal regulation (steady-state conductance; g_s), and the kinetics of endpoint attainment (half response

time, $t_{½}$), may reflect stomatal sensitivity to ABA. Both $t_{½}$ (Assmann & Grantz, 1990; Knapp & Smith, 1990; Whitehead & Teskey, 1995; Franks & Farquhar, 2007; Pearcy & Way, 2012; Creese *et al.*, 2014; Lawson & Blatt, 2014; Hoshika *et al.*, 2016) and g_s (Wilkinson & Davies, 2010; Franks & Britton-Harper, 2016; Horak *et al.*, 2017) are sensitive to plant exposure to VPD and water deficit that involve ABA signaling.

The single origin model is consistent with the ancient origin of ABA-associated genes, signaling networks, and metabolic pathways (Fujii *et al.*, 2009; Cai *et al.*, 2017). The core metabolic components of stomatal regulation (Sakata *et al.*, 2014; Lind *et al.*, 2015) can be traced back through the lineage of land plants to the cyanobacteria (Maršálek *et al.*, 1992; Chater *et al.*, 2011). The number of genes related to ABA signaling in guard cells increased from algae to ferns to gymnosperms to angiosperms (Cai *et al.*, 2017). OST1 homologues from a moss and a lycophyte partially restored ABA sensitivity in Arabidopsis ost1 mutants (Chater *et al.*, 2011; Ruszala *et al.*, 2011) and the ABA response was reduced in the moss, *Physcomitrella patens*, by knockout of its OST1 homologue. By contrast, the aquatic fern, *Ceratopteris richardii*, was found (McAdam *et al.*, 2016) to lack the functional pairing of homologues of OST1 (protein kinase) and of SLAC1 (anion channel) genes that mediate fast stomatal responses to various stimuli in angiosperms (Vahisalu *et al.*, 2008; Lind *et al.*, 2015).

Physiological data have also been inconclusive and often contradictory even within a single species (Ruszala *et al.*, 2011; McAdam & Brodribb, 2012). ABA accumulated in droughted bryophytes, lycophytes, ferns, gymnosperms and angiosperms (Pilate *et al.*, 1989; Brodribb & McAdam, 2011a; Ruszala *et al.*, 2011; McAdam & Brodribb, 2012). However, reopening following rehydration required reduction of tissue ABA concentration only in angiosperms (McAdam & Brodribb, 2012), while in ferns and lycophytes a close association between leaf turgor and g_s suggested a simple hydropassive mechanism, independent of ABA concentration (McAdam *et al.*, 2016). In *Pteridium esculentum*, gas exchange was independent of foliar ABA concentration. Injecting ABA into the xylem did not inhibit stomatal opening of *P. esculentum* or of two other fern species, including *Nephrolepis exaltata* L. Schott (McAdam & Brodribb, 2012). By contrast, Cai *et al.* (2017) found a dose-specific stomatal closing response to ABA in isolated epidermis of two fern species, including *N. exaltata*. Furthermore, nonstomatal responses to ABA are observed in taxa from liverworts (Tougane *et al.* 2010; Rock *et al.*, 2010; Mori & Murata, 2011), to ferns (Swami & Raghavan, 1980; Chia & Raghavan, 1982; Hickok, 1983), to angiosperms.

Uncertainties in the literature regarding the origins of stomatal function reflect a diversity of test organisms (Vaten & Bergmann, 2012) and experimental protocols (Creese *et al.*, 2014; Franks & Britton-Harper, 2016). Under some conditions, the stomatal response to CO_2 was absent in a lycopod, and in ferns from the relatively primitive Osmundaceae, the more advanced Pteridaceae, and the highly evolved eupolypods, Aspleniaceae and Lomariopsidaceae (Brodribb *et al.*, 2009). Similarly, low concentrations of ABA were ineffective in modulating steady-state g_s of these ferns (Brodribb *et al.*, 2009; Brodribb & McAdam, 2011a, b, 2013; McAdam & Brodribb, 2012, 2013; Haworth *et al.*, 2015). Under different conditions, stomatal responses to CO_2 were observed in primitive ferns of the Marattiaceae and in two families of advanced eupolypods, including the Lomariopsidaceae (Franks & Britton-Harper, 2016).

Stomatal kinetics have been underutilised relative to g_s in studies of phylogeny and ecophysiology. The control of stomatal kinetics, and the mechanistic and evolutionary relationships between $t_{½}$ and g_s remain largely unknown in any taxon, yet both are critical for plant adaptation to constantly changing natural environments (Ooba & Takahashi, 2003; Brodribb *et al.*, 2009; Vico *et al.*, 2011; Pearcy & Way, 2012). Response rates have measurable effects on plant carbon acquisition (Kirschbaum *et al.*, 1988; Vico *et al.*, 2011; Vialet-Chabrand *et al.*, 2013), water balance (Lawson & Blatt, 2014; Tougane *et al.*, 2010; Drake *et al.*, 2013), pollutant uptake (Paoletti & Grulke, 2010), and even regional stream flow (McLaughlin *et al.*, 2007). Stomatal $t_{½}$ provides a quantifiable trait that may convey information not attainable from steady-state measurements (Kaiser & Paoletti, 2014).

Studies of stomatal kinetics have increased recently, following a long period of focus on steady-state responses. Demonstration that ABA effects on stomatal dynamics can be separable from effects on g_s suggests their independent regulation, provides a novel tool for phylogenetic analysis, and a promising direction for further study of environmental effects on stomatal responses and signaling networks. This may also represent a route to improvement of plant water-use efficiency (WUE; Lawson & Blatt, 2014; Vico *et al.*, 2011; Qu *et al.*, 2016). The rate of stomatal closure can be linked to WUE (Reich & Lassoie, 1984; Kirschbaum *et al.*, 1988; Paoletti & Grulke, 2005; Lawson *et al.*, 2011; Lawson & Blatt, 2014), with a theoretical potential for 20% improvement (Lawson *et al.*, 2011; Lawson & Blatt, 2014). Accelerated stomatal kinetics associated with changes in guard cell morphology may have facilitated the rapid radiation of grasses during global aridification *c.* 35 Ma (Hetherington & Woodward, 2003).

The present study tests stomatal responses to step changes in light, within the context of differing concentrations of exogenous ABA. We evaluated the effect of ABA treatment on the kinetics of response and on steady-state conductance, across three diverse taxa. We focus on the advanced fern, *N. exaltata*, but apply the same protocols to a gymnosperm, incense cedar (*Calocedrus decurrens* (Torr.) Florin), and to an angiosperm, soybean (*Glycine max* (L.) Merr.). The objectives of the kinetics approach were: (1) to explore a novel means of distinguishing the gradualistic vs single origin models of stomatal regulation using $t_{½}$ rather than g_s; (2) to separate phenotypic expression of $t_{½}$ from expression of g_s; and (3) to explore differences in asymmetry of opening and closing across diverse taxa and exposures to ABA. The phylogenetic approach allowed us: (1) to test whether our experimental approach could resolve stomatal sensitivity to ABA in fern by demonstrating it in other taxa; and (2) to explore commonalities and contrasting aspects of stomatal regulation across diverse taxa. In exploring a novel diagnostic application of stomatal kinetics,

we tested a core distinction between the gradualistic and single origin models.

Materials and Methods

Plant material

Plants were grown from July to October 2015 in a research glasshouse at the University of California, Kearney Agricultural Center (Parlier, CA, USA; 103 masl; 36.598°N, 119.503°W) (Paudel *et al.*, 2016). Maximum irradiance at the glasshouse bench was *c.* 1300 μmol m^{-2} s^{-1}.

The ornamental fern, Boston Fern, *Nephrolepis exaltata* (L.) Schott (Benedict, 1922) Lomariopsidaceae (Nephrolepidaceae) was obtained from a local nursery. *Nephrolepis* is a recently evolved (*c.* 13 Ma) Eupolypod (Schneider *et al.*, 2004) of neotropical distribution, subject to some hybridisation in the New World (Hennequin *et al.*, 2010). Plants were divided into 4 l plastic pots in moist potting medium (Earthgro Potting Soil, Scotts Company, Marysville, OH, USA). Seedlings of cedar (incense cedar; *Calocedrus decurrens* (Torr.) Florin) Cupressaceae, were collected from Lassen National Forest, California (1457 m asl; 40.422°N, 120.702°W). Seedlings were transplanted into the same medium and pots. Seeds of soybean (*Glycine max* (L.) Merr.; cv Fiskeby III; PI 438471) Fabaceae, were planted into the same medium and pots, and thinned to a single seedling per pot at the first true leaf stage. Experiments were performed after more than 6 months acclimation in fern and cedar, and 4–6 wk after planting in soybean.

Young mature leaves were used for all experiments. Part of a pinna from the mid-rachis of a nearly fully unrolled frond (megaphyll) of fern was selected. Fronds of *Nephrolepis* often fail to unroll completely. Part of an upper canopy branchlet fully covered with appressed needles of cedar, and a lateral leaflet of the trifoliate soybean leaf were selected for experimental exposures. Plants were brought from the glasshouse to a temperature-controlled laboratory at 09:00 each morning.

Abscisic acid treatment

Authentic abscisic acid (±ABA, 98.5%, CAS 14375-45-2; Sigma-Aldrich, St Louis, MO, USA) was dissolved in reagent grade methanol (125×10^{-3} M), and stored at −18°C. Three concentrations of ABA were tested, 0 M (0 [ABA], control), 3.9×10^{-6} M (low ABA concentration), and 1.0×10^{-4} M (high ABA concentration), obtained by serial dilution with water immediately before use. The control and high ABA concentration contained 800 ppmv methanol. The low ABA concentration contained 248 ppmv methanol.

ABA was applied exogenously to each test leaf as a mist from a plastic spray bottle. Application continued until all surfaces of the test leaf were dripping with excess ABA solution. For fern, the plant was inclined to extend the sprayed leaf area beyond the pot. For cedar and soybean, the potting medium was protected with a plastic sheet fitted to the stem. All leaf surfaces were treated, although cedar and fern are hypostomataous and only soybean is amphistomatous.

Experiments were performed after whole plants were preconditioned to 1000 μmol m^{-2} s^{-1} on a laboratory bench using a bank of three white light-emitting diode (LED) lamps for 30 min. After this consistent light preconditioning, ABA was applied to the test frond, branchlet or leaflet, and the plant remained exposed to this light for an additional 25 min. The treated foliage was then placed in the cuvette (2.0 × 3.0 cm; 6.0 cm^2) of a steady-state gas exchange system (LI-6400XT; LiCor Inc., Lincoln, NE, USA). Light within the cuvette was 1000 μmol m^{-2} s^{-1} for fern, 1800 μmol m^{-2} s^{-1} for cedar, and 1500 μmol m^{-2} s^{-1} for soybean, provided by a red (80%) and blue (20%) LED source (LI 6400-02B). These constituted the high light conditions and elicited near-maximum g_s in each species. After equilibration to cuvette conditions and attainment of a stable g_s, gas exchange measurements were recorded. Consecutive sequences from high light to shade to high light allowed measurement of initial and final g_s as well as $t_{½}$ for the stomatal closing and opening responses. The shade conditions were 35 μmol m^{-2} s^{-1} for fern, 100 μmol m^{-2} s^{-1} for cedar, and 50 μmol m^{-2} s^{-1} for soybean, eliciting near minimum g_s. Illumination of the entire plant was switched off and on simultaneously with the step change of light in the cuvette.

Gas exchange measurements

The projected area of fern and cedar foliage enclosed in the cuvette was determined before the measurement. The soybean leaflets covered the entire 6.0 cm^2, extending beyond the margins of the cuvette. Stomatal conductance was not measured before application of ABA, to avoid unnecessary tactile stimulation before the tests of kinetics.

Gas exchange measurements were initiated at *c.* 10:30 h and concluded by *c.* 16:00 h each day; diurnal gas exchange rhythms over this period were not observed. Automated measurements of stomatal conductance, net carbon assimilation, and environmental parameters within the cuvette were obtained at 15 s intervals.

CO_2 was removed with soda lime and added back to the air stream at 400 μmol mol^{-1}. Block temperature of the cuvette was maintained at 25.0°C, with leaf temperatures generally 3–4°C warmer, depending on leaf area and prevailing stomatal conductance. The change in leaf temperature during a response was generally < 2°C with few exceptions, limiting changes in VPD to 0.1–0.5 kPa. The humidity of the air entering the cuvette was not controlled and the flow rate was maintained at 500 μmol s^{-1}.

Data analysis

Half times ($t_{½}$) for stomatal closing and opening responses to step changes in light were calculated for fern and cedar, and for closing responses in soybean, by fitting single exponential equations to g_s vs time (SigmaPlot v.11.0; Systat Software Inc., San Jose, CA, USA), as

$$t_{½} = (\log_e (2))/\lambda \quad \text{Eqn 1}$$

where λ is the time constant, fitted for stomatal closing as

$$g_s(t) = a + (b)e^{-\lambda t} \quad \text{Eqn 2a}$$

and for stomatal opening as

$$g_s(t) = a - (b)e^{-\lambda t} \quad \text{Eqn 2b}$$

in which a and b are fitted parameters related to the initial and final magnitude of g_s, and t is time after the step change.

Half times for stomatal opening of soybean were not well-fitted by Eqn 2b. Therefore, $t_{½}$ values were extracted by fitting sigmoidal equations to g_s vs time as

$$g_s = g_{min} + (g_{max} - g_{min})/((1 + e^{(-(t-t_{1/2})/c)})) \quad \text{Eqn 2c}$$

where c is a fitted parameter related to the slope near the midpoint (Hill Slope).

Data were analysed using one-way analysis of variance (ANOVA) (PROC GLM; SAS v.9.2.1). Mean separation was by Duncan's New Multiple Range Test. Significant differences are reported at P-values < 0.05.

Results

Stomatal response kinetics

Following step decreases in light, the time courses of stomatal closure in fern, cedar, and soybean (Fig. 1; black circles) were well described by first order exponential kinetics (red lines; fitted to these representative curves). The reopening kinetics following step increases in light were also well described by first order exponential kinetics for fern and cedar (Fig. 1), while opening in soybean was better described by sigmoidal functions. These relationships were consistently observed, and were not altered by exposure to ABA (compare Fig. 1a,c,e and Fig.1 b,d,f).

Fern stomatal closure was accelerated by both low ABA concentration (3.9×10^{-6} M) and high ABA concentration (1.0×10^{-4} M) (Fig. 2a). High ABA concentration had no further effect beyond that of low ABA concentration. Low ABA elicited a $t_{½}$ for opening in fern of about 6 min, with $t_{½}$ declining at low ABA concentration and increasing at high ABA concentration (Fig. 2b). The unexpected increase of $t_{½}$ for opening at high ABA concentration was observed consistently.

In the absence of exogenous ABA, closing was slowest in fern (Fig. 2a; mean $t_{½}$ = 43 min), intermediate in cedar (Fig. 2c; mean $t_{½}$ = 16 min), and fastest in soybean (Fig. 2e; mean $t_{½}$ = 5.5 min). By contrast, opening at 0 [ABA] (Fig. 2b,d,f) was considerably slower in soybean (mean $t_{½}$ = 17 min) than in cedar or fern (mean $t_{½}$ = 4.6 and 5.9 min, respectively).

Stomatal closing was significantly accelerated by exogenous ABA in all three taxa (Fig. 2a,c,e). In all taxa examined, $t_{½}$ at high ABA concentration was significantly lower (faster) than in the 0 [ABA] control. The effect was proportional to ABA concentration in cedar (Fig. 2c). The effect of the ABA-induced changes in mean $t_{½}$ (data from Fig. 2) on time courses of declining g_s in the three taxa are presented in Supporting Information Fig. S1(a,c,e).

Stomatal opening kinetics responded differently to ABA in the different taxa (Fig. 2b,d,f). Fern exhibited a biphasic response. There were no significant effects of ABA on stomatal opening in cedar, although a proportional declining trend of $t_{½}$ with increasing ABA concentration was observed (Fig. 2d). In soybean, the $t_{½}$ for opening at high ABA concentration was significantly greater than at 0 [ABA]. Mean time courses of increasing g_s are shown in Fig. S1(b,d,f). The slowing of opening at high ABA concentration indicates stomatal sensitivity to ABA in fern and soybean, but the biphasic response in fern complicates interpretation of the regulatory responses.

Asymmetry of stomatal opening and closing

Opening and closing kinetics were significantly asymmetric in all three taxa (Fig. 2). The ratio of $t_{½}$ for closing to $t_{½}$ for opening was 7.6 for fern, 3.5 for cedar, and 0.3 for soybean. The relationship between $t_{½}$ for opening and $t_{½}$ for closing, as both varied with ABA concentration and taxon, did not conform to a hypothesised 1 : 1 relationship (not shown). This reflected the greater $t_{½}$ of opening than closing in soybean, greater $t_{½}$ for closing than opening in fern and cedar, and the greater response to ABA of $t_{½}$ for opening in soybean and for closing in fern and cedar. The mechanistic basis of these differences remains to be characterised.

Steady-state stomatal conductance (g_s)

The gas exchange sequence, step decrease in light followed by step increase in light, induced stomata to close from an initial steady-state conductance and then to reopen to a second steady state, presenting two values of open stomata for analysis of g_s ($g_{s,\ light\ 1}$ and $g_{s,light\ 2}$; Fig. 3). At 0 [ABA], g_s (Fig. 3a,c,e) was larger in soybean (for example $g_{s,light\ 1}$; 0.24 mol m^{-2} s^{-1}) than in cedar (0.14 mol m^{-2} s^{-1}) or fern (0.041 mol m^{-2} s^{-1}). In fern there were no significant effects of ABA at either concentration on steady-state g_s, although g_s trended downward with increasing ABA concentration (Fig. 3a,b). There were no significant effects of ABA on g_s of cedar (Fig. 3c,d). In contrast with fern and cedar, g_s of soybean was significantly reduced by ABA (Fig. 3e,f). The higher ABA concentration reduced g_s of soybean by 70% relative to 0 [ABA] at both $g_{s,light\ 1}$ and $g_{s,\ light\ 2}$.

Discussion

Support for a single origin model of ABA-mediated stomatal control

We evaluated the effects of ABA on the kinetics ($t_{½}$) as well as the steady-state endpoint (g_s) of stomatal response in an advanced fern, a gymnosperm, and an angiosperm. The experiment focused on fern. The tests of the additional taxa demonstrated

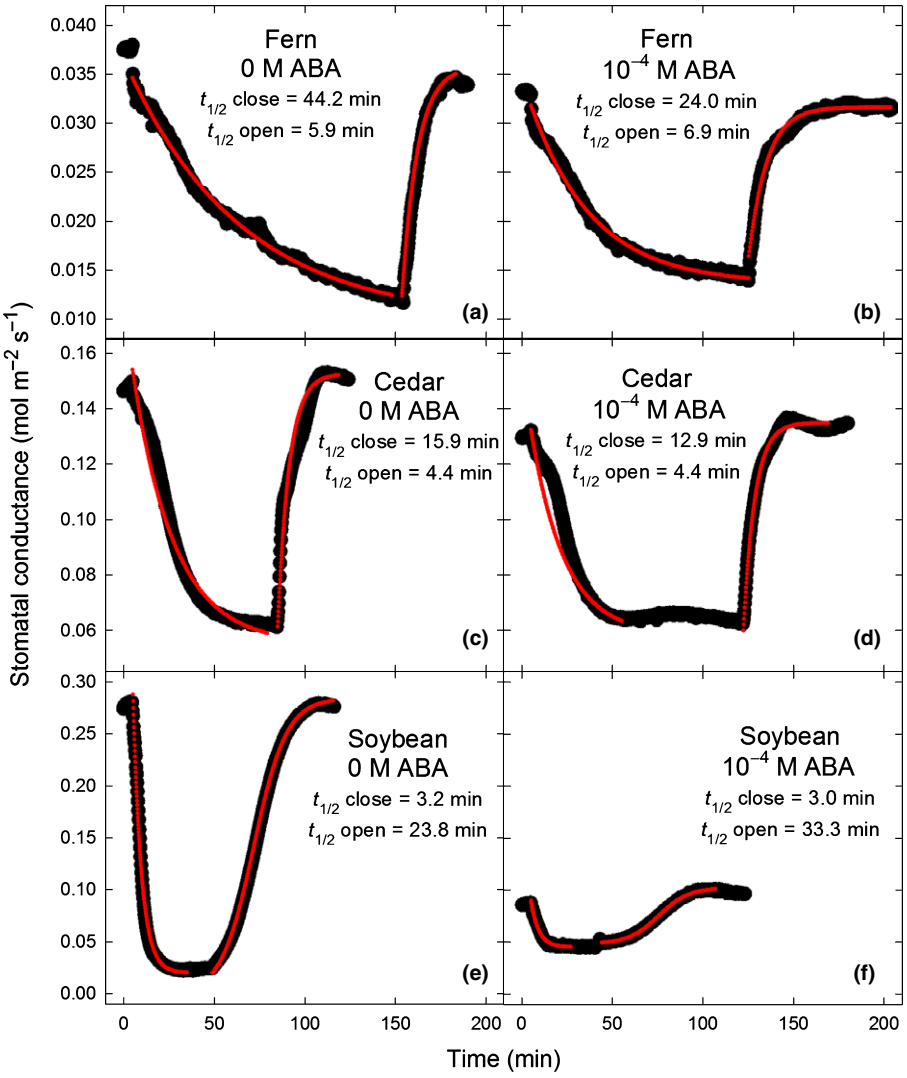

Fig. 1 Representative time courses of stomatal conductance (g_s; black circles) and fitted curves (red lines) in leaves exposed to 0 M abscisic acid (ABA) or 10^{-4} M ABA following step reduction in light followed by a step increase to the original light intensity. The time scales are identical in all panels, demonstrating the differences in kinetics. The g_s scales differ between species, for clarity, but are identical within a species showing the effect of ABA on the magnitude of g_s. The half response time ($t_{½}$) is shown for each curve.

that our techniques and kinetic approach are sufficiently powerful to resolve ABA effects on both $t_{½}$ and g_s. In cedar, g_s was unaffected by ABA, but closing kinetics were accelerated. In soybean, g_s and $t_{½}$ were both reduced substantially at high ABA concentration. The potential limitations of a test involving exogenous application of ABA are mitigated in this case by the similar apoplastic transport of both endogenous and exogenous ABA to guard cells (Christmann *et al.*, 2007; Endo *et al.*, 2008).

In fern, we observed significant sensitivity of $t_{½}$ to ABA but no significant sensitivity of g_s, as observed for g_s in some previous studies (McAdam & Brodribb, 2012; Haworth *et al.*, 2015). The major effect on closing kinetics was at low ABA concentration, with little further effect at the higher concentration. This suggests substantial stomatal sensitivity to ABA in this Eupolypod, although generalisation to less advanced fern lineages should be undertaken with caution. Our comparisons across taxa showed that responses of stomatal kinetics and steady-state endpoints to ABA may be separable and independent of each other. The sensitivity of $t_{½}$ to ABA in fern supports a single origin model of ABA-mediated metabolic control of stomatal conductance. Stomata and their responsiveness to ABA likely evolved together, early in the phylogeny of land plants, arising with the tracheophytes or earlier with the bryophytes (Rensing, 2018).

Rates of stomatal response have been suggested to be mechanistically linked to steady-state conductance (Caird *et al.*, 2007; Vico *et al.*, 2011; Drake *et al.*, 2013; Vialet-Chabrand *et al.*, 2013) but this was not apparent in fern. The absence of ABA effects on g_s in fern in our study is consistent with some previous results with ferns (Brodribb & McAdam, 2011a,b; McAdam & Brodribb, 2012, 2013; Haworth *et al.*, 2015) and hornworts (Lucas & Renzaglia, 2002). However, the consistent but nonsignificant decline in g_s we observed in fern with increasing ABA suggests that sensitivity of g_s to ABA cannot be ruled out. Indeed, these steady-state results contrast with significant closing responses to ABA observed under different experimental conditions in ferns (Cai *et al.*, 2017), hornworts (Hartung *et al.*, 1987; lycopods (Ruszala *et al.*, 2011), and mosses (Chater *et al.*, 2011). These latter results have also supported a single origin model.

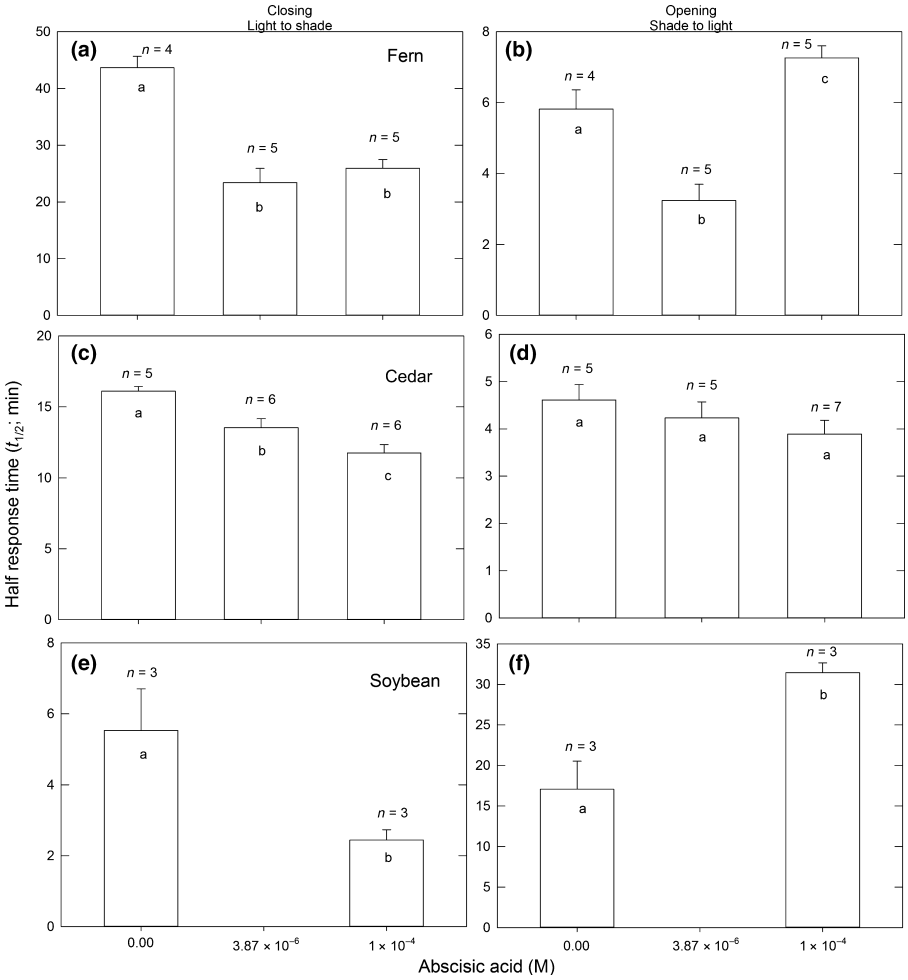

Fig. 2 The effect of abscisic acid (ABA) on the half time ($t_{½}$) of stomatal response to a step decrease (a, c, e) or increase (b, d, f) in light, of fern (a, b), cedar (c, d), and soybean (e, f). Mean $t_{½}$ (± SE) is shown with number of replications (*n*). Bars with different letters within a panel denote significance at $P < 0.05$. Note different $t_{½}$ scales. Data for soybean at low ABA concentration were not available.

Stomatal kinetics

There is considerable variability within and across taxa for both opening and closing kinetics (Haworth *et al.*, 2018b) and for g_s (Ng & Jarvis, 1980; Knapp & Smith, 1987; Franks & Farquhar, 2007; Grulke *et al.*, 2007a; Handley & Grulke, 2008; Grantz *et al.*, 2014). Meta-analyses (Ooba & Takahashi, 2003; Brodribb *et al.*, 2009; Vico *et al.*, 2011) suggest that both closing and opening are slower in ferns than in gymnosperms and angiosperms. The slower responses of ferns have been attributed to differences in stomatal morphology and ionic relations (Franks & Farquhar, 2007), and to the putative passive (that is ABA-insensitive) response mechanism (Brodribb & McAdam, 2011a; McAdam & Brodribb, 2012). The role of ABA insensitivity may need to be revisited.

The sigmoidal time course observed in stomatal opening of soybean has been observed in some other angiosperms, (snap bean, *Phaseolus vulgaris*, Paoletti & Grulke, 2010; *Tradescantia*, Franks & Farquhar, 2007; *Zea mays*, Raschke, 1970). Some dynamic stomatal models (Kirschbaum *et al.*, 1988; Vialet-Chabrand *et al.*, 2013) have treated opening as sigmoidal, while others (Knapp, 1993; Whitehead & Teskey, 1995; Vico *et al.*, 2011) have adopted the single exponential response, as observed in the present study in stomatal opening of fern and cedar and in closing of all three taxa.

The kinetics of stomatal opening and closing have been considered closely related to each other (Paoletti & Grulke, 2010; Dumont *et al.*, 2013). We had hypothesised a proportional relationship between opening and closing kinetics, as both varied with taxon and ABA, but this relationship was not observed. Closing was consistently slower than opening in cedar and fern, but much faster than opening in soybean, consistent with previous observations in both angiosperms (Hetherington & Woodward, 2003; Handley & Grulke, 2008; Paoletti & Grulke, 2010; Lawson *et al.*, 2011; Gerardin *et al.*, 2018) and ferns (Brodribb & McAdam, 2011a; Grantz *et al.*, 2014). In cotton (*Gossypium barbadense*) and coneflower (*Rudbeckia laciniata*), closing was slightly slower than opening, but in *Populus* and *Phaseolus*, closing was faster than opening (Grulke *et al.*, 2007b; Paoletti & Grulke, 2010; Grantz *et al.*, 2014). Stomatal closing was consistently more rapid than opening in angiosperms in the meta-analysis of Ooba & Takahashi (2003) but slower in the meta-analysis of Vico *et al.* (2011), although in the latter study, woody angiosperms closed faster.

Stomatal kinetics are more closely tied to plant economy of water than of carbon (Ooba & Takahashi, 2003; Lawson *et al.*,

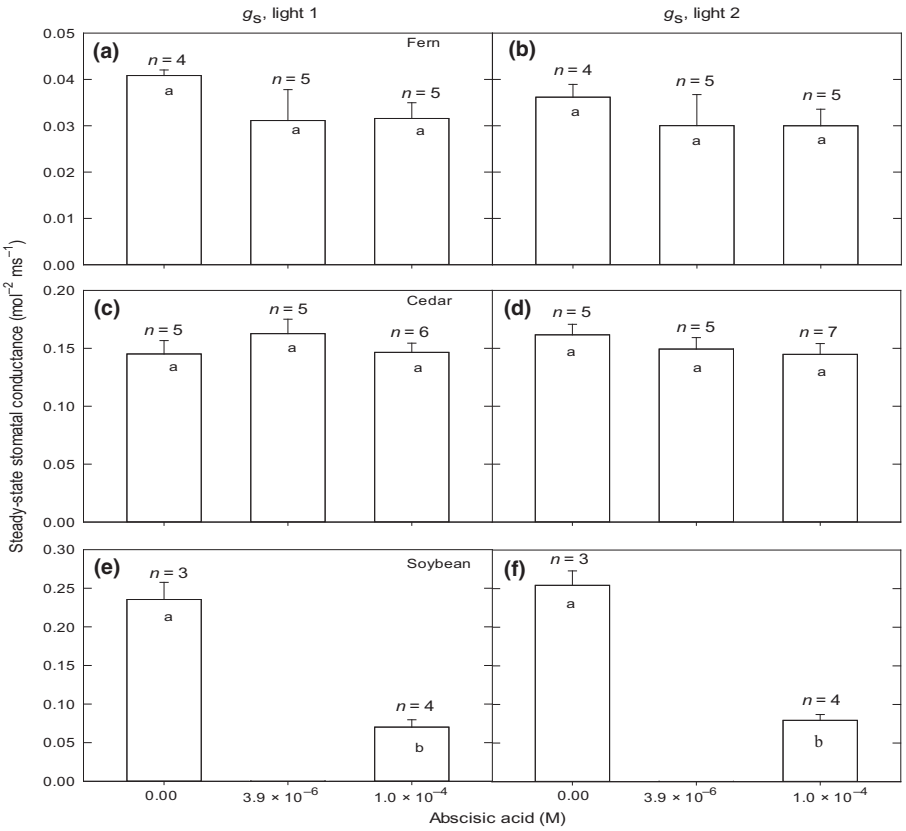

Fig. 3 The effect of abscisic acid (ABA) on steady-state stomatal conductance (g_s; mean ± SE) of fern (a, b), cedar (c, d), and soybean (e, f). g_s was determined in the light, before a step decrease in light ($g_{s,light\ 1}$; a, c, e) and following a step increase returning light to its original value ($g_{s,\ light\ 2}$; b, d, f). Bars with different letters within a panel denote significance at $P < 0.05$. Data for soybean at low ABA concentration were not available.

2011). Differences between opening and closing show that selective pressures on the mechanism or regulation of the two responses are dissimilar. In ferns, changing CO_2 concentration induced a monotonic stomatal response, while in angiosperms it induced an overshoot and recovery (Franks & Britton-Harper, 2016). In ferns, gymnosperms, and angiosperms, g_s increased when CO_2 concentration was reduced, but g_s declined only in angiosperms when CO_2 concentration was increased (Brodribb *et al.*, 2009). These distinctions may have phylogenetic as well as ecophysiological significance. The early kinetic experiments of Raschke (1970), suggested substantial metabolic differences between opening and closing responses. These energetics have been incorporated into recent models of stomatal response (Vico *et al.*, 2011) and may be useful targets for selection of improved crop germplasm. However, optimised values of $t_{½}$ may be required for specific species and environments, as sunfleck duration determines whether biochemical or stomatal limitation dominates productivity (Ooba & Takahashi, 2003; von Caemmerer *et al.*, 2004; Lawson *et al.*, 2011). A survey of diverse rice germplasm yielded only modest relationships between the rate of stomatal closure and WUE or yield (Qu *et al.*, 2016).

Rapid closure and slow opening are associated with tight control of water loss (Vico *et al.*, 2011). Exposure to exogenous ABA accelerated stomatal closing in all three taxa examined here, reducing cumulative conductance and predicted water loss (not shown) following a step decrease in light. ABA application may serve as an experimental surrogate for plant water deficit. In previous studies, drought accelerated both stomatal opening and closing in *Nicotiana tabacum* (Gerardin *et al.*, 2018), diverse rice cultivars (*Oryza sativa*; Qu *et al.*, 2016), and a range of gymnosperm and angiosperm taxa (Vico *et al.*, 2011). In *Arundo donax* (Poaceae), drought accelerated stomatal closing but slowed opening, in proportion to tissue ABA concentration (Haworth *et al.*, 2018a).

In fern, significant acceleration of stomatal opening was observed at low ABA concentration, which was reversed at high ABA concentration. While this biphasic response does not lead to straightforward interpretation, we suggest that the consistent acceleration of closing and the unexpected reversal of opening at high ABA concentration may simulate a proportional response to plant water deficit, enhancing evaporative cooling under mild deficit (low ABA concentration) (Radin *et al.*, 1994) and conserving water at high ABA concentration. Stomatal opening was also slowed in soybean at high ABA concentration. These observations are consistent with the accepted role of ABA in reducing g_s during drought and the role of rapid closure in enhancing WUE (Reich & Lassoie, 1984; Kirschbaum *et al.*, 1988; Paoletti & Grulke, 2005; Lawson *et al.*, 2011; Lawson & Blatt, 2014).

Conclusions

The current results support previous conclusions that stomata of ferns and gymnosperms do not exhibit steady-state stomatal responses to ABA, although this result may depend on experimental condition. They provide novel data showing that stomatal kinetics are sensitive to ABA in plants without a concomitant steady-state response to ABA and demonstrate that the separation of kinetic from steady-state responses to ABA and other stimuli may have heuristic value in phylogenetic, ecophysiological, and

crop improvement studies. Increasing evidence for clade-specific loss and gain of specific regulatory features, including stomatal responses, requires that phylogenetic analyses be based on the expression of a number of phenotypic traits. Conclusions regarding stomatal origins based on steady-state responses may be misleading without consideration of response kinetics. Stomatal sensitivity to ABA, including modulation of response kinetics, appears to have developed quite early in the lineage of land plants, facilitating radiation and adaptation to diverse terrestrial habitats. This situation suggests that manipulation of stomatal kinetics for crop improvement may be advantageous in theory, but may prove difficult in practice.

Acknowledgements

The authors are grateful for funding from the University of California at Riverside Research Allocation Process, from USDA NIFA Project No. CA-R-BPS-5005-H, and from the US Department of Agriculture, Forest Service, Cooperative Agreement Order AG-04T0-P-13-0052. The authors thank K. Burkey for provision of the seed of soybean and H-B. Vu for skilled assistance with plant growth and execution of the gas exchange experiments.

Author contributions

DAG and NEG conceived of the experiments. DAG and BSL conducted the gas exchange experiments. All authors contributed to collection of environmental data, and to data analysis and interpretation. DAG wrote the paper with considerable input from NEG and BSL.

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Supporting Information

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

Fig. S1 Effect of ABA on the time courses of stomatal conductance (g_s; relative units) generated from the mean half response time ($t_{½}$) of each treatment.

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