Allocation of the epidermis to stomata relates to stomatal physiological control: Stomatal factors involved in the evolutionary diversification of the angiosperms and development of amphistomaty

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ABSTRACT

The proportion of the leaf epidermis allocated to stomata (EP\textsubscript{50%}) and stomatal function (the capacity to adjust stomatal pore area to regulate stomatal conductance: \(G_s\)) are key components in leaf gas exchange, and have likely played a major role in plant evolution. We examined the velocity of change in \(G_s\) (\(G_{SD50\%}\)) during a transition from steady state conditions in the light to darkness and \(E_{P50\%}\) in 31 vascular plants with diverse evolutionary origins. Across all species, \(E_{P50\%}\) correlated to \(G_{SD50\%}\) and the magnitude of \(G_s\) reduction (\(G_{AS50\%}-G_{AD50\%}\)) after the cessation of illumination. Those species with higher absolute and relative \(G_{SD50\%}\) values tended to distribute stomata more evenly over the abaxial and adaxial leaf surfaces, whereas species with lower \(G_{SD50\%}\) utilised only one leaf surface for gas exchange. Groups that diverged at relatively early stages in plant phylogeny, including ferns, gymnosperms and basal angiosperms, exhibited lower \(E_{P50\%}\) and \(G_{SD50\%}\), and took longer to achieve the initial 50% reduction in \(G_s\) (\(T_{SD50\%}\)) than the more recently diverging angiosperms; in particular, the amphistomatous monocot grasses, which also showed higher absolute rates of photosynthesis and \(G_s\). We propose that selective pressures induced by declining \([CO_2]\) over the past 100 Myr have favoured greater allocation of the epidermis to stomata, increased amphistomaty (the presence of stomata on the abaxial and adaxial surfaces) and faster control of \(G_s\) in the more recently derived angiosperm groups. Modification of photosynthesis to enhance the carbon and water use efficiencies of C3 crops may therefore require concurrent increases in stomatal density and in the capacity of stomata to react quickly to environmental pressures.

1. Introduction

The origination of stomata 410 million years ago (Ma) enabled plants to colonise the land by facilitating the uptake of carbon dioxide (CO\textsubscript{2}) for photosynthesis (A) while allowing the regulation of transpiration to minimise the risk of desiccation (Edwards et al., 1998; Duckett et al., 2009). Stomatal conductance (\(G_s\)) is controlled by physiological adjustment of the size of the stomatal pore and morphological alteration of the number and size of stomata on newly developing leaves. These morphological stomatal responses effectively set the limits for physiological control of \(G_s\) (Woodward, 1987; Fanourakis et al., 2015). The percentage of the leaf epidermis devoted to stomata (EP\textsubscript{50%}), and therefore available for gas exchange, varies widely between species and has likely played a key role in plant evolution (Franks and Beerling, 2009; Assouline and Or, 2013; Boer et al., 2016). A diverse range of physiological stomatal behaviours are also observed, and whether these are associated with plant phylogeny remains a matter of debate (Brodribb et al., 2009; McAdam and Brodribb, 2012; Chater et al., 2013; Hasper et al., 2017). Physiological and morphological stomatal responses operate in tandem to control \(G_s\) following a change in the atmospheric concentration of carbon dioxide ([CO\textsubscript{2}]) (Haworth et al., 2013; Haworth et al., 2015). However, despite the wide range of research undertaken into epidermal patterning and physiological stomatal behaviours, they are often considered in isolation, and it is not
clear whether allocation of the epidermis to stomata is related to the physiological function of stomatal complexes. Any coordination between $EP_{so}$ and stomatal function may have played a role in plant evolutionary history, and may also have implications for the modification of crops to optimise $A$ and water use efficiency (WUE).

The allocation of the epidermis to gas exchange is determined by the stomatal pore area (SPA) and stomatal density (SD) on the abaxial and adaxial leaf surfaces (Cowran, 1977). Observations of living (Hetherington and Woodward, 2003) and fossil (Franks and Beuling, 2009) plants indicates that there is an inverse relationship between stomatal size and density. More recently diverged angiosperm groups tend to possess higher densities of smaller stomata than more basal groups with ancient evolutionary origins such as ferns and gymnosperms (Franks and Beuling, 2009). This trend towards higher densities of smaller stomata in groups that originated more recently may recently reflect the influence of declining $[CO_2]$ over the past 100 million years (Myr) (Haworth et al., 2017). As the availability of $CO_2$ for $A$ declined, stomatal morphologies that enabled a high rate of $CO_2$ di

et al., 2013; Haworth et al., 2015).

closely spaced, might have been favoured (Assouline and Or, 2013; Mott et al., 1982; Peat and Fitter, 1994; Muir, 2018). To the best of our knowledge, previous studies of stomatal distribution have not accounted for the role of physiological stomatal regulation (i.e. the speed of stomatal aperture adjustment) in the occurrence of amphistomaty. Physiological regulation of stomatal aperture ranges from active (where osmolytes are pumped across the cell membrane of the guard cells following a stimulus) to passive (where the turgor of the guard cells follows that of the leaf). Stomatal opening in response to sub-ambient $[CO_2]$ has been observed in epidermal strips detached from the mesophyll layer, while closing to super-ambient $[CO_2]$ involves a signal from the mesophyll layer (Fujita et al., 2013). An evolutionary transition from passive to active stomatal behaviour has been proposed to have contributed towards the expansion of the angiosperms (Brodribb et al., 2009; McAdam and Brodribb, 2012). However, genetic (Chater et al., 2011; Ruszala et al., 2011), guard cell membrane transporter protein (Chen et al., 2017) and gas exchange (Ruszala et al., 2011; Haworth et al., 2013; Franks and Britton-Harper, 2016; Hasper et al., 2017) analyses suggest that active physiological stomatal behaviours originated in early plant lineages. Nonetheless, the more recently derived Poaceae monocots (grasses) exhibit morphologically and mechanically divergent stomatal complexes (termed ‘dumb-bell’ stomata) in comparison to other angiosperms and gymnosperms (termed ‘kidney-shaped’ stomata). The stomata of grasses tend to be capable of more rapid adjustments of SPA (Franks and Farquhar, 2007). This enables grasses to adjust $G_a$ more rapidly to changes in light and $[CO_2]$ than other vascular plants which have kidney-shaped stomata (Haworth et al., 2013; Haworth et al., 2015).

To elucidate potential relationships between epidermal patterning and stomatal function, we assessed $EP_{so}$, the time to achieve the initial 50% of the overall reduction in $G_a$ ($T_{50\%}$) and the velocity in the change of $G_a$ over time (hereafter termed $G_{so50\%}$) during a transition from light to darkness. The $G_a$ response to darkness has been used to investigate evolutionary patterns in physiological stomatal behaviour (McAdam and Brodribb, 2012; Elliott-Kingston et al., 2016; Xiong et al., 2018) and stomatal function in plants grown under elevated $[CO_2]$ (Haworth et al., 2016) and drought (Haworth et al., 2018). Stomatal closure during a transition from light to dark conditions has been shown to be more effective in differentiating plant groups (McAdam and Brodribb, 2012; Xiong et al., 2018) and characterising the impact of drought on physiological stomatal function (Haworth et al., 2018) than a transition from dark to light to stimulate stomatal opening. We hypothesise that those species that devote a larger proportion of their epidermis to gas exchange will exhibit more rapid control of $G_a$. This study specifically aimed to: i) examine $G_{so50\%}$, $T_{50\%}$, and $EP_{so}$ in plants with diverse evolutionary origins; ii) investigate the influence of $EP_{so}$, $T_{50\%}$, and $G_{so50\%}$ on $A$ under steady-state conditions in the light, and; iii) explore possible evolutionary patterns in $EP_{so}$ and stomatal function which may relate to the diversification of the angiosperms, and whether these attributes can be utilised to enhance the carbon and water use efficiencies of crop plants.

2. Materials and methods

The area of the epidermis allocated to stomata and physiological stomatal functionality was assessed in 31 species. These vascular plants represented species with diverse evolutionary lineages, and for the purposes of the present study were categorised as: ferns (Osmunda regalis, Cyatheae cooperi, Cyrtomium fortunei, Matteucea orientalis and Dicksonia antarctica), gymnosperms (Lepidozamia peroffskyana, Cycas siamensis, Ginkgo biloba, Agathis australis, Nageia nagi and Podocarpus macrophyllus), basal angiosperms (Amborella trichopoda, Schisandra grandiflora, Magnolia stellata and Magnolia grandiflora), eudicots (Solanum lycopersicum, Moricandia marianodix, Coffea arabica, Helianthus annuus, Gossypium hirsutum, Chenopodium quinoa, Populus nigra, Capsicum frutescens and Salix alba) and monocots (Avena sativa, Triticum aestivum, Hordeum vulgare, Fargesia robusta, Phragmites australis, Typha latifolia and Arundo donax). The term “basal angiosperm” refers in the present work to angiosperms whose lineages diverged, according to a consensus of recent molecular phylogenetic studies (Byng et al., 2016), prior to the most recent common ancestor of eudicots and monocots.

The magnitude of the overall $G_a$ response ($G_{so50\%}$), the time to achieve the first 50% of the total reduction in $G_a$ ($T_{50\%}$) and the velocity of change in $G_a$ ($G_{so50\%}$) as stomata close following a cessation of illumination were used to determine physiological stomatal functionality following Haworth et al. (2018). A PP-Systems Ciras-2 attached to a PLC6(U) leaf cuvette and LED light unit (PP-Systems, Amesbury, Massachusetts, USA) was used to measure $G_a$. The size of cuvette was chosen in relation to the shape/size of leaf. In all cases, the leaf filled the entire cuvette; the cuvette plate size for each species is provided in Supplementary data Table 1. Leaves were placed inside the cuvette under conditions of 400 ppm $[CO_2]$, 25°C, 60–65% relative humidity and saturating photosynthetically active radiation (PAR) (values for each species are given in Supplementary data table S1) for 30 min until $G_a$ was stable ($G_{so50\%}$). Stomatal conductance was then recorded every 10 s for a further 10 min. After ensuring stomatal stability, the lights within the cuvette were switched off and $G_a$ was recorded every 10 s for a minimum of 60 min, or until the full extent of stomatal closure had been achieved and $G_a$ had remained stable for 20 min ($G_{so50\%}$) (Fig. 1). The $G_{so50\%}$ was determined as the velocity by which 50% of the $G_{so50\%}$ was reduced (Fig. 1). After the onset of darkness, this was expressed as absolute and relative values (assuming 100% $G_a$ at the point where lights were switched off). This parameter, and the time needed to achieve the initial 50% reduction in

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Fig. 1. The response of stomatal conductance \( G_s \) during a transition from steady state conditions in the light to darkness (indicated by a vertical dashed line and change from yellow to black in the upper horizontal bar) of the evolutionarily diverse species analysed in this study: a) each line represents the mean \( G_s \) response of each species (a minimum of four replicates for each species); b) the mean \( G_s \) response of each plant group (absolute values of \( G_s \)); and c) the mean relative \( G_s \) response of each group assuming 100\% \( G_s \) at the point when illumination ceased. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

\( G_s \) \( (T_{50\%}) \) were measured following the protocol of Haworth et al. (2018) (a schematic illustration is provided in Supplementary data Fig. S1). The \( G_s \) decrease is in effect a rate of change of velocity (i.e. in this case a deceleration) and expressed as mmol m\(^{-2}\)s\(^{-2}\). The \( G_{s50\%} \) was measured on one leaf per plant from a minimum of four replicate plants per species.

Epidermal micro-morphology was examined on the same leaves used for gas exchange analysis. Dental impression gel was used to create negative impressions of the abaxial and adaxial leaf surfaces. Clear nail varnish was then applied to the dental impression gel (Weyers and Lawson, 1985). The nail varnish positives were then mounted on glass microscope slides and imaged using a Leica DM2500 microscope at-tached to a Leica DFX300FX camera (Leica Microsystems, Wetzlar, Germany). A 0.4 \( \times \) 0.4 mm grid was digitally superimposed over each image to calculate SD. The mean of 10 images was used to determine SD for each leaf surface. Rarefaction analysis indicated that SD values stabilised after five to six images. The average SD for the abaxial and adaxial leaf surfaces was then determined. The SD for each replicate plant was then averaged to produce the mean value per species. Stomatal pore area during full stomatal opening was calculated assuming an ellipse shape where stomatal width is half stomatal pore length (Beering and Chaloner, 1993). Stomatal pore length was measured from a minimum of 40 stomatal complexes per species (i.e. 10 per replicate). The SD and SPA were then used to determine \( EP_n \) (see Haworth et al., 2015). The values of \( EP_n \) presented within the manuscript represent the maximum proportion of the epidermis devoted to gas exchange over both the abaxial and adaxial leaf surfaces.

Fig. 2. The relationship between photosynthesis \( (A) \) and stomatal conductance \( (G_s) \) of vascular plants with diverse evolutionary origins. Ferns (green): Osmunda regalis (solid square), Cyathea cooperi (solid diamond), Cyrtomium fortunei (solid triangle), Matteuccia orientalis (solid circle) and Dicksonia antarctica (solid inverted triangle). Gymnosperms (yellow): Lepidostrobus par-oxiflynea (solid square), Cypess siamensis (solid diamond), Ginkgo biloba (solid triangle), Agathis australis (solid circle), Nageia nagi (solid inverted triangle) and Podocarpus macrophyllus (square white-fill). Basal angiosperms (blue) Amborella trichopoda (solid square), Schisandra grandiflora (solid diamond), Magnolia stellata (solid triangle) and Magnolia grandiflora (solid circle). Eudicots (black): Solanum lycopersicum (solid square), Moricandia moricandiodes (solid diamond), Coffee arabica (solid triangle), Helianthus annuus (solid circle), Gossypium hirsutum (solid inverted triangle), Chenopodium quinoa (square white-fill), Populus nigra (diamond white-fill), Capsicum frutescens (triangle white-fill) and Salix alba (circle white-fill). Monocots (red): Avena sativa (solid square), Triticum aestivum (solid triangle), Hordeum vulgare (solid triangle), Fargasia robusta (solid circle), Phragmites australis (solid inverted triangle), Typha latifolia (square white-fill) and Arundo donax (diamond white-fill). The black line indicates the line of best fit and the two grey lines either side indicate the 95\% confidence intervals of the mean. Linear regression was used to assess the significance of any relationship between \( G_s \) and \( A \). Error bars indicate one standard error either side of the mean. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

Photosynthesis was positively related to \( G_s \) under saturating PAR in the evolutionarily diverse vascular plants analysed in this study (Fig. 2). The ferns, gymnosperms and basal angiosperms exhibited similarly low levels of \( A \) and \( G_s \), although the basal angiosperms showed a wider range of \( A \) values (Fig. 3a and b). Stomatal conductance under saturating PAR was on average 32.9\% greater in the monocots than the eudicots, and \( G_s50\% \) and lower \( G_s \) when averaged for each group were most rapid in the eudicots and monocots. The fast growing amphistomatous grasses \( P. \) australis, \( T. \) latifolia and \( A. \) donax exhibited the highest levels of \( G_{s50\%} \), \( G_{sLIGHT-G_{sDARK}} \) and \( EP_n \), and the lowest levels of \( T_{50\%} \). The eudicot angiosperms exhibited respective 60.8\% and 54.7\% lower average \( G_{s50\%} \) and \( EP_n \) values, and 88.7\% longer \( T_{50\%} \) than the monocots. The ferns, gymnosperms and basal angiosperms exhibited the lowest rates of \( G_s \) response and maximum allocation of the epidermis to gas exchange (Fig. 3c and f). Data presented in Figs. 2 and 3 is given in Supplementary information Table 1.

The 31 species analysed in this study showed a negative relationship between SPA and SD (Fig. 4), similar to those reported for SD with guard cell length (Hetherington and Woodward, 2003) and the size of the stomatal complex (Franks and Beerling, 2009). The magnitude of the total \( G_s \) response \( (G_{sLIGHT-G_{sDARK}}) \) was not associated with SPA (Fig. 5a) or the SD:SPA ratio (Fig. 5c). However, \( G_{sLIGHT-G_{sDARK}} \) was positively related to SD (Fig. 5b) and \( EP_n \) (Fig. 5d). The time to achieve
the initial 50% of the overall $G_s$ response was negatively related to SPA (Fig. 5e), SD (Fig. 5f), the SD:SPA ratio (Fig. 5g) and $EP_a$% (Fig. 5h). These relationships were statistically significant, although $R^2$ values ranged from 0.04 to 0.26 signifying comparatively low fit. Relative and absolute values of $G_{s50\%}$ were not related to SPA (Fig. 5i and m), but were positively correlated to SD (Fig. 5j and n). Relative (linear regression: $R^2 = 0.0005; F_{1,29} = 0.013; P = 0.910$) and absolute (linear regression: $R^2 = 0.032; F_{1,29} = 0.974; P = 0.332$) $G_{s50\%}$ was also not related to the length of the guard cell. The ratio of SD to SPA was weakly correlated to relative $G_{s50\%}$ ($R^2 = 0.174; Fig. 5k$), but not associated with absolute $G_{s50\%}$ in the species analysed (Fig. 5o), indicating that species with large numbers of small stomata did not alter $G_s$ more rapidly than those with low densities of large stomata. Relative values of $G_{s50\%}$ were positively associated with $EP_a$ (Fig. 5i); however, the hypostomatous monocot $F. robusta$ diverged from this relationship, exhibiting low $EP_a$% but comparatively a high relative $G_{s50\%}$. A strong positive correlation was observed between absolute values of $G_{s50\%}$ and $EP_a$% across all species examined (Fig. 5p).
The majority of species analysed in this study were either perfectly hypostomatous (all stomata arranged on the abaxial surface) or amphistomatous (half of stomata on the adaxial surface). Three eudicots \((P. \text{nigra}, C. \text{frutescens} \text{ and } H. \text{annuus})\) did not distribute stomata evenly over the leaf epidermis, with lower proportions of stomata on the adaxial surface. The relationship between the proportion of stomata on the adaxial surface and absolute \(G_{50}\) values (Fig. 6a) followed a rectangular hyperbola. The maximum \(G_{50}\) exhibited by a hypostomatous species was 0.173 mmol m\(^{-2}\) s\(^{-1}\), beyond which any further increase in \(G_{50}\) was not associated with higher \(A\). Stomatal conductance showed similar relationships, being linearly related to \(G_{50}\) (Fig. 7a and i) and \(EP_{\%}\) (Fig. 7j) to a level of \(\sim 800\) mmol m\(^{-2}\) s\(^{-1}\). Further increases in \(G_{50}\) or \(EP_{\%}\) were not associated with greater \(G_{s}\) in any of the species examined.

A note of caution must be observed due to the occurrence of self-correlation in the relationships observed between steady-state \(G_{s}\) under saturating PAR and parameters such as \(G_{s LIGHT- G s DARK}\) which utilise \(G_{s LIGHT}\) values in their calculation.

Principal component analysis of the physiological and morphological stomatal characteristics measured in this study indicated a high degree of overlap in the multi-variate space occupied by ferns, gymnosperms and basal angiosperms (one-way ANOVA with LSD post-hoc test of eigenvalues: component 1, \(F_{2,52} = 1.197, P = 0.310\); component

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**Fig. 4.** Relationship between stomatal pore area and stomatal density of the species analysed in this study. The black line indicates a logarithmic best-fit line and the two grey lines either side indicate the 95% confidence intervals of the mean. Non-linear regression was used to assess the significance of any relationship between stomatal pore area and stomatal density. Symbols as in Fig. 2.

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Levels of \(A\) and \(G_{s}\) under saturating PAR exhibited positive relationships to \(G_{s LIGHT- G s DARK}\) (Fig. 7a and 7f) and negative relationships with \(T_{50}\) (Fig. 7b and g). Photosynthesis was positively related to both \(G_{50}\) (Fig. 7c and d) and \(EP_{\%}\) (Fig. 7e); in all cases the response of \(A\) followed a rectangular hyperbola, reaching a plateau between 25 and 30 mmol m\(^{-2}\) s\(^{-1}\), beyond which any further increase in \(G_{50}\) or \(EP_{\%}\) was not associated with higher \(A\). Stomatal conductance showed similar relationships, being linearly related to \(G_{50}\) (Fig. 7a and i) and \(EP_{\%}\) (Fig. 7j) to a level of \(\sim 800\) mmol m\(^{-2}\) s\(^{-1}\). Further increases in \(G_{50}\) or \(EP_{\%}\) were not associated with greater \(G_{s}\) in any of the species examined.

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**Fig. 5.** Relationships between the magnitude of the \(G_{s}\) response (\(G_{s LIGHT- G s DARK}\)) time to achieve the initial 50% of the overall \(G_{s}\) response (\(T_{50}\)) and the absolute and relative velocity of stomatal conductance response during a transition from light to dark (\(G_{50}\)) and stomatal pore area (a, e, i, m), stomatal density (b, f, j, n), ratio of stomatal density to stomatal pore area (c, g, k, o) and the proportion of the epidermis devoted to stomata (d, h, l, p). The black line indicates the line of best fit and the two grey lines either side indicate the 95% confidence intervals of the mean. Linear regression was used to assess the significance of any relationships. Symbols as in Fig. 2.
The black line indicates the line of best fit and the two grey lines either side indicate the 95% confidence intervals of the mean. Linear (a, c, d and e) and non-linear (b) regression was used to assess the significance of any relationships. Symbols as in Fig. 2.

2, F_{2,52} = 1.6, P = 0.207 (Fig. 8). The more recently evolved eudicots and monocots had statistically identical eigenvalues for the first component but not for the second (one-way ANOVA: component 1, F_{1,66} = 0.172, P = 0.682; component 2, F_{1,66} = 34.4, P = 2.043 \times 10^{-7}). Moreover, despite partly over-lapping with the space occupied by ferns, gymnosperms and basal angiosperms, the eudicots and monocots occupied significantly distinct multi-variate space (one-way ANOVA: component 1, F_{1,133} = 133.1, P = 6.227 \times 10^{-21}; component 2, F_{1,133} = 14.7, P = 0.0002).

### Discussion

The proportion of the epidermis allotted to stomata is a major factor controlling leaf gas exchange and photosynthetic capacity (Kaiser, 2009). Photosynthesis was positively related to G_{s}, (Fig. 2) in the 31 species studied, with the highest rates of A observed in eudicots and monocots, rather than the basal angiosperm, gymnosperms and ferns (Haworth et al., 2011; Mott et al., 2012). Modelling of theoretical maximum G_{s} on the basis of epidermal micro-morphology often correlates to observed G_{s} using gas-exchange (Ohsumi et al., 2007; Kaiser, 2009). Scaling relationships based on physical characteristics (eg. Brown and Escombe, 1900; Cowan, 1977) will largely account for the strong correlation between G_{s} and EP_{50} found within this study (Fig. 5p). These relationships between leaf micro-morphology (Fig. 5i and p) and gas exchange characteristics (Fig. 7) were also closely related to the physiological ability of stomata to regulate G_{s} via adjustment of SPA.

The highest EP_{50} values were found in the amphistomatous eudicots and monocots (Fig. 6d). The capacity to utilise both the abaxial and adaxial leaf surfaces for gas exchange was associated with greater conductance to CO\textsubscript{2} and A (Fig. 3 and 6) (Parkhurst, 1978; Mott et al., 1982). Interference between adjacent stomatal complexes (Parlange and Wagoner, 1970) and structural constraints (Franks and Farquhar, 2007) may prevent hypostomatous species (including hyperstomatous species possessing only adaxial stomata) from achieving equivalent levels of EP_{50} to those observed in amphistomatous species. Amphistomaty generally occurs in fast growing species in open, high-light habitats where uptake of CO\textsubscript{2} may limit A (Mott et al., 1982; Peat and Fitter, 1994). The results of the present study are consistent with this interpretation (Fig. 6a), but also indicate that a high level of physiological stomatal regulation of G_{s} is a necessary component of amphistomaty (Fig. 6a). The selective pressures that have led to amphistomaty are complex and multifactorial (Parkhurst, 1978; Muir, 2015, 2018). It is noteworthy that there were comparatively few ‘intermediate’ species over a narrow range of G_{s50\%} and EP_{50\%} values (Fig. 6a), consistent with selective pressures to optimise G_{s50\%} and the proportion of stomata on the adaxial surface resulting in a restricted range of ‘optimal’ outcomes (Muir, 2015). This would suggest that selective pressures favouring increased EP_{50} through amphistomaty would also induce increased physiological regulation of SPA. Differential abaxial and adaxial stomatal responses to the same environmental signals in amphistomatous plants (Pospisilova and Solarova, 1980) also likely play a major role in the adaptation of amphistomatous plants to growth in high light environments. The hypostomatous monocot, F. robusta, was the exception to this pattern, displaying relative levels of G_{s50\%} consistent with the amphistomatous monocots and eudicots, but lower EP_{50} (Fig. 6b). The leaves of closely related members of the genus Fargesia are amphistomatous (eg. Wang, 2017), raising the possibility that the costs of amphistomaty, such as pathogens and occlusion with water in a humid habitat (see Muir, 2015), have resulted in hypostomaty in F. robusta.

A number of studies have suggested that evolutionary patterns in leaf epidermal micro-morphology (Hetherington and Woodward, 2003; Franks and Beerling, 2009; Boer et al., 2016) and stomatal function (Brodrrib, 2009; Haworth et al., 2011; Elliott-Kingston et al., 2016) have been driven by declining [CO\textsubscript{2}] from the Cretaceous to the present. The results of this study (Fig. 3) show that the phylogenetically basal ferns, gymnosperms and basal angiosperms exhibited lower levels of G_{s50\%} and EP_{50\%} than the more recently derived eudicots and monocots that originated during periods characterised by lower atmospheric [CO\textsubscript{2}] (Haworth et al., 2011; Elliott-Kingston et al., 2016). Nonetheless, there is overlap in the values of A, G_{s}, EP_{50\%}, T_{s50\%} and G_{s50\%} between the more recently derived eudicot and monocot groups and the more basal lineages (ie. greater EP_{50\%} necessitates more rapid adjustment of G_{s}), suggesting a degree of scaling in these relationships that may preclude
High densities of small stomata may facilitate diffusion of CO$_2$ into the leaf (Boer et al., 2016) and allow for more rapid adjustment of $G_s$ (Giday et al., 2013; Raven, 2014). The SD:SPA ratio and SPA were negatively related to $T_{50\%}$ (Fig. 5e and g), suggesting that smaller stomata can regulate $G_s$ more rapidly. However, these relationships should be treated with caution as they were comparatively weak and were not reflected in the magnitude ($G_{S\text{LIGHT}}$-$G_{S\text{DARK}}$) (Fig. 5a and c) or velocity ($G_{S\text{LIGHT}}$, Fig. 5i, m, k and o) of $G_s$ adjustment. Positive relationships have been observed between $G_{50\%}$ and stomatal size during stomatal opening in five closely related Banksia species (Drake et al., 2013) and in the dehydration response of Rosa hybrida (Giday et al., 2013), but not during a transition from light to dark conditions in a more diverse range of species (Fig. 5i and m) (Haworth et al., 2015; Haworth et al., 2016). This disparity may reflect the differential mechanical (Franks and Farquhar, 2007) and signalling (Haworth et al., 2016) mechanisms that operate in the stomata of the species represented in the present study. As observed in the genus Banksia (Drake et al., 2013), a positive correlation was also found between SD and $G_{50\%}$ in this study (Fig. 5j and n), suggesting that the number of stomata, and not stomatal size, is the dominant factor in the relationship between the velocity of $G_s$ response and $EP_{n}$ (Fig. 5p).

The higher $G_{50\%}$ found in many of the more recently derived eudicot and monocot groups, in comparison to ferns and gymnosperms, may be associated with differential mechanisms of stomatal movement (Franks and Farquhar, 2007). It is noteworthy, that the lowest relative $G_{50\%}$ values occurred in the ferns, consistent with observations of comparatively slower $G_s$ responses to light and [CO$_2$] (McAdam and Brodribb, 2012; Haworth et al., 2015; Franks and Britton-Harper, 2016). This may be indicative of a difference in the physiological function between the stomata of fern and seed plants (eg. McAdam and Brodribb, 2012); however, this would not be consistent with genetic, gas exchange and biochemical analyses (Ruszala et al., 2011; Chater et al., 2013). A comprehensive review of the hypotheses regarding the evolution of physiological stomatal function can be found in Franks et al. (2017). Increased $G_{50\%}$ and lower $T_{50\%}$ (Fig. 3), alongside greater complexity in leaf vein architecture (Roth-Nebelsick et al., 2001), higher rates of water transport in xylem vessels (as oppose to xylem tracheids) (Sperry et al., 2006; Meinzer et al., 2009) and higher responsiveness of leaf hydraulic conductance to light transitions (Xiong et al., 2018) may have enabled greater allocation of the epidermis towards gas exchange in the more recently derived angiosperm groups (Fig. 5p). As [CO$_2$] declined over the past 100 Myr, increased $EP_{n}$ would have facilitated diffusion of CO$_2$ into the leaf (Fig. 7j). Previous gas exchange analyses (Ruszala et al., 2011; Haworth et al., 2013; Haworth et al., 2015; Franks and Britton-Harper, 2016; Hasper et al., 2017) are not consistent with an evolutionary transition from generally passive physiological stomatal behaviours in more ancient plant groups to stomatal behaviours considered to be active in angiosperms (Brodribb et al., 2009; Brodribb and McAdam, 2011; McAdam and Brodribb, 2012). However, the results of this study would suggest that an increase in stomatal functionality, allowing more rapid $G_s$ adjustment and greater allocation of the cuticle to gas exchange may have conferred a selective advantage to more recently derived eudicot and monocot angiosperm groups in terms of greater photosynthetic capacity (Figs. 5p and 7c). This difference in stomatal functionality and epidermal allocation to gas exchange is evident from the principal component analysis presented here (Fig. 8), in which differential groupings of the more recently derived eudicots and monocots, with respect to the more phylogenetically basal groups, were observed. The increased capacity for gas exchange in the more recently derived angiosperm groups may have contributed to their expansion since the Late Cretaceous (Haworth et al., 2011; de Boer et al., 2012). Despite the absence of a clear phylogenetic delineation in active and passive physiological stomatal behaviours between the more recently evolved angiosperm
the preceding 400 Myr (Berner, 2006) may be the capacity for more water-loss when water availability is low (eg. Robinson, 1994) or suggest that modified morphological traits (Flexas, 2016). The results of this study suggest the capacity for more rapid stomatal movements, allowing greater $E_{w}$ through amphistomatomy to sustain $A$ (Fig. 7e), optimise WUE and protect against excessive water-loss when water availability is low (eg. Robinson, 1994) or evaporotranspirative demand is high (eg. Schulze et al., 1974). The greater $E_{w}$ and $G_{S_{50\%}}$ over a wider range of $G_{s}$ values in the monocots may also act as a selective advantage in allowing more rapid alteration of $G_{s}$ to exploit fluctuations in growth conditions over brief time-scales and optimise carbon gain in the short-term. It is noteworthy that leaf-level $A$ does not increase any further beyond an $E_{w}$ of 4% (Fig. 7e). This suggests that high rates of carbon gain are restricted on a leaf area basis by diffusion resistance to $CO_{2}$-uptake in the mesophyll (eg. Veronmann-Jürgenson et al., 2017) or photosynthetic biochemical and/or chemical capacity (eg. Gu et al., 2014). The fast growing monocots ($P$. australis, $T$. latifolia and $A$. donax) may possess levels of $E_{w}$ and $G_{S_{50\%}}$ sufficient to accompany greater mesophyll conductance and improved biochemical efficiency of $CO_{2}$ assimilation, whereas the eudicot angiosperms with lower rates of $G_{s}$ and $A$ may not exhibit sufficient levels of $E_{w}$ to cope with enhanced $A$ (eg. Flexas, 2016). A common relationship is observed between the flux of gases and the total surface area of chloroplasts in plants (Evans and Loreto, 2000), suggesting that an increase in light harvesting (Sakowska et al., 2018) may allow for greater gas exchange in the monocots.

Declining $[CO_{2}]$ over much of the Cretaceous and Tertiary may have favoured species with rapid stomatal movements and higher $E_{w}$ (Fig. 5p). However, rising $[CO_{2}]$ over the past 200 years (Keeling et al., 2005; Monastersky, 2013), and the predicted increases over the coming century (Prentice et al., 2001; Meinshausen et al., 2009), may reduce the influence of selective pressures favouring high $E_{w}$ and fine control of $G_{s}$. Nevertheless, high $G_{s}$ is associated with increased yield in C3 crop species (Fischer et al., 1998; Roche, 2015). Attempts to increase food security via modification of $A$ have been restricted by the inter-connectivity of photosynthetic biochemistry and other physiological and morphological traits (Flexas, 2016). The results of this study suggest that modification of epidermal patterning to increase $E_{w}$ and improved physiological stomatal functionality would be required alongside any modification in the photosynthetic physiology of C3 species. Improved $G_{S_{50\%}}$ may be particularly relevant in the utilisation of drought prone dry-lands in the production of food and biomass crops (Turner, 2004). The fast growing monocots ($P$. australis, $T$. latifolia and $A$. donax) and eudicots ($Chenopodium quinoa$, $Populus nigra$ and $Salix alba$) may serve as useful case studies in the analysis of species with $G_{s}$ responses and epidermal patterning conducive to increased yield.

Author contributions

MH conceived the study. MH, GM, TMGG, CD, MC, JF and CPS conducted the experiment. All authors contributed to the writing of the manuscript.

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Appendix A. Supplementary data

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References


