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Co-ordination in morphological leaf traits of early diverging angiosperms is maintained following exposure to experimental palaeo-atmospheric conditions of sub-ambient O_2 and elevated CO_2

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Abstract

In order to be successful in a given environment a plant should invest in a vein network and stomatal distribution that ensures balance between both water supply and demand. Vein density (D_v) and stomatal density (SD) have been shown to be strongly positively correlated in response to a range of environmental variables in more recently evolved plant species, but the extent of this relationship has not been confirmed in earlier diverging plant lineages. In order to examine the effect of a changing atmosphere on the relationship between D_v and SD, five early-diverging plant species representing two different reproductive plant grades were grown for seven months in a palaeo-treatment comprising an O_2:CO_2 ratio that has occurred multiple times throughout plant evolutionary history. Results show a range of species-specific D_v and SD responses to the palaeo-treatment, however we show that the strong relationship between D_v and SD under modern ambient atmospheric composition is maintained following exposure to the palaeo-treatment. This suggests strong inter-specific co-ordination between vein and stomatal traits for our study species even under relatively extreme environmental change. This co-ordination supports existing plant function proxies that use the distance between vein endings and stomata (D_m) to infer plant palaeo-physiology.

Introduction

Global diversity in plant and leaf architecture reflects a plasticity in morphology that allows plants to survive in a range of environments (Díaz et al., 2016). In this current era of rapid climate change, understanding the relationships between plant morphological traits and how they might be influenced by the surrounding environment is of the utmost importance, enabling predictions of plant responses over the coming decades as atmospheric carbon dioxide (CO_2) rises. Plants are a critical component of the hydrological cycle, influencing the amount of water vapour that is returned to the atmosphere via the process of transpiration (Rodell et al., 2015). The predicted future increases in CO_2 and global temperatures will have an impact on plant physiological function and morphological traits and will consequently influence the hydrological cycle (Gedney et al., 2006; Betts et al., 2007). The present study focuses on vein and stomatal density, two plant morphological traits that play a pivotal role in the transpirational pathway, and attempts to understand how one may influence the other as a plant encounters environmental change.

Stomata are microscopic pores on a leaf surface that regulate gas exchange. CO_2 from the atmosphere which is essential for photosynthesis is exchanged for water vapour from the inside of the leaf (Jones, 1992). Stomata respond to environmental cues, opening in response to increasing light, low carbon dioxide, and high humidity (Assmann 1999; Outlaw 2003; See review by Roelfsema and Hedrich 2005; Vavasseur and Raghavendra 2005; Shimazaki et al., 2007; Lawson 2009). Stomatal opening results in an increase in stomatal pore aperture which leads to an increase in both carbon uptake and water loss from the leaf. Stomatal density (SD) is the number of stomata per mm^2 of leaf tissue and it is determined by various genetic (Nadeau and Sack 2002; Shpak et al., 2005) and environmental factors (McElwain and Chaloner 1995; Woodward and Kelly 1995; Casson and Gray 2008). A change in SD alters gas exchange along the plants' diffusional pathway, influencing transpiration and therefore water demand. Veins are found in the leaves of plants, and are differentiations of the vascular bundles that transport water and nutrients from the soil to leaves, as well as sucrose from leaves to the storage sites of the plant (Sack and Holbrook 2006). A network of major and minor veins (some species only have major veins) carries water throughout the leaf tissue to the stomata where it is lost to the atmosphere as water vapour. Vein density (D_v) is the length of veins per leaf area ($mm\ mm^{-2}$), and in angiosperms it is determined predominantly by the minor veins, as they make up > 80% of the total vein length of the leaf (Sack et al., 2012). Minor vein density has been shown to be an important functional plant trait, exerting a strong influence over xylem conductivity (K_x) and outside xylem conductivity (K_{ox}), parameters that determine leaf hydraulic conductance (K_{leaf}) (Sack and Frole 2006; McKown et al., 2010). Thus it could be said that in the same way that stomatal density and size influence the water demands of a plant, the vein architecture influences its water supply.

In order to be successful in a given environment a plant should invest in a vein network and stomatal distribution that ensures balance between both water supply and demand. Maintaining this balance via co-ordinated shifts in venation and stomatal traits should ensure that the plant is operating at optimal efficiency in terms of carbon uptake and water loss, conforming to the optimality principle (Sack and Scoffoni 2013). Previous studies have found a strong relationship between D_v and SD in response to light (Brodribb and Jordan 2011) and vapour pressure deficit (Carins Murphy et al., 2014) in certain derived plant species and between SD and transpiration (T) across a range of ferns, conifers, and angiosperms from both tropical and temperate ecosystems (Boyce et al., 2009). D_v and SD have been shown to be strongly correlated with modelled maximum theoretical stomatal conductance (g_{max}) in a diverse range of Proteaceae species (Brodribb et al., 2013). Furthermore, a recent study using a range of modern and basal plant species grown in greenhouse conditions has also reported a strong correlation between D_v and g_{max} (McElwain et al., 2016a), suggesting that this balance does indeed exist. Moreover, other studies combine anatomical and physiological measurements to uncover the links between the architectural properties of a leaf and photosynthetic potential. For example, a proxy for leaf photosynthetic capacity has been developed based on the mesophyll path length (D_m) between vein endings and stomata. In multi-veined species, veins should be optimally placed to minimise D_m ensuring maximum photosynthetic capacity (Brodribb et al., 2007). These results together demonstrate the potential link between leaf hydraulic morphology and photosynthetic physiology and also highlight the ability of plants to maintain a balance between leaf phenotypic traits under environmental change.

The co-ordination between water supply (D_v) and demand (SD) traits is critical to plant success and the relationship between the two seems to be conserved across the major plant groups under present day atmospheric conditions (Boyce et al., 2009; Brodribb and Jordan 2011; Brodribb et al., 2013; Carins Murphy et al., 2014; McElwain et al., 2016a). However, it is not known whether this relationship is maintained when levels of oxygen (O_2) and CO_2 in the atmosphere change. Co-ordination between these two morphological traits may have been critical throughout the past 400 million years during times of fluctuating atmospheric O_2 and CO_2. Maintaining this balance between water supply and demand may have allowed certain species to operate more efficiently in their environment. For example, it has been widely proposed that the co-evolution of leaf traits (an increase in D_v and SD) during the Cretaceous decline in atmospheric CO_2 allowed angiosperms to outcompete other plant groups as they transitioned from predominantly moist to drier habitats (Boyce and Zwieniecki 2012; de Boer et al., 2012; McElwain et al., 2016a).

Stomatal density has been shown to be inversely proportional to atmospheric CO_2 (McElwain and Chaloner 1995; Woodward and Kelly 1995; Beerling and Woodward 1996; Royer 2001; Konrad et al., 2008; Franks and Beerling 2009a, 2009b) and it has been accepted as a palaeo-environmental proxy for CO_2 on this basis. Studies examining SD responses to concurrent changes in atmospheric O_2 and CO_2 are scarce, as are those investigating D_v responses to atmospheric change. In one of the few studies, examining both living and herbarium material of *Acer monspessulanum* L. and *Quercus petraea* Liebl, no change was observed in D_v in response to an increase in CO_2 from 280ppm to 350ppm (Uhl and Mosbrugger 1999). However, in other studies D_v has been shown to respond to environmental change (Brodribb and Jordan 2011; Sack and Scoffoni 2013; Carins Murphy et al., 2014) and has also been used in models to predict both atmospheric carbon dioxide partial pressure and temperature (Blonder and Enquist 2014). Furthermore, D_v has the potential to be a useful palaeo-environmental proxy, as venation networks are often preserved in fossilised plant material. For example, studies have shown an increase in D_v in angiosperms during the Cretaceous period when CO_2 was declining (Feild et al., 2011b; Boyce and Zwieniecki 2012).

Using a range of early diverging plant species (Figure 1), this study examines for the first time the effect of changing atmospheric conditions on the relationship between D_v , SD, and g_{max}. Using four angiosperm and one fern species, the plasticity and co-ordination of these morphological plant traits was assessed in a low O_2/high CO_2 atmosphere. In the context of this study, co-ordination in plant traits refers to either inter-specific or intra-specific co-ordination. Inter-specific co-ordination is taken to mean an observable trend in morphological plant traits across the experimental species. Intra-specific co-ordination on the other hand, is when the direction of change of plant traits is the same within a single species. We acknowledge that the number of species studied here is relatively small and experimental conditions limited to one palaeo-treatment, and as such, results discussed here are merely intended to be a suggestion of the possible behaviour of early diverging plant species under changing atmospheric conditions. Further studies using a wider range of species and palaeo-treatments will build on the current study and will allow more robust conclusions to be drawn.

Results were used to determine the robustness of plant function proxies that rely on co-ordination in morphological traits (such as the use of D_m as a proxy for palaeo-assimilation rate (Brodribb et al., 2007; Wilson et al., 2015)), specifically when applied at times of the geological past when the atmospheric composition was different from that of today. Theoretical maximum stomatal conductance (g_{max}) is a plant functional trait calculated using both SD and anatomical measurements of stomatal geometry (Franks and Beerling 2009a). This trait has been used in palaeo-CO_2 proxy models (Franks et al., 2014) to infer past CO_2 levels from the stomatal conductance (g_s) of ancient fossil species. The extent of the relationship between g_{max} and operational stomatal conductance (g_{op})

has been shown in two angiosperm species (Franks et al., 2009; Dow et al., 2014) and more recently in a range of basal angiosperm, gymnosperm, and fern species (McElwain et al., 2016a). Across this range of basal species g_{max} and g_{op} were found to be strongly related ($r^2 = 0.54$), with a scaling relationship of $g_{\mathrm{op}} = 0.25\ g_{\mathrm{max}}$. Therefore g_{max} was used in the current study as a means to infer changes in physiological behaviour with a change in the concentration of O_2 and CO_2 and to relate this to changes in D_{v}.

Materials and Methods

Species and growth conditions

Plant species from two different evolutionary plant groups, ferns (*Cyathea australis*) and angiosperms (*Chimonanthus praecox*, *Magnolia delavayi*, *Cornus capitata*, and *Zantedeschia aethiopica*), were grown for approximately 7 months in Conviron (Winnipeg, Manitoba, Canada) BDW-40 walk-in plant growth chambers at PÉAC (Programme for Experimental Atmospheres and Climate), Rosemount Environmental Research Station, University College Dublin. For each of these plant groups, the earliest diverging species obtainable from each plant family was used in order to follow the nearest living relative (NLR) protocol (Mosbrugger 2009), whereby the responses of extant plant species can be said to reflect the responses of their extinct relatives. Growth conditions were set to ambient (2 chambers at 21% O_2 and 400 ppm CO_2, O_2:CO_2 ratio of 525) and a palaeo-treatment of low O_2/high CO_2 (3 chambers at 16% O_2 and 1900 ppm CO_2, O_2:CO_2 ratio of 84.21). These conditions represent prehistoric modelled atmospheres (Bergman et al., 2004; Berner 2009) that likely occurred multiple times throughout the last 400 million years, for example in the Devonian (~ 419-359 mya), the late Triassic (~ 218-201 mya), and Jurassic periods (~ 201-145 mya) (Willis and McElwain 2014). Plants were given water and nutrients according to the individual species requirements (See Table S1). Chambers were set to a 16h day/8h night schedule, with day/night temperatures of 20°C /15°C, relative humidity of 65% and a photosynthetic photon flux density (PPFD) of 600 μmol $m^{-2}\ s^{-1}$.

SD, D_{v}, and g_{max} quantification

SD and D_{v} were quantified on three leaves per plant, and four plants per species per treatment using a modified vein density protocol (Berlyn and Miksche, 1976; Perez-Harguindequy et al., 2013). Leaves were cleared in 5% NaOH, bleached, and then brought through a series of 30%, 50%, 70%, and 100% ethanol. Leaves were then stained using Safranin and Fast Green before being brought back through the ethanol series in the reverse order (100% - 30% ethanol). Leaves were then suspended in distilled water before being mounted on glass slides for microscopy. For SD quantification, multiple images (on average 6 images) were taken over an area of approximately 1cm^2 per leaf and stomatal counts performed using Image J software inside a superimposed grid of 0.09 mm^2 on each image (this area was already determined to be the most representative of the entire leaf using the protocol of Poole and Kürschner from Jones and Rowe (1999)). For D_{v} quantification, images were taken on three areas of each leaf (an area of approximately 2mm^2 near the tip, centre, and bottom of the leaf near the petiole) and using Image J software the length of veins in these areas was manually traced (using a Wacom Intuos4 pen tablet) and D_{v} calculated in Excel. Minor D_{v} is independent of leaf size and accounts for the majority (>80%) of total vein length per area in most angiosperms (Sack et al., 2012), therefore vein length per area was calculated only on minor veins (quaternary orders upwards), major veins being excluded from analysis. Images were taken using a Leica DM2500 microscope with Leica DFC300FX camera (Leica® Microsystems, Wetzlar, Germany) attached and

Syncroscopy Automontage (Synchroscopy, Cambridge, United Kingdom) digital imaging software was used to impose grids and scale bars on each image.

For g_{max} quantification, anatomical measurements of 120 stomata per species and per treatment were obtained using the same images used for SD determination (See Table 1 for parameter values). g_{max} was calculated using the following diffusion equation (Parlange and Waggoner 1970; Franks and Beerling 2009a):

$$g_{max} = \frac{\left(\frac{\mathrm{dw}}{v} \cdot \mathrm{SD} \cdot pa_{\mathrm{max}}\right)}{pd + \frac{\pi}{2} \cdot \sqrt{pa_{\mathrm{max}}/\pi}}$$

dw = diffusivity of water vapour at 25°C (0.0000249 $m^2 s^{-1}$), v = molar volume of air (0.0224 $m^3 mol^{-1}$), SD = Stomatal density (m^{-2}), pa_{max} = max stomatal pore area (m^2), pd = stomatal pore depth (m).The maximum stomatal pore area was calculated (treating the pore as an ellipse) by using stomatal pore length as the long axis, pore length/2 as the short axis and taking the stomatal pore depth as being equal to the width of a fully turgid guard cell (Franks and Beerling 2009a, 2009b). It is important to note here that guard cells examined in the current study were not experimentally maintained at maximum turgor before anatomical measurements were made. Even though this might lead to a slight underestimation of g_{max}, this approach is in line with that used in many other palaeo-studies (Franks and Beerling 2009a, 2009b).

Vein density responses of a selection of non-angiosperm species from 2009 palaeo-experiment

In order to assess whether other non-angiosperm species show a change in D_{v} under different atmospheric compositions, dried plant material from a previous palaeo-experiment was analysed for D_{v}. Representatives of both the gymnosperms (*Agathis australis*, *Lepidozamia peroffskyana*, and *Gingko biloba*) and ferns (*Osmunda regalis*) were grown for 18 months in walk-in Conviron growth chambers in both an ambient and low O_2/high CO_2 atmosphere (Ambient treatment: 20.9 % O_2 and 380ppm CO_2, low O_2/ high CO_2 treatment: 13% O_2 and 1500 ppm CO_2, O_2:CO_2 ratios 552.63 and 86.67 respectively). These species have either parallel or dichotomously branching major veins, therefore it was not necessary to clear and stain leaves for D_{v} observation. D_{v} was calculated on one leaf per plant and three plants per species per treatment. Leaves were imaged using a Nikon SMZ1000 stereomicroscope with Leica DFC490 camera attached (Leica® Microsystems, Wetzlar, Germany), and using Image J software the length of veins was manually traced and D_{v} calculated in Excel in an area between 20mm^2 and 60mm^2.

Statistical analysis

Data were first checked for normal distribution and Generalised Linear Models were run in Minitab (version 16.1.1) statistical software to investigate differences in SD, D_{v}, and g_{max} between treatments (Figure 2). Minitab (version 16.1.1) statistical software was also used for correlation tests (Figure 4), boxplot representation of data (Figure 2, Figure 5), and to graphically display percent changes in SD, D_{v}, and g_{max} (Figure 3). RStudio (version 0.99.489) was used for Standardised Major Axis (SMA) regression analysis and for scatterplot representation of data (Figure 4).

Results

Species show a varied and species-specific response in SD, D_v, and g_{max} to the palaeo-treatment (Figure 2, Table 1). A significant decrease in SD is seen in two species (*Chimonanthus praecox* shows a 24% and *Zantedeschia aethiopica* a 34% decrease), *Magnolia delavayi* and *Cyathea australis* show a non-significant yet noticeable decrease (8% and 15% respectively), and another angiosperm (*Cornus capitata*) shows a small (9%), but non-significant increase (Figure 2A, Figure 3). D_v shows a similar mix of responses, three of the species show a significant decrease (*Chimonanthus praecox*, *Zantedeschia aethiopica*, and *Cyathea australis* decrease by 15%, 12%, and 17% respectively), *Magnolia delavayi* shows a very slight (2%) yet non-significant increase, and *Cornus capitata* shows a significant (14%) increase (Figure 2B, Figure 3). Two of the angiosperm species (*Chimonanthus praecox* and *Zantedeschia aethiopica*) show a significant decrease in g_{max} (16% and 32% respectively) in response to the palaeo-treatment, with *Magnolia delavayi* showing a non-significant decrease (11%), *Cornus capitata* a non-significant increase (15%), and *Cyathea australis* a non-significant decrease (2%) (Figure 2C, Figure 3). See Table S2 for results of generalised linear models (F and associated p-values).

Three out of four angiosperm species (*Chimonanthus praecox*, *Cornus capitata*, and *Zantedeschia aethiopica*) and the fern species *Cyathea australis* demonstrate intra-specific co-ordination of D_v, SD, and g_{max} in response to the palaeo-treatment (Figure 3). This co-ordination is evident even though one angiosperm species (*Cornus capitata*) shows an increase in all three parameters while the remaining species show a decrease. *Magnolia delavayi* shows co-ordination in two plant traits in response to the palaeo-treatment (SD and g_{max}), however co-ordination is lacking between both of these parameters and D_v.

The positive relationship between D_v and SD (Figure 4A) is strong under ambient conditions (Pearson's correlation coefficient: $r = 0.91$, SMA regression: $r^2 = 0.82$) and it persists in the palaeo-treatment ($r = 0.86$, $r^2 = 0.73$). Similarly, D_v and g_{max} (Figure 4B) show a strong relationship under both the ambient ($r = 0.87$, $r^2 = 0.76$) and palaeo-treatment ($r = 0.73$, $r^2 = 0.53$). The slopes of the regression lines between D_v and SD and D_v and g_{max} are not significantly different in the ambient and palaeo-treatments ($D_v = 0.01SD + 2.53$ for ambient and $D_v = 0.01SD + 2.39$ for palaeo-treatment; $D_v = 0.003g_{max} + 2.85$ for ambient and $D_v = 0.003g_{max} + 2.66$ for palaeo-treatment.

Discussion

SD, D_v, and g_{max} responses to low O_2/high CO_2

Results of the current study reflect variability in SD responses to atmospheric change. The inverse relationship between SD and CO_2 has been well documented in the literature using both fossil, herbarium, and living plant material (Woodward and Kelly 1995; McElwain and Chaloner 1995; Beerling and Woodward 1996; Royer 2001; Konrad et al., 2008; Franks and Beerling 2009a, 2009b). However, this inverse relationship is not universal across all species (Beerling and Kelly 1997; Haworth et al., 2013). Stomatal responses to O_2 are not well documented in the literature to date. The few existing studies however show an increase in stomatal index (ratio of stomata to epidermal cells or SI) in response to growth in 35% O_2 (Beerling et al., 1998), and a range of SD and SI responses to growth in a combined low O_2/high CO_2 treatment, as well as to separate low O_2 and high CO_2 treatments (Haworth et al., 2013).

The observed decrease in D_v in the majority of species exposed to the palaeo-treatment is likely a consequence of an overall lower water demand due to stomatal optimisation in a high CO_2

atmosphere. Reduced g_s in response to high CO_2 has been shown in previous studies (Haworth et al., 2013). This overall reduction in SD and D_v reflects a balance between water supply and demand in the palaeo-treatment, and the overall result would likely be a reduction in allocation of resources to non-essential veins and stomata, and a maximisation of resource use. It is important to acknowledge that the SD and D_v responses observed in the current study cannot be attributed to either low O_2 or high CO_2 alone without undertaking additional and separate sub-ambient O_2 and elevated CO_2 growth experiments using the same species. For the purposes of this analysis, suffice it to say that any SD and D_v responses are the result of the specific O_2:CO_2 ratio in the palaeo-treatment growth chambers.

It is noteworthy that only two of the studied species (*Chimonanthus praecox* and *Magnolia delavayi*) have vein densities higher than 6mm mm^{-2} (Figure 2B), the 'critical vein density' (Feild et al., 2011b; de Boer et al., 2012) that angiosperms are thought to have surpassed as they rose to dominance in the Cretaceous. This emphasises the similarity between our chosen study species and those very early evolving angiosperms that had vein densities as low as non-angiosperms (Brodribb and Feild 2010; Boyce and Zwieniecki 2012). The significant D_v decrease seen in the fern species (*Cyathea australis*) is interesting (Figure 2B), as it is thought that non-angiosperm species are incapable of altering their vein architecture in the same way that angiosperm species can (de Boer et al., 2012; Boyce et al., 2009). Non-angiosperms seem to exhibit limited plasticity in D_v when exposed to a long-term palaeo-atmospheric treatment (Figure 5). Examination of archived leaf material of three gymnosperms and one fern species from a 2009 palaeo-experiment (Haworth et al., 2013) shows that growth in a low O_2/high CO_2 atmosphere results in a change in D_v in one gymnosperm species (*Agathis australis*), but has no effect on D_v in the remaining species (two gymnosperms and one fern). These non-angiosperms have vein densities below 2mm mm^{-2} and all have either parallel or dichotomously branching major vein networks, implying that this vein configuration may lack developmental plasticity. The major veins of non-angiosperms are generally thicker in diameter and their xylem anatomy is distinct from that of the angiosperms, lacking an important feature that is believed to be paramount in the proliferation of minor veins, vessels with simple perforation plates. Only angiosperms evolved these less resistive perforation plates, and this in conjunction with the development of thinner minor veins may have allowed this plant group to outperform non-angiosperms (Feild and Brodribb 2013). Angiosperms also possess vein endings that are diffuse or dispersed throughout the leaf allowing them to develop more reticulate venation patterns, whereas gymnosperms with their marginal vein endings lack this ability (Boyce 2005). An important implication of the current findings is that some angiosperm species are able to alter their vein density on a developmental time-scale in response to a change in atmospheric composition; studies to date have only discussed CO_2-driven D_v changes across evolutionary time-scales (Brodribb and Feild 2010; Boyce and Zwieniecki 2012; McElwain et al., 2016a). Results of the current study indicate that at least in some early diverging species, D_v is a plant functional trait that can respond dynamically to atmospheric change.

Relationship between D_v, SD, and g_{max}

The strong relationship observed in both the ambient and palaeo-treatment between D_v and SD (Figure 4A) demonstrates inter-specific co-ordination in two morphological plant traits that determine hydraulic supply and demand across different plant lineages and under a changing atmosphere. Furthermore, the robust relationship observed between D_v and g_{max} (Figure 4B) demonstrates that morphology has the potential to influence the physiological behaviour of these species, via the strong relationship already found between g_{max} and g_{op} (Franks et al., 2009; Dow et al., 2014; McElwain et al., 2016a). Examining the direction of change in the morphological traits for each species it is clear that a high degree of intra-specific co-ordination is also occurring (Figure 3). Three out of four angiosperm species and the fern species show intra-specific co-ordination in D_v, SD, and g_{max} in response to the palaeo-treatment. Co-ordination between traits that determine the water relations (supply and demand) of a plant is critical for its survival. For example, an increase in SD and/or g_{max} would increase the evaporative demands of the plant and without a corresponding increase in D_v (to match the increase in water demand with an increase in hydraulic supply) the plant would be mal-adapted to its environment and would most likely not survive. The opposite scenario would not be as detrimental to plant survival, however a decrease in SD and/or g_{max} without a corresponding decrease in D_v would result in a waste of resources, the construction of veins being costly to the plant (Sack and Scoffoni 2013). This ability to co-ordinate morphological traits under a changing atmosphere likely occurred throughout plant evolutionary history as the composition of atmospheric O_2 and CO_2 fluctuated, allowing certain plant species to adapt and survive. During the Cretaceous decline in atmospheric CO_2 for example, it is thought that angiosperms were able to increase their gas exchange capacity (thereby increasing photosynthetic rates) by evolving smaller stomata (Franks and Beerling 2009a), and by increasing both the density of stomata on the leaf surface and the density of veins (Boyce et al., 2009; Brodribb and Feild 2010; de Boer et al., 2012; McElwain et al., 2016a). Furthermore, angiosperms that surpassed the ‘critical vein density’ of 6mm mm^{-2} were able to out-compete the gymnosperms and ferns in niches with high evapotranspirational demand where an increase in water supply to the leaf would have been necessary for survival (de Boer et al., 2012). Higher vein densities have been suggested to confer a higher capacity for CO_2 uptake and an increased range of g_{op}; this would explain the ability of angiosperms to expand to such diverse habitats and to outcompete species that are more constrained in their venation and hence gas exchange capacity (McElwain et al., 2016a). A recent study suggests that angiosperms are indeed hydraulically optimised for a diverse range of environments, achieving this by maintaining an equal vein to vein and vein to evaporative surface distance in the leaf (Zwieniecki and Boyce 2014). Ferns are under-invested hydraulically due to their thin leaves and large vein to vein distances, and while some gymnosperms do approach optimal investment by producing thicker leaves in more water-demanding environments, they are as a group sub-optimal in terms of vein placement (Zwieniecki and Boyce 2014).

The current study supports these theories by demonstrating a higher degree of plasticity in D_v in some early diverging angiosperms in response to a changing O_2:CO_2 ratio, compared to the studied gymnosperms and ferns (Figure 5). It is interesting however, that examined non-angiosperm species from the 2009 palaeo-experiment show limited plasticity in D_v as well as in SD (see SD results for these non-angiosperm species reported in Haworth et al., 2013). This demonstrates that while these species do not show a high degree of morphological plasticity in response to a changing atmosphere on experimental time-scales comparable to the studied angiosperms, they do demonstrate similar co-ordination in leaf morphological traits.

Furthermore, these results suggest that angiosperms are not only capable of showing morphological plasticity in response to rising O_2 and declining CO_2 (Boyce et al., 2009; Feild et al., 2011b; de Boer et al., 2012), but also to declining O_2 and high CO_2 conditions. The robust positive relationship observed here between the density of veins and stomata strongly supports the theory suggested by Brodribb et al., (2007), whereby multi-veined leaves optimise the placement of veins in relation to stomata so that the distance water needs to travel through the resistive mesophyll (D_m) is minimised. Although D_m was not directly measured in this study, the morphological co-ordination observed suggests that any change in D_v will elicit a corresponding change in SD or vice versa, allowing the leaf to minimise the distance between veins and stomata, and to maximise photosynthetic performance and operational efficiency of the leaf.

Implications for past plant-atmosphere interactions

The experimental species examined show a species-specific and varied response to growth in the palaeo-treatment, yet the strong positive relationship between D_v and SD persists (Figure 4A). Furthermore, the positive relationship observed between D_v and g_{max} (Figure 4B) demonstrates the link between hydraulic and gas exchange/diffusional processes in these species, as shown in previous studies (Sack et al., 2003; Boyce et al., 2009; Brodribb and Field 2010; Feild et al., 2011a; McElwain et al., 2016a). The finding that D_m is most likely maintained under changing atmospheric conditions (due to the intra-specific co-ordination between D_v and SD) has important implications when attempting to understand plant-atmosphere interactions throughout the last 400 million years of plant evolution. A lack of co-ordination in D_v and SD on developmental time-scales would result in a plant that is morphologically and physiologically out of sync, negatively impacting operational efficiency and overall fitness under changing atmospheric conditions. Furthermore, plant species that exhibited plasticity in these morphological traits under a changing atmosphere would likely have had an ecological advantage over plant species that were morphologically inflexible, being able to maximise their photosynthetic capacity as the surrounding environment changed (McElwain et al., 2016a). The finding that a proxy for photosynthetic capacity (D_m) (Brodribb et al., 2007) remains stable under changing atmospheric conditions is important for accurate initial parameterisation of mechanistically based models used to predict palaeo-CO_2 (Franks et al., 2014) since these require robust estimates of palaeo-assimilation (Franks et al., 2014; McElwain et al., 2016b). The current study focuses on plant responses to an experimentally imposed low O_2/high CO_2 atmosphere. This O_2:CO_2 ratio occurred multiple times throughout plant evolutionary history based on model and proxy estimates (Royer 2001; Bergman et al., 2004; Royer et al., 2004; McElwain et al., 2005; Berner 2006, 2009; Steinthorsdottir et al., 2016) however, it would be beneficial to investigate the effect of other atmospheric O_2:CO_2 ratios on these plant trait relationships in order to test their linearity. Further experiments examining plant responses to a range of palaeo-atmospheric conditions will build on these results, providing a better picture of plant-atmosphere interactions over the past 400 million years and allowing predictions of future plant responses to global climate change.

Conclusions

Species show a varied response in SD, D_v, and g_{max} to growth in an experimental low O_2/high CO_2 palaeo-atmosphere. Regardless of this variation in responses, a strong relationship is observed between D_v and SD and D_v and g_{max} under both the ambient and palaeo-atmosphere. Gymnosperms studied here appear to lack the same degree of developmental plasticity in D_v compared to the angiosperms, at least on short experimental time-scales. The ability to increase their range of D_v values may have contributed to the success of angiosperms during the Cretaceous decline in CO_2; a high degree of plasticity in this trait possibly provided early diverging angiosperms with a competitive advantage over other seed plant groups in more changeable environments. The tight relationship observed between D_v and SD in the palaeo-treatment suggests that D_m is likely maintained under environmental change and lends confidence to existing palaeo-CO_2 proxies that use this parameter in their models. Further studies examining the robustness of these plant trait relationships under a range of O_2:CO_2 ratios are needed in order to elucidate the full spectrum of plant-atmosphere interactions throughout the last 400 million years.

Table 1. Measured parameters (± standard deviation) for experimental species. SD = stomatal density, D_v = Vein density, g_{max} = maximum theoretical stomatal conductance. n = 12 for all species except *Zantedeschia aethiopica* where n = 9.

	SD mm^{-2}		D_v mm mm^{-2}		Pore length μm		Pore depth μm		g_{max} mmol $m^{-2}s^{-1}$	
	Ambient	Palaeo	Ambient	Palaeo	Ambient	Palaeo	Ambient	Palaeo	Ambient	Palaeo
	(n = 12)	(n = 12)	(n = 12)	(n = 12)	(n = 12)	(n = 12)	(n = 12)	(n = 12)	(n = 12)	(n = 12)
Chimonanthus praecox	513±103.2	390±102.2	8.3±0.8	7.0±1.0	10.4±1.0	10.9±1.0	3.8±0.3	3.4±0.3	2300±279.0	1923±298.9
Magnolia delavayi	343±48.2	316±72.7	6.6±0.5	6.7±1.1	8.9±0.9	8.7±0.8	5.8±0.9	5.6±0.9	1018±210.8	908±201.3
Cornus capitata	193±21.8	210±34.6	4.4±0.3	5.1±0.5	10.3±0.5	10.3±0.5	6.5±0.6	5.9±0.6	671±74.3	774±156.5
Zantedeschia aethiopica	104±20.4	68±14.7	4.3±0.5	3.8±0.2	15.9±1.7	16.0±0.9	8.6±0.5	8.3±0.8	599±108.6	406±75.0
Cyathea australis	171 ±50.3	153±33.2	3.3±0.2	2.7±0.2	12.4±1.4	13.6±1.7	9.4±0.8	9.8±0.5	649±213.4	676±230.8

Figure 1. Diagram illustrating the phylogenetic placement of selected experimental plant species.

Figure 2. Changes in (A) SD, (B) D_v and (C) g_{max} of experimental species in response to the palaeo-treatment. Different letters signify a significant difference between treatments. A = ambient treatment, P = palaeo-treatment.

Figure 3. Percent change in SD, D_v, and g_{max} of experimental species.

Figure 4. SMA regression showing the relationship between (A) D_v and SD, $D_v = 0.01SD + 2.53$ for ambient and $D_v = 0.01SD + 2.39$ for palaeo-treatment, and (B) D_v and theoretical maximum stomatal conductance (g_{max}), $D_v = 0.003g_{max} + 2.85$ for ambient and $D_v = 0.003g_{max} + 2.66$ for palaeo-treatment. Each data point represents a single leaf.

Figure 5. Comparison of the D_v responses of gymnosperms, ferns, and angiosperms to growth in low O_2/high CO_2 conditions. Different letters signify a significant difference between treatments. A = ambient treatment, P = palaeo-treatment.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

CEF carried out vein density and stomatal density analysis, anatomical stomatal measurements for g_{max} calculation, statistical analysis, and drafted the manuscript. ASP contributed stomatal density data. CEK was involved in the 2009 palaeo-experiment and therefore provided dried leaf material for vein density analysis. JCM is the principal investigator. All authors read, revised, and approved the final manuscript.

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