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RESEARCH PAPER

Uncorrelated evolution of leaf and petal venation patterns across the angiosperm phylogeny

Adam B. Roddy[1,*], C. Matt Guilliams[1,2], Terapan Lilittham[1], Jessica Farmer[1], Vanessa Wormser[1], Trang Pham[1], Paul V.A. Fine[1], Taylor S. Feild[3] and Todd E. Dawson[1]

[1] Department of Integrative Biology, University of California, Berkeley, USA
[2] Jepson Herbarium, University of California, Berkeley, USA
[3] School of Marine and Tropical Biology, James Cook University, Townsville, Queensland, Australia

* To whom correspondence should be addressed. Email: adam.roddy@gmail.com

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Abstract

Early angiosperm evolution, beginning approximately 140 million years ago, saw many innovations that enabled flowering plants to alter ecosystems globally. These included the development of novel, flower-based pollinator attraction mechanisms and the development of increased water transport capacity in stems and leaves. Vein length per area (VLA) of leaves increased nearly threefold in the first 30–40 million years of angiosperm evolution, increasing the capacity for transpiration and photosynthesis. In contrast to leaves, high water transport capacities in flowers may not be an advantage because flowers do not typically contribute to plant carbon gain. Although flowers of extant basal angiosperms are hydrated by the xylem, flowers of more recently derived lineages may be hydrated predominantly by the phloem. In the present study, we measured leaf and flower VLA for a phylogenetically diverse sample of 132 species from 52 angiosperm families to ask (i) whether flowers have lower VLA than leaves, (ii) whether flowers of basal angiosperm lineages have higher VLA than more recently derived lineages because of differences between xylem and phloem hydration, and (iii) whether flower and leaf VLA evolved independently. It was found that floral structures had lower VLA than leaves, but basal angiosperm flowers did not have higher VLA than more derived lineages. Furthermore, the independent evolution of leaf and petal VLA suggested that these organs may be developmentally modular. Unlike leaves, which have experienced strong selection for increased water transport capacity, flowers may have been shielded from such selective pressures by different developmental processes controlling VLA throughout the plant bauplan.

Key words: Angiosperms, floral evolution, hydraulics, vein density.

Introduction

Evolution of the modern angiosperm flower represents an innovation that has reverberated across ecosystems globally. Flowers have co-evolved with specialized pollinators, contributing to early angiosperm success and to increases in animal diversification (Thien *et al.*, 2000; Hu *et al.*, 2008; Thien *et al.*, 2009). While major insights about developmental evolution in early angiosperms have focused primarily on flowers (Bowman, 1997; Soltis *et al.*, 2007; Endress, 2011; Mathews & Kramer, 2012; Zhang *et al.*, 2012), understanding floral evolution in the broader context of whole-plant physiology could reveal new sources of selection acting on plant reproduction. For example, constraints on reproductive investment influence vegetative architecture, such as branch ramification (Harris & Pannell, 2010) and leaf size (Bond & Midgley, 1988).

Abbreviations: PIC, Phylogenetic independent contrasts; VLA, vein length per area.

Despite their unique developmental processes, flowers and leaves are subject to the same environmental and resource limitations, requiring carbon and water to be supplied throughout development. As a result, trade-offs exist between investments in vegetative and reproductive structures (Bazzaz *et al.*, 1987; Reekie & Bazzaz, 1987*a*,*b*,*c*). While some flowers may be able to contribute substantially to the carbon costs of reproduction (Bazzaz & Carlson, 1979; Bazzaz *et al.*, 1979; Galen *et al.*, 1993), water must be supplied to flowers and fruits by roots and stems, potentially at the cost of vegetative function (Nobel, 1977; Galen *et al.*, 1999; Lambrecht & Dawson, 2007). Thus, understanding the ecophysiological mechanisms of floral water balance may reveal new insights into the ecology and evolution of flowers (Galen, 2000; Chapotin *et al.*, 2003; Feild *et al.*, 2009*a*,*b*).

Despite the influence of floral water balance on vegetative physiology (Galen *et al.*, 1999; Galen, 2000), reproductive development (Patiño & Grace, 2002) and pollinator attraction and manipulation (Bertsch, 1983; von Arx *et al.*, 2012), surprisingly little work has focused on understanding the water dynamics of flowering. In general, larger flowers within a species require more water (Galen *et al.*, 1999), but xylem sap flow rates through flower-bearing branches are highly variable among species (Roddy & Dawson, 2012). How flowers are plumbed into the vegetative system probably varies among species as well. Some authors have assumed that higher, less-negative water potentials of flowers compared with leaves means that flowers are hydrated by the phloem (Trolinder *et al.*, 1993; Chapotin *et al.*, 2003). In contrast, blooming (anthesis) flowers of extant, early-diverging angiosperm lineages are hydrated by the xylem (Feild *et al.*, 2009*a*,*b*), which may be why these flowers wilt and die even under slight droughts. The distinction between phloem and xylem hydration is complicated because in one early-diverging extant lineage, *Magnolia grandiflora*, water potential gradients indicate that the inner whorl of tepals is phloem hydrated while the outer whorl is xylem hydrated (Feild *et al.*, 2009*b*). Supplying water via the phloem rather than the xylem during anthesis may allow flowers to access a greater level of hydraulic autonomy and release from drought stress occurring in the rest of the plant, potentially facilitating pollinator attraction under otherwise adverse conditions. Phloem hydration may also indicate low water requirements of flowers because flux rates through the phloem are much lower than those through the xylem.

In leaves and leaf homologues, such as sepals and petals, xylem and phloem are organized into vascular bundles or veins. One of the major advances among early angiosperm lineages compared with non-angiosperms was the development of densely veined leaves, expressed as the amount of vein length per unit of leaf surface area (Feild & Arens, 2007; Brodribb & Feild, 2010). Increased vein length per area (VLA; also commonly referred to as 'vein density') brings xylem-based liquid water closer to the sites of evaporation and carbon fixation inside the leaf. Such increased hydraulic supply capacity yields a greater capacity for transpiration and photosynthesis (Sack & Frole, 2006; Brodribb *et al.*, 2007). Based on Cretaceous fossil angiosperm leaves as well as on phylogenetic reconstructions across extant basal lineages, angiosperm VLA is estimated to have increased nearly threefold during the first 30–40 million years of angiosperm diversification (Brodribb & Feild, 2010; Feild *et al.*, 2011). Increasing the upper limit of leaf VLA allowed angiosperms to assimilate carbon and accumulate biomass more rapidly than non-angiosperms, perhaps facilitating their ecological domination beginning in the Cretaceous (Brodribb & Feild, 2010).

How floral venation influences floral water balance and evolution, however, remains unexamined. Because flowers generally require less water than leaves (Blanke & Lovatt, 1993; Higuchi & Sakuratani, 2005; Feild *et al.*, 2009*b*; Lambrecht *et al.*, 2011; Roddy & Dawson, 2012), selection may have acted in different directions on petal and leaf venation. In contrast to selection for increased water transport capacity in leaves, selection probably favoured reduced water transport in flowers. Alternatively, modular developmental processes could have buffered floral hydraulic traits from the strong selection for increased VLA in leaves. Unlike leaves, most petals do not synthesize substantial amounts of carbon, mitigating the need for high transpiration rates normally requisite for maintaining high rates of photosynthesis. If petals and leaves experienced different constraints on their water balance, then their hydraulic traits may have evolved independently and may be developmentally modular and possibly also functionally modular. Developmental modularity would occur if the underlying developmental processes causing vein branching frequency, and thus VLA, were different for flowers and leaves. Alternatively, correlated evolution of leaf and petal VLA would suggest that a common developmental programme underlies VLA throughout the plant bauplan. In this case, the strong selective advantage of high VLA in leaves carried along petal VLA by overwhelming any selection for low petal VLA. Thus, any reduction in the water requirements of flowering may not be reflected in petal VLA, a trait more associated with water supply than water loss. Whether petal VLA decreased while leaf VLA increased depends on the extent to which floral and foliar VLA experienced selection in different directions.

In the present study, we examined VLA evolution in flowers and leaves across the angiosperm tree of life and test three main hypotheses. First, because flowers generally require less water than leaves, we predicted that floral structures would possess lower VLA than associated leaves. Secondly, because flowers of more recently derived angiosperm species may be phloem hydrated, we hypothesized that flowers of these species would have lower VLA than flowers of basal angiosperm lineages. Thirdly, although patterns of venation in different structures are related in many lineages (Melville 1960, 1969), we hypothesized that if flowers and leaves experienced different selection regimes for water transport capacity, then flower and leaf VLA evolved independently.

Materials and methods

Sampling

The majority of sampled taxa were collected from living specimens in the University of California Botanical Garden and the Tilden Botanical Garden, both in Berkeley, CA, USA. Collecting primarily from common gardens ensured that species were well watered and had grown in similar environmental conditions. We targeted plants with large, recently opened flowers on exposed, sunlit branches, and collected flowers and fully expanded leaves from one to three individuals per species. Other early-diverging lineages were sampled from natural populations in the field (see Supplementary Table S1 at *JXB* online). Despite the diversity of our sampling, there remained three large phylogenetic gaps in our dataset, the graminoid monocots, the Asteraceae, and the Orchidaceae. Although these groups account for a substantial amount of all angiosperm diversity, their floral morphologies are markedly different from most other clades. Furthermore, species with small, wind-pollinated flowers were largely excluded.

Leaves and flowers were collected simultaneously and transported to the laboratory for sample processing. Approximately 1 cm^2 sections were taken from midway between the leaf midrib and margin, midway between the base and tip of the leaf and placed in 2–4% NaOH for clearing. Except for the field-collected specimens, we did not remove the leaf epidermis before imaging. Because of the high variability in vein density within a petal, we collected multiple 1 cm^2 sections from all parts of the petals and sepals and placed them in 2–4% NaOH. For structures that were smaller than 1 cm^2, we placed the entire petal or sepal into NaOH for clearing. For all structures, sections were taken from multiple leaves or flowers per species and pooled into one vial for each structure of each species. After 2–4 weeks, leaves were briefly washed in dH_2O, transferred to a 3% bleach solution for a few minutes, washed again in dH_2O, and then placed into 95% ethanol. Sepals, petals, and tepals were similarly transferred to ethanol, except that most of them did not require clearing for as long, nor did they require bleaching to complete the clearing process. Once in ethanol, most samples were quickly stained with Safranin O. Except for the few field-collected samples, we used a Leica DM2500 microscope outfitted with a Nikon DS-Fi1 camera at 5–20× magnification. We captured one to two images per section from each of five to 12 sections per species. Vein densities were measured using ImageJ (version 1.44o; Rasband, 2012) and the mean was determined. Based on our estimates, at least five samples needed to be measured to reduce the variance to within 5% of the mean of 20 fields (data not shown).

Statistical and comparative analyses

To perform analyses of trait evolution, we grouped some structures together based on their presumed function and on their trait values. Sepals, bracts, and outer whorl tepals did not differ significantly in VLA and are collectively referred to here as 'sepals'. Petals, hypanthia, and inner whorl tepals of basal angiosperms did not differ significantly in their vein densities and were grouped together and are referred to as 'petals'. Differentiated perianths have evolved as many as six times among the angiosperms (Zanis *et al.*, 2003), and distinct petals may be derived either from stamen-like structures or bract- or leaf-like structures (Irish, 2009). These distinctions, like our groupings of structures, are based on morphological characters, such as the number of vascular traces. To determine whether VLA differed between structures, we used a linear mixed-effects model that treated plant structure (leaf, sepal, petal, flower) as a fixed effect and plant structure nested within species as a random effect. We used Bonferroni-adjusted, Tukey post-hoc pairwise comparisons to compare VLA between plant structures, using the *glht* function in the package *multcomp* for R (version 2.14.1; R Core Team, 2012). To compare differences in vein density between major clades, we used analysis of variance with Tukey post-hoc comparisons to estimate pairwise differences in vein density between clades. For all subsequent phylogenetic analyses, we used ln-transformed vein densities.

We obtained a phylogenetic supertree of our sampled taxa using the online version of Phylomatic (Webb & Donoghue, 2005). The resulting undated ultrametric tree was imported into Phylocom 4.2 (Webb *et al.*, 2008) so that recent node age estimates could be written into the tree file using the *bladj* function. We obtained node age estimates for as many nodes as possible in our undated supertree from Bell *et al.* (2010). Note that *bladj* was used primarily to write these node age estimates into our tree file, rather than to anchor a few nodes and evenly distribute the remaining, unanchored nodes. This dated supertree is poorly resolved for recent divergences (e.g. divergences within genera) but represents, we believe, the best approach to analysing trait evolution on such a phylogenetically broad dataset. Polytomies in this tree were resolved by adding short branches that represented approximately 1 million years.

Because we lacked measurements for some structures of some species, trees were pruned to include only tips with non-missing data in both traits for each pairwise trait comparison. Phylogenetic independent contrasts (PICs; Felsenstein, 1985) were calculated using the *pic* function in the package *ape* for R (version 2.14.1; R Core Team, 2012). PICs quantify the amount of trait disparity that occurs at each node in a phylogeny based on the trait values of the descendent taxa or nodes and the branch lengths between the parent and daughter nodes. PICs are a way to control for the non-independence of sampling related lineages. Independent contrast methodology allows one to test whether two traits repeatedly co-evolve in a coordinated way. For example, a significant, positive correlation between PICs for two traits would mean that large divergences in one trait repeatedly occur at the same nodes as large divergences in the second trait. Thus, significant correlations between PICs of two traits are commonly used to determine whether two traits have undergone correlated evolution. The conservative, non-parametric Spearman rank test was used to test for pairwise correlations between ln-transformed traits and for pairwise correlations between PICs. All reported *P* values have been adjusted for the number of simultaneous comparisons using the Bonferroni correction.

Results

In total, our dataset included 132 species from 90 genera and 52 families (Supplementary Table S1). Plant structure was a significant predictor of VLA (F=133.79, P <0.0001), and all pairwise, post-hoc, Bonferroni-adjusted comparisons were also highly significant (all pairwise P <0.0001; Fig. 1). Leaf VLA ranged from 1.09 for *Disporopsis pernyi* to 12.68 mm mm^{-2} for *Calycanthus occidentalis*, sepal VLA ranged from 0.50 for *Smilacina stellata* to 12.70 mm mm^{-2} for *Scutellaria californica*, and petal VLA ranged from 1.00 for *Disporopsis pernyi* to 6.65 mm mm^{-2} for *Calystegia stebbinsii*. Despite substantial overlap in the ranges of VLA for these three structures, mean leaf VLA (5.47 mm mm^{-2}; P <0.001) was significantly higher than mean sepal VLA (3.78 mm mm^{-2}; P <0.001), which was higher than mean petal VLA (2.44 mm mm^{-2}; P <0.001).

Some clades differed significantly in their VLA values (Fig. 2). Tukey post-hoc analyses showed that monocot leaf VLA was significantly lower than leaf VLA of the Ranunculales, fabids, malvids, and asterids (all pairwise P <0.001). While slightly higher than the monocots, leaf VLA of basal angiosperms fell within the lower tails of the ranges for all the other major clades. Sepal VLA showed similar patterns to leaf VLA. Tukey post-hoc analyses showed fabid, malvid, and asterid sepal VLA to be

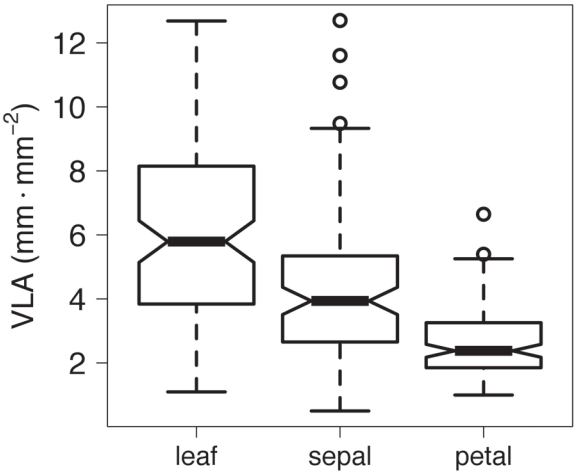

Fig. 1. Boxplot of VLA for leaves, sepals, and petals. All pairwise differences are highly significant (P <0.001). Median values are indicated by the solid line in the middle of the boxes.

significantly higher than monocot sepal VLA (all pairwise P <0.001). Malvid sepal VLA was also marginally higher than Ranunculales sepal VLA (P=0.088). Interestingly, there were no significant differences between any pairwise clade comparisons of petal VLA, although the sampled non-graminoid monocots had generally lower petal VLA than the other major clades.

All pairwise correlations between ln-transformed traits were also significant (Fig. 3A–C); species with higher leaf VLA generally also had higher sepal and petal VLA. The highest correlation coefficient was between leaves and sepals [r=0.57, degrees of freedom (df)=84, P <0.001]. Leaf and petal VLA (r=0.34, df=102, P <0.001) were more strongly correlated than sepal and petal VLA (r=0.32, df=90, P <0.01). However, correlations of independent contrasts showed somewhat different patterns (Fig. 3D–F). There was no significant correlation between leaf and petal contrasts (r=0.13, df=102, P=0.19), although there were significant correlations between leaf and sepal VLA contrasts (r=0.40, df=84, P <0.001) and between sepal and petal VLA contrasts (r=0.27, df=90, P <0.05). These patterns of correlations observed between traits and PICs across the entire phylogeny were consistent with cladewise trait and PIC correlations. In particular, no clade showed a significant correlation between leaf and petal VLA contrasts (data not shown).

Discussion

Our results strongly support the idea that VLA has evolved independently in petals and leaves (Fig. 3), demonstrating that vegetative and reproductive organs are developmentally modular. Furthermore, because VLA is so critical to water supply in leaves, flowers and leaves may also be physiologically modular. These results support observations that flower and fruit water status can remain relatively unaffected by large variation in plant water status (Trolinder *et al.*, 1993).

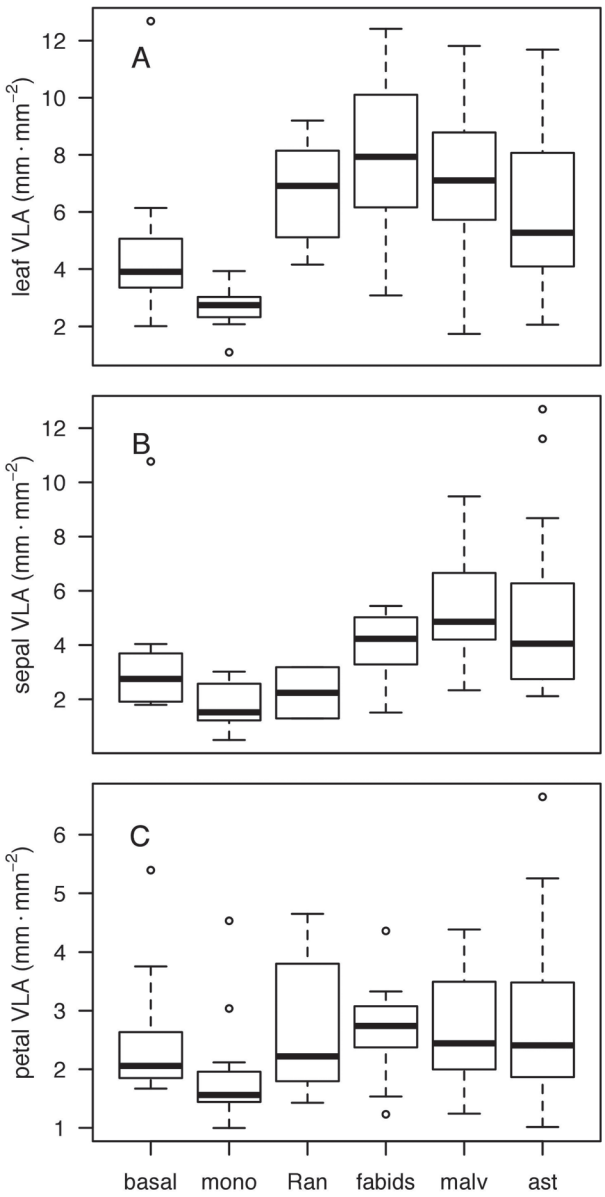

Fig. 2. Boxplots of VLA for (A) leaves, (B) sepals, and (C) petals for each major clade included in this study. Basal, magnoliids and Austrobaileyales; mono, monocots; Ran, Ranunculales; malv, malvids; ast, asterids. See Supplementary Table S1 for list of species in each clade.

Consistent with our first hypothesis, floral structures exhibited significantly lower VLA than leaves (Fig. 1). In particular, mean petal VLA was less than half that of leaf VLA. The maximum measured leaf and sepal VLA values were similar, reflecting the functional similarities of many leaves and sepals. However, the maximum petal VLA was approximately half that of either leaves or sepals. In leaves, a high VLA is associated with higher efficiency because it enables higher rates of transpiration and photosynthesis per unit leaf area and because conduits in leaves with high VLA have higher intrinsic hydraulic conductance (Brodribb *et al.*, 2007; Feild and Brodribb, 2013). In contrast, a low VLA in non-photosynthetic petals may translate into higher efficiency because less water

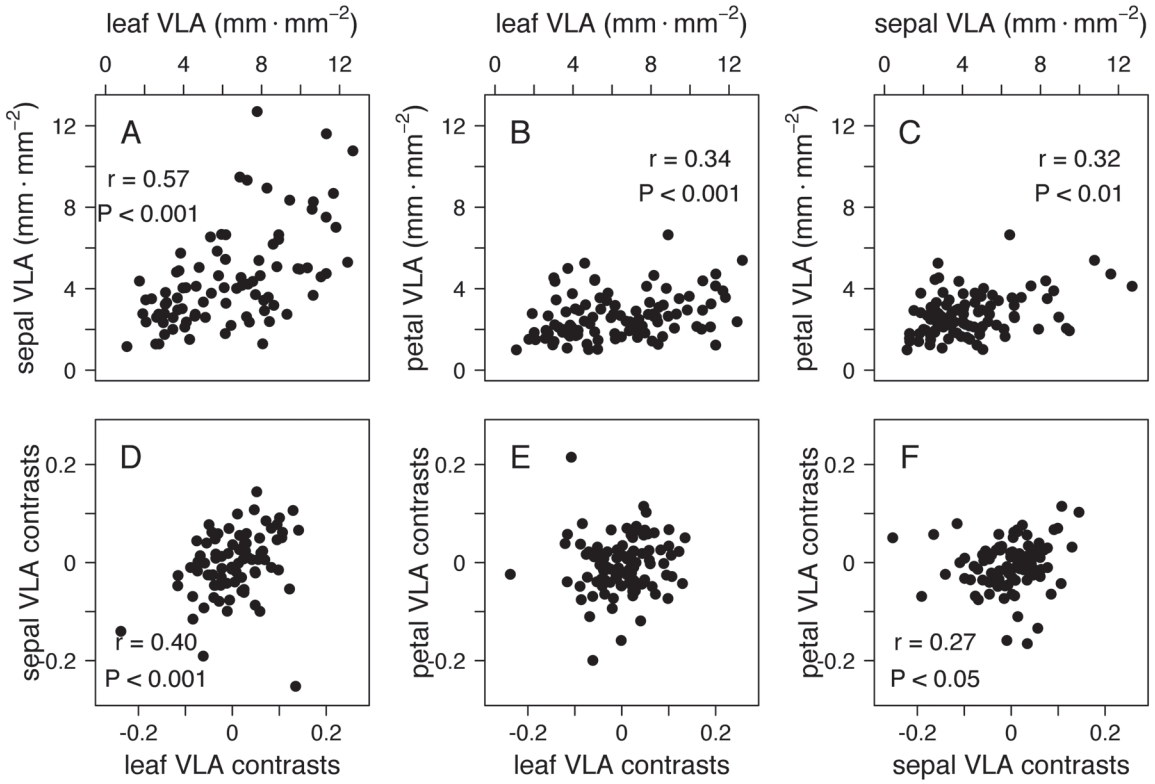

Fig. 3. Pairwise (A–C) trait and (D–F) phylogenetic independent contrast correlations. Correlation coefficients and *P* values are shown for statistically significant correlations based on Spearman rank tests.

and carbon are needed for a given floral display area. However, if the intrinsic hydraulic conductance of conduits in petals is associated with VLA as it is in leaves, then the carbon costs of a high VLA in petals may not be that large. Recent work on petal development in *Arabidopsis thaliana* suggests that leaf and petal shape may be controlled by variations on the same underlying developmental process. Such commonality may constrain the range of possible forms in both structures while allowing selection to act on each structure independently (Sauret-Güeto *et al.*, 2013), therefore producing often similar venation patterns in different structures (Melville, 1960, 1969).

Our second hypothesis that basal angiosperm flowers developed higher VLA than flowers of more recently derived eudicot lineages was not supported (Fig. 2). The mean and maximum vein densities of basal angiosperm tepals were not any higher than those of petals from the other major clades. Such a result is perhaps not surprising because ancient angiosperm lineages (i.e. Austrobaileyales) possess very low leaf VLA (Boyce *et al.*, 2009; Feild *et al.*, 2009*a*). The difference between leaf and tepal VLA among basal lineages (Austrobaileyales and magnoliids) and the monocots was much smaller than the differences between leaf and petal VLA of the more recently derived eudicot clades (Fig. 2). Leaf VLA of these more recently derived lineages increased dramatically during the Cretaceous (Boyce *et al.*, 2009; Brodribb & Feild, 2010), leading to large differences between leaf and petal VLA. Thus, in contrast to our hypothesis, selection may not necessarily have favoured reductions in petal VLA. Rather, petals may not have been exposed to the strong selection for high VLA in leaves if different developmental processes give rise to leaf and petal VLA.

The large range of petal VLA among the eudicots may reflect the greater diversity of ecological contexts in which these species exist compared with the magnoliids and Austrobaileyales (Feild *et al.*, 2009*a*). Among the eudicots, there was remarkable variation in petal VLA even within some genera, further supporting the evolutionary lability of this trait. Alternatively, the large range of petal VLA among all eudicot clades may reflect the myriad developmental origins of the petal (Zanis *et al.*, 2003). Petals are derived from stamen-like structures (andropetaloidy) in some lineages or from bract- or leaf-like structures (bracteopetaloidy) in other lineages (Irish, 2009). Apart from their often similar gross morphologies, stamen-derived and bract-derived petals may differ in some key anatomical or physiological traits.

Although petals of reportedly phloem-hydrated eudicot flowers (Trolinder *et al.*, 1993; Chapotin *et al.*, 2003) and *Magnolia* tepals have lower VLA than xylem-hydrated *Magnolia* tepals (Feild *et al.*, 2009*b*), there were no significant differences in petal VLA between major clades (Fig. 2C), implying that petal VLA may not reliably indicate xylem versus phloem hydration. Despite reports that a variety of fruits (Ho *et al.*, 1987; Greenspan *et al.*, 1994; Dichio *et al.*, 2002) and some flowers (Trolinder *et al.*, 1993; Chapotin *et al.*, 2003) are phloem hydrated, there is no general consensus on the frequency of phloem hydration of flowers. Furthermore, reports of phloem hydration of flowers have been equivocal. Flowers are not better hydrated than subtending stem xylem,

nor is it clear whether maintaining higher water potentials of flowers compared with subtending stems requires hydration by the phloem. Interestingly, recent work on grapes (Choat *et al.*, 2009) and tomatoes (Windt *et al.*, 2009), both of which were previously reported as being phloem hydrated (Ho *et al.*, 1987; Greenspan *et al.*, 1994), has shown that phloem-delivered water may buffer fruit water status from variation in xylem import rather than supplying the predominant amount of water to the fruit. The water supply dynamics of flowers and fruits may be more complicated than the simplistic model of hydration by either the xylem or the phloem, and water may flow bidirectionally to and from reproductive organs (Johnson *et al.*, 1992). For example, while xylem sap flows towards mango (*Mangifera indica*) inflorescences during the day (Higuchi & Sakuratani, 2005), xylem sap flows away from developing fruits during the day and towards them at night (Higuchi & Sakuratani, 2006).

Our third hypothesis that VLA has evolved independently in flowers and leaves was also mostly supported by our results (Fig. 3D–F). Patterns of venation in petals and leaves have been thought to be correlated with venation patterns in leaves (Melville, 1960, 1969). Indeed, our results showed that, when not controlling for phylogeny, all pairwise correlations of VLA traits were highly significant. However, phylogenetic independent contrast correlations were not similarly significant. The stronger correlation between sepal and leaf contrasts than between sepal and petal contrasts suggested that functional constraints may trump developmental constraints. These nuanced results illustrate the interplay between evolution, development, and physiology. We speculate that sepal and bract VLA have evolved with both leaves and petals because sepals and bracts commonly perform vegetative functions, such as photosynthesis and protecting the developing flower, yet develop at the same time as reproductive structures. These results generally support the idea of functional modularity between vegetative and reproductive structures (Fig. 3E; Berg, 1959; 1960). Despite the expectation under the Berg hypothesis that selection should favour stronger correlations among floral traits than between floral and leaf traits, our results showed that sepal VLA contrasts and leaf VLA contrasts were more strongly correlated than were sepal and petal VLA contrasts (Fig. 3D, F). Such a result may be expected if the function of VLA is different in petals than it is in sepals and leaves. For example, phloem hydration of petals may alleviate the need for veins to function for water transport, as they probably do in sepals and leaves.

Studies of morphological trait variation have shown similar results and have highlighted the genetic basis for reproductive and vegetative modularity. In *Dalechampia scandens*, floral bract length was more coupled to variation in floral traits related to pollination than it was to variation in leaf traits, including leaf size (Pélabon *et al.*, 2011). Quantitative trait loci mapping of leaf and flower size traits in *A. thaliana* showed large, positive genetic correlations among either flower or leaf traits, but not between flower and leaf traits (Juenger *et al.*, 2005). The degree to which floral and foliar traits are decoupled may vary unpredictably among species (Armbruster *et al.*, 1999; Hansen *et al.*, 2007) and may depend on the traits in question. Many of these studies, including those of Berg (1959, 1960), have focused on variation in the size and shape of flowers with little regard for physiological traits. Athough they may share developmental motifs that define the ranges of possible variation, physiological traits of leaves and petals may arise from uncorrelated selection pressures.

In conclusion, new insights into the water relations of reproduction are transforming our understanding of angiosperm evolution and plant water transport (Higuchi & Sakuratani, 2005, 2006; Feild *et al.*, 2009*a*,*b*). In the present study, we used a novel dataset to address long-standing questions about the comparative evolution of flower and leaf traits, focusing on vein length per area, a trait functionally important to leaves and that evolved rapidly among angiosperm lineages (Brodribb & Feild, 2010; Feild *et al.*, 2011). Despite increased leaf VLA among recently derived angiosperm lineages, petal VLA has evolved independently and has remained relatively low. These results suggest that vegetative and reproductive structures may be developmentally modular. Future studies characterizing the linkages between floral physiological traits, such as VLA or stomatal density, and hydraulic functioning throughout a flower's lifespan would further refine our understanding of the evolution and ecophysiology of flowers.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Table S1. List of species, their families, their major clade, and the collection sites analysed in this study.

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