New Phytologist

Research

Similar geometric rules govern the distribution of veins and stomata in petals, sepals and leaves

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Received: *4 December 2017*
Accepted: *9 April 2018*

New Phytologist (2018)
doi: 10.1111/nph.15210

Key words: epidermal cell size, floral evolution, hydraulics, stomatal density, vein density.

Summary

- Investment in leaf veins (supplying xylem water) is balanced by stomatal abundance, such that sufficient water transport is provided for stomata to remain open when soil water is abundant. This coordination is mediated by a common dependence of vein and stomatal densities on cell size. Flowers may not conform to this same developmental pattern if they depend on water supplied by the phloem or have high rates of nonstomatal transpiration.
- We examined the relationships between veins, stomata and epidermal cells in leaves, sepals and petals of 27 angiosperms to determine whether common spacing rules applied to all tissues.
- Regression analysis found no evidence for different relationships within organ types. Both vein and stomatal densities were strongly associated with epidermal cell size within organs, but, for a given epidermal cell size, petals had fewer veins and stomata than sepals, which had fewer than leaves.
- Although our data support the concept of common scaling between veins and stomata in leaves and flowers, the large diversity in petal vein density suggests that, in some species, petal veins may be engaged in additional functions, such as the supply of water for high cuticular transpiration or for phloem delivery of water or carbohydrates.

I. Introduction

Flowers are one of the key evolutionary innovations that characterize angiosperm diversification, with selection of floral traits by pollinators considered to be an important driver of this diversity (Rosas-Guerrero *et al.*, 2014). However, because the capacity to match hydraulic supply in the leaf with demand for liquid water is emerging as an important driver of plant adaptation (Brodribb *et al.*, 2013), it seems timely to consider whether these processes also affect floral traits. An understanding of the evolution and development of floral traits in the broader context of whole-plant physiology would provide new insights into why flowering plants have prevailed across ecosystems globally. Despite this, many gaps remain in our understanding of the fundamental links between floral structure and physiological function. One such gap is the relationship between the tissues regulating flower hydration through water supply (the venation) and those that determine the transpiration rate in flowers.

Flowers are exposed to the same evaporative environment as leaves, and often possess stomata (Lipayeva, 1989), necessitating a water supply throughout their lifespan. In leaves, water supply, as determined by the vein density (total minor vein length per unit area; $mm\,mm^{-2}$) (Sack & Frole, 2006; Brodribb *et al.*, 2007), is well coordinated with the potential for evaporation, as determined by the stomatal density (total number of stomata per unit area; mm^{-2}) (Brodribb & Jordan, 2011; Carins Murphy *et al.*, 2012, 2014, 2016; Blonder & Enquist, 2014; Blonder *et al.*, 2017). This coordination appears to maintain a balance between the investment in vascular tissue and the production of stomatal guard cells (Edwards, 2006; Dunbar-Co *et al.*, 2009; Carins Murphy *et al.*, 2012, 2014, 2016; Zhang *et al.*, 2012). Having too many veins per stomata means that carbon uptake cannot compensate for investment in the production of relatively costly xylem (Lambers & Poorter, 1992) and displacement of photosynthetic tissue, whereas having too few veins per stomata reduces carbon uptake because insufficient water is supplied to allow stomata to open even when water is abundant. Despite considerable interest in the influence of floral water balance on whole-plant fitness (Galen, 2000), the evolution of the vascular system of flowers has only recently received focused scientific attention (Roddy *et al.*, 2013, 2016), and little is known about the relationships between the tissues responsible for the regulation of plant water status (the veins and stomata) in flowers.

Flowers are distinguished from leaves, in part, by being relatively short-lived and fixing only limited amounts of carbon. This lessens the need for the high porosity to CO_2 (and water vapor) required to maintain high rates of photosynthesis (Roddy & Dawson, 2012; Teixido & Valladares, 2014). Yet, to attract pollinators, and thus promote pollination success, flower turgor must be maintained throughout flowering often under desiccating conditions that can lead to wilting. Thus, an understanding of whether veins that supply water to flowers are coordinated with stomatal densities, in a similar manner to those in leaves, could indicate whether flowers can be considered to function as leaves in terms of water relations. Alternatively, differences in the developmental geometry of these tissues in flowers may indicate evolutionary differences in the demands on flower veins, beyond carrying xylem water for stomatal transpiration. In addition to their divergent functions, there are some other reasons to suspect that the structure/function relationships associated with the maintenance of flower and leaf water balance may be under different selective constraints. First, the large range of vein densities found in leaves is not replicated in petals (Roddy *et al.*, 2013). Furthermore, previous studies have shown that phloem can supply water to the flowers of many species (Trolinder *et al.*, 1993; Chapotin *et al.*, 2003; De la Barrera & Nobel, 2004; Galen, 2005). This could alter the relationship between veins and water demand because phloem may provide an alternative pathway for transport. If petals were using phloem as the primary water transport mechanism, more veins would be required in petals than in leaves to maintain the same number of open stomata, because water transport through the phloem is much less efficient than through xylem cells.

There is some evidence to suggest that the flowers from members of the early-divergent angiosperm clades *Illicium*, *Magnolia* and *Calycanthus* (basal ANA groups and magnoliids of angiosperms) are hydrated predominantly by the xylem (Trolinder *et al.*, 1993; Chapotin *et al.*, 2003; De la Barrera & Nobel, 2004; Galen, 2005; Roddy *et al.*, 2018). Thus, xylem hydration of flowers may be the ancestral state in angiosperms (Feild *et al.*, 2009). The petals of eudicot species also show evidence of good hydraulic connection with the rest of the plant, with xylem cavitation in petals occurring synchronously or earlier than in leaves (Zhang & Brodribb, 2017). Synchronous cavitation suggests that leaves and petals are hydrated by a common basal hydraulic network, and that the water potential of these two organs is coordinated, reflecting some hydraulic continuity between them. However, many authors have suggested that the flowers of eudicots may be hydrated by phloem tissue (Chapotin *et al.*, 2003), meaning that the costs of water supply (in terms of investment in vascular infrastructure) in these species would differ between leaves and flowers, as well as the structure/function relationships that maintain water balance. Indeed, flowering plants may use a variety of mechanisms to keep their flowers turgid and functional during flowering. For example, some flowers have high hydraulic capacitance (Chapotin *et al.*, 2003; Roddy *et al.*, 2018), which can delay water potential declines and help isolate flower water status from changes in the water status of other parts of the plant.

Coordination in the density of veins and stomata in leaves is expected because of the xylem-dominated delivery of water to leaves. Changes in epidermal cell size during light acclimation facilitated the coordination between leaf vein and stomatal density in the subtropical rainforest tree, *Toona ciliata* (Carins Murphy *et al.*, 2012). Thus, larger epidermal cells in leaves grown under low irradiance led to larger leaves with lower densities of veins and stomata than leaves grown under high irradiance. This critical coordination of cell size during plastic adaptation of leaves to different evaporative and photosynthetic conditions of high and low irradiance gave rise to the suggestion that changing cell size could be a key mechanism for plant ecological evolutionary adaptation (Brodribb *et al.*, 2013). Furthermore, the association between epidermal cell size, vein density and stomatal density in leaves may be a general developmental rule in vascular plants, and allow coordinated evolution of these traits (Carins Murphy *et al.*, 2016, 2017). Recently, leaf vein density, stomatal density and cell size have been linked to large-scale changes in genome size through evolutionary time, with genome downsizing in angiosperms providing a lower limit for cell size, and thus allowing greater densities of veins and stomata (Simonin & Roddy, 2018). However, in floral organs, basic information about the correlation between vein density, stomatal density and cell size remains unclear. In flowers, in which the tissue responsible for water delivery remains a matter of debate, knowledge of the relationships between xylem, stomatal and epidermal tissues could be used to support different models of water delivery. For example, the very low hydraulic conductance of the phloem would mean that tissues depending on phloem for the delivery of water would be expected to require a much higher density of venation to support an equivalent transpiration rate in a xylem-supplied tissue.

To address this deficiency in our understanding of flower water supply, we examined the relationships between vein density, stomatal density and epidermal cell size in the leaves, sepals and petals of a diverse sample of 27 species of angiosperm. Veins are known to be more widely spaced in petals and sepals than in leaves (Roddy *et al.*, 2013). We hypothesized that, if flowers shared a similar xylem-dominated water supply as leaves, the density of stomata and veins would remain proportional in petals and sepals, and hence both would be more widely spaced in floral structures than in leaves. Furthermore, a close geometric relationship between stomata, veins and epidermal cells within leaves and flowers would demonstrate dilution to be a general coordinating pattern in plant organs. To test this, we examined the relationships between veins and stomata among organs, and then compared these observed relationships between veins, stomata and epidermal cells within leaves, sepals and petals with modeled relationships which assumed that vein and stomatal density were 'passively diluted' by epidermal cell expansion to maintain a constant ratio between vein and stomatal density.

II. Materials and Methods

Plant material

Plant material was collected from living specimens growing at the Sandy Bay campus of the University of Tasmania and the Royal Tasmanian Botanical Gardens, both located in Hobart,

Tasmania, Australia. In total, 27 angiosperm species (Table 1) were sampled. These species were chosen for three reasons: they were from morphologically and ecologically diverse groups that varied in distribution; they (or their close relatives) were represented in molecular phylogenies, thus allowing phylogenetically independent analysis of relationships; and they had different flower types (e.g. the petals of some species had stomata, whereas other species had petals without stomata). Thus, these species were selected to obtain a substantial amount of morphological and phylogenetic diversity from within the angiosperm phylogeny. Healthy, recently opened flowers and fully expanded leaves were collected from three to five individuals per species.

Anatomical traits

Leaves and flowers were collected simultaneously, preserved in FAA (formalin : acetic acid : alcohol : distilled water = 10 : 5 : 50 : 35) and transported to the laboratory for microscopic analysis. Vein density ($mm\ mm^{-2}$), stomatal density (mm^{-2}), epidermal cell size (mm^2), stomatal size (mm^2) and stomatal index were quantified from paradermal sections. A sample of *c.* 100 mm^2 was taken from midway between the midrib and margin of three to five leaves per species. Entire sepals and petals were sampled when they were smaller than 100 mm^2. Multiple sections were collected from three to eight petals and sepals of *Passiflora tarminiana* because vein densities in the sepals and petals of this species were highly variable. Sections were prepared by soaking in commercial household bleach (50 $g\ l^{-1}$ sodium hypochlorite and 13 $g\ l^{-1}$ sodium hydroxide) until cleared of all pigment. Bleach was removed by thoroughly rinsing in water and sections were stained with 1% toluidine blue for 30 s to color the lignin-rich veins. Sections were then mounted in phenol glycerine jelly. Five fields of view at ×10 magnification (field of view area, 0.56 mm^2) were photographed from each section using a Nikon Digital Sight DS-L1 camera (Melville, NY, USA) mounted on a Leica DM 1000 microscope (Nussloch, Germany). Sepals and petals of species with very low vein density were scanned at 2400 dpi using a flatbed scanner. Vein density was measured as the total length of vascular tissue per mm^2 of surface area, and stomatal density as the total number of stomata per mm^2 of surface area, using the image analysis software ImageJ (National Institutes of Health, Bethesda, MD, USA). Partial stomata and epidermal cells were included in density counts if visible along the top and right-hand edge of photomicrographs and discarded if visible along the bottom and left-hand edge (Kubínová, 1994). Stomatal size was determined as the mean area of five stomata (the combined area of a pair of guard cells) per field of view. Epidermal cell size was subsequently calculated as in Carins Murphy

Table 1 A list of the studied species

Family	Genus	Species	Geographic distribution	Growth form
Schisandraceae	*Illicium*	*floridanum*	Southeastern USA	Shrub
Magnoliaceae	*Magnolia*	*grandiflora*	Southeastern USA	Woody
Orchidaceae	*Cymbidium*	*hookerianum*	India, China and Vietnam	Herb
Amaryllidaceae	*Agapanthus*	*campanulatus*	Southern Africa	Herb
Ranunculaceae	*Aquilegia*	*vulgaris*	Europe	Herb
Aphanopetalaceae	*Aphanopetalum*	*resinosum*	Eastern Australia	Vine
Saxifragaceae	*Bergenia*	*purpurascens*	Southwestern and eastern China, southern Tibet	Herb
Hydrangeaceae	*Philadelphus*	*purpurascens*	Yunnan and Sichuan in southwestern China	Shrub
Ericaceae	*Rhododendron*	*camtschaticum*	China, Kashmir, Nepal, Bhutan and Sikkim	Shrub
Primulaceae	*Cyclamen*	*persicum*	Greece, Syria and Lebanon	Herb
Pittosporaceae	*Pittosporum*	*tenuifolium*	New Zealand	Woody
Caprifoliaceae	*Weigela*	*florida*	China	Shrub
Scrophulariaceae	*Alonsoa*	*meridionalis*	Peru	Herb
Apocynaceae	*Vinca*	*minor*	Central and southern Europe	Herb
Solanaceae	*Solanum*	*lycopersicum*	South America	Herb
Onagraceae	*Fuchsia*	*magellanica*	Southern South America	Sub-shrub
Tropaeolaceae	*Tropaeolum*	*majus*	South America, Peru and Brazil	Herb
Rutaceae	*Correa*	*pulchella*	South Australia	Shrub
Rutaceae	*Crowea*	*exalata*	Australia	Herb
Rosaceae	*Rosa*	*rugosa*	Eastern Asia	Shrub
Rosaceae	*Prunus*	*cerasus*	Europe and southwest Asia	Woody
Fabaceae	*Pisum*	*sativum*	Southwest Asia and northeast Africa	Herb
Polygalaceae	*Polygala*	*myrtifolia*	South Africa	Shrub
Picrodendraceae	*Micrantheum*	*hexandrum*	Australia	Shrub
Passifloraceae	*Passiflora*	*tarminiana*	Tropical South America	Vine
Oxalidaceae	*Oxalis*	*acetosella*	Europe and Asia	Herb
Cunoniaceae	*Bauera*	*rubioides*	Australia and Tasmania	Shrub

et al. (2016) using the following equation:

$$\text{Epidermal cell size} = (1 - (\text{stomatal density} \times \text{mean stomatal size}))/\text{epidermal cell density},$$

where traits are expressed per mm^2. The stomatal index was calculated according to Salisbury (1927).

To examine the relationships between genome size and epidermal cell size in leaves, sepals and petals, we obtained genome size data for the species included in our study (Supporting Information Table S1). Genome size data from congeneric species were used when data were not available for the actual species from the Kew plant DNA C-values database (Release 6.0, December 2012) (Bennett & Leitch, 2012).

'Passive dilution' models

Expected values of vein density and epidermal cell size assuming passive dilution were calculated separately for leaves, sepals and petals, following the method outlined in Carins Murphy *et al.* (2016). The 'passive dilution' hypothesis argues that vein and stomatal density are predominantly coordinated by the expansion of epidermal cells (Carins Murphy *et al.*, 2016). The models of expected values assume that vein and stomatal densities are uniquely related to epidermal cell size and that the epidermis comprises only epidermal and stomatal cells with a constant ratio between them (reflected by a stable stomatal index). Data were transformed to ensure that the relationships expected under a model of passive dilution were linear (i.e. 1/epidermal cell size was compared with stomatal density, $1/\sqrt{}$epidermal cell size with vein density and vein density with $\sqrt{}$stomatal density). Species with petals without stomata were omitted from the analysis of relationships between vein and stomatal density, and epidermal cell size and stomatal density, in petals. Consequently, epidermal cell size was calculated for the range of stomatal densities found in the observed dataset using the mean stomatal index of each organ. According to previous studies (Franks & Beerling, 2009; Carins Murphy *et al.*, 2016) and the correlation observed between stomatal size and stomatal density in leaves and sepals in this study (Fig. S1), we fitted a linear regression to the data (stomatal size = $a \times$ stomatal density^b) based on the pooled data from all species to account for this association. Mean stomatal size was used when modeling relationships in petals, as, in this organ, stomatal size was independent of changes to stomatal density (Fig. S1). The relationship between vein density and epidermal cell size was modeled based on the assumption that vein density was uniquely driven by dilution by cell expansion, and thus that vein length is associated with a fixed proportion of the perimeter of an epidermal cell. According to this assumption, epidermal and xylem cells would expand in unison. Therefore, if a theoretical epidermal cell was square-shaped and an associated xylem cell was the length of one side of the epidermal cell, they would always remain in contact on that side as they expanded. Thus, a geometric model of vein density as a function of epidermal cell size was determined for a fixed stomatal index (mean per organ) incorporating either a predicted stomatal size according to the stomatal density in leaves and sepals, or mean stomatal size for all petals as outlined above. Finally, the expected relationship between vein density and stomatal density was modeled by combining the relationships above (Fig. S2).

Statistical analysis

All statistical analyses were performed in R (R Core Team, 2014). Coordination of stomatal and vein density across species and organ types (leaf, sepal or petal) was tested using analysis of covariance (ANCOVA), with vein density as the response variable and $\sqrt{}$stomatal density, organ type and their interaction as effects. The effect of organ type on stomatal size across species was assessed using two-way analysis of variance (ANOVA) and Tukey's Honest Significant Difference (HSD) in R. Log and square root transformations were applied to normalize the data. We obtained a phylogenetic relationship of all our studied species using the online version of Phylomatic (Smith *et al.*, 2011). Equal branch length was assumed in the present study to minimize type I error rates (Ackerly, 2000; Fu *et al.*, 2012). As we lacked some traits (e.g. stomata) in some species, for each pair of traits, the trees were trimmed to only include non-missing data of species. Possible phylogenetic correlations were assessed via phylogenetically independent contrasts (PICs) analysis using the 'pic' function in the R package PICANTE (Felsenstein, 1985; Webb *et al.*, 2008).

To test whether relationships between stomatal density and epidermal cell size were consistent across organs, we performed ANCOVA with stomatal density as the response variable and 1/epidermal cell size, organ type and their interaction as effects. We performed similar analyses on vein density and $1/\sqrt{}$epidermal cell size. To test whether the observed vein and stomatal densities followed relationships consistent with passive dilution across species in all three organs, we used linear regression to compare the difference between observed and expected values with observed values. Specifically, we compared the difference in observed and expected epidermal cell size with observed stomatal density, and the difference in observed and expected vein density with the $\sqrt{}$observed epidermal cell size. Thus, the presence of significant regressions would be evidence for processes other than passive dilution operating on stomatal or vein density.

III. Results

Variation in vein density, stomatal density and epidermal cell size among leaves, sepals and petals

At the broadest level, we found that the overall mean values of vein and stomatal density ranked from highest in leaves to lowest in petals, and that these values varied in a clearly proportional fashion (Fig. 1). Specifically, vein densities in leaves (mean ± SE = 6.05 ± 0.53, $n = 27$) were 1.8 times greater than those in sepals (3.45 ± 0.29, $n = 20$) ($P < 0.001$) and 2.7 times

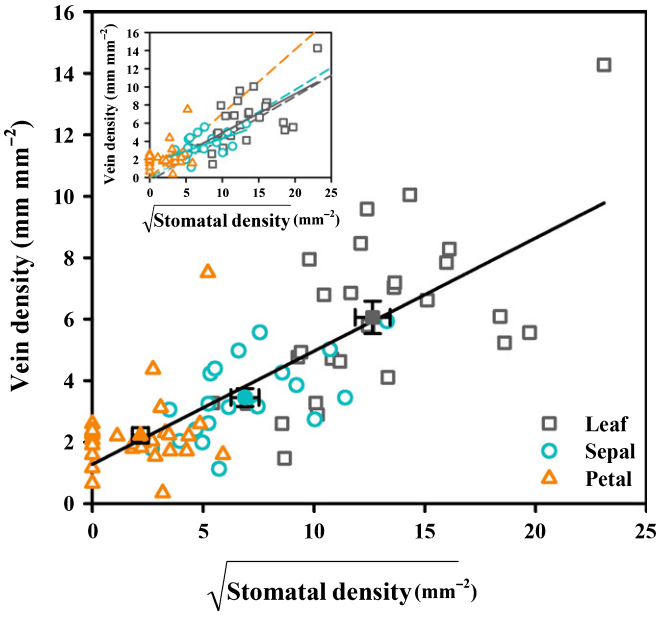

Fig. 1 Observed relationships between vein and √stomatal density in the leaves, sepals and petals of 27 angiosperm species. Analysis of covariance (ANCOVA) indicated that there was a highly significant positive linear relationship between vein and √stomatal density across all organ types (solid line) ($r^2 = 0.61$, $P < 0.001$), but found no evidence of different slopes or intercepts among organ types (Supporting Information Table S2). The insert shows the observed relationships between vein and √stomatal density within all organ types (solid lines) compared with modeled relationships (dashed lines). Individual regressions for organ types found that highly significant linear relationships are described in leaves ($r^2 = 0.41$, $P < 0.001$) and sepals ($r^2 = 0.36$, $P < 0.01$), but not petals.

greater than those in petals (2.22 ± 0.25, $n = 27$) ($P < 0.001$) (Figs 2, 3a). However, vein densities in sepals were 1.6 times greater than those in petals ($P < 0.05$). Similarly, stomatal densities in leaves (175.75 ± 22.36, $n = 27$) were 3.2 times greater than those in sepals (55.06 ± 10.09, $n = 20$) ($P < 0.001$) and 14.5 times greater than those in petals (12.09 ± 2.13, $n = 18$) ($P < 0.001$) (Figs 2, 3b). Stomatal densities in sepals were 4.6 times greater than those in petals ($P < 0.001$). Epidermal cell size did not vary significantly between leaves (1.56 ± 0.23, $n = 27$) and sepals (1.66 ± 0.19, $n = 20$) ($P > 0.05$), or sepals and petals (2.19 ± 0.33, $n = 27$) ($P > 0.05$). However, epidermal cells were significantly larger in petals than in leaves ($P < 0.05$) (Fig. 3c). Epidermal cell size tended to increase from leaves to sepals to petals.

Geometric relationships between veins, stomata and epidermal cells

There was a significant positive correlation between vein density and √stomatal density among all species in both leaves and sepals, but not petals, whether or not phylogeny was considered (insert in Fig. 1; Table 2). However, ANCOVA (Table S2) showed that there was no evidence that the relationship between vein density and √stomatal density differed among organ types (when species with and without stomata on their petals were considered). In particular, the mean scores for leaves, petals and sepals showed identical ratios of veins to √stomata (Fig. 1). Similar positive correlations were found between vein density and 1/√epidermal cell size (Fig. 4; Table 2) and between stomatal density and 1/epidermal cell size (Fig. 5; Table 2) in all organs. However, the relationship between stomatal density and 1/epidermal cell size was weaker in sepals and petals than in leaves, and stomatal density and stomatal index were positively correlated among all species in sepals and petals (Fig. 6; Table 2). This relationship was absent in leaves, as would be predicted from the strong association between

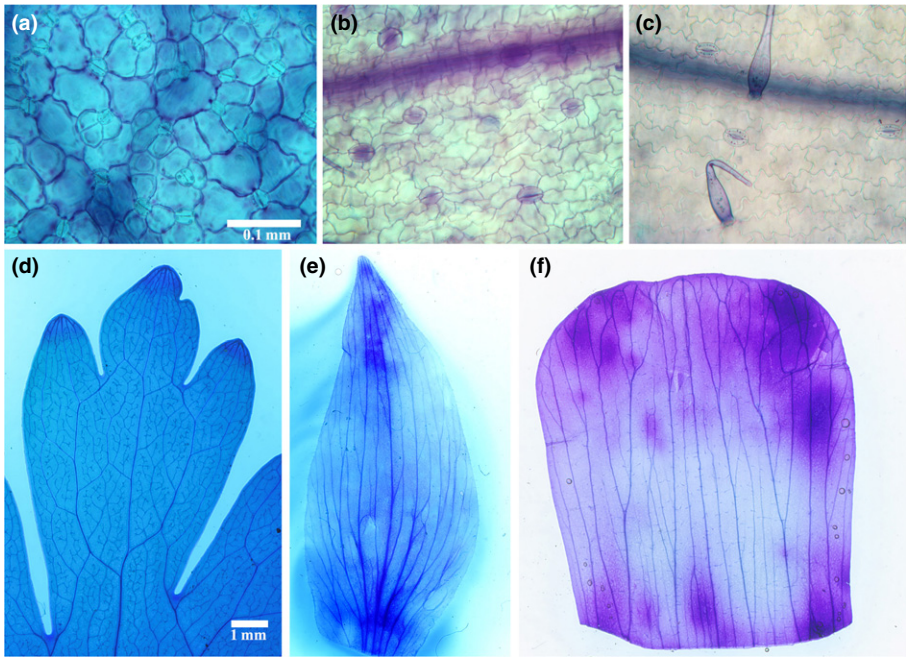

Fig. 2 Photomicrographs showing the stomata and vascular structure of (a, d) a cleared leaf, (b, e) sepal and (c, f) petal from a representative species *Aquilegia vulgaris*.

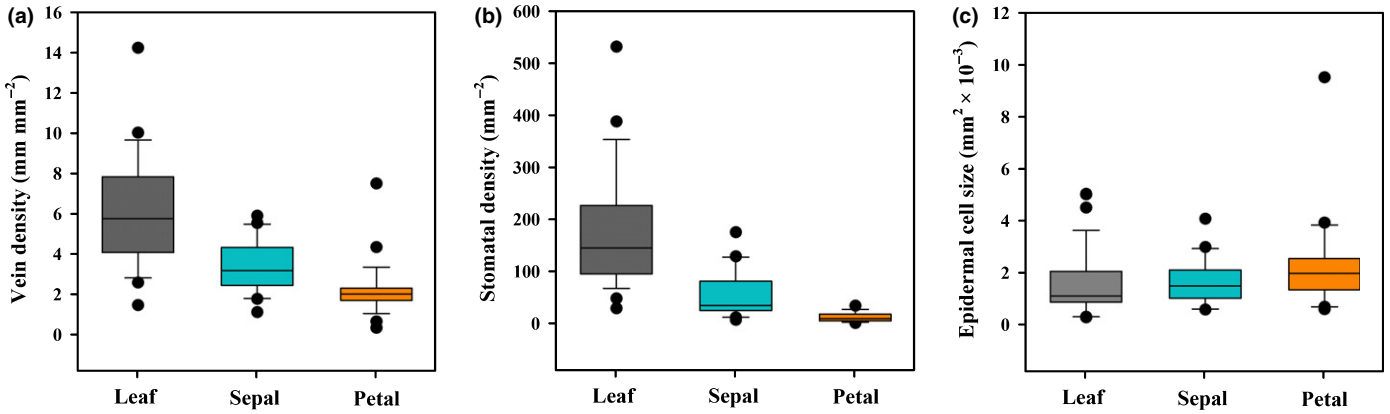

Fig. 3 Variation in (a) vein density, (b) stomatal density and (c) epidermal cell size among leaves, sepals and petals of 27 angiosperm species. The median for each dataset is indicated by the black center transverse line, and the black first and third transverse lines are the upper and lower margin lines. The circles represent outliers.

Table 2 Pearson correlation coefficients and phylogenetic independent contrast correlations among pairs of traits

Trait pair	Organ type	Pearson correlation coefficient (r^2)	Phylogenetic correlation coefficient (r^2)
Vein density and √stomatal density	Leaf	0.41***	0.58***
	Sepal	0.36**	0.38**
	Petal	0.10ns	0.09ns
Stomatal density and 1/epidermal cell size	Leaf	0.73***	0.77***
	Sepal	0.30**	0.31**
	Petal	0.33**	0.41***
Vein density and 1/√epidermal cell size	Leaf	0.28**	0.46***
	Sepal	0.22*	0.23*
	Petal	0.37**	0.48***
Stomatal density and stomatal index	Leaf	0.002ns	0.04ns
	Sepal	0.52***	0.47***
	Petal	0.49***	0.27*
Stomatal size and epidermal cell size	Leaf	0.002ns	0.04ns
	Sepal	0.35**	0.21*
	Petal	0.56***	0.49**

***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, $P > 0.05$.

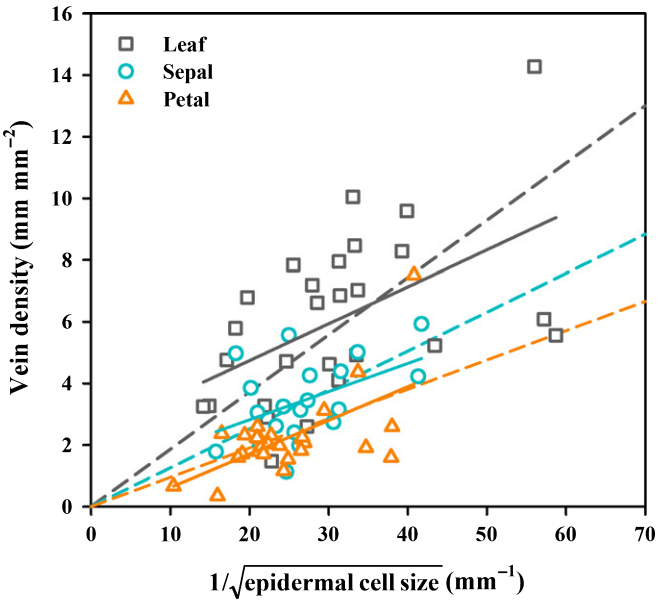

Fig. 4 Observed relationships between vein density and 1/√epidermal cell size in the leaves, sepals and petals of 27 angiosperm species (solid lines) compared with modeled relationships (dashed lines). Positive linear relationships between vein density and 1/√epidermal cell size are present in leaves ($r^2 = 0.28$, $P < 0.01$), sepals ($r^2 = 0.22$, $P < 0.05$) and petals ($r^2 = 0.37$, $P < 0.01$). Analysis of covariance (ANCOVA) showed that the intercepts of these relationships were highly significantly different ($F_{2,59} = 16.8$, $P < 0.001$), but with no difference in slopes ($F_{2,59} = 0.1$, $P > 0.05$). The differences between the observed and predicted vein density were not correlated with the observed epidermal cell size in leaves, sepals or petals ($r^2 = 0.005$, 0.01 and 0.14; $P > 0.05$, respectively).

stomatal density and cell size in leaves. Stomatal size varied independently from epidermal cell size in leaves, but was positively correlated with epidermal cell size among all species in sepals and petals (Fig. 7; Table 2). Furthermore, there was a negative correlation between stomatal density and stomatal size among all species in leaves ($r^2 = 0.25$, $P = 0.01$) and sepals ($r^2 = 0.26$, $P = 0.02$), but not in petals ($r^2 = 0.04$, $P > 0.05$) (Fig. S1). Stomatal size varied among organ types in some species ($F_{2,181} = 74.03$, $P < 0.001$), but a significant species by organ type interaction effect ($F_{2,36} = 15.55$, $P < 0.001$) indicated that stomatal size was not consistently related to organ type within species (Table 3). In addition, there were significant relationships between epidermal cell size and genome size in leaves ($r^2 = 0.28$, $P < 0.05$) and sepals ($r^2 = 0.36$, $P < 0.05$), but not in petals ($r^2 = 0.12$, $P > 0.05$) (Fig. S3).

In all organs (leaves, sepals and petals), the observed relationships between veins, stomata and epidermal cells broadly agreed with modeling which assumed that veins and stomata are passively diluted by cell expansion (observed relationships used in these analyses did not include those species lacking petal stomata) (Figs 4, 5). Decreases in epidermal cell size in petals and sepals were associated with the addition of fewer stomata than expected under passive dilution, but these differences were small compared with the overall trend (Fig. 5). However, the existence of nine species with veins but no stomata on their petals (Table 3) indicates decoupling of coordination between veins and stomata in the petals of these species.

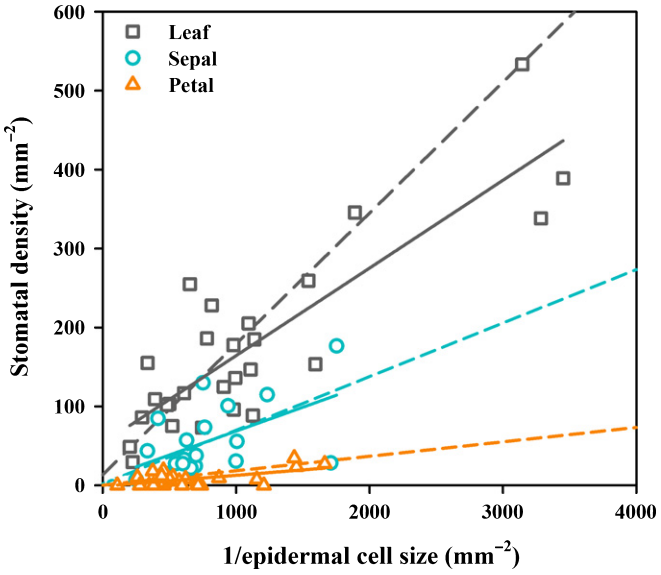

Fig. 5 Observed relationships between stomatal density and 1/epidermal cell size in the leaves, sepals and petals of 27 angiosperm species (solid lines) compared with modeled relationships (dashed lines). Positive linear relationships between vein density and 1/epidermal cell size are described in leaves ($r^2 = 0.73$, $P < 0.001$), sepals ($r^2 = 0.30$, $P < 0.05$) and petals ($r^2 = 0.33$, $P < 0.01$). Analysis of covariance (ANCOVA) showed highly significant differences in the intercepts ($F_{2,59} = 44.5$, $P < 0.001$) and slopes ($F_{2,59} = 6.8$, $P < 0.01$) of these relationships. The differences between the observed and predicted epidermal cell size were not correlated with the observed stomatal density in leaves ($r^2 = 0.02$; $P > 0.05$), but showed significant negative relationships in sepals and petals ($r^2 = 0.62$ and 0.34; $P < 0.05$, respectively).

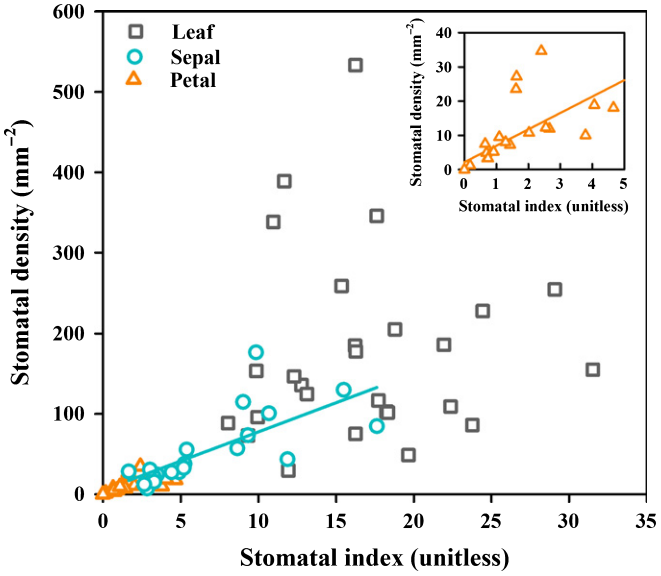

Fig. 6 Relationships between stomatal density and stomatal index in the leaves, sepals and petals of 27 angiosperm species. The insert shows the relationship in petals in greater detail. Positive linear relationships are described in sepals ($r^2 = 0.52$, $P < 0.001$) and petals ($r^2 = 0.49$, $P < 0.001$), but not in leaves ($r^2 = 0.002$, $P = 0.83$).

IV. Discussion

Complex organisms depend on coordinated mechanisms to respond to changing external conditions. Developmental

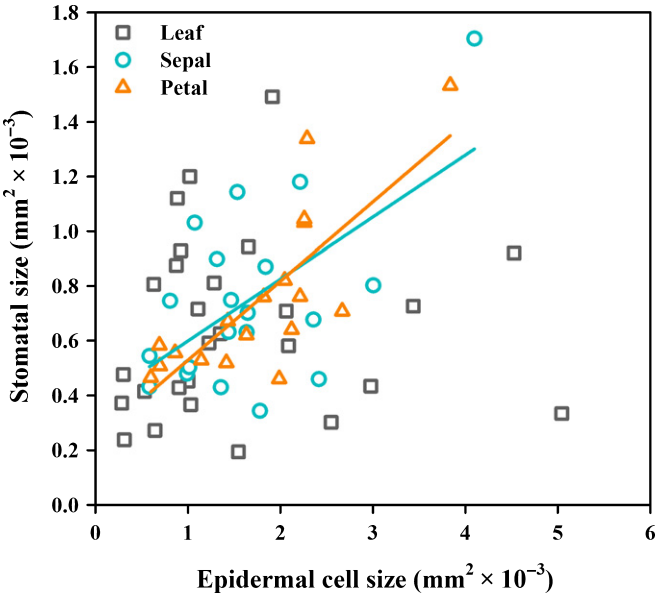

Fig. 7 Relationships between stomatal size and epidermal cell size in the leaves, sepals and petals of 27 angiosperm species. Positive linear relationships are described in sepals ($r^2 = 0.35$, $P = 0.007$) and petals ($r^2 = 0.56$, $P < 0.001$), but not in leaves ($r^2 = 0.002$, $P = 0.83$).

coordination is particularly vital for plants because their primordial tissues have indeterminate growth. Our observations of the vascular system, stomatal tissues and epidermal cell size provide some answers to important questions about water delivery and loss in flowers. We found that, although vein and stomatal densities in flowers were both low, and vein densities were related to epidermal cell size in a similar fashion to that in leaves, the low and variable stomatal densities in petals were not significantly correlated with vein density. Species with petals containing veins but no stomata represent an obvious case in which vein–stomatal coordination was absent (insert in Fig. 1).

Consistent with our first hypothesis, floral structures exhibited significantly lower vein density and stomatal density than leaves (Fig. 3), with mean petal vein and stomatal density less than half that of leaves. In leaves, high vein and stomatal densities are associated with high water transport efficiency and rates of water loss, because leaves with high vein density have higher hydraulic conductance than those with low vein density (Brodribb *et al.*, 2007; Feild & Brodribb, 2013), which supports high rates of transpiration and photosynthesis per unit leaf area. By contrast, low vein and stomatal densities in non-photosynthetic petals almost certainly reflect a smaller demand for water (Roddy *et al.*, 2013). A recent study of petal development in *Arabidopsis thaliana* has suggested that leaf and petal shape may be controlled by modifications of a common underlying developmental program (Sauret-Güeto *et al.*, 2013). Such commonality may control the range of shapes possible in both leaves and petals, whilst allowing selection to act on each structure independently, therefore generating often similar venation patterns in different structures (Melville, 1960, 1969).

We took a phylogenetically comparative approach to examine the correlated evolution between vein density and stomatal

Table 3 Mean size of leaf, sepal and petal stomata of 27 angiosperm species

Species	Leaf ($mm^2 \times 10^{-3}$)	Sepal ($mm^2 \times 10^{-3}$)	Petal ($mm^2 \times 10^{-3}$)
Illicium floridanum	0.92 ± 0.03^{a}	1.71 ± 0.19^{b}	-
Magnolia grandiflora	0.88 ± 0.04^{a}	-	0.71 ± 0.05^{a}
Cymbidium hookerianum	1.20 ± 0.02	-	-
Agapanthus campanulatus	1.49 ± 0.44^{a}	-	1.30 ± 0.09^{a}
Aquilegia vulgaris	0.59 ± 0.01^{a}	0.75 ± 0.05^{b}	0.82 ± 0.03^{b}
Aphanopetalum resinosum	0.37 ± 0.02^{a}	0.63 ± 0.01^{b}	0.51 ± 0.07^{ab}
Bergenia purpurascens	0.63 ± 0.02^{a}	0.87 ± 0.01^{b}	0.62 ± 0.02^{a}
Philadelphus purpurascens	0.37 ± 0.01^{a}	-	0.52 ± 0.01^{b}
Rhododendron camtschaticum	0.48 ± 0.01^{a}	-	0.76 ± 0.08^{b}
Cyclamen persicum	0.71 ± 0.01^{a}	0.43 ± 0.03^{b}	-
Pittosporum tenuifolium	0.42 ± 0.02^{a}	0.43 ± 0.01^{a}	0.47 ± 0.01^{a}
Weigela florida	0.95 ± 0.01^{a}	1.14 ± 0.03^{ab}	1.34 ± 1.09^{b}
Alonsoa meridionalis	0.73 ± 0.03^{a}	0.80 ± 0.01^{b}	0.76 ± 0.01^{ab}
Vinca minor	0.58 ± 0.01^{a}	0.70 ± 0.04^{b}	0.64 ± 0.04^{ab}
Solanum lycopersicum	0.27 ± 0.01^{a}	0.75 ± 0.05^{b}	0.56 ± 0.04^{c}
Fuchsia magellanica	0.30 ± 0.01^{a}	0.48 ± 0.05^{a}	-
Tropaeolum majus	0.20 ± 0.01^{a}	0.68 ± 0.06^{b}	-
Correa pulchella	1.49 ± 0.44^{a}	-	1.53 ± 0.09^{a}
Crowea exalata	0.81 ± 0.01^{a}	1.03 ± 0.03^{b}	1.03 ± 0.05^{b}
Rosa rugosa	0.81 ± 0.04	-	-
Prunus cerasus	0.43 ± 0.02^{a}	0.90 ± 0.02^{b}	0.67 ± 0.05^{c}
Pisum sativum	0.44 ± 0.02^{a}	0.46 ± 0.01^{a}	
Polygala myrtifolia	1.12 ± 0.02^{ab}	1.18 ± 0.02^{a}	1.05 ± 0.05^{b}
Micrantheum hexandrum	0.93 ± 0.03^{a}	0.55 ± 0.11^{b}	0.59 ± 0.01^{b}
Passiflora tarminiana	0.24 ± 0.01^{a}	0.50 ± 0.01^{b}	0.53 ± 0.02^{b}
Oxalis acetosella	0.34 ± 0.01^{a}	0.35 ± 0.02^{a}	-
Bauera rubioides	0.72 ± 0.03^{a}	0.63 ± 0.01^{a}	-

Different superscript letters show significant differences within species ($P < 0.05$). The values are presented as the mean ± SE.

density, and found that vein density had an evolutionary association (consistent after adjusting for phylogenetic association) with stomatal density in leaves and sepals, presumably reflecting the functional similarities of many leaves and sepals. This relationship reflects an efficient balance between investment in liquid and vapor conductances in leaves and sepals (Brodribb & Jordan, 2011; Carins Murphy *et al.*, 2012). In petals, however, vein and stomatal density showed independent evolution, reflecting much weaker geometric relationships between stomatal density and epidermal cells size in floral structures than in leaves. A probable explanation for this is that, in petals, stomatal density may not be strongly correlated with the surface conductance to water vapor. Petal stomata may have a limited ability to prevent water loss (Hew *et al.*, 1980). Instead, water loss through the cuticle may play an important role in determining water loss, and hence hydraulic demand for water, in petals (Roddy *et al.*, 2016). Another explanation is that the maintenance of hydration in flowers has been traditionally attributed to water delivery from the phloem (Nobel, 1983; Trolinder *et al.*, 1993; Chapotin *et al.*, 2003; Galen, 2005). This would result in a different relationship between vein density and water supply than that which exists in leaves in which water is delivered by the xylem (Blanke & Lovatt, 1993; Higuchi & Sakuratani, 2005; Feild *et al.*, 2009; Lambrecht *et al.*, 2011; Roddy & Dawson, 2012). Mass flow through xylem cells is the only means by which sufficient water can be carried internally to support transpiring leaves. However, because rates of water supply and water loss from petals can be low (Feild *et al.*, 2009; Roddy & Dawson, 2012; Roddy *et al.*, 2018), the phloem contribution of water might be a relatively larger fraction of the total water needed by the flower. Variation of water transport in flowers suggests that different species may employ different hydraulic conductance strategies to remain turgid and to maintain function during flowering (Chapotin *et al.*, 2003; Feild *et al.*, 2009; Roddy *et al.*, 2018). However, recent studies have suggested that flowers may be hydrated by xylem, at least in basal angiosperms (Chapotin *et al.*, 2003; Roddy *et al.*, 2018).

The stomatal density of petals was correlated with epidermal cell size as in leaves, but was also influenced by the ratio of guard cells to epidermal cells (the stomatal index). These findings suggest that there is some selective pressure to achieve a balance

between tissues regulating water loss and water transport in flowers by fixing the ratio of the number of xylem cells to guard cells during adaptive variation, but not to the same extent as in leaves. Coordination between stomatal and vein density provides important functional advantages in terms of balancing investment in these key tissues to achieve efficient use of carbon in leaves (Brodribb *et al.*, 2009), and this pressure may be the adaptive driver that has led to cell size-dependent coordination between xylem and stomatal tissues in leaves and flowers. We found significant relationships between epidermal cell size and genome size in leaves and sepals, but not in petals (Fig. S3). This suggests that adaptive changes to cell size (perhaps through modification of genome size) have led to similar changes in the densities of veins and stomata in leaves and sepals. By contrast, cell size is independent of genome size in petals, indicating the adaptive adjustment of cell size of different tissues, independent of genome size (Jordan *et al.*, 2015).

Previous published studies have highlighted the importance of stomatal cell size in regulating the density of stomata in the leaf, because constraints on packing density mean that fewer large stomata than smaller stomata can fit on the leaf (Beaulieu *et al.*, 2008; Franks & Beerling, 2009; Brodribb *et al.*, 2013). We found a similar relationship in leaves and sepals among the measured species, but not in petals (Fig. S1). Given the connection between size and density of stomata, a critical implication of reduced stomatal cell size is an increase in the amount of water and CO_2 that can be exchanged over the leaf epidermis, because stomatal density is allowed to increase, and pore depth is reduced. This, together with the positive relationship between stomatal size and epidermal cell size in petals and sepals (Fig. 7), suggests that, in flowers, unlike leaves, stomatal size is intrinsically linked with the size of neighboring cells. However, in leaves, the independence of stomatal size from the size of other cells in the leaf and the tight association with stomatal density may be another adaptive avenue for increasing leaf porosity to CO_2.

In conclusion, the new information presented here on the balance of transport and evaporative tissues in flowers is useful to understand angiosperm evolution and plant water transport in organs with disparate functions. In the present study, we have addressed the long ignored questions of water supply and water loss in flowers, focusing on the vascular system, stomatal tissues and cell size, traits that are functionally important in leaves (Brodribb & Feild, 2010; Brodribb *et al.*, 2013; Carins Murphy *et al.*, 2016). Although the mean stomatal and vein densities of flower parts were low and similarly proportional to those of leaves in our sample, the correlation between these two tissues was not present in petals. Our results suggest that, although plant evolution to enhance photosynthetic rates in leaves can be easily visualized as a process of selection for small cell size, leading to high densities of stomata and veins, in flowers, selective pressure to maximize the benefit to cost ratio of investment in vascular and stomatal tissue appears to be less intense, most likely because of their different function. Weaker linkages between veins and stomata in petals, however, do not suggest that water supply and demand are not balanced, but instead hint at the potential contribution of the cuticle to water loss from petals, and/or the phloem-driven water supply. Future research into the influence of these traits on flower physiological functions is needed to understand how flowers maintain turgor and delay desiccation.

Acknowledgements

The authors thank Natalie Tapson of the Royal Tasmanian Botanical Gardens for granting access to the gardens, and for help in collecting plant material. This study was supported by the National Natural Science Foundation of China (31670415), the Australian Research Council, Yunnan Key Laboratory for Wild Plant Resources (52Y777234261), Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, the visiting scholarships of the Chinese Academy of Sciences, and the Brazilian agency FAPEMIG.

Author contributions

T.J.B. and F-P.Z. designed the study; F-P.Z., A.A.C. and G.J.J carried out the experiments. F-P.Z, M.R.C.M. and T.J.B analyzed the data; F-P.Z., M.R.C.M., T.J.B., G.J.J. and A.A.C. wrote and revised the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Relationships between stomatal density and stomatal size in the leaves, sepals and petals of 27 angiosperm species.

Fig. S2 The expected relationship between cell size, vein density, stomatal density, water supply and water loss in leaves, sepals and petals, assuming that vein (water supply) and stomatal (water

loss) densities change due to a common dilution by cell expansion.

Fig. S3 Positive linear relationships between epidermal cell size and genome size are described in leaves and sepals, but not in petals.

Table S1 Species for which genome size was derived from the Kew plant DNA C-values database

Table S2 Results of analysis of covariance (ANCOVA) to determine whether relationships between vein and stomatal density are consistent across organ types

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