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Hydraulic tuning of vein cell microstructure in the evolution of angiosperm venation networks

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Summary

• High vein density (D_V) evolution in angiosperms represented a key functional transition. Yet, a mechanistic account on how this hydraulic transformation evolved remains lacking. We demonstrate that a consequence of producing high D_V is that veins must become very small to fit inside the leaf, and that angiosperms are the only clade that evolved the specific type of vessel required to yield sufficiently conductive miniature leaf veins.

• From 111 species spanning key divergences in vascular plant evolution, we show, using analyses of vein conduit evolution in relation to vein packing, that a key xylem innovation associated with high D_V evolution is a strong reduction in vein thickness and simplification of the perforation plates of primary xylem vessels.

• Simple perforation plates in the leaf xylem occurred only in derived angiosperm clades exhibiting high D_V (> 12 mm mm^{-2}). Perforation plates in the vessels of other species, including extant basal angiosperms, consisted of resistive scalariform types that were associated with thicker veins and much lower D_V.

• We conclude that a reduction in within-vein conduit resistance allowed vein size to decrease. We suggest that this adaptation may have been a critical evolutionary step that enabled dramatic D_V elaboration in angiosperms.

Introduction

The hydraulic function of the leaf vein network is linked to the maximum capacity for photosynthesis across vascular plants (Sack & Frole, 2006; Brodribb *et al.*, 2007; McKown *et al.*, 2010; Brodribb & Jordan, 2011). Xylem hydraulics and photosynthesis are linked because the xylem supplies water to the photosynthetic tissues to prevent their desiccation during photosynthetic CO_2 exchange with the atmosphere. Increasing the investment in vein plumbing can support greater photosynthetic capacity because more water can be delivered to the sites of evaporation within the leaf, thus allowing a greater exchange of CO_2. High water transport capacity in leaves requires a high density of vein branching within the leaf lamina, expressed as millimeters of vein length per square millimeter of leaf area (D_V). Indeed, D_V has been shown to act as a principal determinant of leaf hydraulic supply capacity across land plant diversity (Brodribb *et al.*, 2007).

Given the photosynthetic benefits associated with higher D_V, it is curious that, among the diverse venation architectures that have evolved over the 380-million-yr history of leaves, the appearance of leaves with dense venation (> 8 mm mm^{-2}) occurred uniquely in the angiosperm clade during the mid to Late Cretaceous (Boyce *et al.*, 2009; Brodribb & Feild, 2010; Feild *et al.*, 2011). By contrast, all other nonangiosperm leaves since the origin of vascular plants diversified within a low and narrow band of D_V averaging *c.* 3 mm mm^{-2} (Boyce *et al.*, 2009; Feild *et al.*, 2011). The high D_V found across modern angiosperms is thought to represent a critical adaptation in the rise of angiosperms to ecological dominance in productive environments, by enabling a greater capacity for photosynthesis and growth (Brodribb & Feild, 2010; de Boer *et al.*, 2012). By triggering an increase in the potential maximum rate of leaf and plant gas exchange, the innovation of high D_V in angiosperms potentially acted as the mechanistic fulcrum linking angiosperm evolution to large-scale changes in ecosystem processes during the Cretaceous (Bond & Scott, 2010; Boyce & Lee, 2010, 2011; Feild *et al.*, 2011). Among these hypothesized changes are an increase in the size of terrestrial primary productivity base, altered terrestrial carbon sequestration processes as well as mineral weathering and nutrient runoff, enhanced fire frequency and intensified vegetation–climate interactions that favored the origin and ecological spread of angiosperm-dominated lowland tropical rainforests (Boyce *et al.*, 2009; Bond & Scott, 2010; Boyce & Lee, 2010, 2011; Boyce & Zwieniecki, 2012).

Despite the recognition that the evolution of high D_V represented a major transition in early angiosperm physiological evolution, the structural mechanisms responsible for the evolution of high-D_V angiosperm leaves remain unexamined. No mechanistic account has been provided for why angiosperms were the only clade to strike upon the benefits of high D_V, and how angiosperms increased D_V without incurring a dramatic inflation in

direct synthetic costs of vein tissue, or other indirect costs (Feild *et al.*, 2009, 2011; McKown *et al.*, 2010). One such indirect cost is that increasing the number of veins displaces space in the leaf that could be used for photosynthetic tissues or other structures – thus representing a cost in functional opportunity. Such constraints suggest that there is likely to be a strong evolutionary pressure for miniaturizing vein dimensions to evolve high D_V. Paradoxically, many of the most densely veined angiosperm leaves known bound the lower limits of leaf cost, with low leaf mass per area (Boyce *et al.*, 2009; Poorter *et al.*, 2009; Brodribb & Feild, 2010).

A viable hypothesis explaining the origin of high D_V is that evolutionary innovation in the anatomy of angiosperm leaf xylem opened up novel options for increasing the maximum density of vein branching without compounding vein network costs. Such a hypothesis flows from observations that major structural differences in the xylem conduits developed from the primary xylem system characterize leaf venation across vascular plants, and particularly within angiosperms (Bailey, 1944; Bierhorst & Zamora, 1965; Zamora, 1966). Vein xylem cells vary diversely in the types of pits on their lateral walls and the extent to which their endwall regions are open to flow. Recent experimental data argue that the structure of overlapping regions shared between individual cells inside angiosperm vessels, called perforation plates, can strongly influence xylem conduit hydraulic conductivity (Christman & Sperry, 2010). Significantly, comparative observations of primary xylem perforation plates conducted across extant basal angiosperms motivate a hypothesis that perforation plates gradually shifted from highly resistive forms composed of dozens of bar-like (scalariform) pits to less hydraulically resistive ones bearing a single large (simple) aperture through angiosperm evolution (Fig. 1; Bailey & Tupper, 1918; Bailey, 1944; Bierhorst & Zamora, 1965; Zamora, 1966; Carlquist, 1975, 2009, 2012). However, an analysis of xylem conduit structural evolution in relation to venation geometry across vascular plants remains absent.

Here, we test the hypothesis that evolutionary innovation in xylem perforation plate structure represented a key trigger that allowed angiosperms to produce the high vein densities that are widespread across most modern ecologically dominant and highly productive trees. By measuring the thickness and density of minor veins in a diversity of angiosperms and nonangiosperms, we examine the role of xylem cell structural variation across vascular plants on the geometry and packing of leaf veins.

Materials and Methods

Plant species and field sites

We sampled 111 species of vascular plants for comparative investigations of vein network variation (Supporting Information Table S1). The taxa selected encompassed the major extant clades of vascular plants (lycophytes, ferns, gymnosperms) plus a broad range of major phylogenetic divergences across extant angiosperm phylogeny. The leaves sampled came primarily from natural populations or plants cultivated outdoors under climatic conditions similar to those experienced by a species in the field. One mature, undamaged leaf from two individual plants per species was sampled for anatomical measurements.

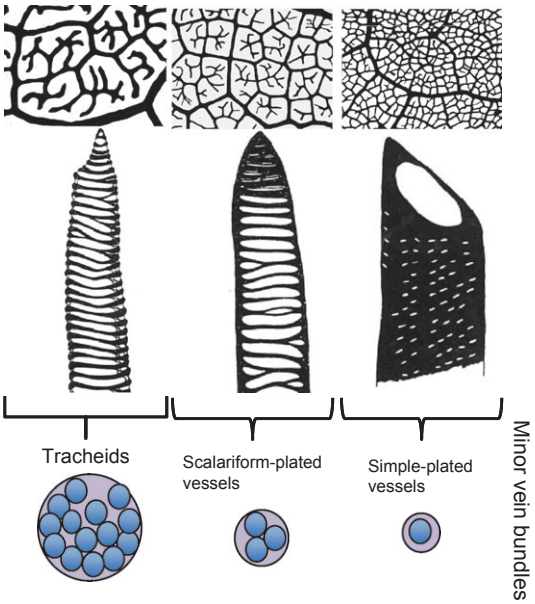

Fig. 1 Overview of the hypothesis that hydraulic tuning of vein tracheary element structure played a decisive role in the evolution of densely veined angiosperm leaves. Evolutionary consolidation in perforation plate form in vessel elements is associated with increased vein density and thinning of minor vein thickness because decreased intraconduit resistance permits miniaturized veins to remain sufficiently conductive. Images of primary metaxylem elements are drawn from our observations of perforation plates under light microscopy. Vein silhouettes from top left to top right include: *Pseudowintera axillaris*, vessel-less (tracheid-only) angiosperm; *Magnolia grandiflora*, magnoliid angiosperm with scalariform perforation plated vessels in the venation; and *Bauhinia hookeri*, eudicot with simple perforation plated vessels throughout the venation.

Vein density and vein surface area measurements

D_V was defined as the length of veins ramifying in a given amount of leaf area (mm mm^{-2}). The vein surface area (SA_V) was taken as the amount of surface area occupied by vein xylem conduits for a given amount of leaf area. SA_V divided by D_V yields an average vein thickness for the network. We determined D_V and SA_V on chemically cleared leaves. Four samples from two leaves for each species, *c.* 5 mm × 7 mm, were cut from the middle portion of the lamina. Before clearing, the adaxial cuticle was removed by hand using a bent double-edged razor blade to more clearly distinguish vein xylem when viewed under a microscope. We cleared leaf pieces in 5% aqueous NaOH and/or 5% bleach overnight at 65°C. Once cleared, leaf pieces were washed in distilled water and then stained in acidified 1% toluidine blue, 0.1% cresyl violet stain or 1% safranin. Leaf pieces were then destained in distilled water or 100% ethanol to maximize xylem contrast. In such a way, we were able to discriminate nonvein tissues (e.g. sclereids, bundle sheath extensions) from tracheary elements. Most species sampled lacked these features.

D_V and SA_V were determined digitally using ImageJ freeware (http://rsb.info.nih.gov/ij/; National Institutes of Health,

Bethesda, MD, USA) on a 1.8 × 2.3 mm^2 area for all species. At such a scale, major (with metaxylem) and primarily minor (protoxylem-dominated) vein orders were included. Our hypothesis is best tested at such a scale for two reasons: (1) minor veins function primarily in hydraulic flow as opposed to major veins that significantly bear static and dynamic mechanical loads in addition to hydraulic flow; and (2) minor veins strongly determine leaf hydraulic conductance (Sack & Frole, 2006; Brodribb *et al.*, 2007; McKown *et al.*, 2010). High-resolution images (4300 × 3800 pixels) were obtained using a digital camera (Nikon D300S, New York, NY, USA) attached to a compound microscope (CH2-Olympus, Mt. Waverly, Vic., Australia).

Compiling the database on primary xylem tracheary element types and vein maceration

Published compendia of primary xylem tracheary elements across vascular plants were used to codify broad patterns in tracheary element form across the majority of the taxa sampled (Table S1; Bierhorst, 1960; White, 1963; Bierhorst & Zamora, 1965; Zamora, 1966; Muhammad & Sattler, 1982; Carlquist & Schneider, 2007). We defined primary xylem development into two categories: (1) protoxylem, which consists of first formed xylem conduits comprising annular and helical lignified rings; and (2) metaxylem, which consists of later formed xylem conduits bearing a secondarily deposited, pitted secondary cell wall (Bierhorst & Zamora, 1965). For protoxylem and metaxylem tracheary elements, each species was scored for the presence of tracheids or vessel elements. If vessel elements occurred, we scored whether vessel elements possessed scalariform or simple perforation plates. Nearly all codes of tracheary element state were based on light microscopy observations. Such observations can present problems in coding tracheary element character states, particularly in the secondary xylem of basal angiosperms and fern primary xylem, where vessel-looking tracheary elements often turn out to be nonperforated, and therefore tracheids, when observed with scanning or transmission electron microscopy (Carlquist & Schneider, 2007; Carlquist, 2009, 2012). The extent that such a phenomenon occurs in the primary xylem of angiosperms remains undocumented. However, such effects do not undermine the testing of the hypothesis that perforation plate evolution influenced D_V evolution. Such is the case for two reasons: (1) intact pit membranes only increase tracheary element endwall resistance; and (2) putative variable pit membrane presence did not obscure the results from hydraulic measurements made on single vessels in the wood of basal angiosperms, which demonstrated that evolutionary streamlining of perforation plate form reduced intravessel hydraulic resistance (Christman & Sperry, 2010).

Many reported patterns of primary xylem tracheary element development have been derived from macerations of petiole or young stem xylem which lack secondary vascular development (Bailey, 1944; Bierhorst, 1960; Bierhorst & Zamora, 1965; Zamora, 1966). We tested whether codifying primary xylem conduit development of a species from young stems or petioles accurately represented that in metaxylem and protoxylem cells of the leaf venation in a sample of 18 genera (see Table S1 for the taxa sampled). To determine vessel element form in veins, major and minor veins were dissected from leaf pieces that contained vein orders identical to those sampled in D_V and SA_V analyses. Veins were dissected by hand using a double-edged razor blade and stereoscope. Veins were next chemically macerated. Leaf veins were immersed in capped 5-ml vials containing a maceration solution (1 : 4 : 5 by volume, 30% H_2O_2 : distilled water : glacial acetic acid) and placed in an oven at 65°C until the tissues became translucent, usually after 16 h. After several washings with distilled water over 4 h, the samples were stained with 0.1% aqueous safranin O (w/v) for 5–30 min. Then the vials were shaken to loosen tracheary elements apart. After settling of the tracheary element macerate, a drop of macerate was pipetted onto a slide and scanned with a compound microscope using differential interference contrast optics to image tracheary element endwalls (Zeiss M1 AxioImager, New York, NY, USA). Digital images of macerated xylem were taken with a camera attached to the microscope. In all 18 comparisons, we found that previously reported developmental patterns based on young stems were the same as those determined using macerated vein protoxylem and metaxylem. For several key taxa not studied previously (see Table S1), we used vein maceration to determine patterns of primary xylem tracheary element variation.

Modeling of vein spatial organization

We consider the case of a typical hypostomatic leaf in which the plane of the leaf minor venation is below the palisade tissue and hence does not displace the photosynthetic palisade volume. To understand the spatial limits on vein size, we used a geometric model to determine the maximum thickness and density combinations allowed before neighboring veins collided. We used a uniform hexagonal matrix as a good approximation of the minor vein geometry (Price *et al.*, 2012) and calculated the maximum line (vein) width that was allowable before all veins coalesced into a single layer with no space between veins. In such a synthetic vein matrix, we added the constraint that a proportion of the space between veins must remain open. If veins became so densely packed as to coalesce together, the vertical connection between the stomata (typically located abaxially on the leaf) and the photosynthetic tissue (typically located adaxially) would be cut off because of the dense, nonporous nature of xylem tissue that is impermeable to CO_2 diffusion. Hence, a solid layer of veins in the middle of the leaf would greatly limit photosynthesis, because CO_2 could not pass internally from the stomata to the sites of photosynthetic carboxylation (Flexas *et al.*, 2012). Measurements of species with high D_V (> 20 mm mm^{-2}) indicated that veins occupied a mean of 42% of the projected leaf area. Hence, we modeled the maximum area of veins in our synthetic hexagonal matrix at 10%, 20%, 30% and 40% of the total area of the leaf to observe the effects on vein maximum thickness.

Results

We found that different vascular plant groups populated distinctive ranges in D_V. Ferns, lycophytes, conifers and cycads

encompassed a narrow range of low D_V values: ferns and lycophytes ranged from 0.4 mm mm^{-2} in *Asplenium obtusatum* to 6.5 mm mm^{-2} in *Dipteris conjugata* ($n = 29$ taxa); conifers and cycads ranged from 0.8 mm mm^{-2} in *Bowenia serrulata* to 1.8 mm mm^{-2} in *Zamia furfuracea* ($n = 5$ species). Basal angiosperm lineages (i.e. Amborellales, Austrobaileyales) also produced a similar range of D_V values to nonangiosperms overall (range from 2.35 mm mm^{-2} in *Austrobaileya scandens* to 4.8 mm mm^{-2} in *Illicium lanceolatum*; $n = 13$ species). However, both magnoliids and eudicot angiosperms exhibited considerably broader ranges in D_V. D_V ranged from 2.7 to 16.7 mm mm^{-2} in magnoliids and 3.2 to 22.7 mm mm^{-2} in eudicots.

Associated with these D_V patterns, vein thicknesses fell into distinctive patterns that were strongly associated with D_V and the types of tracheary element present in the primary xylem (Fig. 2a). Considering all species together, there was a strong relationship between vein thickness and D_V, with vein thickness declining rapidly as a function of $D_V^{-0.6}$. Modeled maximum D_V converged with the observed pattern in all species when the space between veins was set at 30% of the total surface area of the leaf (Fig. 2a).

Different vein anatomies produced different patterns in D_V by vein thickness. For example, low D_V and thick veins characterized the tracheid-only sample (including ferns, lycophytes, conifers, cycads and vessel-less angiosperms), with a mean vein thickness of *c.* 90 μm in this group, and no species produced vein thicknesses of < 50 μm (Fig. 2b). Vessel-less veins exhibited broad overlap with venation systems consisting of scalariform plated vessels in the protoxylem and metaxylem (Fig. 2b). At the other end of the spectrum, eudicots and magnoliid taxa with simple perforation plates throughout the primary xylem produced D_V up to 23 mm mm^{-2} and a mean vein thickness of 17 μm with minimum values of *c.* 10 μm in some tropical species (Fig. 2a,b). Vein thicknesses when simple perforation plates were present in the protoxylem or metaxylem were significantly thinner than those of all other groups possessing tracheids and/or scalariform perforation plates, with one exception, where the small sample size of taxa with tracheids in the protoxylem and metaxylem scalariform perforation plates prevented robust comparisons (Table S2; $P < 0.05$).

Discussion

The evolution of high D_V within angiosperms ranks as one of the most important functional transitions in modern plant history (Boyce *et al.*, 2009; Bond & Scott, 2010; Brodribb & Feild, 2010; de Boer *et al.*, 2012; Boyce & Zwieniecki, 2012; Crisp & Cook, 2012). However, debate surrounds the evolutionary process explaining why angiosperms are uniquely capable of producing enormously branched networks of minor veins in the leaf (Feild *et al.*, 2009, 2011; Brodribb & Feild, 2010; de Boer *et al.*, 2012; Crisp & Cook, 2012). Our results motivate a hypothesis that a key functional requirement for the evolution of high D_V is that veins must become very narrow to fit a densely branched network in the leaf, and that angiosperms represent the only clade that evolved a xylem conduit anatomy sufficiently conductive to permit miniature vessels to maintain water supply.

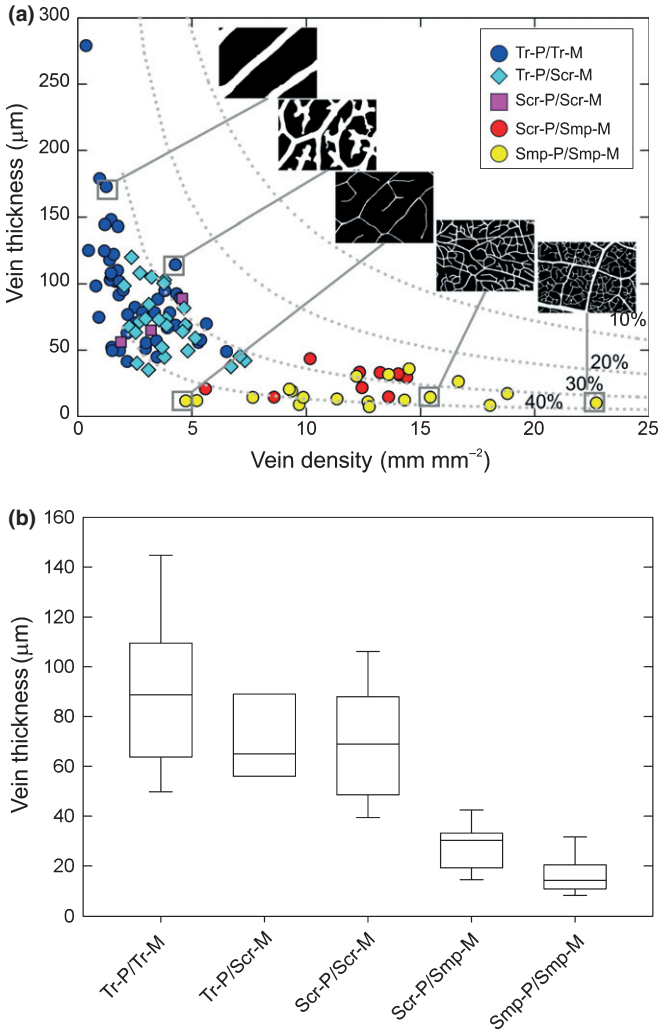

Fig. 2 Relationship of vein thickness with vein density (a) and vein thickness variation across different primary xylem element developmental categories (b) across vascular plants. (a) Vein thickness declines sharply with vein density. The dotted lines illustrate the limits of the maximum area of veins based on a synthetic hexagonal matrix covered by 10%, 20%, 30% and 40% of the total area of the leaf (see Materials and Methods). Vascular plant leaves are grouped into five different categories of primary xylem tracheary element composition: Tr-P/Tr-M, blue circles; Tr-P/Scr-M, light blue diamonds; Scr-P/Scr-M, pink squares; Scr-P/Smp-M, red circles; Smp-P/Smp-M, yellow circles. Abbreviations for these categories are: Tr-P, tracheids present in the protoxylem; Tr-M, tracheids present in the metaxylem; Smp-P, vessel elements with simple perforation plates present in the protoxylem; Smp-M, vessel elements with simple perforation plates present in the metaxylem; Scr-P, vessel elements with scalariform perforation plates present in the protoxylem; Scr-M, vessel elements with scalariform perforation plates present in the metaxylem. Minor venation silhouettes, from left to right, illustrate different points in the vein thickness by density morphospace: a vessel-less fern (*Arostichum aureum*), vessel-less angiosperm (*Zygogynum pancheri*), angiosperm eudicot herbs at low (*Chimifuga racemosa*) and high (*Apocynum cannabinum*) vein density, and high-D_V angiosperm tropical lowland rainforest pioneer tree (*Macaranga quadrifolia*). (b) Box plots of variation in vein thickness across five categories of primary xylem development (abbreviations as in (a)). Box plots depict the variation within each group clade, with the bottom and top of the box indicating the 25th and 75th percentiles, respectively, the two whiskers the 10th and 90th percentiles, respectively, and the horizontal line within the box, the median value.

Given that veins only occupy a single plane in the leaf, the only way to produce high D_V without the intersection of neighboring veins is by miniaturization of minor vein widths. Critically, however, a major functional constraint arises from the miniaturization of the minor vein network. As the vein thickness declines, so too will the size and number of xylem conduits, causing a steep drop in the hydraulic conductivity of the network (Cochard *et al.*, 2004; McKown *et al.*, 2010). Therefore, the hydraulic conductivity of minor veins may become limiting at high D_V, as plants are forced to build minor veins with reduced size and number of conduits to maintain low vein thickness within the spatial constraint of the leaf. The minimization of losses in conduit water transport should therefore become paramount as the density of minor veins increases and their diameter is forced to decline. In strong support of such a concept, we show that only species bearing metaxylem and protoxylem perforation plates reduced to simple, low-resistance pores were capable of developing very high D_V ($> 15\ mm\ mm^{-2}$). Such densities are nearly twice those found in the most densely veined nonangiosperms known (Boyce *et al.*, 2009; Feild *et al.*, 2011). These angiosperms possessing simple plated primary xylem vessels were the same species that had the thinnest veins at any D_V. Thus, we suggest that simple plated primary xylem vessels constitute a critical innovation in xylem hydraulic evolution because intraconduit resistance is lowest in these conduits (Choat *et al.*, 2008; Christman & Sperry, 2010). We propose that such an anatomical feature provides a critical shift in the functional efficiency of venation that allows the production of high D_V.

Apparently only angiosperms evolved vessels with simple perforation plates in the primary xylem (Fig. 3; Bierhorst, 1960; Bierhorst & Zamora, 1965; Muhammad & Sattler, 1982). Primary xylem vessels themselves, however, are widespread across vascular plant clades with reports from some ferns and Gnetales (Fig. 3; Bierhorst, 1960; Bierhorst & Zamora, 1965; Muhammad & Sattler, 1982; Carlquist & Schneider, 2007; Feild & Balun, 2008). Nonangiosperm primary xylem vessels appear to possess low-efficiency perforation plates between vessel elements. For instance, the few known examples of pteridophyte vessel elements consist of very long scalariform perforation plates with partially degraded pit membranes and narrowly spaced bars (Carlquist & Schneider, 2007). Such perforation plates are hydraulically analogous, but not homologous, to the resistive scalariform perforation plates found in basal angiosperm lineages (Carlquist & Schneider, 2007; Christman & Sperry, 2010), and we found that species with scalariform plated vessels only exhibited a small increase in their capacity to miniaturize the minor veins (Fig. 2b). Pteridophyte metaxylem vessels are confined to rhizomes or stipes, with no reports known of vessels with simple perforation plates in the leaf venation (White, 1963; Carlquist & Schneider, 2007). Although simple plated vessels occur in the wood of some Gnetales, gnetalean primary xylem perforation plates consist of narrowly pitted foraminate areas that would exhibit similar flow restrictions to the slits in scalariform plates (Bierhorst, 1960; Muhammad & Sattler, 1982). So far, there is only one report of perforation plate resistance in Gnetales, with *Ephedra viridis* vessel elements exhibiting fairly resistive endwalls

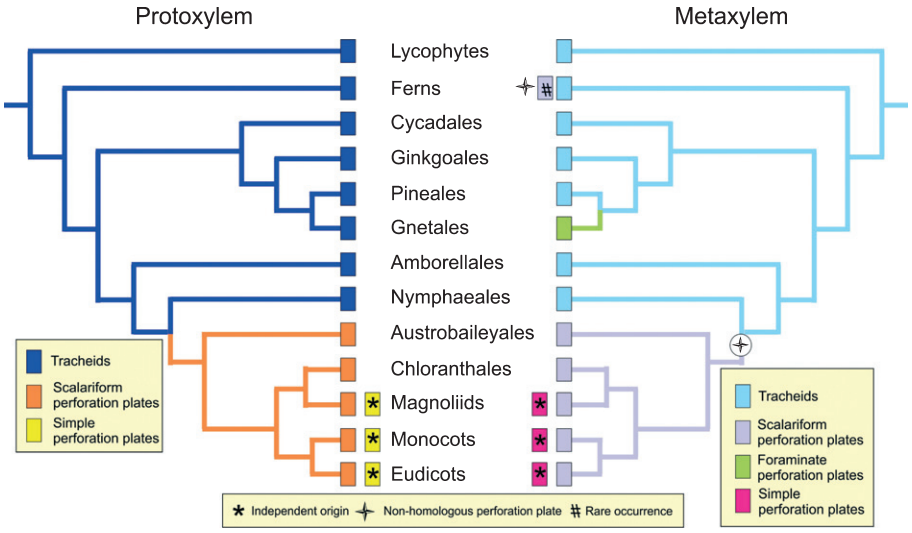

Fig. 3 Summary of the phylogenetic distribution of primary xylem element conduits across vascular plants. The figure represents the summary of a parsimony character reconstruction of primary xylem evolution as described in Supporting Information Fig. S1 (performed using Mesquite 2.75; Maddison & Maddison, 2011). Primary xylem vessels occur in several lineages of vascular plants, including ferns and Gnetales, as well as angiosperms. Presently, metaxylem vessel distribution in ferns appears to be very rare, with most species bearing tracheids only (Carlquist & Schneider, 2007). Previous reports of vessels in lycophytes based on light microscopy require careful re-consideration as discussed in Carlquist & Schneider (2007). At this stage, lycophytes are best taken as vessel-less. At least three independent origins of simple plated vessels occurred in angiosperms, because several successive lineages branching before simple plated eudicots, monocots and magnoliids were found to lack simple perforation plates (Fig. S1). Such a pattern suggests that ancestral nodes of these major lineages possessed scalariform plated vessels. However, the specific number of simple perforation plate origins in the primary xylem remain unknown and will require future sampling of basal eudicot lineages, as well as basal lineages in major core eudicot clades. Trait values were compiled from literature sources and our maceration results (Bailey, 1944; Bierhorst, 1960; White, 1963; Bierhorst & Zamora, 1965; Zamora, 1966; Carlquist, 1975, 2012; Muhammad & Sattler, 1982; Takahashi, 1988).

(Christman & Sperry, 2010). More direct hydraulic measurements on gnetalean vessels would be highly desirable to examine how foraminate plate form influences hydraulic resistance.

The emergent evolutionary pattern is that only angiosperms are capable of producing high-efficiency vessels in the leaf primary xylem. However, within angiosperms, there is a clear evolutionary pattern of vessel element streamlining through the phylogenetic diversification of angiosperms (Bailey, 1944; Bierhorst & Zamora, 1965; Zamora, 1966; Carlquist, 2009, 2012). Amborellales and Nymphaeales are vessel-less (Carlquist, 2009, 2012). We found that primary xylem vessels, where water is sieved through numerous and narrowly spaced slits between elements, were reconstructed as evolving in the common ancestor of Austrobaileyales, Chloranthales, magnoliids, monocots and eudicots (Fig. 3). Scalariform plated angiosperm vessels were associated with thick, low-density vein networks. Such a pattern suggests that early leaf vessels were only marginally more efficient at conducting water than vessel-less networks. This hydraulic pattern parallels that observed in the hydraulic evolution of secondary xylem vessels of angiosperms (Sperry *et al.*, 2007; Feild & Wilson, 2012).

We found that vessels with simple perforation plates evolved multiple times in the leaves of angiosperms – independent within magnoliids, eudicots and monocots (Figs 3, S1). Specifying how many times simple perforation plates evolved within each of these major angiosperm lineages and whether the developmental processes orchestrating pit consolidation into a simple opening are homologous remain open questions requiring future comparative work (Bierhorst & Zamora, 1965; Carlquist, 2012). Nonetheless, from a hydraulic perspective, the important step in terms of acquiring high D_V was the evolution of xylem vessels in the leaf, an innovation that would have initially improved the cost–benefit of the venation system, but importantly also provided a venation network that had very high potential for miniaturization (and hence ramification), because of a later evolved ability to erode perforation plates to form simple perforation plates.

Evolutionary miniaturization of the angiosperm minor veins in the leaf broadly parallels a pattern of stomatal size miniaturization observed during vascular plant evolution (Franks & Beerling, 2009; de Boer *et al.*, 2012; Franks *et al.*, 2012). Like stomata, it is likely that functional-based spacing rules dictate a minimum allowable proximity between elements (Franks *et al.*, 2012), in this case minor veins. For veins, we suggest that the proximity of veins to one another is constrained by the requirement of a minimally obstructed CO_2 diffusional continuum between the stomata and the photosynthetic tissue (Brodribb *et al.*, 2007). Owing to their impermeability for CO_2 diffusion, minor veins cannot become so wide or dense as to coalesce into a single plane in the middle of the leaf. Such a constraint further compounds the spatial limitation for vein thickness. In all species, we found that the observed vein thicknesses allowed only *c.* 30% of the venation plane to remain unobstructed by veins, and that this small space was highly sensitive to changes in vein thickness. Although there are no models at present that predict the role of vein thickness or density variation on internal CO_2 conductance, it is likely that the positive correlation between D_V and A_{max} (photosynthetic capacity) places further pressure on veins to miniaturize. The reason for such a hypothesis is that increasing A_{max} requires increases in both D_V and internal CO_2 conductance (Flexas *et al.*, 2012), yet these two demands will conflict unless veins become disproportionately narrower to open up the space between stomata and photosynthetic tissues.

We speculate that a key mechanism potentially coordinating reductions in stomatal size and primary xylem size with angiosperm diversification is evolutionary down-sizing of genome size. Cutting back genome size, all else being equal, feeds back to shrink cell size (Franks *et al.*, 2012). As such, a future examination of *C*-value evolution with stomatal size and vein conduit size across angiosperms could represent a highly illuminating test of a hypothesis that genome remodeling influenced leaf functional evolution through changes in cell size. Nonetheless, it is clear that structural assembly of terrestrial angiosperm's ability to make enormously productive leaves involved linking high stomatal density together with dense vein branching (Feild *et al.*, 2009; de Boer *et al.*, 2012; Franks *et al.*, 2012).

Our data offer a new explanation for why angiosperms evolved densely veined leaves. A structural novelty – simple perforation plates – arising in the angiosperm leaf vein system unlocked new possibilities in vein packing that permitted an enormously greater reticulated vein network inside the leaf, but at lower spatial cost per vein than tracheid-based networks. Hydraulic efficiency benefits of perforation plate streamlining do not appear to have acted as an immediate hydraulic switch to high D_V, and there apparently were several independent experiments with perforation plate simplification within angiosperms. Reconstruction of the selective drivers for these events during angiosperm diversification will require fascinating future work.

Towards an understanding of these evolutionary drivers, a preliminary association that begs further scrutiny is that vein simple perforation plates and thin minor veins occur in several early diverging herbaceous clades that are characterized by low D_V (Zamora, 1966; Brodribb & Feild, 2010). As such, we hypothesize that decreases in the construction costs of venation may have been linked to the evolution of herbaceous habits during early eudicot evolution. In magnoliids, however, a different evolutionary pattern appears evident with the early evolution of minor vein simple perforation plates. The evolution of simple perforation plates and high D_V appears to be tied together to the evolution of increased plant height and high photosynthetic capacity, because high-D_V magnoliids occur either as tall canopy-dominant lowland rainforest trees and/or lowland tropical rainforest pioneers (Brodribb & Feild, 2010; Feild *et al.*, 2011).

Because the primary xylem forms the plumbing for the fine root system, we speculate that perforation plate reduction also represented a key element in the downsizing of fine root thickness by its influence on fine root xylem cross-sectional area during angiosperm diversification. The evolution of miniaturized fine roots in angiosperms appears to have sparked new functions that influenced mineral weathering processes as well as nutrient uptake and interactions of soil microbes with angiosperms (Comas *et al.*, 2012). Our results make it clear that paying increased attention to xylem hydraulic innovations arising during angiosperm evolution

will yield fundamental insights into the causes and consequences of angiosperm origin and early diversification.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Parsimony character mapping of primary xylem tracheary elements across the sample angiosperm taxa.

Table S1 Taxa and data values used in comparative analyses of venation and tracheary element structure

Table S2 Comparisons of vein thickness variation across tracheary element groups found in vascular plant leaves

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