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Research

Structural and hydraulic correlates of heterophylly in *Ginkgo biloba*

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Summary

• This study investigates the functional significance of heterophylly in *Ginkgo biloba*, where leaves borne on short shoots are ontogenetically distinct from those on long shoots. Short shoots are compact, with minimal internodal elongation; their leaves are supplied with water through mature branches. Long shoots extend the canopy and have significant internodal elongation; their expanding leaves receive water from a shoot that is itself maturing.

• Morphology, stomatal traits, hydraulic architecture, Huber values, water transport efficiency, *in situ* gas exchange and laboratory-based steady-state hydraulic conductance were examined for each leaf type.

• Both structure and physiology differed markedly between the two leaf types. Short-shoot leaves were thinner and had higher vein density, lower stomatal pore index, smaller bundle sheath extensions and lower hydraulic conductance than long-shoot leaves. Long shoots had lower xylem area : leaf area ratios than short shoots during leaf expansion, but this ratio was reversed at shoot maturity. Long-shoot leaves had higher rates of photosynthesis, stomatal conductance and transpiration than short-shoot leaves.

• We propose that structural differences between the two *G. biloba* leaf types reflect greater hydraulic limitation of long-shoot leaves during expansion. In turn, differences in physiological performance of short- and long-shoot leaves correspond to their distinct ontogeny and architecture.

Introduction

The ramified and modular nature of plants can result in their photosynthetic surfaces experiencing significant environmental and developmental heterogeneity. Many species exhibit notable differences in leaf form within a single plant, a phenomenon known as heterophylly. There are different ways of achieving heterophylly, depending on the developmental or genetic origin of variation in leaf form. For example, sun and shade leaves are associated with intra-crown variation in light intensities and evaporative demand (Smith & Nobel, 1977; Hamerlynck & Knapp, 1994). Sun/shade leaf morphology can result from differing constraints during growth: leaves emerge from bud with an equivalent number of major veins and stomata, but restricted hydraulic supply during cell expansion leads to more deeply lobed sun leaves with higher vein and stomatal density (Zwieniecki *et al.*, 2004b; Boyce, 2009). By contrast, heteroblastic plants produce discrete leaf forms during different developmental stages (Moore, 1992; Jones, 2001). Another form of heterophylly is found in plants with different shoot types, such as short shoots (also known as spur, dwarf or reproductive shoots) and long shoots (water, extension or vegetative shoots). While different shoot types occur on a range of angiosperm species (e.g. Yagi, 2000; Zhang *et al.*, 2005), the phenomenon is less well documented among gymnosperms. A notable gymnosperm with shoot-based heterophylly is *Gingko biloba* L., a northern temperate gymnosperm.

Ginkgo biloba has two leaf types borne on different kinds of shoots: abundant short shoots that have essentially no internodal elongation, and less common long shoots that elongate to 10–30 cm in length over a growing season (Fig. 1). Long shoots are produced by the terminal meristem of existing long shoots but can also form laterally through the conversion of short shoots (Fig. 1). Long shoots are associated with branch extension, whereas reproductive organs – male and female strobili on different plants – are borne on short shoots (Chamberlain, 1966). The leaves of short shoots are larger and more abundant and emerge in early spring from overwintering buds (Fig. 2a). By contrast, long-shoot leaves are smaller and often deeply lobed (Fig. 2b); they are not preformed, but instead differentiate as the shoot elongates (Critchfield, 1970). Because of its taxonomic status, unique morphological characteristics and medicinal properties, researchers have had a keen interest in *G. biloba* for decades (Foster, 1938; Gunckel & Wetmore, 1946; Arnott, 1959; Critchfield, 1970; Del Tredici *et al.*, 1992; Royer *et al.*, 2003; Del Tredici, 2007; Bornhoeft *et al.*, 2008; Boyce, 2009). However, *G. biloba* has rarely been studied from a functional viewpoint and virtually nothing is known about the physiological significance of its distinct leaf types.

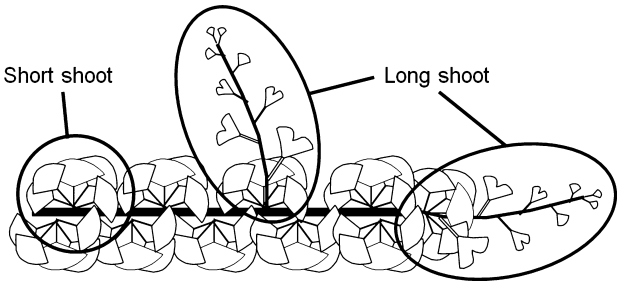

Fig. 1 Diagram of a *Ginkgo biloba* branch, illustrating the arrangement of short and long shoots.

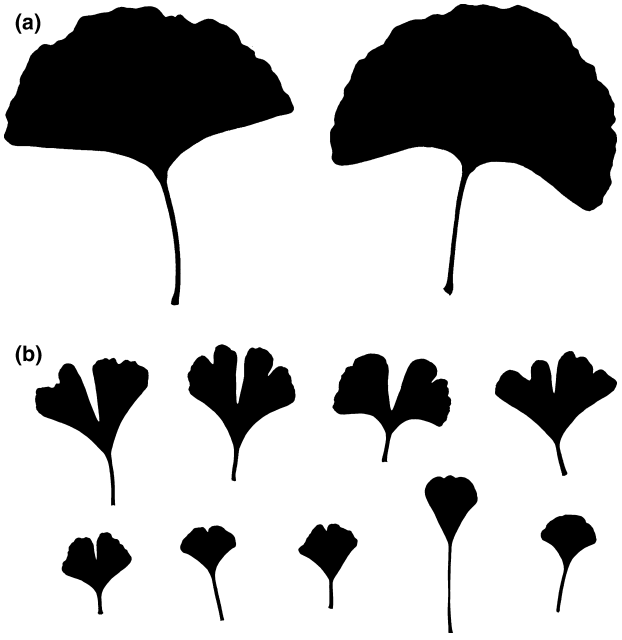

Fig. 2 Examples of the relative sizes and shapes of *Ginkgo biloba* leaves on (a) short shoots and (b) long shoots, highlighting the variability in shape complexity (leaf dissection index) of the long-shoot leaves.

Heterophylly represents one way of overcoming the spatially and temporally varying environmental conditions presented to a plant through its life. Although physiological studies of heterophyllous species are rare, differences between leaf types in gas exchange and water use efficiency have been reported (Mulkey *et al.*, 1992; Brodribb & Hill, 1993; Kitajima *et al.*, 1997). The hydrology of different leaf types on a single plant has received less attention (Sack *et al.*, 2003). Moreover, virtually nothing is known about the comparative physiological function of short- vs long-shoot leaves. Long shoots are associated with canopy expansion through rapid growth into areas of higher light (Yagi, 2000; Kull & Tulva, 2001). This suggests that long- and short-shoot leaves may differ in their rates of gas exchange. Based on the observed scaling between assimilation rate and leaf hydraulic capability (Brodribb & Holbrook, 2005), one might expect hydraulic traits to reflect gas exchange patterns. However, the developmental and anatomical characteristics of long shoots suggest that they might be hydraulically constrained relative to short shoots. For example, long-shoot leaves receive water needed during leaf expansion from a shoot that is itself undergoing elongation, whereas short-shoot leaves are supplied entirely through mature stems. Further, long shoots of *G. biloba* can continue to develop days to weeks after short shoots, with full elongation often finishing in mid-summer (Critchfield, 1970). Because *G. biloba* xylem is constructed entirely of tracheids, limitations on water transport through expanding shoots may influence the development of long-shoot leaves.

The contrasting developmental constraints and architectural arrangements of *G. biloba* long- and short-shoot leaves motivated us to ask how their respective gas exchange strategies are supported in terms of their structural investment in hydraulic capacity. Our approach was to compare the structural and physiological properties of short- vs long-shoot leaves relative to their differing ontogeny and architecture. In particular, we addressed whether *G. biloba* long-shoot leaves display morphological traits related to hydraulic limitations during leaf expansion and to what extent they are able to overcome any such limitations.

Materials and Methods

Study plant material

Leaf morphological and anatomical characteristics were measured on *G. biloba* leaves from two sites. Gas exchange

measurements were conducted on leaves of short and long shoots taken from a large, well-established tree on the Harvard University campus. Hydraulic measurements were made on leaves from this large tree and five younger trees growing in the Harvard University research garden. The majority of leaves used for morphological analyses were collected from 12 well-established trees growing on the University of Chicago campus. To ensure that morphological measurements made on Chicago leaves could be pooled with Harvard leaves, data from both sites were regressed (e.g. vein density × inter-vein distance) and in all cases the regression line for Chicago fell within the 95% confidence limits of the Harvard data (results not shown). Thus, further reference to morphological methods and analyses is for leaves from both sites unless otherwise stated.

Leaf morphology and anatomy

Morphological measurements were made on one leaf from five long shoots and five short shoots from each of the 12 Chicago trees where possible (112 leaves), plus a further 16 leaves (eight of each shoot type) from four branches on the Harvard tree, giving a total of 128 sample leaves. All leaves were collected from the outer, sunlit canopy approx. 2 m from the ground. For each shoot, a fully expanded leaf was selected of approximately the same developmental stage on the shoot; that is, the 3rd–5th last leaf on the short shoots, and the 3rd–8th last leaf on the long shoots. On these leaves we made measurements of size (area), leaf dissection index (LDI: perimeter/$\sqrt{}$area) (cf. McLellan & Endler, 1998), leaf mass per area (LMA), vein density (total vein length per cm^2), inter-vein distance and stomatal coverage. Leaves were scanned individually on a flatbed scanner to obtain area and perimeter measurements. After scanning, stomatal peels were made of the underside of each leaf using clear nail varnish; leaves were then oven-dried and weighed to obtain their dry mass. For each whole leaf, dry mass was divided by leaf area to obtain LMA (g m^{-2}). For the scanned images, vein density was measured near the leaf base and the centre of the distal margin using the IMAGE-J freeware software program (National Institute of Health, Bethesda, MD, USA). The average inter-vein distance at three locations (base, centre and margin) on each leaf was measured by drawing an arc parallel to the leaf margin, counting the number of times a vein intersected this arc and dividing the length of the arc by this number. Stomatal density (the number of stomata per mm^2) was measured by examining the stomatal peels under 200× magnification; stomatal pore length was measured at 400× magnification. To obtain an average stomatal pore length for three locations on each leaf (near the base, centre and margin), pore lengths of one stomate in the centre of each of four quadrants within the field of view were measured and these were averaged for each location on the leaf. The stomatal pore index (SPI = average stomatal pore $length^2$ × stomatal density) was then calculated, providing a measure of stomatal coverage (Sack *et al.*, 2003, 2005).

We measured anatomical traits by examining cross-sections of *G. biloba* leaf tissue taken from the large Harvard tree, embedded in paraffin wax and stained with Safranin (Jensen, 1962). Ninety cross-sections were made on 30 leaves, 15 of each shoot type, at three locations on each leaf: near the base, in the centre and near the margin. Sections were photographed at 400× magnification and measurements of xylem and associated tissue anatomy were made on these images using IMAGE-J. Measurements detailed below were made at three random locations on each cross-section and used to obtain an average measurement for that cross-section. The short and long diameter of every tracheid lumen in a vein cross-section was measured, added together, and divided by two to obtain an average tracheid diameter, d. This measurement was used to calculate a hydraulically weighted average diameter: $\Sigma d^5/\Sigma d^4$ (Sperry & Saliendra, 1994), where tracheids are assumed to be circular. We calculated the relative investment in tracheid walls: the total xylem area (including walls) minus the sum of the area of the tracheid lumen for each vein cross-section. Additionally, the number of tracheids in each vein cross-section was counted. These data, coupled with the short and long tracheid diameters, enabled calculation of the Hagen–Poiseuille vein conductivity (conductance per length) at the base, centre and margin of leaves using the formula for an ellipse (Lewis & Boose, 1995; Cochard *et al.*, 2004; Sack & Frole, 2006):

$$\sum(\pi a^3 b^3/(64\eta\beta(a^2 + b^2))) \qquad \text{Eqn 1}$$

(η, the viscosity of water at 25°C, 8.9×10^{-10} MPa s; β, the mmol volume of water, 1.8×10^{-8} m^3 $mmol^{-1}$; a and b, the short and long internal tracheid diameters.) Thus, for Eqn 1, the units of vein conductivity are mmol m s^{-1} MPa^{-1}. As a measure of hydraulic structural cost, we divided the calculated vein conductivity by tracheid wall area at the base, centre and margin of leaves to obtain an estimate of transport efficiency (mmol m^{-1} s^{-1} MPa^{-1}).

Each section was photographed at 100× and 200× magnification for measurements of leaf thickness and dimensions of the bundle sheath extension (BSE). Leaf thickness was measured near the base, centre and margin and at each location was measured as far from a vein as possible. The bundle sheath extensions were measured for one vein in each of these sections. Because the upper and lower BSEs were approximately rectangular in cross-section, their areas were estimated from the depth and width of the extension measured between the vein and the epidermis. The upper and lower BSE areas were added to provide an estimate of total BSE cross-sectional area at each location.

Branch allometry

Huber values (xylem cross-sectional area divided by the total downstream leaf area) were measured on branches collected from seven small trees growing in the Harvard University research garden. Measurements were made at two stages of development: (1) in spring (April), when shoots were in the final stages of leaf expansion but shoots were still elongating, that is, approx. 1/3 of their final length, and (2) in mid-summer (July), after shoot elongation was complete and long shoots were approx. 30 cm long. One branch per tree, each having several short shoots and one apical long shoot, were sampled. Cross-sections were made at three to five places along the mature portion of the branch and two to three places along the elongating shoot. Xylem cross-sectional area was determined from images taken using a stereoscope microscope; downstream leaf area was measured using a leaf area meter.

Gas exchange

Gas exchange measurements were conducted in summer. Measurements were made on 18 leaves: nine of each shoot type (three leaves of each type from three intact branches). Measurements were conducted using an open system infrared gas analyser (IRGA) (LI-6400; Li-Cor, Lincoln, NE, USA) with a standard chamber-mounted red/blue light source supplying the leaf with 800 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) (the same light intensity used for laboratory hydraulic measurements; see the next section, 'Leaf hydraulic design'.). When the smaller long-shoot leaf sections did not fill the chamber, the area of the measured portion was determined and gas exchange calculations adjusted retrospectively. Measurements were made between 12:00 and 14:00 h; CO_2 supplying the chamber was held at 400 ppm, relative humidity ranged between 39 and 48% and chamber temperature ranged between 24 and 26°C.

Leaf hydraulic design

Hydraulic measurements were made on leaves of short and long shoots during summer. Shoots still attached to a length of branch were cut from the outer (sunlit) canopy. Branches were transported to the laboratory in a black plastic bag, re-cut underwater and placed in a water-filled beaker before being measured. Measurements were made on 16 leaves, nine from long shoots and seven from short shoots. Our aim was to determine whether the two leaf types differed in whole-leaf hydraulic conductance (K_{leaf}) and whether any differences reflected whole-leaf gas exchange. We also sought to understand how the transport capacity was distributed along the delivery pathway of leaves from base to margin for each leaf type.

We measured leaf hydraulic conductance by determining the flow rate into and through the leaf of water supplied to the petiole with positive pressure. The flow rate through the leaf for a given driving pressure was determined by measuring the pressure drop across a known resistance (3.4 m of 0.125 mm internal diameter polyetherketone (PEEK) tubing) in series with a sample leaf (Tyree *et al.*, 1999; Sack *et al.*, 2002). PEEK resistors were calibrated by applying a known pressure head and measuring the resulting flow onto an analytic balance (± 0.01 mg) with resistance calculated as $\Delta P/Q$, where ΔP is the applied pressure above atmospheric and Q is the mass flow rate recorded on the balance. Filtered and degassed 10 mM KCl was supplied to the system at a constant pressure of 0.2 MPa.

Because the resistance of a leaf dominates that of a branch in series with it, measuring the resistance of the leaf via the subtending branch portion effectively provides leaf resistance (Rockwell, 2010) and is now accepted practice (e.g. Brodribb & Cochard, 2009). The petioles of *G. biloba* are relatively flimsy and difficult to seal tightly without damaging the tracheids internally. Thus, the hydraulic measurements here were carried out on a leaf left intact attached to a 1–3-cm branch segment with all other leaves removed. The proximal end of the branch segment was cut under water and wrapped in laboratory film (Parafilm M; Alcan Inc., Montreal, QC, Canada) to provide a snug connection with the apparatus via ¼-inch tubing, again wrapped in Parafilm and secured with a cable tie. The leaf scars and the cut distal end of the branch were sealed with cyanoacrylate (Loctite 409; Loctite Corporation, Hartford, CT, USA). Preliminary experiments, in which the cut distal end of the branch and the leaf scars were sealed after connecting the branch to water pressure, resulted in steadily decreasing flow rates; however, stable flow rates were observed when the cuts were sealed before pressurization. Thus, all hydraulic experiments reported below were conducted using the latter method. A possible explanation for decreasing flow rates when the system was pressurized before sealing the distal end of the branch is that the relatively high volume of this branch segment allowed the measurement solution to flow through the branch and perfuse the leaf. In contrast, given the low absolute flow rates through *G. biloba* leaves (here, $\leq 5 \times 10^{-13}$ kg s^{-1}), for the time course of these experiments (< 4 h) sealing the distal end of the branch before pressurizing the system resulted in only xylem sap present in the branch at the time of excision being pushed through the lamina. We believe that the latter procedure more faithfully replicated the *in vivo* hydraulic conditions.

Pressure measurements were made with an MP50 pressure transducer (Micron Instruments, Simi Valley, CA, USA) located between the PEEK resistors and the sample leaf. Before each measurement, the pressure of the system when there was no flow through the leaf and the system was at equilibrium (initial delivery pressure, P_i) was recorded.

Pressure was recorded again after flow to the leaf was opened and steady-state flow rates were achieved (final pressure, P_0). Flow rate was determined as $((P_i - P_0)/P_0) \times (1/R_{PEEK})$, where R_{PEEK} is the total resistance of the PEEK resistors. During measurements, the sample leaf was supplied with 800 μmol m^{-2} s^{-1} PAR via a grow lamp suspended above a water bath. To prevent transpirational water loss, the leaf remained immersed in water, the temperature of which was monitored, allowing flow rates to be corrected for changes in viscosity resulting from changes in temperature. The temperature of the remainder of the system, measured using a thermocouple attached to the PEEK resistors, remained stable to within 2°C throughout the experimental period. Steady-state flow rates through the leaf were achieved approx. 20 min after being connected to the flow meter, at which point the flow rate was recorded. Leaf hydraulic conductance was calculated as the inverse of the difference in resistance of the intact sample (leaf + attached branch) minus the resistance with the blade removed, normalized by leaf area.

To determine the way in which transport capacity was distributed along the delivery pathway of *G. biloba* leaves, a fresh razor blade was used to make a continuous cut, 5 mm in from and parallel to the leaf margin, thus removing the distal 5-mm portion of the leaf blade. After cutting, steady state was achieved in approx. 5 min, whereupon the flow was recorded and a further portion of leaf blade, 5 mm from the distal edge, was removed. This process was repeated until only the leaf petiole remained (7–10 cuts, depending on the initial leaf size). Thus, measures of hydraulic permeability for a series of points along the water delivery pathway were obtained. Here, hydraulic permeability is defined as the total conductance per length of all veins located at a specific distance from the base of the leaf. The following function, which describes how the normalized hydraulic permeability (f) varies with distance from the base of the lamina, was fitted to the hydraulic data for each leaf type using a numerical iteration:

$$f(x) = (1 - x)^a \qquad \text{Eqn 2}$$

(x, the relative distance along the path length (equation 17, Zwieniecki *et al.*, 2006).)

Data analysis

Data were analysed using generalized linear models in the software package JMP (SAS Institute Inc., Cary, NC, USA). For leaf area, dissection index and LMA, leaf type was included as a fixed effect. For all other anatomical and morphological data, leaf type, position on the leaf, and the interaction between leaf type and position were treated as fixed effects. Student's t-test was used to analyse the difference between leaf types in Huber values. Analysis of gas exchange data included shoot type, branch, and their interaction as fixed effects. For measured hydraulic data, the source of the leaves used (i.e. large tree vs small trees) was included as a factor in analyses and found not to account for significant variation in hydraulic function. Therefore, results reported below are for leaves pooled from all source trees. Calculated resistivity and transport efficiency data were log-transformed, whereas all other data met the assumptions of normality and were not transformed. Analysis of variance was used to analyse differences in whole-leaf measurements of K_{leaf} between the two leaf types.

Results

Leaf morphology and anatomy

In almost every structural variable measured – from gross morphology to anatomy – short- and long-shoot leaves differed significantly from one another. As expected, when leaves from a comparable phyllotactic order were compared, the size of the two leaf types differed significantly, with short-shoot leaves being twice the size (cm^2) of long-shoot leaves (Table 1). Long-shoot leaves had a higher LDI (a measure of shape complexity) than short-shoot leaves; the variation in shape was almost three times greater in long- than short-shoot leaves (Table 1). The greater variation in shape complexity for long-shoot leaves reflects the fact that the number of lobes ranged from zero to four, although most leaves were lobed to some extent (Fig. 2b). Long-shoot leaves had a significantly higher LMA than short-shoot leaves (Table 1). Long-shoot leaves were also thicker than short-shoot leaves, with both leaf types being thicker near the base than at the centre or near the margin (Fig. 3a, Table 1).

SPI (a function of stomatal size and density) was approx. 20% higher in long-shoot leaves than short-shoot leaves (Table 1). Average stomatal pore length did not differ across the lamina or between leaf types; thus, the variation in SPI was driven by stomatal density, which was significantly higher in long- than in short-shoot leaves (Fig. 3b, Table 1). Additionally, in contrast to short-shoot leaves, where stomatal density was uniform across the lamina, stomatal density in long-shoot leaves was greater near the distal margin than at the centre or near the base (Table 1). Short-shoot leaves had higher vein density than long-shoot leaves and vein density was slightly higher near the base than near the leaf margin for both leaf types (Table 1). Average inter-vein distance was significantly greater in long-shoot leaves than in short-shoot leaves and did not differ among the base, centre and marginal regions of the leaf (Fig. 3c, Table 1).

Anatomical traits examined via cross-section varied with distance from the base of the leaf and the nature of this variation often differed between leaf types. The hydraulically weighted average tracheid diameter decreased from base to

Table 1 Differences in *Ginkgo biloba* long- and short-shoot leaf traits

Trait	Leaf type at each position (mean ± SE)			Significance level		
	Short	Long		Leaf type (df = 1)	Position (df = 2)	Leaf type × position (df = 2)
Average leaf area (cm^2) (n = 146)	28.0 ± 1.2	11.0 ± 0.5		***	NA[c]	NA
Dissection index (n = 127)	4.57 ± 0.03	5.04 ± 0.08		***	NA	NA
LMA (g m^{-2}) (n = 130)	0.84 ± 0.02	0.97 ± 0.02		***	NA	NA
Leaf thickness (μm) (n = 30)	278.0 ± 9.3	278.0 ± 14.2	B	*	***	
	206.0 ± 7.6	233.3 ± 11.4	C			
	163.3 ± 7.5	202.7 ± 11.2	M			
Stomatal density (number mm^{-2}) (n = 334)	25.15 ± 0.72	33.14 ± 1.31	B	***	*	**
	26.00 ± 0.59	33.47 ± 1.22	C			
	25.14 ± 0.64	40.07 ± 1.41	M			
Stomatal pore length (μm) (n = 383)	11.00 ± 0.56	10.81 ± 0.58	B			
	10.92 ± 0.54	10.84 ± 0.59	C			
	10.94 ± 0.54	10.54 ± 0.59	M			
Stomatal pore index (pore length × density) (n = 334)	307.7 ± 10.2	407.9 ± 14.8	B	***	**	**
	316.4 ± 8.1	413.5 ± 13.9	C			
	307.9 ± 9.8	485.0 ± 13.5	M			
Vein density[a] (cm cm^{-2}) (n = 254)	15.15 ± 0.22	13.79 ± 0.26	B	***	*	
	14.97 ± 0.26	12.70 ± 0.28	M			
Inter-vein distance (mm) (n = 380)	0.69 ± 0.01	0.80 ± 0.02	B	***		
	0.67 ± 0.01	0.83 ± 0.02	C			
	0.70 ± 0.02	0.85 ± 0.02	M			
Tracheid number per vein (n = 30)	18.3 ± 1.2	23.9 ± 1.6	B	*	***	
	9.4 ± 0.5	12.3 ± 1.7	C			
	7.5 ± 0.3	9.0 ± 1.6	M			
Hydraulically weighted tracheid diameter (μm) (n = 30)	14.1 ± 0.35	10.1 ± 0.26	B	***	***	**
	10.0 ± 0.37	9.04 ± 0.36	C			
	8.94 ± 0.58	8.56 ± 0.49	M			
Total lumen cross-sectional area (μm^2) (n = 30)	1,424.2 ± 109.6	1,210.5 ± 91.9	B		***	
	432.8 ± 35.1	460.6 ± 64.7	C			
	290.7 ± 34.5	301.7 ± 66.2	M			
Tracheid wall investment (μm^2) (n = 30)	1,386.1 ± 66.8	1,593.5 ± 42.7	B	*	***	
	515.3 ± 60.1	781.6 ± 161.3	C			
	407.6 ± 39.8	441.8 ± 58.5	M			
Calculated vein conductivity[b] (mmol m s^{-1} MPa^{-1}) ×10^4 (n = 30)	3.31 ± 0.63	1.66 ± 0.43	B		***	
	0.53 ± 0.10	0.44 ± 0.10	C			
	0.22 ± 0.07	0.24 ± 0.05	M			
Calculated transport efficiency[b] (mmol m^{-1} s^{-1} MPa^{-1}) ×10^{-4} (n = 30)	2.56 ± 0.12	1.09 ± 0.10	B	**	**	
	1.83 ± 0.80	0.67 ± 0.13	C			
	0.82 ± 0.14	0.66 ± 0.12	M			
Bundle sheath extension cross-sectional area (μm^2) (n = 30)	20 957 ± 2314	24 542 ± 2609	B	*	***	
	9496 ± 522	13 577 ± 522	C			
	7563 ± 529	10 075 ± 443	M			

Values are the mean and standard error for whole leaves or for sections made at different positions on the leaf: close to the base (B), centre (C) and margin (M).

[a]Vein density was measured at the base and margin only so df = 1 for position.

[b]ANOVA performed on log-transformed data.

[c]Measurements made on whole leaf.

Significance levels (P < 0.05 = *, 0.001 = ** and 0.0001 = ***) are from one- and two-factor ANOVAs testing for effects of leaf type, position and their interaction.

margin in both leaf types; however, the relative decrease was greater in short-shoot leaves because tracheid diameter near their base was significantly greater than in long-shoot leaves but did not differ near the margin (Figs 3d, 4a–d; Table 1). The average number of tracheids per vein cross-section was significantly greater in long- than short-shoot leaves and in both leaf types decreased from base to margin (Figs 3e, 4a–d; Table 1).

In terms of total vein lumen volume, the greater number of tracheids per vein cross-section in long-shoot leaves offset their relatively lower diameters near the base such that total lumen cross-sectional area did not significantly differ from

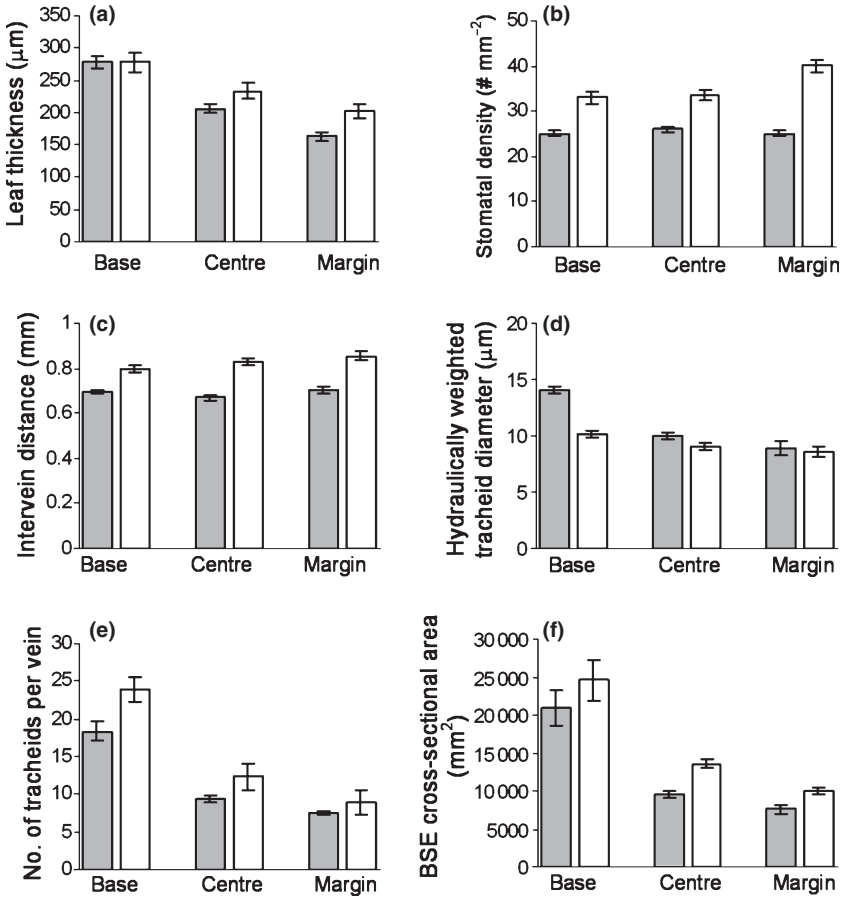

Fig. 3 Morphological and anatomical trait variations between *Ginkgo biloba* short-shoot leaves (shaded bars) and long-shoot leaves (open bars) at different locations on the leaf: near the base, centre and margin. Error bars are the standard error.

that of short-shoot leaves (Table 1). Similarly, calculated Hagen–Poiseuille vein conductivity did not differ significantly between leaf types overall (Table 1) but for all leaves was approx. 5 times higher near the base than at the centre, and 10 times higher near the base than near the margin (Fig. 5a). Vein transport efficiency, calculated as the Hagen–Poiseuille vein conductivity per cross-sectional tracheid wall area, also decreased from the base but was significantly lower for long- than for short-shoot leaves: 50% lower near the base and *c.* 20% lower near the margin (Fig. 5b, Table 1). This difference in transport efficiency was driven by the greater number of small diameter tracheids in long-shoot leaves, which had significantly greater tracheid wall cross-sectional area per vein cross-section than short-shoot leaves (Table 1).

As with tracheid and xylem traits, bundle sheath extension cross-sectional area was significantly greater near the base relative to the centre and near the margin of the lamina in both leaf types (Fig. 3f; Table 1). Additionally, BSE area was higher overall in long- than in short-shoot leaves (Fig. 4e,f; Table 1). Note that, in each transect pair used to calculate total BSE area, one transect ran parallel to the leaf plane and the other ran perpendicular to it. The overall difference in BSE area found here was driven by a significantly longer average parallel transect in long-shoot leaves (results not shown). The perpendicular transect, that is, that associated with leaf thickness, did not differ significantly between leaf types; therefore, the lower BSE area in short-shoot leaves cannot be attributed to their reduced leaf thickness relative to long-shoot leaves.

Branch allometry

Huber values describe the ratio of supporting xylem cross-sectional area to supplied leaf area, independent of position along the branch. During shoot elongation in early spring, short-shoot Huber values were significantly higher than those for long-shoots (Student's *t*-test: short, mean $0.000129 \pm 2.07 \times 10^{-5}$ SE; long, mean $0.000070 \pm 2.68 \times 10^{-5}$ SE; $t = 4.62_{(1,12)}$, $P < 0.001$). However, by the time shoots had fully elongated in mid-summer, this pattern had reversed, with the long-shoot xylem area : leaf area ratio exceeding that of short shoots (short, mean $0.000115 \pm 2.47 \times 10^{-5}$ SE; long, mean $0.000159 \pm 2.47 \times 10^{-5}$ SE; $t = 3.01_{(1,10)}$, $P < 0.05$).

Gas exchange

When measured in summer, long-shoot leaves had higher rates of gas exchange than short-shoot leaves. Independent

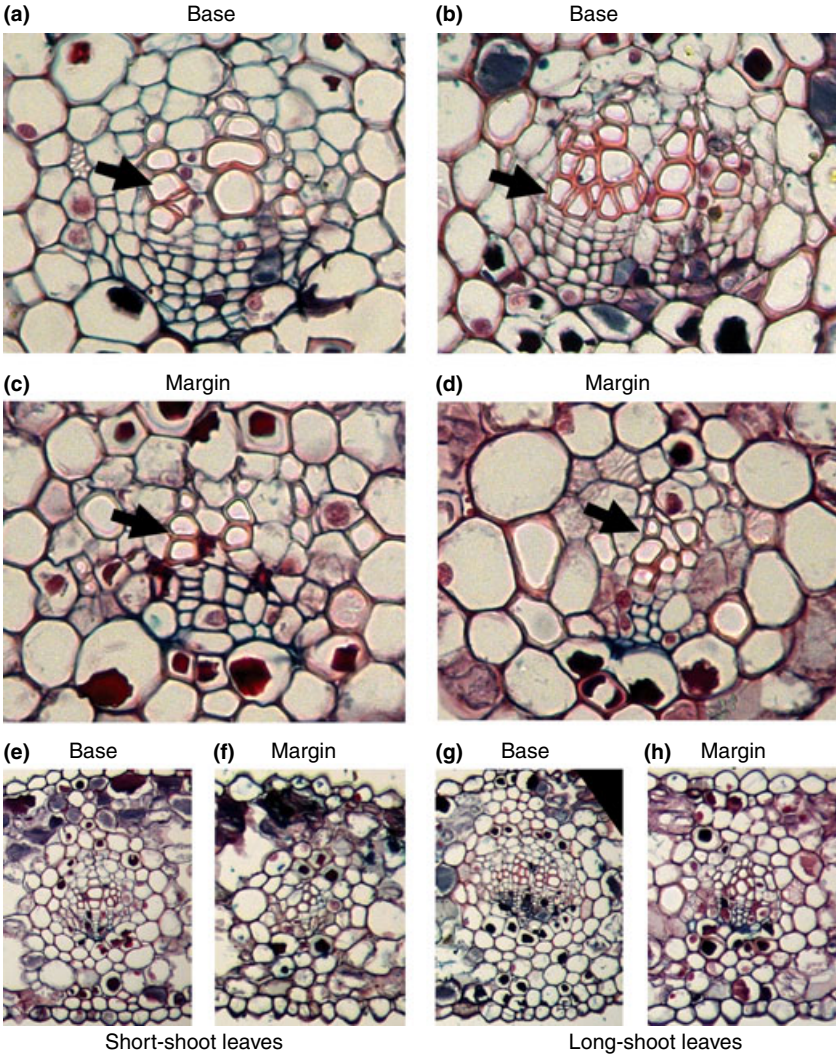

Fig. 4 Cross-sections through *Ginkgo biloba* leaves at 200× magnification, showing tracheids near the base of short-shoot leaves (a) and long-shoot leaves (b); and near the margin of short-shoot leaves (c) and long-shoot leaves (d). Tracheids are located at the centre of each vascular bundle, with their walls stained; the left, lowermost tracheid is indicated with an arrow. Plates (e)–(h) at 100× magnification show bundle sheath extensions (BSEs) near the base of short-shoot leaves (e) and long-shoot leaves (f); and near the margin of short-shoot leaves (g) and long-shoot leaves (h). BSEs are the group of well-defined cells between the vein and the lower and upper epidermis, although they are generally wider on the lower side of the leaf.

of any differences among branches, photosynthetic rate, stomatal conductance to CO_2, and transpiration rate were all significantly higher in long- than in short-shoot leaves (Table 2).

Leaf hydraulic design

As expected for a plant with tracheids, *G. biloba* leaf hydraulic conductance (K_{leaf}), averaging 6.31 mmol MPa^{-1} s^{-1} m^{-2}, was at the low end of the range for land plants but within the range for conifers (Brodribb *et al.*, 2005). Comparing leaf types, K_{leaf} was significantly higher in *G. biloba* long-shoot leaves than in short-shoot leaves (Table 2).

The distribution of hydraulic permeability from base to margin differed dramatically between leaf types (short-shoot leaves, exponent a = 0.0555; long-shoot leaves, exponent a = 0.3807). The optimal distribution of permeability (f) for a linear leaf with consistent water loss along its length (x) is described by $f(x) = (1 - x)^{0.5}$ (equation 17, Zwieniecki *et al.*, 2006), where permeability declines with length to supply progressively less leaf area. In comparison, because of the flared shape of *G. biloba* leaves, water loss should increase with distance from the base of the lamina to supply a progressively greater surface area. Consistent with this, the hydraulic permeability of *G. biloba* leaves exhibited a much shallower decline from base to tip than observed in linear leaves (Zwieniecki *et al.*, 2006). However, whereas base-to-margin permeability decreased markedly in long-shoot leaves, short-shoot leaves had nearly constant permeability across most of their length (Fig. 6).

Discussion

In this study, short- and long-shoot *G. biloba* leaves differed in almost every morphological, anatomical and physiological parameter measured. We believe that these differences are linked to developmental constraints associated

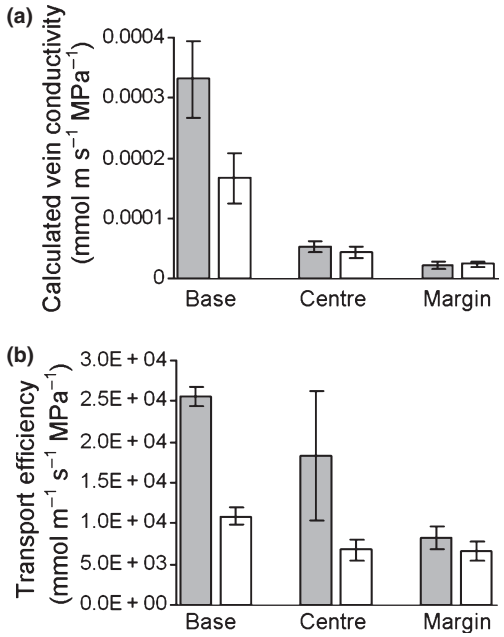

Fig. 5 Calculated Hagen–Poiseuille vein conductivity (a) and transport efficiency (b) at different locations on *Ginkgo biloba* short- (shaded bars) and long-shoot leaves (open bars). The Hagen–Poiseuille vein conductivity was calculated based on the number and internal diameter of tracheids per vein cross-section at each location. Transport efficiency was calculated as Hagen–Poiseuille vein conductivity per structural investment in xylem (estimated from cross-sectional tracheid wall area).

with the relative ontogeny of long vs short shoots. Short-shoot leaves undergo their initial development while protected within the bud and flushing leaves are supplied by xylem that has accumulated over multiple years in the branch supporting them. By contrast, the maturation of long-shoot leaves is dependent on water supplied by xylem that is still under construction. Indeed, we found the branch Huber values for long-shoot leaves during shoot elongation to be lower than those for short-shoot leaves, indicating that long-shoot leaves have less developed xylem in their supporting stems at this early stage. Coupled with reduced structural supply, transpirational water loss from the already formed short-shoot leaves is likely to decrease the water potential in the xylem of the developing long shoots. Thus, long-shoot leaves appear to be under greater hydraulic limitation than short-shoot leaves during development.

Evidence for differing hydraulic constraints on the two *G. biloba* leaf types comes from an analysis of leaf hydraulic supply. The decline in pressure within leaf veins is determined both by axial patterns of hydraulic permeability and by the rate at which water leaks radially from the veins to supply transpiration (Zwieniecki *et al.*, 2004a, 2006). The pronounced decrease in permeability towards the margin of long-shoot leaves could result in stomatal closure at the distal end of the flow path and/or insufficient water reaching distal cells. In this study, the margins of long-shoot leaves had significantly higher stomatal densities relative to other regions of the lamina, suggestive of drought stress during growth (Heckenberger *et al.*, 1998). Drought stress during cell expansion is reported to result in smaller leaves with more lobed margins in exposed, outer canopy leaves (Zwieniecki *et al.*, 2004b; Boyce, 2009). Because *G. biloba* leaves expand from a marginal meristem, hydraulic limitations during leaf expansion may be responsible for the smaller and more highly lobed long-shoot leaves that decrease in leaf size towards the distal end of the shoot.

The induction mechanisms of long-shoot elongation have not been identified but there is some evidence that external cues, such as light or temperature, are involved (Flesch *et al.*, 1991). This suggests that long shoots are initiated when conditions are favourable for vigorous branch growth, as is the case for long shoots of other species, which extend the canopy by capitalizing on high light conditions (Yagi, 2000; Kull & Tulva, 2001). However, achievement of vigorous growth during a short growing period requires high rates of gas exchange supported by an adequate hydraulic supply. In *G. biloba*, initial hydraulic limitations in long-shoot leaves were apparently overcome, as demonstrated by their comparatively strong physiological performance later in the growing season. Their low springtime

Table 2 Differences in gas exchange and whole-leaf hydraulic conductance K_{leaf} between long- and short-shoot leaves of *Ginkgo biloba*

	Leaf type (mean ± SE)		Significance level		
Trait	Short	Long	Leaf type (df = 1)	Branch (df = 2)	Leaf type × branch (df = 2)
Photosynthesis (μm m^{-2} s^{-1})	4.19 ± 0.28	5.12 ± 0.23	**	***	
Stomatal conductance (mol m^{-2} s^{-1})	0.057 ± 0.005	0.078 ± 0.004	**	**	
Transpiration (mmol m^{-2} s^{-1})	1.05 ± 0.07	1.41 ± 0.06	***	*	
Hydraulic conductance (K_{leaf}, mmol MPa^{-1} s^{-1} m^{-2})	4.69 ± 0.37	7.58 ± 0.66	**	NA[1]	NA

[1]Analyses conducted on pooled data from multiple trees.
Values are the mean and standard error. Significance levels ($P < 0.05$ = *, 0.001 = ** and 0.0001 = ***) are from one- and two-factor ANOVAs testing for effects of leaf type, branch and their interaction.

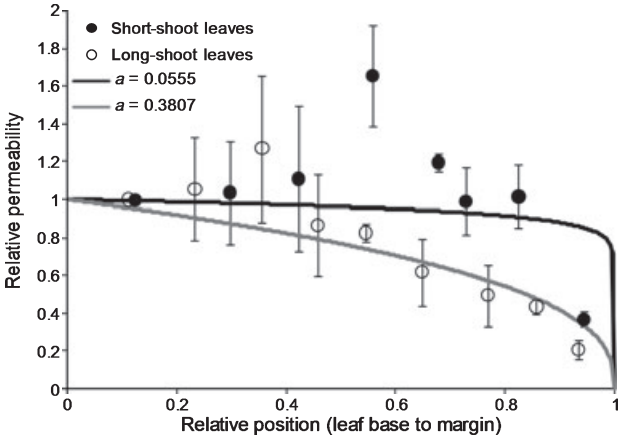

Fig. 6 Total hydraulic permeability, normalized to 1 at the leaf base, vs relative path length in *Ginkgo biloba* short-shoot leaves (solid circles) and long-shoot leaves (open circles). Data points are the mean of measurements made on cut leaf sections (see the body of the text for details), representing hydraulic permeability (conductance per length) at relative distances along the hydraulic pathway, from the base of the leaf just above the petiole ($x = 0$) to the distal edge just inside the outer margin ($x = 1$). Error bars are the standard error. The black line is the curve fitted to the short-shoot leaf data; the grey line is the curve fitted to the long-shoot leaf data. Values shown on the graph are for the exponent of the function $f(x) = (1 - x)^a$ describing how the relative hydraulic permeability (f) varies with distance from the base of the lamina.

Huber values were replaced in mid-summer by significantly higher values than those of short-shoot leaves. This increased xylem area supplying long-shoot leaves enabled higher transpiration and stomatal conductance, in turn supported by higher stomatal densities. Additionally, unlike these experimental gas exchange measurements, which were made under common boundary layer conditions within the IRGA cuvette, in nature the conditions experienced by the two leaf types might be quite different. The smaller and well-spaced long-shoot leaves probably experience smaller boundary layers and thus higher rates of transpiration for the same stomatal aperture than the larger and closely packed long-shoot leaves. These differences in shoot architecture would serve to accentuate the differences in transpiration rates we observed. The high demand of long-shoot leaves was met by significantly higher K_{leaf} than that for short-shoot leaves, consistent with other studies that have found a relationship between leaf hydraulic capacity and rates of water loss (Brodribb *et al.*, 2002, 2005).

High rates of gas exchange potentially could increase the risk of cavitation in *G. biloba* long-shoot leaves, particularly in summer. The average daytime temperature experienced by our study trees during leaf flushing in April is 9°C, whereas shoot development continues well into the summer, with temperatures averaging 23°C (values for both Chicago, IL and Cambridge, MA; CustomWeather, 2010). For even slight increases in vapour pressure deficit, *G. biloba* stomata are highly sensitive (Franks & Farquhar, 1999), suggesting a high safety margin to decrease the risk of xylem cavitation (Franks & Brodribb, 2005). Long-shoot leaves could further reduce cavitation risk through the possession of narrower basal tracheids than short-shoot leaves. The development of narrow conduits in late-wood xylem in response to the changing hydraulic demands during the growing season is well documented (Sass & Eckstein, 1995; Fonti *et al.*, 2010). The narrow, thick-walled tracheids of *G. biloba* long-shoot leaves might thus represent an adaptation for increased safety during periods of high evaporative demand.

Although the hydraulic differences between leaf types may be explained from an ecological standpoint, the anatomical basis for the observed differences in K_{leaf} is less clear-cut. Through a tradeoff between tracheid size and number, *G. biloba* long-shoot leaves had the same calculated vein conductivity as short-shoot leaves. However, the density of these equally conductive veins was lower in long-shoot leaves than in short-shoot leaves, yet they still achieved a higher K_{leaf}. One explanation is that higher K_{leaf} in long-shoot leaves is facilitated by relatively greater radial flow. In most leaves, where there exists a vein size hierarchy, the proportion of total resistance occurring outside the major veins and petiole can be up to 75% (Sack *et al.*, 2004). *Ginkgo biloba* leaves have only one vein order. Their parallel veins dichotomously branch but essentially span the entire length of the leaf, maintaining an equal distance from one another. The bundle sheath extension (BSE) has been recognized as playing an important role in delivering water to the leaf lamina via the epidermis (Sheriff & Meidner, 1974; Byott & Sheriff, 1976). Resistance to flow from the BSE to the epidermis is thought to be low relative to the resistance between the epidermis and the mesophyll (Sheriff & Meidner, 1974). This suggests that there exists a vein–BSE–epidermis water delivery continuum distinct from other compartments within a leaf such as the mesophyll (Zwieniecki *et al.*, 2007). As *G. biloba* leaves lack a minor vein network, the BSE potentially comprises a key component of the water delivery complex. The larger BSEs of long-shoot leaves may be a major factor in increasing K_{leaf} by increasing radial flow from the veins.

There are various ways of achieving heterophylly and many result in distinct leaf types best suited to their particular environmental conditions. In *G. biloba* long-shoot leaves, the small size, deep and numerous lobes, and high gas exchange rates beg comparison to sun leaves. However, heterophylly derived from the ontogenetic development of long and short shoots should not be considered analogous to sun/shade heterophylly arising from variation in canopy placement (Niklas & Cobb, 2010). Recent work on short-shoot leaves of *G. biloba* showed that reduced lamina area and higher vein density occur at the top of the canopy, demonstrating spatially induced sun/shade-leaf characteristics *within* a shoot type, rather than *between* shoot types

(Boyce, 2009). Yet when comparing between shoot types, we found that vein density was significantly lower in long-shoot leaves than in short-shoot leaves. Thus, *G. biloba* possesses two forms of heterophylly: sun and shade leaves at different regions of the canopy (Boyce, 2009), and long- and short-shoot leaves arising from distinct shoot types. While much has been written on the presumed function of sun vs shade leaves, this study demonstrates the ecological significance of shoot-derived heterophylly. Like sun and shade leaves, long- and short-shoot leaves display an equally dramatic divergence in structure and function. However, although aspects of morphology may arise as a consequence of differing hydraulic constraints during growth, the association of the two leaf types with preformed and *in situ* bud development suggests a certain degree of hard-wired differentiation.

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References

Arnott HJ. 1959. Anastomoses in the venation of *Ginkgo biloba*. *American Journal of Botany* **46**: 405–411.

Bornhoeft G, Maxion-Bergemann S, Matthiessen PF. 2008. External validity of clinical trials for treatment of dementia with *Ginkgo biloba* extracts. *Zeitschrift Fur Gerontologie Und Geriatrie* **41**: 298–312.

Boyce CK. 2009. Seeing the forest with the leaves – clues to canopy placement from leaf fossil size and venation characteristics. *Geobiology* **7**: 192–199.

Brodribb TJ, Cochard H. 2009. Hydraulic failure defines the recovery and point of death in water-stressed conifers. *Plant Physiology* **149**: 575–584.

Brodribb T, Hill RS. 1993. A physiological comparison of leaves and phyllodes in *Acacia melanoxylon*. *Australian Journal of Botany* **41**: 293–305.

Brodribb TJ, Holbrook NM. 2005. Water stress deforms tracheids peripheral to the leaf vein of a tropical conifer. *Plant Physiology* **137**: 1139–1146.

Brodribb TJ, Holbrook NM, Gutierrez MV. 2002. Hydraulic and photosynthetic co-ordination in seasonally dry tropical forest trees. *Plant, Cell & Environment* **25**: 1435–1444.

Brodribb TJ, Holbrook NM, Zwieniecki MA, Palma B. 2005. Leaf hydraulic capacity in ferns, conifers and angiosperms: impacts on photosynthetic maxima. *New Phytologist* **165**: 839–846.

Byott GS, Sheriff DW. 1976. Water movement into and through *Tradescantia virginiana* (L.) leaves. 2. Liquid flow pathways and evaporative sites. *Journal of Experimental Botany* **27**: 634–639.

Chamberlain CJ. 1966. *Gymnosperms: structure and evolution*. New York, NY, USA: Dover Publications, Inc.

Cochard H, Nardini A, Coll L. 2004. Hydraulic architecture of leaf blades: where is the main resistance? *Plant, Cell & Environment* **27**: 1257–1267.

Critchfield WB. 1970. Shoot growth and heterophylly in *Ginkgo biloba*. *Botanical Gazette* **131**: 150–162.

CustomWeather 2010. [WWW document].URL http://www.customweather.com/ [accessed on 7 August 2010].

Del Tredici P. 2007. The phenology of sexual reproduction in *Ginkgo biloba*: ecological and evolutionary implications. *Botanical Review* **73**: 267–278.

Del Tredici P, Hsieh L, Guang Y. 1992. The *Ginkos* of Tian Mu Shan. *Conservation Biology* **6**: 202–209.

Flesch V, Jacques M, Cosson L, Petiard V, Balz JP. 1991. Effects of light and temperature on growth of *Ginkgo biloba* cultivated under controlled long day conditions. *Annales Des Sciences Forestieres* **48**: 133–147.

Fonti P, von Arx G, Garcia-Gonzalez I, Eilmann B, Sass-Klaassen U, Gartner H, Eckstein D. 2010. Studying global change through investigation of the plastic responses of xylem anatomy in tree rings. *New Phytologist* **185**: 42–53.

Foster AS. 1938. Structure and growth of the shoot apex in *Ginkgo biloba*. *Bulletin of the Torrey Botanical Club* **65**: 531–556.

Franks PJ, Brodribb TJ. 2005. Stomatal control and water transport in the xylem. In: Holbrook NM, Zwieniecki MA, eds. *Vascular transport in plants*. Burlington, MA, USA: Elsevier Academic Press, 69–89.

Franks PJ, Farquhar GD. 1999. A relationship between humidity response, growth form and photosynthetic operating point in C-3 plants. *Plant, Cell & Environment* **22**: 1337–1349.

Gunckel JE, Wetmore RH. 1946. Studies of development in long shoots and short shoots of *Ginkgo biloba* L. 1. The origin and pattern of development of the cortex, pith and procambium. *American Journal of Botany* **33**: 285–295.

Hamerlynck EP, Knapp AK. 1994. Leaf-level responses to light and temperature in two co-occurring *Quercus* (Fagaceae) species – implications for tree distribution patterns. *Forest Ecology & Management* **68**: 149–159.

Heckenberger U, Roggatz U, Schurr U. 1998. Effect of drought stress on the cytological status in *Ricinus communis*. *Journal of Experimental Botany* **49**: 181–189.

Jensen W. A. 1962. *Botanical Histochemistry: principles and Practice*. San Francisco, CA, USA: W.H. Freeman and Company.

Jones CS. 2001. The functional correlates of heteroblastic variation in leaves: changes in form and ecophysiology with whole plant ontogeny. *Boletin Sociedad Argentina de Botanica* **36**: 171–186.

Kitajima K, Mulkey SS, Wright SJ. 1997. Seasonal leaf phenotypes in the canopy of a tropical dry forest: photosynthetic characteristics and associated traits. *Oecologia* **109**: 490–498.

Kull O, Tulva I. 2001. Shoot structure and growth along a vertical profile within a *Populus tilia* canopy. *Tree Physiology*, **22**: 1167–1175.

Lewis AM, Boose ER. 1995. Estimating volume flow rates through xylem conduits. *American Journal of Botany* **82**: 1112–1116.

McLellan T, Endler JA. 1998. The relative success of some methods for measuring and describing the shape of complex objects. *Systematic Biology* **47**: 264–281.

Moore PD. 1992. Ecology – a leaf for all seasons. *Nature* **360**: 110–111.

Mulkey SS, Smith AP, Wright SJ, Machado JL, Dudley R. 1992. Contrasting leaf phenotypes control seasonal variation in water loss in a tropical forest shrub. *Proceedings of the National Academy of Sciences, USA* **89**: 9084–9088.

Niklas KJ, Cobb ED. 2010. Ontogenetic changes in the numbers of short- vs. long-shoots account for decreasing specifica leaf area in *Acer rubrum* (Aceraceae) as trees increase in size. *American Journal of Botany* **97**: 27–37.

Rockwell FE. 2010. *Leaf water transport*. Doctoral thesis, Harvard University Cambridge, Cambridge, MA, USA.

Royer DL, Hickey LJ, Wing SL. 2003. Ecological conservatism in the "living fossil" *Ginkgo*. *Paleobiology* **29**: 84–104.

Sack L, Cowan PD, Jaikumar N, Holbrook NM. 2003. The 'hydrology' of leaves: co-ordination of structure and function in temperate woody species. *Plant, Cell & Environment* **26**: 1343–1356.

Sack L, Frole K. 2006. Leaf structural diversity is related to hydraulic capacity in tropical rain forest trees. *Ecology* **87**: 483–491.

Sack L, Melcher PJ, Zwieniecki MA, Holbrook NM. 2002. The hydraulic conductance of the angiosperm leaf lamina: a comparison of three measurement methods. *Journal of Experimental Botany* **53**: 2177–2184.

Sack L, Streeter CM, Holbrook NM. 2004. Hydraulic analysis of water flow through leaves of sugar maple and red oak. *Plant Physiology* **134**: 1824–1833.

Sack L, Tyree MT, Holbrook NM. 2005. Leaf hydraulic architecture correlates with regeneration irradiance in tropical rainforest trees. *New Phytologist* **167**: 403–413.

Sass U, Eckstein D. 1995. The variability of vessel size in beech (*Fagus sylvatica* L.) and its ecophysiological interpretation. *Trees-Structure and Function* **9**: 247–252.

Sheriff DW, Meidner H. 1974. Water pathways in leaves of *Hedera helix* L. and *Tradescantia virginiana* L. *Journal of Experimental Botany* **25**: 1147–1156.

Smith WK, Nobel PS. 1977. Temperature and water relations for sun and shade leaves of a desert broadleaf, *Hyptis emoryi*. *Journal of Experimental Botany* **28**: 169–183.

Sperry JS, Saliendra NZ. 1994. Intra-plant and inter-plant variation in xylem cavitation in *Betula occidentalis*. *Plant, Cell & Environment* **17**: 1233–1241.

Tyree MT, Sobrado MA, Stratton LJ, Becker P. 1999. Diversity of hydraulic conductance in leaves of temperate and tropical species: possible causes and consequences. *Journal of Tropical Forest Science* **11**: 47–60.

Yagi T. 2000. Morphology and biomass allocation of current-year shoots of ten tall tree species in cool temperate Japan. *Journal of Plant Research* **113**: 171–183.

Zhang C, Tanabe K, Fumio T, Itai A, Wang S. 2005. Spur characteristics, fruit growth and carbon partitioning in two late-maturing Japanese pear (*Pyris pyrifolia* Nakai) cultivars with contrasting fruit size. *Journal of the American Society for Horticultural Science* **130**: 252–260.

Zwieniecki MA, Boyce CK, Holbrook NM. 2004a. Functional design space of single-veined leaves: role of tissue hydraulic properties in constraining leaf size and shape. *Annals of Botany* **94**: 507–513.

Zwieniecki MA, Boyce CK, Holbrook NM. 2004b. Hydraulic limitations imposed by crown placement determine final size and shape of *Quercus rubra* L. leaves. *Plant, Cell & Environment* **27**: 357–365.

Zwieniecki MA, Brodribb T, Holbrook NM. 2007. Hydraulic design of leaves: insights from rehydration kinetics. *Plant, Cell & Environment* **30**: 910–921.

Zwieniecki MA, Stone HA, Leigh A, Boyce CK, Holbrook NM. 2006. Hydraulic design of pine needles: one-dimensional optimization for single-vein leaves. *Plant, Cell & Environment* **29**: 803–809.