

ORIGINAL ARTICLE

Bundle sheath lignification mediates the linkage of leaf hydraulics and venation

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Abstract

The lignification of the leaf vein bundle sheath (BS) has been observed in many species and would reduce conductance from xylem to mesophyll. We hypothesized that lignification of the BS in lower-order veins would provide benefits for water delivery through the vein hierarchy but that the lignification of higher-order veins would limit transport capacity from xylem to mesophyll and leaf hydraulic conductance (K_{leaf}). We further hypothesized that BS lignification would mediate the relationship of K_{leaf} to vein length per area. We analysed the dependence of K_{leaf} and its light response, on the lignification of the BS across vein orders for 11 angiosperm tree species. Eight of 11 species had lignin deposits in the BS of the midrib, and two species additionally only in their secondary veins, and for six species up to their minor veins. Species with lignification of minor veins had a lower hydraulic conductance of xylem and outside-xylem pathways and lower K_{leaf} . K_{leaf} could be strongly predicted by vein length per area and highest lignified vein order ($R^2 = .69$). The light-response of K_{leaf} was statistically independent of BS lignification. The lignification of the BS is an important determinant of species variation in leaf and thus whole plant water transport.

KEYWORDS

bundle sheath extensions, high pressure flow metre method, irradiance, leaf hydraulic conductance, leaf venation architecture

1 | INTRODUCTION

Leaf water transport capacity is a critical determinant of rates of transpiration and carbon gain. The leaf vein xylem and outside-xylem pathways account for the leaf hydraulic resistance ($\text{m}^2 \text{ s MPa mmol}^{-1}$, inverse of leaf hydraulic conductance, K_{leaf}), which, in turn, contributes a substantial fraction of plant hydraulic resistance (Brodribb, Feild, & Jordan, 2007; Sack et al., 2013; Sack & Holbrook, 2006; Scoffoni et al., 2016). Within the leaf, water moves through xylem in the petiole and axially throughout the vein system and radially out of the veins via apoplastic and symplastic routes through the bundle sheath (BS), BS extensions (BSEs) in certain species, mesophyll, and epidermal cells to sites where water evaporates before diffusing from the stomata (Buckley, John, Scoffoni, & Sack, 2017; Nardini & Salleo, 2003). This study focuses on the role of lignification of the BS as a potentially major contributor to leaf hydraulic capacity.

Many recent studies have shown that species variation in K_{leaf} is related to the morphological traits of vein and parenchyma tissues

(Buckley, Sack, & Gilbert, 2011; Sack & Frole, 2006; Scoffoni et al., 2016; Xiong et al., 2015) and especially the vein length per leaf area (VLA; also known as “vein density”; Brodribb et al., 2007; Kawai & Okada, 2016; Sack et al., 2013; Sack & Frole, 2006; Scoffoni et al., 2016; Xiong et al., 2015). A high VLA enables rapid water transport via the xylem, providing more parallel pathways through the reticulate vein network and a reduced distance outside the xylem to evaporation sites (Brodribb et al., 2007; Buckley et al., 2017; McKown, Cochard, & Sack, 2010; Sack & Frole, 2006) and would thus reduce both xylem and outside-xylem hydraulic resistances (R_x and R_{ox} , respectively; Sack & Frole, 2006; Brodribb et al., 2007). Indeed, differences among species in VLA scales up to driving variation in stomatal conductance and light-saturated photosynthetic rate across some sets of diverse species, and in an evolutionary context among closely related species (Brodribb et al., 2007; Sack et al., 2013; Scoffoni et al., 2016; Walls, 2011). However, a number of studies have found K_{leaf} to be statistically independent of VLA across other diverse species sets (Nardini, Gortan, & Salleo, 2005; Nardini, Peda, & La Rocca, 2012;

Scoffoni et al., 2015; Scoffoni, Rawls, McKown, Cochard, & Sack, 2011; Somerville, Sack, & Ball, 2012; Taneda, Kandel, Ishida, & Ikeda, 2016). This discrepancy has often been attributed to the multiple xylem and outside-xylem traits that influence K_{leaf} , including, for example, vein cross-sectional conductivity and vein parenchyma or BS membrane permeability (Caringella, Bongers, & Sack, 2015; Volkov et al., 2007), in particular due to the differential activity of aquaporins that control membrane permeability (Cochard et al., 2007; Cohen, Attia, & Moshelion, 2011; Lopez et al., 2013).

A potentially major determinant of variation among species in K_{leaf} is the deposition of lignin in cell walls in the BS cells surrounding the vein xylem and phloem. Lignification of cell walls enhances the mechanical strength (Niklas, 1992), prevents infected pathogens from spreading into other cells (Sticher, Mauch-Mani, & Metraux, 1997), and reduces the capacity to conduct water (North & Peterson, 2005). In the primary tissues of the root, lignification of cellulose microfibrils in the endodermis cell walls, known as Casparian bands, renders the walls hydrophobic (Enstone, Peterson, & Ma, 2003; Geldner, 2013) and inhibits the apoplastic transport of water and inorganic ions from the cortex to the stele, requiring water to pass through cell membranes and increasing root hydraulic resistance (Donaldson, 2001; North & Peterson, 2005; Steudle, 2000). Several studies have focused on developmental and functional commonalities between the root endodermis and leaf BS (Mertz & Brutnell, 2014; Sack & Scoffoni, 2013; Slewinski, Anderson, Zhang, & Turgeon, 2012); notably, lignification of cell walls can occur in both. Indeed, the lignification of leaf BS cells or the formation of a lignified leaf vein endodermis is common pteridophytes and conifers, though rarely investigated in angiosperms (Lersten, 1997; Liesche, Martens, & Schulz, 2011). A recent study showed that a number of *Rhododendron* species possessed lignified BS and BSEs and that lower-altitude species had more lignification and lower K_{leaf} (Taneda et al., 2016), consistent with reduced radial flow of water out of the veins. Models of roots, leaves, and shoots have shown that changes in axial versus radial transport through “leaky” systems influence the overall hydraulic resistance (Cochard, Nardini, & Coll, 2004; Landsberg & Fowkes, 1978; McKown et al., 2010; North, Lynch, Maharaj, Phillips, & Woodside, 2013; Taneda & Taten, 2011). We hypothesized that BS

lignification would lead to reduced radial water transport from the vein xylem. Thus, lignification of the lower-order veins should increase R_x , by forcing water through a longer axial transport pathway to the higher-order veins, whereas lignification of lower- and/or higher-order veins should increase R_{ox} , by impeding transport from vein xylem to parenchymatous tissues. Consequently, BS lignification should reduce K_{leaf} . Further, species with BS lignification extending to higher vein orders should have higher R_x and R_{ox} and lower K_{leaf} .

We tested for a potential role of the lignification of leaf vein BS cells on K_{leaf} for diverse angiosperm trees. We examined the extent of lignification and its relationship to R_x and R_{ox} and lower K_{leaf} . In addition, we tested for an influence of lignification on the response of K_{leaf} to irradiance, which has been attributed to aquaporin expression at BS cells in response to light. Previous hypotheses suggested that reduced K_{leaf} in low irradiance may relate to water leaving the xylem via the apoplast and that high irradiance driving aquaporin expression in the BS would cause increased transmembrane flow and higher K_{leaf} (Cochard et al., 2007; Cohen et al., 2011). Such hypotheses would imply that greater apoplast resistance would cause a lower K_{leaf} in low irradiance and thus a greater K_{leaf} light response. Conversely, if water leaves the xylem through the BS and apoplast in series, then a greater apoplast resistance would lead to a lower overall effect of aquaporin expression in the BS, causing a lower K_{leaf} light response. The objective of this work is to clarify the role of vein BS lignification as a determinant of leaf and whole plant water transport.

2 | MATERIALS AND METHODS

2.1 | Plant materials

We focused on 11 forest angiosperm species of temperate forests in Japan diverse in leaf habit and structure (Table 1): deciduous species *Acer rufinerve* Siebold et Zucc., *Cerasus speciosa* (Koidz.) H. Ohba, *Cornus controversa* Hemsl. ex Prain, *Magnolia kobus* DC., *Mallotus japonicus* (L.f.) Müll. Arg., and *Quercus serrata* Murray, and evergreen

TABLE 1 Plant species used in this study

Species	Symbols	Leaf habit	SLA (cm ² g ⁻¹)	Leaf area (cm ²)	VLA (mm ⁻¹)
<i>Quercus serrata</i> Murray	Qs	D	138 ± 4.31 ^{a,b,c}	32.3 ± 2.8 ^{a,b}	10.5 ± 0.631 ^a
<i>Magnolia kobus</i> DC.	Mk	D	155 ± 4.05 ^{d,e}	66.2 ± 1.4 ^{c,d}	5.67 ± 0.340 ^b
<i>Acer rufinerve</i> Siebold et Zucc.	Ar	D	165 ± 11.8 ^{d,e}	119 ± 5.4 ^e	7.82 ± 0.469 ^{c,d}
<i>Quercus acuta</i> Thunb.	Qa	E	96.1 ± 1.06 ^{f,g}	57.6 ± 2.1 ^{a,f}	6.84 ± 0.410 ^{b,d}
<i>Cinnamomum camphora</i> (L.) J.Presel	Cca	E	113 ± 6.76 ^{c,f,g}	23.8 ± 1.3 ^b	10.8 ± 0.645 ^a
<i>Ligustrum lucidum</i> Aiton	LI	E	118 ± 6.16 ^{b,c,f}	41.0 ± 2.4 ^{a,g}	6.86 ± 0.412 ^{b,d}
<i>Quercus myrsinaefolia</i> Blume	Qm	E	83.3 ± 1.05 ^g	49.7 ± 2.5 ^{f,g}	9.95 ± 0.597 ^{a,e}
<i>Cornus controversa</i> Hemsl. ex Prain	Cco	D	177 ± 6.63 ^d	76.4 ± 4.6 ^c	5.47 ± 0.328 ^b
<i>Viburnum odoratissimum</i> Ker Gawl. var. <i>awabuki</i> (K. Koch) Zabel	Vo	E	104 ± 3.65 ^{f,g}	32.0 ± 1.4 ^{a,b}	8.56 ± 0.514 ^{c,d,e}
<i>Mallotus japonicus</i> (L.f.) Müll. Arg.	Mj	D	215 ± 8.65 ^h	114 ± 4.6 ^e	14.8 ± 0.889 ^f
<i>Cerasus speciose</i> (Koidz.) H. Ohba	Cs	D	147 ± 5.91 ^{a,b,d}	39.4 ± 2.1 ^{e,g}	9.19 ± 0.551 ^{a,c,e}

Note. SLA and VLA are specific leaf area (=leaf area/leaf dry mass) and vein length per area (=total vein length/area), respectively. D and E are deciduous and evergreen trees, respectively. Data are means ± 1 SE (n = 8–16). Superscript letters indicate significant differences among species in analysis of variance and Tukey's multiple tests at p = .05.

species *Cinnamomum camphora* (L.) J.Presel, *Ligustrum lucidum* Aiton, *Quercus acuta* Thunb. var *yanagitae* Makino, *Quercus myrsinifolia* Blume, and *Viburnum odoratissimum* Ker Gawl. var. *awabuki* (K. Koch) Zabel (Satake, Hara, Watari, & Tominari, 1989). Sun-exposed branches were sampled from one to three mature trees of each species planted on the Hongo campus of the University of Tokyo (35° 42' 48"N, 139° 45' 44"E, 20 m above sea level). The mean annual air temperature was 15.4 °C, and the annual precipitation was 1,528 mm (Tokyo weather station, 1981–2010, Japan Meteorological Agency, <http://www.jma.go.jp/jma/index.html>). These trees were growing under similar soil moisture. Hydraulic conductance was measured between June and October 2014, and leaf anatomical traits between June 2014 and May 2017.

2.2 | Measuring leaf lamina hydraulic conductance

We determined the leaf hydraulic resistance, its partitioning between xylem and outside-xylem compartments, and their light responses using the high-pressure-flow-metre (HPFM) method (Nardini, Tyree, & Salleo, 2001; Sack, Streeter, & Holbrook, 2004; Tyree, Patino, Bennink, & Alexander, 1995). We present overall leaf water transport capacity as leaf hydraulic conductance (K_{leaf}) but partition this capacity in terms of resistances (i.e., $1/\text{conductances}$) of xylem and outside-xylem pathways.

Branches were sampled the evenings before measurements. More than four sun-exposed branches with mature leaves were cut 75 cm proximally to the branch tip and transported to the laboratory in a plastic bag, recut under water by 3 cm and rehydrated overnight under plastic. To examine the light response of K_{leaf} , half the branches were exposed to $\sim 300 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (high light [HL]) in a growth chamber (LP-1.75P2, NKsystem, Osaka, Japan) for 3 to 5 h before K_{leaf} measurement, whereas others were subjected to ambient laboratory irradiance ($< 3 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$, low light [LL]).

In the HPFM, the sample leaf is connected by its petiole via silicone tubing in series with tubing of high-resistance (R_{ref}) as a reference resistor running to a reservoir of solution (TM5R, Unicontrol, Chiba, Japan) of 20 mM KCl solution filtered through a 0.2- μm membrane filter (Sartorius, Göttingen, Germany). The high-resistance tubing was polyethyletherketone tube (PEEK tube, Shimadzu GLC, Tokyo, Japan) with 0.13 mm inner diameter. The length of the high-resistance tubing was 3, 7, or 10 m, depending on the desired flow rate through the leaf, as the measurement gains in signal relative to noise, that is, in precision, by matching R_{ref} to the hydraulic resistance of the leaf. R_{ref} was measured as the ratio of the applied pressure of 0.25 MPa to the mass flow rate through the tubing to an electronic balance (AUW220, Shimadzu, Kyoto, Japan). During the measurement, to avoid extra transpiration, the sample leaf was placed in water with bubbled air maintained at 25 ± 2 °C temperature. The flow of KCl solution through the HPFM apparatus was generated by applying 0.25 MPa of a compressed air (high pressure foot-pump; BALOhashi, Osaka, Japan). After the flow rate reached a steady state, the pressures applied to the flow solution before and after the high-resistance tubing (p_1 and p_2) were measured with pressure

transducers (AP-13S, Keyence, Osaka, Japan), and the hydraulic resistance was determined as $p_2/(p_1 - p_2) \times R_{ref}$.

The partitioning of hydraulic resistance was measured using the vein cutting method (Nardini et al., 2005; Sack et al., 2004; Sack & Frole, 2006; Scoffoni et al., 2016). This method assumes that water passes through the primary-, second-, third-, and higher-order veins and parenchyma tissues before leaving the vein xylem and thus that the hydraulic resistances of these pathways can be considered as arranged in series following Ohm's law (see Section 6 for further consideration of this assumption). After measurement of whole-leaf hydraulic resistance, the fourth- and higher-order veins were cut with 2–3 mm excisions until the flow rate was constant, and the hydraulic resistance of leaves with the fourth-order vein cuts (R_{fourth}) was determined. Then, first-order veins were cut at their bases for determination of the hydraulic resistance of the petiole ($R_{petiole}$). The leaf-lamina hydraulic resistance (R_{leaf}) was estimated by subtracting $R_{petiole}$ from whole-leaf hydraulic resistance ($R_{whole-leaf} = R_{whole-leaf} - R_{petiole}$). The hydraulic resistance of the outside-xylem pathway (R_{ox}) and of vein xylem pathway (R_x) was estimated as $R_{whole-leaf} - R_{fourth}$ and $R_{fourth} - R_{petiole}$, respectively. The leaf was digitized with a scanner (PX-602F, EPSON, Nagano, Japan), and leaf area was obtained with image analysis software (ImageJ, <http://rsbweb.nih.gov/ij/>). Leaves were oven dried at 80 °C overnight, and specific leaf area ($\text{cm}^2 \text{ g}^{-1}$) was determined as leaf lamina area divided by dry mass. The values of R_{leaf} , R_{ox} , and R_x were normalized by the leaf lamina area and standardized for the thermal effect on the viscosity of water by correcting to a value for 25 °C (Ionenko, Anisimov, & Dautova, 2010).

2.3 | Leaf anatomical traits

Anatomical traits were measured on sun-exposed leaves from the same trees used for the hydraulics measurements. Leaves were cleared using the method of Berlyn and Miksche (1976). Leaf segments (5 mm on a side) were excised from the middle part of the leaf lamina avoiding first- and second-order veins and soaked in 99.5% ethanol for 2 days to clear the chlorophyll, then soaked in a 10% NaOH solution for 1–2 days, placed in chlorine bleach for 1–5 min, and washed several times with distilled water. The cleared leaf segments were stained with 0.1% safranin (Division Chroma, Münster, Germany) in distilled water, immersed in chloral hydrate (Wako, Osaka, Japan) in 50% glycerine (Wako), and photographed with a digital camera (DP71, Olympus, Tokyo, Japan) mounted on a light microscope (BX50, Olympus). The length of veins, virtually all minor veins, was measured in a 3.2×2.5 mm area with image analysis software (ImageJ). The VLA was determined as the ratio of vein length to the projected area in the analysed image.

To examine the extent of vein BS lignification, thin cross sections of the midrib-, second-, and third-order and minor veins (i.e., fourth or higher) were hand-sectioned and stained with phloroglucinol (Wako, Osaka, Japan), which specifically stains lignin red (Pomar, Merino, & Barcelo, 2002). The thin sections were stained with 1% phloroglucinol in 17% hydrochloric acid and observed with a light microscope (BX50, Olympus) and photographed under the digital camera. For the lignin depositions of the highest-order veins, thin sections were made after the samples were embedded in resin and observed at higher resolution

using the method of Brundrett, Enstone, and Peterson (1988). Leaf segments of 3×3 mm were fixed in formalin/acetic acid/ethanol/water (2:1:12:5), dehydrated in ethanol, and embedded in 2-hydroxyethyl methacrylate-based resin (Technovit 7100, Heraeus Holding, Hanau, Germany). The 5- μm -thick sections were made using an ultramicrotome (Ultracut B, Leica Microsystems, Tokyo, Japan), stained in 0.5% aniline blue (Sigma Aldrich, St. Louis, Missouri, USA) for 45 min, soaked in Lugol solution (Wako) for 20 s, and washed several times with distilled water. The section was mounted in 0.1% FeCl_3 (Wako, Osaka, Japan) in 50% glycerine and observed with a fluorescence microscope (BX50, Olympus, Tokyo, Japan) under ultraviolet illumination (330–385 nm) and photographed with a digital camera (DP71, Olympus) mounted on the microscope. Veins higher than fourth order are referred to as “minor” veins given that the order of these smallest veins could not be distinguished accurately in resin-embedded samples. We also defined the “highest-order veins” as those with the smallest xylem in analysed images.

2.4 | Statistical analyses

Statistical differences in K_{leaf} between treatments (light effect of K_{leaf}) and species groups (life form: evergreen versus deciduous species) were tested using Welch's t-test applied to all data for K_{leaf} measured at LL versus HL for each species and for the evergreen versus deciduous tree species. The difference in $\%R_{\text{ox}}$ and $\%R_x$ were tested by Student's t-test for both LL and HH. Correlations were examined using Pearson's correlation tests. Interspecific differences in specific leaf area, leaf area, and VLA were tested using one-way analysis of variance (ANOVA). To explore effects of BS lignification and light treatment on leaf hydraulic variables, one-way or two-way ANOVAs were conducted, followed by Tukey's multiple comparisons at $p = .05$. Multiple regressions were applied to determine the ability to predict K_{leaf} from VLA and the highest lignified vein order (LVO), or from VLA and the presence or absence of lignification of the minor vein BS (LMV). LVO was determined from first-order vein to minor veins being designated as “4” to be conservative, given that the highest order of lignified minor veins could not be resolved except for *C. camphora*, which was assigned “5” (Table 2). LMV was assigned “1” if minor veins had lignified BS, or otherwise “0”. The predicted regression parameters were selected to minimize the AIC values by stepwise parameter selection. All statistical analyses were made using R statistics software (<http://www.r-project.org/>).

3 | RESULTS

The leaf-lamina hydraulic conductance (K_{leaf}), the partitioning of resistances between the xylem and outside-xylem pathways (R_x and R_{ox} , respectively), and the light response of K_{leaf} varied strongly among the 11 woody species (Figures 1 and 2). K_{leaf} varied from 2.39 to $14.8 \text{ mmol m}^{-2} \text{ s}^{-1} \text{ MPa}^{-1}$ at $3 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (LL) and 2.61 to $21.0 \text{ mmol m}^{-2} \text{ s}^{-1} \text{ MPa}^{-1}$ at $300 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (HL), and eight of the 11 species showed significant increases in K_{leaf} under HL. On average across species, R_{ox} was dominant under LL (Figure S1, $\%R_{\text{ox}}$: $64 \pm 4.7\%$, mean ± 1 SE, Student's t-test: $p < .001$) and R_{ox} and R_x were

TABLE 2 The highest order of vein with lignified bundle sheath or bundle sheath extensions from first-order veins to minor veins

Species	Bundle sheath	Bundle sheath extensions
<i>Quercus serrata</i>	4	4
<i>Magnolia kobus</i>	4	NA (4)
<i>Acer rufinerve</i>	4	NA (4)
<i>Quercus acuta</i>	4	H
<i>Cinnamomum camphora</i>	H	H
<i>Ligustrum lucidum</i>	0	NA (3)
<i>Quercus myrsinifolia</i>	4	H
<i>Cornus controversa</i>	0	NA (4)
<i>Viburnum odoratissimum</i>	2	NA (2)
<i>Mallotus japonicus</i>	2	NA (4)
<i>Cerasus speciosa</i>	0	NA (4)

Note. All veins up to the order assigned showed lignified bundle sheath (BS) and/or BS extensions (BSEs). The species with unlignified BS cells in the midrib were assigned “0”. The species with lignified BS or BSEs in the highest-order vein resolved were assigned “H.” If the veins had no lignified BSEs in the second- and higher-order veins, “NA” was assigned in the column for the species. The figures in parentheses indicate the highest order of veins with unlignified BSEs.

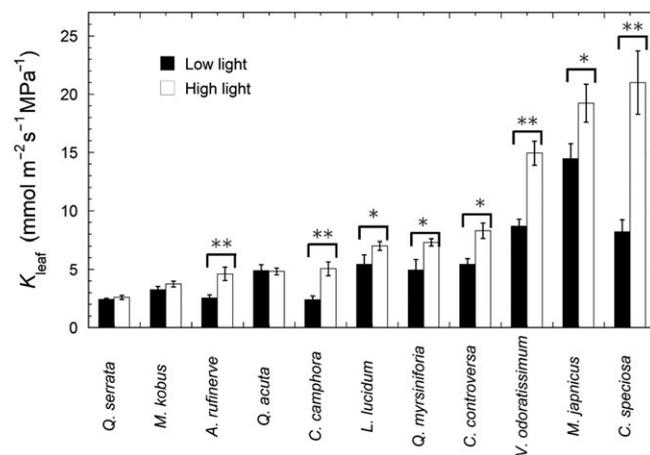


FIGURE 1 Leaf lamina hydraulic conductance (K_{leaf}) of the 11 woody species. Closed and open column show K_{leaf} measured under $300 \mu\text{mol m}^{-2} \text{ s}^{-1}$ (high light) and $3 \mu\text{mol m}^{-2} \text{ s}^{-1}$ (low light). * and ** indicate significant differences at $p < .05$ and $.01$ by Welch's t-tests, respectively. Bars beside each column are ± 1 SE ($n = 6-9$)

similar on average under HL (Figure S1, $\%R_{\text{ox}}$: $49 \pm 6.2\%$, Student's t-test: $p = .15$). A significant light response was found only for R_{ox} and not for R_x for all species (Figure 2, ANOVA and Tukey's multicomparison test at $p = .05$). There were no significant effects of deciduous versus evergreen leaf habit on K_{leaf} or on $\%R_{\text{ox}}$ (Student's t-tests: $p = .21$ or $.86$, respectively). Neither K_{leaf} nor the magnitude of its light response (i.e., the ratio of K_{leaf} measured in HL relative to LL) was significantly related to VLA (Figure S2).

To test the potential influence of BS lignification on leaf hydraulics, the lignin depositions on BS and BSEs were observed with phloroglucinol staining (Figure 3, Table 2). In six of the 11 species, lignified cell walls were observed in the BS of veins from midrib-, second-, third-, and fourth-order veins (*A. rufinerve*, *C. camphora*, *M. kobus*, *Q. acuta*, *Q. myrsinifolia*, and *Q. serrata*; Figure 3a–e, Table 2). In two

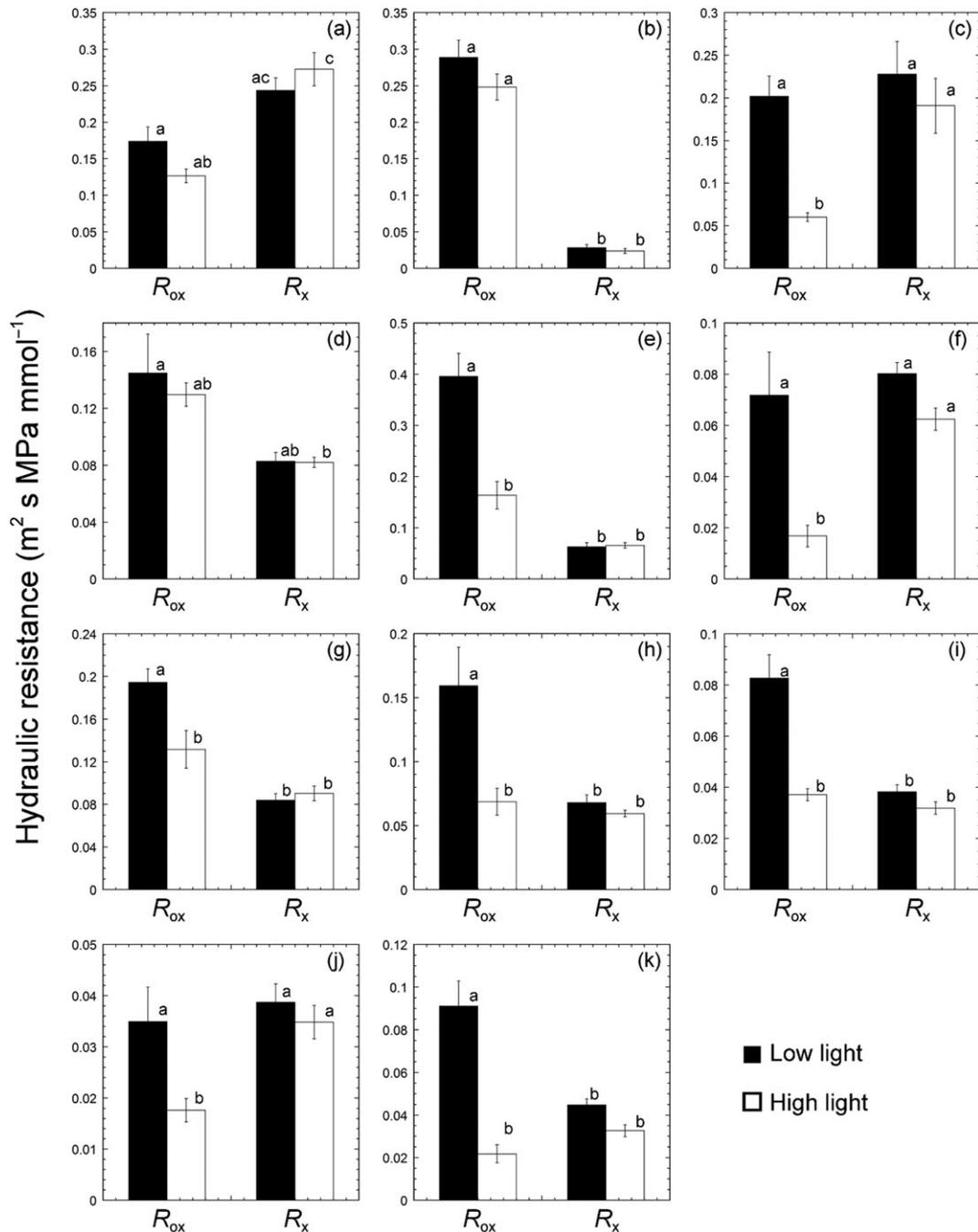


FIGURE 2 Partitioning of hydraulic resistance of outside-xylem and vein xylem pathways among 11 woody species: (a) *Quercus serrata*, (b) *Magnolia kobus*, (c) *Acer rufinerve*, (d) *Quercus acuta*, (e) *Cinnamomum camphora*, (f) *Ligustrum lucidum*, (g) *Quercus myrsinifolia*, (h) *Cornus controversa*, (i) *Viburnum odoratissimum*, (j) *Mallotus japonicus*, and (k) *Cerasus speciosa*. R_{ox} and R_x are hydraulic resistance of outside-xylem and vein xylem pathways, respectively. Black and white column show results under low light and high light conditions, respectively. Bars beside each column are ±1 SE (n = 6-9). Letters beside column are significant difference at p < .05 by analysis of variance and Tukey's multiple comparisons

additional species, *M. japonicus* and *V. odoratissimum*, lignified sclerenchyma cells were found at the periphery of the vascular bundle in the second-order veins but not in the third- and higher-order veins (Figure 3i,j). In *C. controversa*, *C. speciosa*, *L. lucidum*, and *M. japonicas*, BSEs with sclerenchyma and/or collenchyma cells were found in the third- and fourth-order veins, but the primary compound cell walls were not lignified (Figure 3f,h,j,k). Lignin deposition was detected on the primary compound walls (i.e., containing the primary wall and middle lamella) of parenchyma and/or sclerenchyma cells. The intensity of staining varied across species and across vein orders for given species.

Thus, for example, the BS cells of *C. camphora* and *M. kobus* were stained strongly, whereas signals were weak in the second-order veins for *M. japonicus* and *V. odoratissimum*. For all the species with lignified BS, the lower-order veins contained more layers of lignified cells than higher-order veins. Thin cross sections of the highest-order veins were also observed under UV illumination to assess distributions of lignin in detail (Figure 4, Table 2). In leaves of *A. rufinerve*, *M. kobus*, and *Q. serrata*, BSEs did not develop and the cell walls of the BS cells of the highest-order veins were unlignified (Figure 4a-c). By contrast, for leaves of *C. camphora*, *Q. acuta*, and *Q. myrsinifolia*, lignified BSEs



FIGURE 3 Cross sections of the primary-, second-, third-, and fourth-order veins stained with phloroglucinol in 11 woody species: (a) *Quercus serrata*, (b) *Magnolia kobus*, (c) *Acer rufinerve*, (d) *Quercus acuta*, (e) *Cinnamomum camphora*, (f) *Ligustrum lucidum*, (g) *Quercus myrsinifolia*, (h) *Cornus controversa*, (i) *Viburnum odoratissimum*, (j) *Mallotus japonicus*, and (k) *Cerasus speciosa*. In each figure, the four panels represent the primary-, second-, third-, and fourth-order veins from the left. Phloroglucinol stained red in lignified cell walls. The scale bars for midrib veins are 1 mm, and the second panel from left of (a) shows 200 μm and applies to all figures for the second-order vein; the scale bar in third panel from left of (a) shows 100 μm and applies to all figures for the third- and fourth-order veins except for the fourth-order veins in (a), (b), and (h), in which cases the scale bar in the right panel of (a) applies, which shows 50 μm

developed in the highest-order veins and in *C. camphora* but not in *Quercus* species, lignin was detected in BS cells of the highest-order veins (Figure 4d–f).

Across species, R_{leaf} was higher for species with lignified BS cells than those without lignified BS cells (Figure 5a, ANOVA, and Tukey's multicomparison test at $p = .05$). On average, R_{leaf} for species with lignification of the higher vein orders was 2.3- and 2.8-fold higher than that for species without lignification of the minor vein BS under LL and HL, respectively. Species with minor vein BS lignification had higher R_{ox} and R_x under both light treatments (Figure 5b,c, ANOVA and Tukey's test). No significant interaction was found of the effects of the light treatment and the minor vein BS lignification on K_{leaf} or R_{ox} (two-way ANOVA; Table 3).

As described previously, there was no significant relationship between K_{leaf} and VLA across our species set measured at both LL and HL (Figure S2). K_{leaf} was weakly negatively correlated with the highest LVO ($R^2 = .25$ and $p = .12$ under LL, and $R^2 = .36$ and $p = .051$

under HL). However, measured K_{leaf} was strongly predicted from multiple regressions based on VLA and LVO ($R^2 = 67\%$ and 69% under LL and HL, respectively; Figure 6a,c) or VLA and the presence/absence of LMV ($R^2 = .77$ and $.74$ for LL and HL, respectively; Figure 6b,d).

4 | DISCUSSION

Across 11 species of woody angiosperms, leaf hydraulic conductance (K_{leaf}) was negatively related to the presence of lignin depositions on BS cells of minor veins (Figures 5 and 6). Because lignin is hydrophobic and fills the pores formed by cellulose microfibrils, lignified cell walls would inhibit apoplastic water transport (North & Peterson, 2005). Species with the lignified BS differed from those with unlignified BS in both the hydraulic resistance of the outside-xylem pathways (R_{ox}) and the xylem pathway (R_x). Lignification of the BS mediated the relationship between K_{leaf} and leaf vein length per area, as the

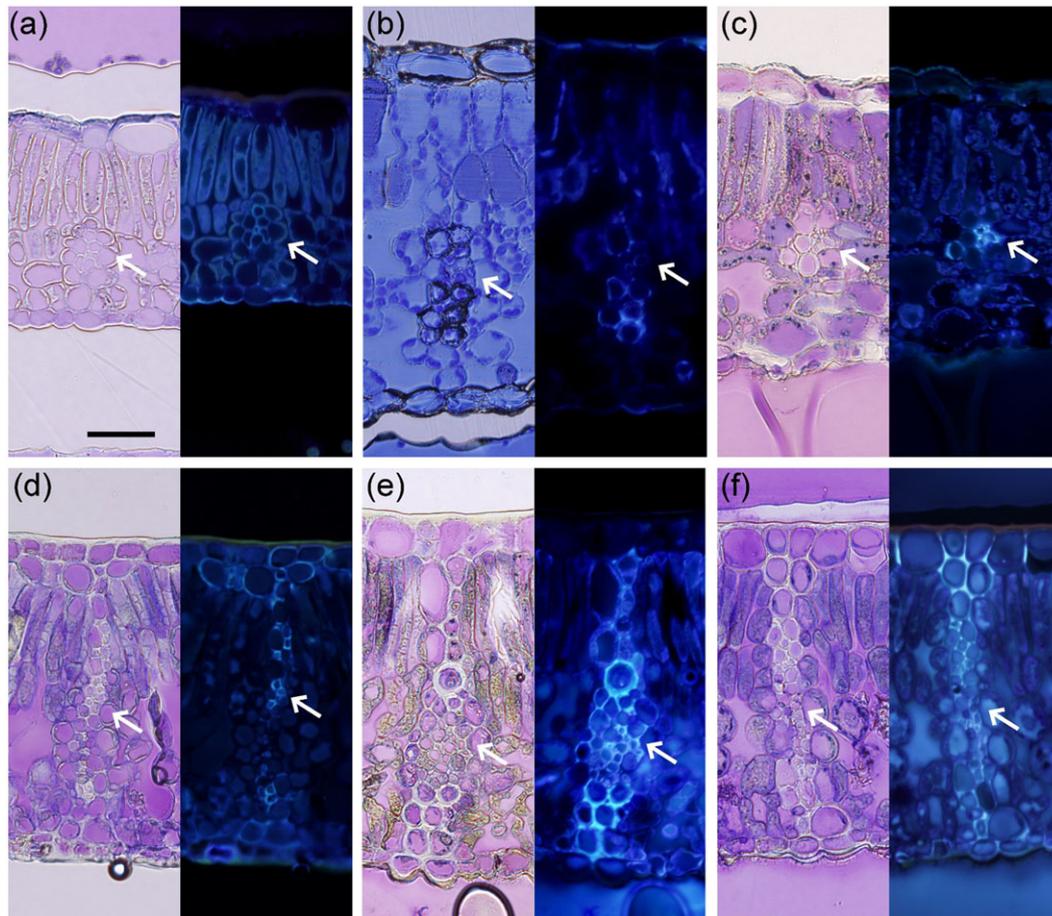


FIGURE 4 Cross sections of the highest-order veins of species with the lignified bundle sheath in minor veins: (a) *Quercus serrata*, (b) *Magnolia kobus*, (c) *Acer rufinerve*, (d) *Quercus acuta*, (e) *Cinnamomum camphora*, and (f) *Quercus myrsinifolia*. Left panels show the images observed on a light microscope, and right panels show the images observed on a fluorescent microscope under UV illumination, with the lignified cell walls coloured pale blue. Arrows show xylem conduits. Scale bar in (a) shows 25 μm and applies to all figures [Colour figure can be viewed at wileyonlinelibrary.com]

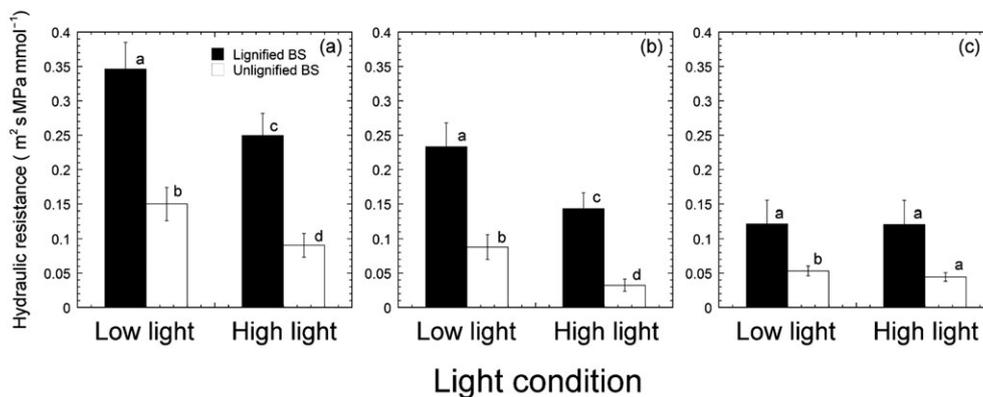


FIGURE 5 Effects of lignification of minor veins on hydraulic resistance of (a) whole-lamina, (b) outside-xylem, and (c) vein xylem pathways measured under low light and high light. Black and white columns are mean hydraulic resistance of species with lignified and unlignified bundle sheath (BS) cells, respectively. Bars beside each column are ± 1 SE ($n = 5$ and 6). Letters beside column are significant difference at $p < .05$ by ANOVA and Tukey's multiple comparisons

combination of VLA and presence/absence of LMV explained about 75–80% of species variation in K_{leaf} under both high and low light, whereas VLA alone was not significantly correlated with K_{leaf} , and LMV was a weak predictor alone (Figure S1 and Figure 6). These results suggest that the lignified BS resulted in lower K_{leaf} by increasing resistance in the apoplastic pathway.

4.1 | Lignin deposition and leaf water transport

For six of the 11 woody species, lignin deposition was found in the minor vein BS and BSE cell walls (Figures 3 and 4). Lignification was found in BS cells of even the highest-order veins in *C. camphora*. Lignin was deposited in the compound primary walls (i.e. the primary walls

TABLE 3 *F*-values for two-way analysis of variance for light treatment, minor-vein lignification, and their interaction

	Light treatment	Lignification	Interaction
R_{leaf}	33.0***	222***	0.235
R_{ox}	41.6***	124***	0.079
R_x	0.243	49.9***	0.712

Note. R_{leaf} , R_{ox} , and R_x are hydraulic resistance of leaf lamina, of outside-xylem, and of vein xylem pathways, respectively. The degrees of freedom were 175 for all analyses.

*** $p < .001$.

and middle lamella; Figure 3), as has been observed in the Casparian bands of root endodermis (Enstone et al., 2003). Sclerenchyma cells had lignified walls, but the secondary walls were not lignified. Because Casparian bands in roots develop only on the lateral sides of endodermis cells, water and ions permeate through the cell membranes of the radial sides of cells. In contrast, in the BS and BSEs, lignin is deposited on all sides of the cells (Figures 3 and 4). Thus, the lignified BS cells would inhibit the water transport through not only the apoplastic route but also additionally, in series with the flow of water through cell membranes. We expect lignification to increase resistance but not to

completely block water flow, especially as in the highest-order veins the staining was weaker and there were fewer lignified cell layers than the lower-order veins (Figures 3e and 4e).

The lignin deposition mediated the relationship between K_{leaf} and VLA (Figure 6). The fundamental assumption of a positive relationship between K_{leaf} and VLA assumes that the veins of any order deliver water equally to the mesophyll tissues (Brodribb et al., 2007). However, the highest LVO differed among the 11 woody species, and the importance of minor vein lignification as a statistical determinant of R_{ox} and K_{leaf} suggests that minor veins with unlignified BS distribute water more effectively than those with lignified BS. Notably, the regression of K_{leaf} against VLA and LMV explained about 70% of the variance in K_{leaf} (Figure 6) although K_{leaf} was not correlated significantly with VLA among the 11 woody species (Figure S2). We hypothesize that in published studies of species sets showing a strong correlation of K_{leaf} and VLA, the species would show similarity in their BS lignification. We also note that other traits such as vein conduit numbers and diameters and membrane permeability would further contribute to species variation in K_{leaf} (Sack & Scoffoni, 2013). In species sets assembled from across diverse plant groups (e.g., mosses, cycads, conifers, and angiosperms; Brodribb et al., 2007), the relationship of K_{leaf} and VLA also reflects variation in such additional

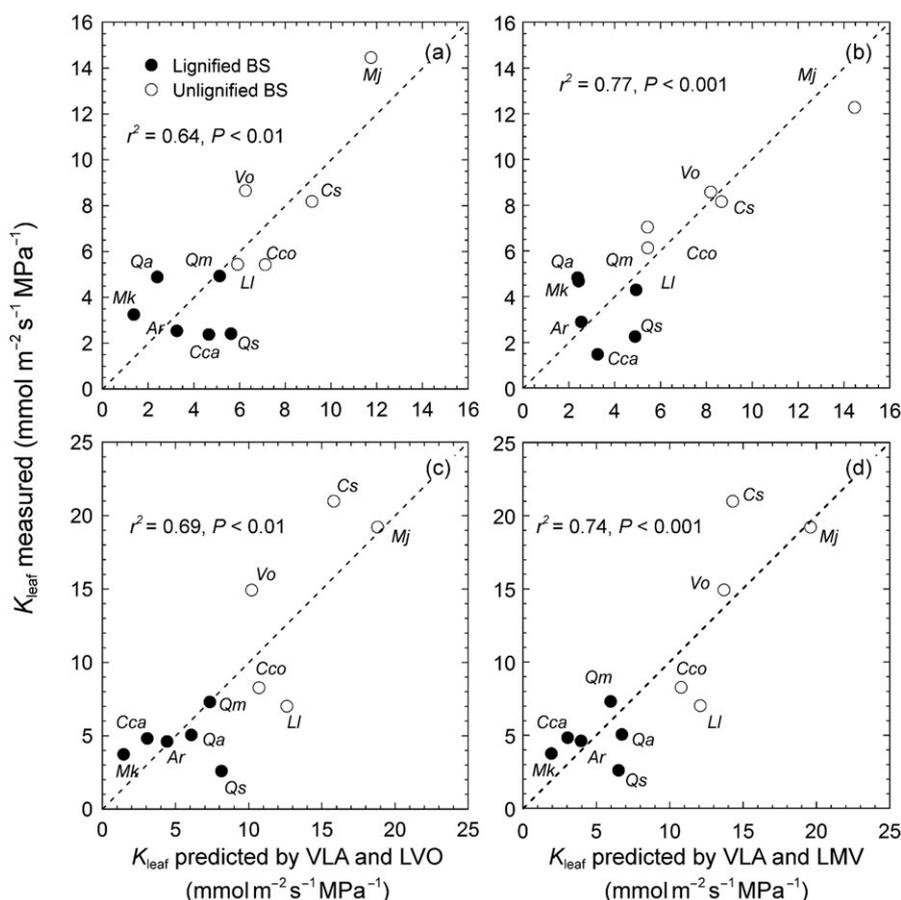


FIGURE 6 Prediction of leaf lamina hydraulic conductance (K_{leaf}) from vein length per area (VLA) and bundle sheath (BS) lignification under low light in (a) and (b) and under high light in (c) and (d). The predicted K_{leaf} was estimated from multiple regression equations based on VLA and the highest lignified vein order (LVO); (a) $K_{\text{leaf}} = 0.876 \text{ VLA} - 1.18 \text{ LVO} + 1.11$, $r = .82$, $p < .01$; (c) $K_{\text{leaf}} = 1.38 \text{ VLA} - 2.38 \text{ LVO} + 3.17$, $r = .83$, $p < .01$) and based on VLA and the presence/absence of lignification of the minor vein BS (LMV); (b) $K_{\text{leaf}} = 0.658 \text{ VLA} - 4.78 \text{ LMV} + 2.53$, $r = .89$, $p < .001$; (d) $K_{\text{leaf}} = 0.943 \text{ VLA} - 9.03 \text{ LMV} + 5.61$, $r = .86$, $p < .001$). Closed and open circles are species having with lignified and unlignified BS cells of the minor veins, respectively. Dashed line is 1:1. Letters beside each point represent species (Table 1)

traits, as vein conduit numbers and diameters increase across those groups (Sack & Scoffoni, 2013). Among angiosperms, lignification of the minor vein BS can have quantitative importance, corresponding to a twofold to threefold difference in K_{leaf} .

4.2 | Lignin depositions and light response of K_{leaf}

The increase of K_{leaf} from low to high irradiance did not differ between species with or without lignified minor vein BS (Figure S1c and Figure 5, Table 3). This finding did not support the hypothesis that the K_{leaf} light response was related to greater apoplastic resistance caused by lignification. Notably, in contrast to the root endodermis cells, the lignified cell walls in BS covered all sides of the cell, thereby preventing rapid water transport both in apoplastic and cell-to-cell routes in these cells (Figures 3 and 4). Thus, the effect of increasing aquaporin activity and membrane permeability would be in series with the apoplastic resistance conferred by BS lignification.

Some species also showed lignification of their BSEs, a tissue that provides the shortest routes for water transport between vein xylem and epidermis, bypassing the mesophyll. Indeed, BSEs tend to confer higher leaf water transport capacity within and across species (Buckley, 2015; Zsögön, Alves Negrini, Pereira Peres, Hoa, & Ball, 2015). Consistent with those findings, in our study, *C. speciosa* and *M. japonicus* had distinct unlignified BSEs from their higher-order veins and a high K_{leaf} relative to other species (Figures 1 and 3). Previous studies reported that heterobaric species possessing BSEs at the minor veins tended to show greater K_{leaf} light responses than homobaric species possessing no BSEs (Scoffoni, Pou, Aasamaa, & Sack, 2008). In our species set, all the species with minor vein BSEs had lignified BS, and lignification was even found in cell walls of the minor vein BSEs for *C. camphora* and the two *Quercus* species (Figures 3d–f and 4d–f). Thus, given that lignification would reduce apoplastic flow, the lignification of the BS and BSEs of these species can explain why among these species, BSEs were not related overall to greater K_{leaf} or to greater K_{leaf} light responses. Further, our species included only two homobaric species, *L. lucidum* and *V. odoratissimum*, and these showed about 30% and 70% increases in K_{leaf} from 3 to 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Additional studies are needed of the relationship of the K_{leaf} light response to anatomy, considering variation in the lignification of BSEs and also including measurement over a wider range of irradiances.

4.3 | Leaf hydraulic distribution and bundle sheath lignification

Across the study species, the spatial patterns of lignin deposition on BS cells differed across vein orders (Figures 3 and 4, Table 2). While in six species, lignified BS cells were found in the third- and fourth-order veins, and no lignin depositions were detected in the BS cells of highest-order veins for any species except *C. camphora*. In *M. japonicas* and *V. odoratissimum*, lignified sclerenchyma cells were found in the second-order but not in the third- or higher-order veins (Figure 3i,j). These spatial patterns of lignin depositions would allow the higher-order veins to provide more water from veins to mesophyll than the lower-order veins, which would enhance the functional specialization of lower-order veins for long-distance transport and the higher-order

veins for the irrigation of the mesophyll (Zwieniecki, Melcher, Boyce, Sack, & Holbrook, 2002).

In particular, the lignification of the BS in lower vein orders would be expected to contribute to the distribution of more water to higher vein orders, that is, reducing radial flow out of the veins. Such an effect would add to effect of reduced permeability of BS membranes in lower-order veins shown by probe studies in tobacco, which would also contribute to greater axial relative to radial transport in lower-order than higher-order veins (Lee, Chung, Zwiazek, 2009). Indeed, the impermeability of low-order vein BS and the increasing vein length of higher vein orders led to the hypothesis that the bulk of water exits the xylem via the minor veins, and this concept was supported by modelling approaches (Cochard et al., 2004; McKown et al., 2010), and our study indicates that BS lignification of the BS cells would also contribute to this effect in many species. Species diversity in BS lignification also may explain why dye studies have shown that in some species, water can exit major veins across the BS, whereas in others, water apparently exits only minor veins (Nardini & Salleo, 2003).

Our finding that lignification of the BS reduced R_x indicates that variation in radial transport related to BS lignification is a factor influencing axial conductance through the xylem in addition to, for example, vein xylem conduit numbers and dimensions and vein densities. It is important also to consider that water exiting from low-order veins directly to the mesophyll could have impacts on the accuracy of methods for leaf hydraulic partitioning. The vein cutting method used here and in earlier studies (Nardini et al., 2005; Sack & Frole, 2006; Sack et al., 2004; Scoffoni et al., 2016) assumes that negligible water exits the xylem radially from lower-order (major) veins and thus that the vein orders can be considered to be arranged in series, and in turn, in series with the outside-xylem pathways. If water exits radially from the lower-order veins to the mesophyll, then the xylem hydraulic resistance (R_x) as estimated from cutting the minor veins may be a coarse estimate, with a complex relationship to the actual resistance network, given that it neglects the axial versus radial water pathways out of the lower-order veins. Thus, the method would be most accurate given complete lignification of lower-order veins and would bias towards overestimating $\%R_x$ with greater radial leakage from lower-order veins. However, the lack of a relationship across species in this study between either R_x or $\%R_x$ and the highest lignified vein order (Pearson's correlation test; $r = .27$, $p = .42$ for HL; $r = .075$, $p = .83$ for LL) implies that such a bias may be small. Future studies should combine BS with vein anatomy into spatially explicit models of the vein system (Cochard et al., 2004; McKown et al., 2010; Scoffoni et al., 2011) to determine the partitioning of hydraulic resistances in xylem and outside-xylem compartments, accounting for varying radial permeabilities of vein orders due to lignification.

4.4 | Functional significance of lignified bundle sheath

New studies are needed of the functional significance of variation in lignification of BS and BSEs across major and minor veins, and in particular, of the potential benefits that would compensate or overcompensate for the disadvantages associated with reduction of K_{leaf} . Lignifying the major veins may enhance distribution of water to

the minor veins, leading to improved distribution across the leaf, whereas reduced lignification of the major veins, enhancing radial leakage, may increase conductive capacity. However, the lignification of minor vein BS evidently increases the hydraulic resistance of the entire system. We propose several hypotheses for a potential advantage. First, lignification of minor veins may provide benefits for the tolerance of the leaf hydraulic system to extreme dehydration. Lignification of the minor vein BS may reduce or delay the collapse of minor vein conduits during leaf dehydration, which may maintain hydraulic conductance (Blackman, Brodribb, & Jordan, 2009). Such collapse has been recently reported for strongly dehydrated *Quercus* leaves, where it may contribute to the prevention of cavitation in the lower-order veins (Zhang, Rockwell, Graham, Alexander, & Holbrook, 2016), and thus species with minor veins that do not collapse must avoid embolism through other mechanism, for example, reduction of hydraulic conductance in the outside-xylem pathways during dehydration (Scoffoni et al., 2017). Second, lignification may contribute additional benefits beyond the hydraulic system. Studies in *Arabidopsis* of knockout mutants of lignin synthesis showed that lignification is related to numerous functions, including not only xylem water transport but also mechanical support, resistance to pests and disease infections, and dormancy of seeds (Barros, Serk, Granlund, & Pesquet, 2015). Lignin depositions on the BS and BSE cells would be expected to enhance mechanical stability and resistance to leaf diseases or herbivores and potentially contribute to longer leaf longevity (Kitajima et al., 2012; Onoda et al., 2011), even as lignin depositions would inhibit leaf water transport and thus potentially reduce potential photosynthetic capacity. In that case, lignification of the BS may be a mechanism contributing to the frequently observed trade-off between leaf lifespan and photosynthetic capacity. Notably, such a trade-off has been found among evergreen species and among deciduous species and across both (Onoda et al., 2011; Reich, Walters, & Ellsworth, 1997), and similarly, we found lignification of the minor vein BS not only in evergreen species with long leaf lifespans but also in deciduous species with short leaf lifespans. Detailed study of the multiple functional roles of vein BS lignification would likely shed light on many aspects of leaf and whole plant design and function.

5 | CONCLUSIONS

We provide new evidence for lignin deposition in the leaf vein BS for diverse species and support for its potential role in constraining leaf water transport capacity. Our analyses suggested that lignification would impose a substantial resistance to water movement through BS. The lower-order veins were more strongly lignified than higher-order veins, which would enhance the functional specialization of the vein hierarchy with lower-order veins acting principally for long-distance transport and higher-order veins for irrigating mesophyll cells. Vein BS lignification and vein length per area together enabled a strong quantitative prediction of K_{leaf} across the diverse species. Future studies are needed to determine other potential benefits of BS lignification, for example, in enhancing leaf mechanical toughness and/or resistance to disease infection, which may contribute to leaf life longevity.

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