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Effects of major vein blockage and aquaporin inhibition on leaf hydraulics and stomatal conductance

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The density and architecture of leaf veins determine the network and efficiency of water transport within laminae and resultant leaf gas exchange and vary widely among plant species. Leaf hydraulic conductance (K_{leaf}) can be regulated by vein architecture in conjunction with the water channel protein aquaporin. However, our understanding of how leaf veins and aquaporins affect leaf hydraulics and stomatal conductance (g_s) remains poor. By inducing blockage of the major veins and inhibition of aquaporin activity using $HgCl_2$, we examined the effects of major veins and aquaporins on K_{leaf} and g_s in species with different venation types. A vine species, with thick first-order veins and low vein density, displayed a rapidly declined g_s with high leaf water potential in response to vein blockage and a greatly reduced K_{leaf} and g_s in response to aquaporin inhibition, suggesting that leaf aquaporins are involved in isohydric/anisohydric stomatal behaviour. Across species, the decline in K_{leaf} and g_s due to aquaporin inhibition increased linearly with decreasing major vein density, possibly indicating that a trade-off function between vein architecture (apoplastic pathway) and aquaporin activity (cell-to-cell pathway) affects leaf hydraulics.

1. Introduction

Plants cannot survive without water, and it is crucial to plants for water to be transported to the leaves; therefore, plant hydraulic properties strongly influence plant performance [1–3]. Leaves account for 30% or more of whole-plant hydraulic resistance, constituting an important hydraulic bottleneck [4,5]. Leaf hydraulic conductance (K_{leaf} = inverse of hydraulic resistance) has a strong influence on stomatal conductance (g_s) and photosynthetic capacity (A_{max}) [6,7], and ultimately on plant ecology [8–10]. Leaf venation, whose architecture varies widely among plant species [11], forms the transport network for water within a lamina and thus affects K_{leaf} [5]. Water flow in the xylem of a leaf vein has lower resistance per length than water flow outside the xylem between xylem and the intercellular space. Therefore, in comparison to leaves with low vein density, leaves with a high vein density have a shorter path length of outside xylem, potentially leading to higher K_{leaf} and A_{max} [6,12]. The ratio of hydraulic resistance inside and outside the xylem in a whole leaf varies among species [5]. Among the 10 species studied in a tropical rain forest, on average, 50% of the resistance was from inside the veins and 39% from outside the veins; in comparison to species that were not sun-adapted, those that were sun-adapted had a higher proportion inside the xylem [13].

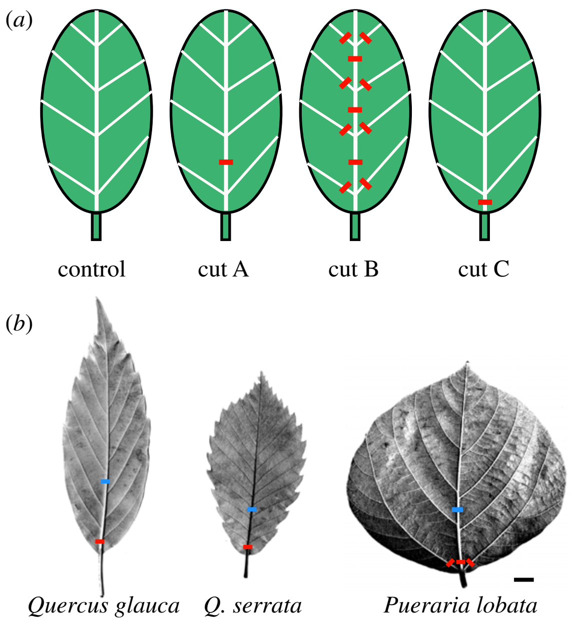

Figure 1. (*a*) Experimental vein blockage design according to Nardini & Salleo [25] and (*b*) images of laminae from study species. Cut surfaces indicated by red and blue lines were sealed with cyanoacrylate. (*a*) Control: intact leaves. Cut A: the midrib was cut at a quarter of the length. Cut B: the midrib was cut at three points, and all second-order veins were cut approximately 4 mm from their base. Cut C: the midrib was cut approximately 2 mm from its base; in *Pueraria lobata*, the large two second-order veins nearest to the base of the lamina were additionally cut. (*b*) Blue and red lines indicate cutting points in cuts A and C, respectively. A bar represents 1 cm.

While a steady-state maximum K_{leaf} strongly correlates with g_s and A_{max} among species, K_{leaf} is not constant and is highly dynamic in response to environmental stimuli [5]. During drought, for example, a decrease in leaf water potential (Ψ_{leaf}) can result in embolism formation in the xylem of leaf veins, thereby reducing their hydraulic conductivity and K_{leaf} [14,15]. Leaf vascular redundancy (i.e. high vein density) can enhance tolerance against hydraulic disfunction in veins because water can flow around the dysfunctional vein through nearby functioning veins [16]. In addition, hydraulic conductance outside the xylem can also be modified [5]. One of the main pathways of the outside xylem is a cell-to-cell pathway through cell membranes, regulated by water channel proteins called aquaporins [17,18]. Aquaporin gene expression in a leaf can potentially be stimulated by light [19], defoliation [20] and rewatering after drought stress [21–23], resulting in an increase in K_{leaf}. A decline in K_{leaf} associated with the deactivation of aquaporins in response to environmental stimuli can result in a decline in g_s [24]. Accordingly, K_{leaf}, and thus g_s, are regulated by the network structure of leaf veins and water channel aquaporins; however, the mutual involvement of leaf veins and aquaporins in K_{leaf} and g_s remains poorly understood.

In this study, we conducted two experiments to artificially decrease K_{leaf} by blocking or inhibiting water flow through major veins and aquaporins in five species with various leaf vein densities and architectures. In the major vein blocking experiments, where three vein blockage patterns were applied to mimic the embolisms in major veins with different intensities [25] (figure 1), we investigated the response of g_s with respect to Ψ_{leaf} in dehydrated leaves in an open field. In the aquaporin inhibition experiments [26], we investigated the decrease in K_{leaf} and g_s by aquaporin inhibition in fully hydrated leaves in a laboratory set-up. By integrating the results of the two experiments, we discuss the effects of leaf aquaporins and leaf venation on the interspecific difference in leaf hydraulics and g_s from physiological and ecological points of view. We hypothesized that species with lower vein density may experience greater impacts of K_{leaf} and g_s on aquaporin inhibition because a low vein density can provide a sensitive K_{leaf} response against hydraulic disfunction in veins [16], and aquaporin upregulation can offset the decline in K_{leaf}. This hypothesis may contribute to delayed g_s reduction and prolonged photosynthesis (higher C acquisition) in vulnerable leaves with low vein density and may be connected to the trade-off function between vein architecture and aquaporin activity. The study species had different arrangements of thick leaf veins (i.e. pinnate and pinnipalmate venation; figure 1*b*), which can differentially affect K_{leaf} and g_s responses to hydraulic failure in the midrib of leaves [16]. The *Quercus* genus was also included because some *Quercus* species have shown an irradiance-dependent increase in K_{leaf} [27–29], which was related to the expression of PIP1 (Plasma membrane Intrinsic Protein) aquaporin [29].

2. Material and methods

Two sets of experiments, major vein blockage and aquaporin inhibition, were conducted using fully expanded current-year leaves from sunlit shoots of trees or vines grown in the arboretum of the Forestry and Forest Products Research Institute, eastern Japan (36°00′ N, 140°08′ E, 20 m.a.s.l. [30]). We studied five angiosperm species, two evergreen tree species (*Quercus acuta* Thunb. and *Quercus glauca* Thunb.) and two winter deciduous tree species (*Castanea crenata* Siebold et Zucc. and *Quercus serrata* Murray) trees and a winter deciduous vine species (*Pueraria lobata* [Willd.] Ohwi), all common in the warm-temperate forests of Japan. The leaf longevities of the evergreen *Q. acuta* and *Q. glauca* were 26 and 14 months, respectively [30]. Two to four individual trees were studied. Leaves from the vine were selected at least 5 m apart from each other because individual vines could not be identified. All trees were 20–25 years old, and their height and diameter at breast height were 10–15 m and 20–30 cm, respectively. 'Leaf' refers to 'leaflet' of the compound leaf of *P. lobata*, for brevity.

(a) Measurement of leaf hydraulic conductance

A vacuum chamber method [31–33] was used to measure K_{leaf}. Briefly, a leaf was placed in a vacuum chamber (7 l) connected to a vacuum pump and a pressure gauge. The petiole of the leaf was attached to a Tygon tube filled with 20 mM KCl solution filtered with a 0.22 µm membrane filter and degassed overnight via a vacuum pump. The other end of the Tygon tube was placed into a KCl solution container, which was placed on a balance (model AG204 ± 0.1 mg sensitivity; Mettler Toledo Japan, Tokyo, Japan). Five vacuum levels in the range 0.035–0.065 MPa were applied (0.005 MPa interval). At each pressure level, the mass of water on the balance was logged every 30 or 60 s at stable flow rates (F). K_{leaf} was calculated as the slope of the flow rate against vacuum pressure, normalized for leaf area, which was determined by a digital image analysis after scanning. Leaves were illuminated with ≈500 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR), provided as white and blue (9 : 1) LED light, starting one hour before measurements and lasting until the measurements were complete. The room temperature was maintained at ≈25°C.

(b) Major vein blockage experiment

Vein blockage experiments were conducted according to Nardini & Salleo [25] in three species with different life forms—the evergreen tree *Q. glauca*, the deciduous tree *Q. serrata* and the deciduous vine *P. lobata*—between August and September 2003. In the evening of the day before the K_{leaf} measurements, sunlit shoots were sampled, immediately recut under water and then transported to the laboratory. The cut ends of the shoots were kept in the water until the measurements started. Three different patterns of vein cuttings were performed with a fresh razor blade (figure 1): (1) the midrib was cut at one-quarter of the length (cut A); (2) the midrib was cut at three points, and all second-order veins were cut at approximately 4 mm from their base (cut B); and (3) the midrib was cut at approximately 2 mm from the base in *Q. glauca* and *Q. serrata*, and the midrib and the two large second-order veins nearest the lamina base were cut at approximately 2 mm from the base in *P. lobata* (cut C). In cut A, water flow was interrupted at a quarter of the midrib, but water could pass through the lateral secondary veins connected to up to a quarter of the midrib. In cut B, water flow through all second-order veins was interrupted, but water could flow through a quarter of the midrib and via minor veins and outside-xylem pathways. In cut C, water inlet into the leaf was extremely limited, and the only path for water flow was through minor veins and outside-xylem pathways. After vein cutting, all cut surfaces were immediately sealed with cyanoacrylate to prevent water flow [25]. On the next day, K_{leaf} of the treated and untreated leaves was measured. Five or six leaves per treatment per species were measured, except for four leaves for cut C in *Q. serrata*.

To investigate the effects of vein blockage on stomatal conductance (g_s) *in situ*, treatments were conducted on intact leaves of the trees and vine in the field during the evening. The next day, treated and untreated leaves were measured (10.00–14.00) for g_s and leaf water potential (Ψ_{leaf}) using a steady-state porometer (Li1600, Li-Cor, Lincoln, USA) and a pressure chamber (Soilmoisture Equipment, Santa Barbara, USA). g_s was measured at the distal third of the lamina. Six to eight leaves per treatment per species were measured. The percentage loss of conductance (PLC) of K_{leaf} and g_s was calculated as follows:

$$\text{PLC} = 100 \times \left(1 - \frac{K_{leaf} \text{ or } g_s \text{ of treated leaves}}{K_{leaf} \text{ or } g_s \text{ of control (i.e. untreated leaves)}}\right). \tag{2.1}$$

Leaf water relations were analysed by the pressure–volume technique [34], and the leaf water potential at the turgor loss point ($\Psi_{w.tlp}$), osmotic potential at full turgor ($\Psi_{s.sat}$) and bulk modulus of elasticity (ϵ_{max}) were calculated [35]. Sunlit shoots were collected in the evening and rehydrated overnight. Seven to ten leaves of each species were measured.

(c) Aquaporin inhibition experiment

The inhibition of water flow through aquaporins was performed using the common aquaporin inhibitor $HgCl_2$ in all study species between August and September 2009. Sunlit shoots were sampled before 09.00, immediately recut under water, and transported to the laboratory. A leaf was connected to a Tigon tube filled with degassed and filtered 20 mM KCl solution, and g_s was monitored using a portable open gas exchange system (Li-6400, Li-Cor, Lincoln, USA) equipped with a 2×3 cm broadleaf chamber and an integrated light source (Li-6400-02B; Li-Cor) until g_s stabilized. The chamber conditions were set as follows: 700 μmol m^{-2} s^{-1} PAR, 370 μmol mol^{-1} CO_2, 25°C block temperature and roughly 60% relative humidity. After g_s stabilization, the leaf was removed and connected to a Tigon tube filled with a degassed and filtered 20 mM KCl + 0.2 mM $HgCl_2$ solution (this $HgCl_2$ concentration can fully inhibit the water channel function of aquaporin [23,26]), and g_s was re-monitored and measured under the same chamber conditions. The $HgCl_2$ solution was perfused to the leaf by a transpiration stream for 1 h, and then, g_s was recorded. No infiltration of water into intercellular spaces was observed during the g_s measurement. After the g_s measurement with $HgCl_2$, the leaf was placed in the vacuum chamber, and K_{leaf} was measured. We repeated the same procedure with a 20 mM KCl solution without 0.2 mM $HgCl_2$ and examined whether the experimental water inflow to the leaves over the course of 1 h affected g_s ($n = 5$ per species); no significant difference was observed between the g_s readings before and after perfusion ($p = 0.37 - 0.95$, paired *t*-test). After the g_s measurement, K_{leaf} was measured for the control. The PLC of K_{leaf} and g_s was calculated by equation (2.1); g_s before the $HgCl_2$ treatment served as the control. After the experiment, whole leaf area and dry mass were measured, and leaf dry mass per area (LMA) was calculated.

(d) Leaf vein density and vessel area

The major and minor vein densities and largest vessel area in the midrib were measured using ImageJ 1.43u (National Institutes of Health, USA https://imagej.nih.gov/ij/). The major vein density (midrib and second- and third-order veins) was measured from a whole leaf image taken by a digital scanner. Minor vein density was measured from a digital image of a 4–10 mm^2 leaf section at the centre of the lamina taken by a digital camera attached to a light microscope. We did not apply a leaf clearing treatment for the minor vein observation because all study species had heterobaric leaves with bundle sheath extensions, which enabled us to observe the highest order veins (5th) from fresh leaves [36]. The largest vessel area at the base of the midrib was measured from a digital image of the section of the midrib, which was fixed in a formalin-acetic acid-alcohol solution. Five to seven leaves per species were measured.

(e) Statistical analysis

All statistical analyses were performed with R v. 3.2.2 [37] at a level of statistical significance of $\alpha = 0.05$. One-way analysis of variance (ANOVA) with a post hoc Tukey test was used to test the differences in the major and minor vein density, largest vessel area in the midrib, leaf area, LMA and leaf water relation parameters among species and in K_{leaf}, g_s and Ψ_{leaf} among the vein blockage treatment within a species. Two-way ANOVA was used to test differences in K_{leaf}, g_s and Ψ_{leaf} among the species and veins or aquaporin inhibition treatments. Weibull functions were fitted to the relationships between Ψ_{leaf} and g_s with the R package fitplc [38]. The maximum g_s in each species was calculated from the control leaves to fit a Weibull function.

3. Results

(a) Leaf properties in the study species

Among the studied species, *P. lobata*, a deciduous vine with a pinnipalmate venation pattern, had the lowest LMA and density of major and minor veins, the largest leaf area and vessel area in the midrib (ANOVA with post hoc Tukey test; electronic supplementary material, table S1). *Quercus glauca*, an evergreen tree, had the highest densities of the major and minor veins. LMA was higher in the evergreen trees than in the deciduous trees, while there was no distinct difference between evergreen and deciduous trees for the other leaf properties (electronic supplementary material, table S1).

Among the three species tested in the vein blockage experiment, $\Psi_{w.tlp}$ and $\Psi_{s.sat}$ were higher in *P. lobata* than in *Q. glauca* and *Q. serrata* (electronic supplementary material, table S2).

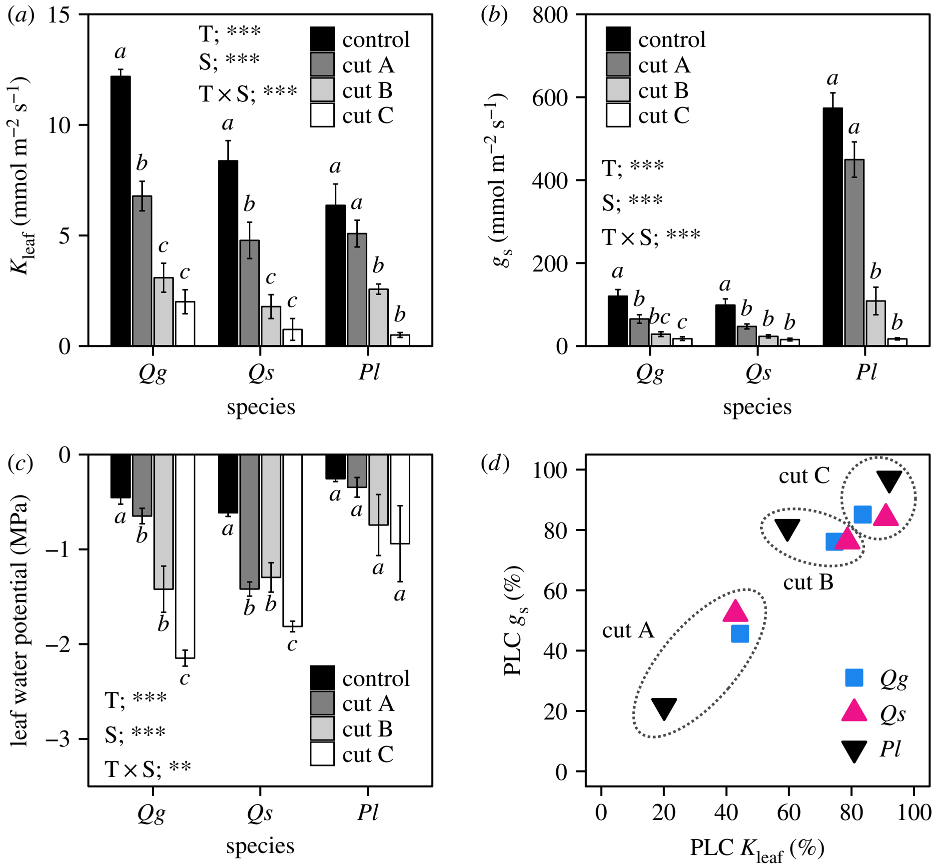

Figure 2. Effects of vein blockage on (*a*) leaf hydraulic conductance (K_{leaf}, measured in the laboratory), (*b*) stomatal conductance (g_s, measured in the field), (*c*) leaf water potential at midday (measured in the field) and (*d*) the relationship between PLC of K_{leaf} and g_s. *Qg*: *Quercus glauca* (evergreen tree); *Qs*: *Q. serrata* (deciduous tree); *Pl*: *Pueraria lobata* (deciduous vine). Bars represent $\pm$ s.e.m. (K_{leaf}; $n = 4$–6, g_s and leaf water potential; $n = 6$–8). Asterisks in panels (*a*–*c*) represent the results of the two-way ANOVA (T, blockage treatment; S, species; T × S, interaction; ***$p < 0.001$, **$p < 0.01$). Letters in panels (*a*–*c*) represent significant differences among blockage treatments within species (Tukey–Kramer multiple comparison, $p < 0.05$).

The value of ϵ_{max} was highest in the evergreen *Q. glauca* and lowest in the vine *P. lobata*.

(b) Leaf major vein blockage experiment

The three types of vein blockage caused similar decreases in K_{leaf} and g_s in the three species (figure 2*a*,*b*), although statistical significance was not observed between some blockage treatments. Of the cut types, cut A had the least effect on K_{leaf} and g_s, and the PLCs of the K_{leaf} and g_s were 40–50% in *Q. glauca* and *Q. serrata* and ≈20% in *P. lobata* (figure 2*d*). Cut B had an intermediate effect, and the PLCs of K_{leaf} and g_s were 80% for all three species (except for K_{leaf} in *P. lobata*, which was approx. ≈60%). Cut C had the greatest effect, and the PLCs of K_{leaf} and g_s were ≈90% in all species. Leaf water potential at midday (Ψ_{leaf}) also tended to decline in the order of control < cut A < cut B < cut C in *Q. glauca* (figure 2*c*); however, the effect of vein blockage was unclear in comparison to the effects on K_{leaf} and g_s (i.e. the orders of cut A and cut B were reversed in *Q. serrata*, and there was no significant difference in Ψ_{leaf} between the cutting treatments in *P. lobata*).

In *Q. glauca*, g_s gradually decreased with decreasing Ψ_{leaf}, which decreased below turgor loss points in leaves with the cut C treatment (figure 3). In *Q. serrata*, g_s also gradually decreased with decreasing Ψ_{leaf}, but Ψ_{leaf} did not decrease below the turgor loss point in any leaves. In *P. lobata*, g_s rapidly decreased in most leaves even with cut C, where Ψ_{leaf} was more than −0.5 MPa, and almost all leaves maintained Ψ_{leaf} above the turgor loss point.

(c) Aquaporin inhibition experiment

K_{leaf} and g_s were significantly affected by aquaporin inhibition in all species (figure 4*a*,*b*). The effects of aquaporin blockage on K_{leaf} and g_s were relatively small in the evergreen and deciduous trees, with a 10–35% reduction in K_{leaf} and 5–19% in g_s on average, but the effects were higher in the deciduous vine *P. lobata*, with a 57% reduction in K_{leaf} and 69% in g_s. There was a significant interaction between species and $HgCl_2$ treatment for g_s (figure 4*b*). Pooling the data by species, the PLC of K_{leaf} by aquaporin blockage was positively correlated with the PLC of g_s ($r^2 = 0.84$, $p = 0.028$; figure 4*c*). In evergreen and deciduous tree species, the PLC of K_{leaf} tended to be higher than the PLC of g_s, whereas in the deciduous vine *P. lobata*, the PLC of K_{leaf} was lower than the PLC of g_s.

The PLCs of K_{leaf} and g_s by aquaporin blockage were significantly negatively correlated with major vein density, but not correlated with minor vein density (i.e. species with lower major vein density experienced greater effects on K_{leaf} and g_s; figure 5).

4. Discussion

The results of the major vein blockage treatment showed a similar tendency to those found in previous studies [16,25]. When water flow was blocked a quarter of the way along the midrib in the cut A treatment, *P. lobata* with large and long

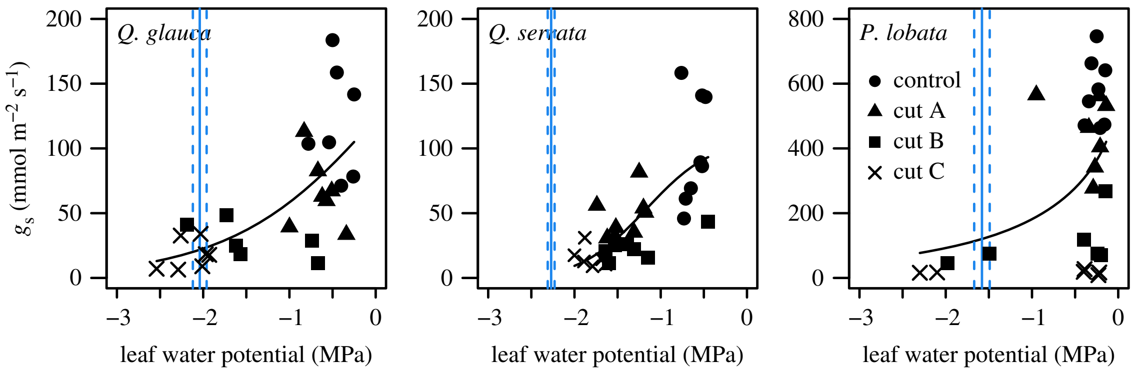

Figure 3. Relationships between leaf water potential and stomatal conductance (g_s) at midday in vein blockage experiments. Blue solid and dashed lines represent the mean ± s.e.m. of leaf water potential, respectively, at the turgor loss point in each species ($n = 7-10$). Weibull functions are fitted.

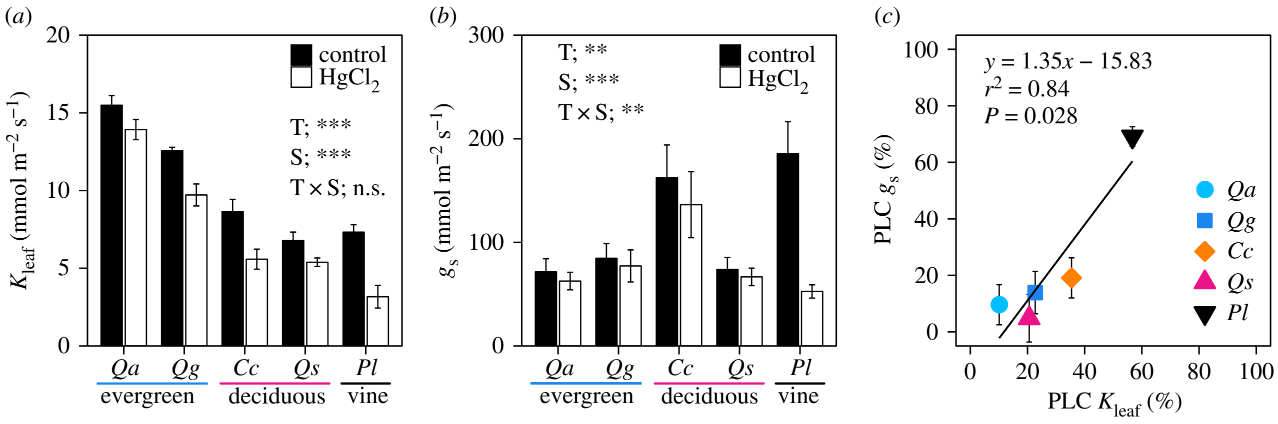

Figure 4. Effects of aquaporin inhibition by $HgCl_2$ on (*a*) leaf hydraulic conductance (K_{leaf}) and (*b*) stomatal conductance (g_s) and (*c*) the relationship between PLC of K_{leaf} and g_s by aquaporin blockage. Evergreen trees: *Qa*, *Quercus acuta*; *Qg*, *Q. glauca*. Deciduous trees: Cc, *Castanea crenata; Qs*, *Q. serrata*. Deciduous vine: *Pl*, *Pueraria lobata*. Bars represent ± s.e.m. (K_{leaf}: $n = 5-7$; g_s: $n = 5-7$). Asterisks in panels (*a*) and (*b*) represent results of two-way ANOVA (T, blockage treatment; S, species; T × S, interaction; ***$p < 0.001$, **$p < 0.01$, n.s., $p > 0.05$).

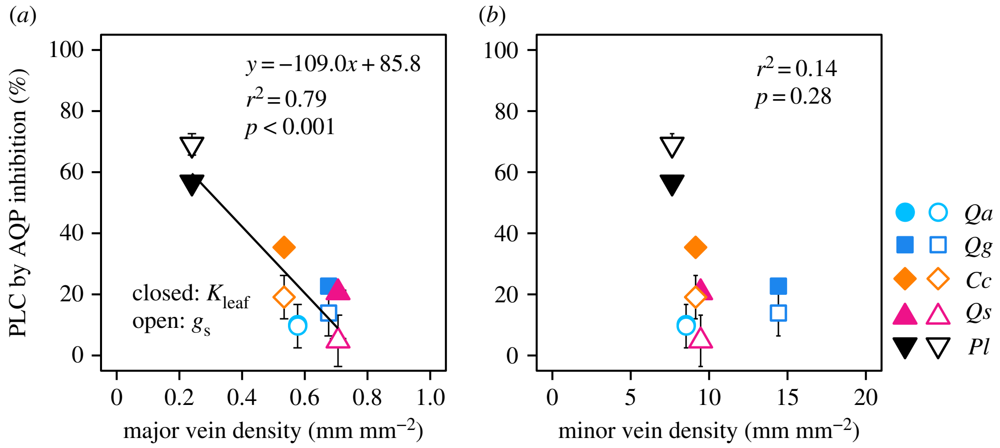

Figure 5. Relationships between PLC by aquaporin inhibition and (*a*) major vein density and (*b*) minor vein density. Species abbreviations are the same as in figure 4. Closed symbols: K_{leaf}, open symbols: g_s. Bars represent ± s.e.m. ($n = 5-7$). (Online version in colour.)

second-order veins extending from the base of the midrib to the outer margins of the leaf (i.e. pinnipalmate venation, figure 1*b*) showed a lower reduction in K_{leaf} and g_s than that in *Q. glauca* and *Q. serrata* with pinnate leaves (which have a single midrib). A minimal impact against a blockage of the middle position of the midrib has also been reported in palmate leaves, which have three or more primary veins at the base of the leaf blade [16]. In addition, recent studies have shown that in comparison with minor veins, lower-order veins with larger conduits were more vulnerable to embolism during leaf dehydration, suggesting a trade-off between efficiency and safety in the leaf venation network, where larger conduits can transport water more efficiently but are more vulnerable to losing their hydraulic function by embolism [15,39,40]. Therefore, leaves with the largest vessel in *P. lobata* would be most vulnerable to xylem embolism; that is, they would be likely to experience hydraulic failure, such as in the cut B and cut C treatments during leaf dehydration. In leaves with the cut C treatment, a higher PLC of K_{leaf} and g_s was observed in *P. lobata* with a lower density of minor veins, and a lower PLC was observed in *Q. glauca* with a higher density of minor veins (figure 2*d*). This result may have partially occurred due to the longer outside-xylem pathway with high hydraulic resistance from the lower minor vein density in *P. lobata*. Our results suggest that the specific difference in venation properties, such as a large venation arrangement, minor vein density and vessel area, can affect a g_s decline due to midrib damage [16] and vein embolism during dehydration.

While aquaporin inhibition by the $HgCl_2$ solution reduced both K_{leaf} and g_s in all species studied (figure 4*a*,*b*), the extent of the decrease varied among species, ranging from a 10% to 57% reduction in K_{leaf} and a 5% to 69% reduction in g_s. Similar reductions in K_{leaf} by a $HgCl_2$ treatment have been reported in other species: *Populus* (22–67% reduction [41,42]), deciduous trees (32–60% reduction [43]), *Vitis* (25 and 61% reduction [21]), and *Arabidopsis* (50% reduction [44]). A physiological mechanism of stomatal closure mediated by aquaporin during drought was proposed; drought-induced abscisic acid (ABA) in the xylem sap flows into the laminae from the petiole, and then, ABA downregulates the activity of aquaporins in bundle sheath cells surrounding veins, leading to reduced K_{leaf}. In turn, this results in rapid Ψ_{leaf} reduction and consequently in stomatal closure [44–46]. Thus, ABA can have dual effects on stomatal closure: a biochemical direct effect on the guard cells and an indirect hydraulic effect through aquaporin-mediated decline in K_{leaf} [45]. Since aquaporin inhibition experiments would cause effects analogous to aquaporin downregulation, our results, in which species with higher reductions in K_{leaf} by the aquaporin blocker $HgCl_2$ tended to have a greater reduction in g_s (figure 4*c*), would support the aquaporin-mediated and hydraulically induced stomatal closure.

Notably, the specific difference in aquaporin activity within a leaf might involve differences in water-use strategies via isohydric or anisohydric stomatal regulation [45,47]. Isohydric plants rapidly close their stomata under drought, which results in the maintenance of a constant Ψ_{leaf} and avoids the risk of catastrophic cavitation, as observed in *P. lobata* (figure 3). By contrast, anisohydric plants slowly close their stomata during drought, resulting in a lower Ψ_{leaf}, which, although increasing the risk of cavitation, maintains photosynthetic production [48], as observed in *Q. glauca* and *Q. serrata*. Scoffoni *et al.* reported that water flow through the outside-xylem pathway was more vulnerable to drought than that through leaf vein xylem in eight species with diverse phylogenies, origins, drought tolerances and life forms, and that reduced aquaporin activity would be the main determinant of the decline in outside-xylem conductance from a model analysis [49]. Therefore, our results that large declines in K_{leaf} and g_s following aquaporin inhibition were found in isohydric *P. lobata* and that small declines in K_{leaf} and g_s were found in anisohydric *Q. acuta* and *Q. serrata* support the hypothesis that hydraulic regulation through aquaporin downregulation might be involved in specific water-use strategies, such as isohydric and anisohydric stomatal regulation. In addition, the low ϵ_{max} in *P. lobata* (electronic supplementary material, table S2) would be susceptible to mesophyll shrinkage during drought, possibly resulting in rapid reduction in K_{leaf} via a reduction in hydraulic conductivity in another outside-xylem pathway, a mesophyll route [50]. The high vulnerability of the outside-xylem pathway could lead to rapid stomatal closure that would protect against catastrophic hydraulic failure in the stem xylem during drought, which would induce plant death [49]. Because the stem of *P. lobata* can be highly sensitive to drought-induced cavitation, such as when the mean cavitation pressure is −0.58 MPa [51], the rapid stomatal closure involved in aquaporin as well as mesophyll shrinkage would protect the plant from not only major vein embolism but also stem embolism and subsequent plant death during drought.

In comparison with minor veins, major veins potentially have greater water transport capacity and a greater carbon cost, and the higher major vein density potentially contributes to higher K_{leaf} and gas exchange rates under moist conditions [52]. We found that species with lower major vein density had a higher PLC for K_{leaf} and g_s due to aquaporin inhibition in a rehydrated leaf (figure 5*a*). This result may suggest that a trade-off function exists between vein architecture (apoplastic pathway) and aquaporin activity (cell-to-cell pathway) with respect to leaf hydraulics. That is, species with higher costs of the major veins can supply enough water to the whole lamina, such that they do not necessarily need to significantly increase the outside-xylem hydraulic conductivity by the aquaporins, whereas species with lower costs of the major veins need to increase the outside-xylem hydraulic conductivity by increasing the aquaporin expression level in a hydrated leaf. Higher major vein density can influence higher LMA and longer leaf spans across species [7]. In addition, high aquaporin dependence on K_{leaf} in *P. lobata* may be related to the life form of the species. Enhanced aquaporin expression and activity in roots and shoots promote an increase in whole-plant hydraulic conductance, thus mitigating Ψ_{leaf} decline and facilitating stomatal opening [47]. A great extent of aquaporin regulation in *P. lobata* would be advantageous for plastic and rapid responses of plant water use to the changing environment because *P. lobata* is a vine species; thus, in comparison with woody species, a vine can easily move to a new expanding environment by extending the new shoots. More studies are needed to clarify the underlying constraint in the trade-off relationship related to leaf hydraulics.

Overall, our results suggest that specific differences in leaf venation architecture, especially major vein density and arrangement, and in the activity of leaf aquaporin, can affect K_{leaf} and g_s, potentially due to their ecological properties, such as growth form, isohydry/anisohydry and vulnerability to embolism, possibly providing a trade-off function between vein architecture and aquaporin activity. Leaf vulnerability to embolism is one of the most central traits for plant drought tolerance [53]; therefore, regulation of K_{leaf} by aquaporins and tolerance of embolism in major veins (and leaf venation architecture) are important traits that determine drought tolerance in angiosperms. Further studies of aquaporins in relation to leaf hydraulics across a wider range of phylogeny and species will provide a deeper understanding of specific drought tolerance and drought-induced vegetation mortality.

Data accessibility. All data generated or analysed during this study are included in this published article and its electronic supplementary material.

Authors' contributions. H.H. and A.I. designed the research; H.H. collected and analysed the data; H.H., M.K., E.A. and A.I. wrote the manuscript.

Competing interests. The authors declare no competing interests.

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