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 HgCl2, 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].

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While a steady-state maximum $K_{\text{leaf}}$ strongly correlates with $g_s$ and $A_{\text{max}}$ among species, $K_{\text{leaf}}$ is not constant and is highly dynamic in response to environmental stimuli [5]. During drought, for example, a decrease in leaf water potential ($\Psi_{\text{leaf}}$) can result in embolism formation in the xylem of leaf veins, thereby reducing their hydraulic conductivity and $K_{\text{leaf}}$ [14,15]. Leaf vascular redundancy (i.e. high vein density) can enhance tolerance against hydraulic dysfunction 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 $K_{\text{leaf}}$. A decline in $K_{\text{leaf}}$ associated with the deactivation of aquaporins in response to environmental stimuli can result in a decline in $g_s$ [24]. Accordingly, $K_{\text{leaf}}$ 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 $K_{\text{leaf}}$ and $g_s$ remains poorly understood.

In this study, we conducted two experiments to artificially decrease $K_{\text{leaf}}$ 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 $\Psi_{\text{leaf}}$ in dehydrated leaves in an open field. In the aquaporin inhibition experiments [26], we investigated the decrease in $K_{\text{leaf}}$ 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 hydraulicities and $g_s$ from physiological and ecological points of view. We hypothesized that species with lower vein density may experience greater impacts of $K_{\text{leaf}}$ and $g_s$ on aquaporin inhibition because a low vein density can provide a sensitive $K_{\text{leaf}}$ response against hydraulic dysfunction in veins [16], and aquaporin upregulation can offset the decline in $K_{\text{leaf}}$. 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 1b), which can differentially affect $K_{\text{leaf}}$ 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 $K_{\text{leaf}}$ [27–29], which was related to the expression of PIPI (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 vine species (Pueraria lobata [Wild.] 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 $K_{\text{leaf}}$. 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 by the network structure of leaf veins and water channel aquaporins; however, the mutual involvement of leaf veins and aquaporins in $K_{\text{leaf}}$ and $g_s$ remains poorly understood.

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tree Quercus glauca, the deciduous tree Quercus serrata and the deciduous vine P. lobata—between August and September 2003. In the evening of the day before the $K_{\text{leaf}}$ 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 flow was 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 outside the xylem. In cut C, water flow into the leaf was extremely limited, and the leaf water potential at the midrib and via minor veins and outside-xylem pathways. After the leaf of the treated and untreated leaves were measured (10.00–14.00) for the minor vein observation because all study species had heteroblastic 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.

(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 ($d$), minor 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 mm² 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 heterobasic 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 $K_{\text{leaf}}$, $g_s$, and $\Psi_{\text{sat}}$ among the leaf blockage treatment within a species. Two-way ANOVA was used to test differences in $K_{\text{leaf}}$, $g_s$, and $\Psi_{\text{sat}}$ among the species and veins or aquaporin inhibition treatments. Weibull functions were fitted to the relationships between $\Psi_{\text{sat}}$ 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 pinnate palmate 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, $\Psi_{\text{sat}}$ and $\Psi_{\text{w.tlp}}$ were higher in P. lobata than in Q. glauca and Q. serrata (electronic supplementary material, table S2).
The value of $\epsilon_{\text{max}}$ 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 $K_{\text{leaf}}$ and $g_s$, in the three species (figure 2a,b), although statistical significance was not observed between some blockage treatments. Of the cut types, cut A had the least effect on $K_{\text{leaf}}$ and $g_s$, and the PLCS of the $K_{\text{leaf}}$ and $g_s$ were 40–50% in Q. glauca and Q. serrata and ≈20% in P. lobata (figure 2d). Cut B had an intermediate effect, and the PLCS of $K_{\text{leaf}}$ and $g_s$ were 80% for all three species (except for $K_{\text{leaf}}$ in P. lobata, which was approx. 60%). Cut C had the greatest effect, and the PLCS of $K_{\text{leaf}}$ and $g_s$ were ≈90% in all species. Leaf water potential at midday ($\Psi_{\text{leaf}}$) also tended to decline in the order of control < cut A < cut B < cut C in Q. glauca (figure 2c); however, the effect of vein blockage was unclear in comparison to the effects on $K_{\text{leaf}}$ 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 $\Psi_{\text{leaf}}$ between the cutting treatments in P. lobata).

In Q. glauca, $g_s$ gradually decreased with decreasing $\Psi_{\text{leaf}}$, which decreased below the turgor loss points in leaves with the cut C treatment (figure 3). In Q. serrata, $g_s$ also gradually decreased with decreasing $\Psi_{\text{leaf}}$ but $\Psi_{\text{leaf}}$ 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 $\Psi_{\text{leaf}}$ was more than −0.5 MPa, and almost all leaves maintained $\Psi_{\text{leaf}}$ above the turgor loss point.

(c) Aquaporin inhibition experiment

$K_{\text{leaf}}$ and $g_s$ were significantly affected by aquaporin inhibition in all species (figure 4a,b). The effects of aquaporin blockage on $K_{\text{leaf}}$ and $g_s$ were relatively small in the evergreen and deciduous trees, with a 10–35% reduction in $K_{\text{leaf}}$ and 5–19% in $g_s$, on average, but the effects were higher in the deciduous vine P. lobata, with a 57% reduction in $K_{\text{leaf}}$ and 69% in $g_s$. There was a significant interaction between species and HgCl$_2$ treatment for $g_s$ (figure 4b). Pooling the data by species, the PLC of $K_{\text{leaf}}$ by aquaporin blockage was positively correlated with the PLC of $g_s$ ($r^2 = 0.84$, $p = 0.028$; figure 4c). In evergreen and deciduous tree species, the PLC of $K_{\text{leaf}}$ tended to be higher than the PLC of $g_s$, whereas in the deciduous vine P. lobata, the PLC of $K_{\text{leaf}}$ was lower than the PLC of $g_s$.

The PLCS of $K_{\text{leaf}}$ 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 $K_{\text{leaf}}$ 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

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**Figure 2.** Effects of vein blockage on (a) leaf hydraulic conductance ($K_{\text{leaf}}$ 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 $K_{\text{leaf}}$ and $g_s$. Qg: Quercus glauca (evergreen tree); Qs: Q. serrata (deciduous tree); Pl: Pueraria lobata (deciduous vine). Bars represent ± s.e.m., $K_{\text{leaf}}$ 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, $p < 0.05$).
second-order veins extending from the base of the midrib to the outer margins of the leaf (i.e. pinnipalmate venation, figure 1b) showed a lower reduction in $K_{\text{leaf}}$ 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 $K_{\text{leaf}}$ and $g_s$ was observed in $P. \text{lobata}$ with a lower density of minor veins, and a lower PLC was observed in $Q. \text{glauca}$ with a higher density of minor veins (figure 2f). This result may have partially occurred due to the longer outside-xylem pathway with high hydraulic resistance from the lower minor vein density in $P. \text{lobata}$. 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 $K_{\text{leaf}}$ and $g_s$ in all species studied (figure 4a,b), the extent of the decrease varied among species, ranging from a 10% to 57% reduction in $K_{\text{leaf}}$ and a 5% to 69% reduction in $g_s$. Similar reductions in $K_{\text{leaf}}$ 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 *Amphilopsis* (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 $K_{\text{leaf}}$. In turn, this results in rapid $\Psi_{\text{leaf}}$ 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 $K_{\text{leaf}}$ [45]. Since aquaporin inhibition experiments would cause effects analogous to aquaporin downregulation, our results, in which species with higher reductions in $K_{\text{leaf}}$ by the aquaporin blocker HgCl$_2$ tended to have a greater reduction in $g_s$ (figure 4c), 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 $\Psi_{\text{leaf}}$ and avoids the risk of catastrophic cavitation, as observed in $P. \text{lobata}$ (figure 3). By contrast, anisohydric plants slowly close their stomata during drought, resulting in a lower $\Psi_{\text{leaf}}$ 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 $K_{\text{leaf}}$ and $g_s$, following aquaporin inhibition were found in isohydric $P. \text{lobata}$ and that small declines in $K_{\text{leaf}}$ 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 $\epsilon_{\text{max}}$ in $P. \text{lobata}$ (electronic supplementary material, table S2) would be susceptible to mesophyll shrinkage during drought, possibly resulting in rapid reduction in $K_{\text{leaf}}$ 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 $P. \text{lobata}$ 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 $K_{\text{leaf}}$ and gas exchange rates under moist conditions [52]. We found that species with lower major vein density had a higher PLC for $K_{\text{leaf}}$ and $g_s$ due to aquaporin inhibition in a rehydrated leaf (figure 5e). 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 $K_{\text{leaf}}$ in $P. \text{lobata}$ 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 $\Psi_{\text{leaf}}$ decline and facilitating stomatal opening [47]. A great extent of aquaporin regulation in $P. \text{lobata}$ would be advantageous for plastic and rapid responses of plant water use to the changing environment because $P. \text{lobata}$ 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 $K_{\text{leaf}}$ 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 $K_{\text{leaf}}$ 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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