Leaf anatomy mediates coordination of leaf hydraulic conductance and mesophyll conductance to CO₂ in Oryza

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Summary
- Leaf hydraulic conductance ($K_{leaf}$) and mesophyll conductance ($g_m$) both represent major constraints to photosynthetic rate (A), and previous studies have suggested that $K_{leaf}$ and $g_m$ is correlated in leaves. However, there is scarce empirical information about their correlation.
- In this study, $K_{leaf}$, leaf hydraulic conductance inside xylem ($K_x$), leaf hydraulic conductance outside xylem ($K_{ox}$), A, stomatal conductance ($g_s$), $g_m$, and anatomical and structural leaf traits in 11 Oryza genotypes were investigated to elucidate the correlation of H₂O and CO₂ diffusion inside leaves.
- All of the leaf functional and anatomical traits varied significantly among genotypes. $K_{leaf}$ was not correlated with the maximum theoretical stomatal conductance calculated from stomatal dimensions ($g_{s,max}$), and neither $g_s$ nor $g_{s,max}$ were correlated with $K_x$. Moreover, $K_{ox}$ was linearly correlated with $g_m$ and both were closely related to mesophyll structural traits.
- These results suggest that $K_{leaf}$ and $g_m$ are related to leaf anatomical and structural features, which may explain the mechanism for correlation between $g_m$ and $K_{leaf}$.

Key words: leaf hydraulic conductance ($K_{leaf}$), leaf vein density, mesophyll conductance ($g_m$), mesophyll structure, rice (Oryza), stomata.

Introduction
In order to keep stomata open for capturing CO₂ during photosynthesis, leaves need a continuous water flow through the leaf hydraulic system to replace the water lost by transpiration (Sack & Holbrook, 2006). By measuring leaf hydraulic conductance ($K_{leaf}$, mmol m⁻² s⁻¹ MPa⁻¹), it is possible to know the efficiency of water transportation through the leaf. However, still little is known about the water transport pathways inside leaves, and particularly outside the xylem (Cochard et al., 2004; Buckley, 2015; Sack et al., 2015). In higher plants, water from the stem enters the petiole and moves through xylem in different vein orders, then exits into the bundle sheath and moves through mesophyll tissue before evaporating into the intercellular airspace and diffusing though stomata. Therefore, $K_{leaf}$ consists of at least two components: inside and outside xylem ($K_x$ and $K_{ox}$ respectively; mmol m⁻² s⁻¹ MPa⁻¹), and hence, $K_{leaf}$ would be limited by both leaf vein density and distribution as well as outside xylem compartment traits (Sack & Holbrook, 2006; Buckley et al., 2015).

A number of studies have dealt with the partitioning of hydraulic resistance within leaves across a broad range of species, and a large variability has been observed. Sack et al. (2005) and Scoffoni & Sack (2015) found that 8–77% of whole leaf hydraulic resistance was situated within the xylem in dicotyledonous species. Other studies reported that leaf hydraulic resistance inside xylem was of minor importance compared with outside xylem (Salleo et al., 2003; North et al., 2013). Further resolution of this question is complicated by the difficulty in measuring $K_x$ or $K_{ox}$ directly, and particularly in a way that gives comparable results for the wide range of types of leaf vein systems that occur in higher plants (Cochard et al., 2004; Nardini et al., 2005, 2010; Sack et al., 2012; North et al., 2013; Sack & Scoffoni, 2013).

Leaf vein systems, as distinct water transport systems, vary greatly from species to species in their arrangement, density, vascular bundle features and xylem conduits within the bundles (Ueno et al., 2006; Blonder et al., 2011; Sack et al., 2012; Sack & Scoffoni, 2013). An empirical correlation of $K_{leaf}$ and venation density, expressed as vein length per area (VLA, mm mm⁻²) was suggested in early studies. VLA is predicted to correlate positively with $K_{leaf}$ by providing more parallel flow paths through the vein system per leaf area, which increases $K_x$ and by decreasing flow path length from veins to evaporation sites, which increases $K_{ox}$ (Sack & Frol, 2006; Brodribb et al., 2007; Sack et al., 2013; Buckley et al., 2015). Although studies over the past two decades have found positive, negative or even no correlations between $K_{leaf}$ and VLA (Nardini et al., 2012, 2014; Sack & Scoffoni, 2012; Flexas et al., 2013b; Xiong et al., 2015d), the majority of data show positive
correlations, particularly for minor vein length per unit area (Sack et al., 2015). The correlation between $K_{\text{leaf}}$ and VLA might be affected not only by leaf vein features, but also by aspects of anatomy outside of the xylem, such as size and hydraulic permeability of bundle sheath cells, mesophyll thickness and cell wall thickness (Buckley et al., 2015). Features of outside-xylem anatomy that are not directly related to VLA may also be important in determining $K_{\text{leaf}}$, especially through effects on the partitioning of transport among apoplastic, transmembrane and vapor phase pathways (Sheriff & Meidner, 1974; Buckley, 2015; Scoffoni, 2015). Recent model approaches have suggested that water transport in leaves occurs mainly within the liquid phase, but for leaves with low tissue density, significant water transport may also happen in the vapor phase, especially under high irradiance and high temperature conditions (Rockwell et al., 2014; Buckley, 2015). More recently, modeling by Buckley et al. (2015) predicted that the contribution of vapor transport to $K_{\text{leaf}}$ increased from 16% to 65% on average across 14 dicot species when the temperature gradient inside leaves rose from 0 to 0.2°C.

Many efforts have been made to reveal the correlation between $K_{\text{leaf}}$ and physiological variables related to photosynthesis. For instance, relationships between $K_{\text{leaf}}$ and photosynthetic rate ($\tilde{A}$), and $K_{\text{leaf}}$ and stomatal conductance ($g_{\text{s}}$) were often observed (Brodribb et al., 2007; Xiong et al., 2015d). In C₃ plants, it has been demonstrated that under current atmospheric conditions $A$ is often strongly limited by CO₂ diffusion conductance, which comprises $g_{\text{s}}$ and mesophyll conductance ($g_{\text{m}}$) (Flexas et al., 2008, 2012; Evans et al., 2009). The $g_{\text{s}}$ is determined by both stomatal features (size and density) and opening status, and the opening status might be related to $K_{\text{leaf}}$ (Brodribb & Holbrook, 2004; Brodribb & McAdam, 2011; Guyot et al., 2012; Ocheltree et al., 2012; McAdam & Brodribb, 2013). Many studies have investigated the effects of leaf anatomical features on $g_{\text{m}}$ in rice (Xiong et al., 2015d). The mesophyll cell wall thickness, mesophyll cell surface area exposed to intercellular air space per leaf area ($S_{\text{m}}$) and mesophyll cell surface area occupied by chloroplasts exposed to intercellular air space per leaf area ($S_{\text{cl}}$) were suggested to be the key traits limiting $g_{\text{m}}$ (Evans et al., 2009; Tomás et al., 2013; Xiong et al., 2015b). However, although very few studies have investigated the correlation of $K_{\text{leaf}}$ and $g_{\text{m}}$ empirically, a positive correlation was found across species (Flexas et al., 2013b) and Oryza genotypes (Xiong et al., 2015d). This correlation was hypothesized to result from a common pathway for water and CO₂ inside the mesophyll and/or a role of aquaporins in both water and CO₂ transport (Flexas et al., 2013b). The possibility that vapor phase transport can contribute substantially to measured $K_{\text{leaf}}$ suggests that the transport properties of leaf intercellular air spaces may also contribute to coordination between $g_{\text{m}}$ and $K_{\text{leaf}}$. To our knowledge, there have been no previous studies of the correlation of $K_{\text{leaf}}$ and $g_{\text{m}}$ in which leaf anatomy was analyzed in parallel.

Wild rice (genus Oryza) is an important germplasm resource and is being utilized to improve rice production. Despite sharing a recent common ancestor, Oryza species are ecologically and phenotypically diverse. Thus, revealing the coordination of leaf functional, structural and biochemical traits across the Oryza genus will help crop breeders to develop new varieties for sustainable agriculture. Giuliani et al. (2013) investigated the diversity of leaf structure and how it relates to photosynthesis and transpiration by using 24 rice and wild relatives. Their results highlight that significant correlations occur among structural and functional traits associated with photosynthesis and transpiration. Here, we investigated the impacts of leaf anatomical traits on $K_{\text{leaf}}$ and CO₂ diffusion across 11 Oryza genotypes to reveal the intrinsic mechanism of correlation between leaf functional and structural traits. The objectives of this study were: to identify the most limiting fraction of $K_{\text{leaf}}$ in rice and rice relatives by partitioning it into $K_{\text{a}}$ and $K_{\text{w}}$ to investigate the impact of variation in leaf anatomical traits on $K_{\text{leaf}}$, $g_{\text{m}}$ and $g_{\text{s}}$ in rice and rice relatives; and to clarify the role of leaf anatomy in coordination of $K_{\text{leaf}}$ and $g_{\text{m}}$.

### Materials and Methods

#### Plant materials

This study was conducted simultaneously with a study of the importance of leaf morpho-anatomical traits in determining leaf hydraulic conductance ($K_{\text{leaf}}$) in rice (Xiong et al., 2015d), and we note that the gas exchange and $K_{\text{leaf}}$ values were taken from the previous one, and the data of new parameters that estimated in the current study were collected from the same plants. Eleven genotypes across five Oryza genus were used (Table 1). Plants were grown outdoors in 15-l pots (with 13 kg soil) with a density of three plants per pot. Nitrogen (N), phosphorus (P) and potassium (K) were applied as basal fertilizers at a rate of 3.0 g, 1.95 g and 1.95 g per pot, respectively. During the growth period, plants were well watered (at least 2 cm water level was kept) and pests were controlled using chemical pesticides. Measurements were taken on plants between 50 and 70 d after planting.

#### Gas exchange

Gas exchange measurements were performed between 09:30 h and 15:30 h on non-senescent fully expanded leaves using an LI-6400XT portable photosynthesis system equipped with a 6400-40 leaf chamber (Li-Cor Inc., Lincoln, NE, USA), in an environmental controlled room (air temperature of 27.8 ± 2.1°C, the photosynthetic photon flux density (PPFD) at leaf surface of 1200 ± 47 μmol m⁻² s⁻¹, and relative humidity of 77.4 ± 5.3%). Inside the leaf cuvette, leaf temperature was maintained at 28°C, PPFD at 1500 μmol m⁻² s⁻¹ and, leaf-to-air vapor pressure deficit at 1.1–1.4 kPa, and CO₂ concentration was adjusted to 400 μmol mol⁻¹ with a CO₂ mixture. After equilibration to a steady state, gas exchange parameters, steady-state fluorescence ($F_{\text{m}}$) and maximum fluorescence ($F_{\text{m}}'$) were recorded. $\Phi_{\text{PSII}}$ was calculated as follows:

$$\Phi_{\text{PSII}} = \frac{F_{\text{m}}' - F_{\text{s}}}{F_{\text{m}}}$$

Potential electron transport rate (J) was computed as follows:

$$J = \Phi_{\text{PSII}} \cdot \text{PPFD} \cdot \alpha \beta$$

($\alpha$, leaf absorptance; $\beta$, partitioning of absorbed quanta between photosystems II and I). The product $\alpha \beta$ was determined,
Table 1 Mean values ± SD of photosynthesis, leaf hydraulic conductance and CO2 diffusion conductance (N = 3–9 plants)

<table>
<thead>
<tr>
<th>Species</th>
<th>Genotype</th>
<th>A (µmol m⁻² s⁻¹)</th>
<th>K₁ (mmol m⁻² s⁻¹ MPa⁻¹)</th>
<th>K₂ (mmol m⁻² s⁻¹ MPa⁻¹)</th>
<th>gₘ (mmol m⁻² s⁻¹)</th>
<th>Kᵢ (mmol m⁻² s⁻¹)</th>
<th>Kᵢ-model (mmol m⁻² s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oryza sativa L.</td>
<td>Shanyou 63</td>
<td>22.6 ± 3.1</td>
<td>7.20 ± 0.29</td>
<td>156 ± 2.7</td>
<td>156 ± 2.7</td>
<td>1.19 ± 0.78</td>
<td>1.19 ± 0.78</td>
</tr>
<tr>
<td>Oryza sativa L.</td>
<td>Huanghuazhan</td>
<td>31.8 ± 1.9</td>
<td>7.77 ± 0.73</td>
<td>151 ± 3.1</td>
<td>151 ± 3.1</td>
<td>1.88 ± 0.19</td>
<td>1.88 ± 0.19</td>
</tr>
<tr>
<td>Oryza sativa L.</td>
<td>N22</td>
<td>25.9 ± 1.2</td>
<td>7.30 ± 1.19</td>
<td>152 ± 2.4</td>
<td>152 ± 2.4</td>
<td>1.70 ± 0.17</td>
<td>1.70 ± 0.17</td>
</tr>
<tr>
<td>Oryza sativa L.</td>
<td>Nipponbare</td>
<td>25.9 ± 1.2</td>
<td>7.30 ± 1.19</td>
<td>152 ± 2.4</td>
<td>152 ± 2.4</td>
<td>1.70 ± 0.17</td>
<td>1.70 ± 0.17</td>
</tr>
<tr>
<td>Oryza sativa L.</td>
<td>ASU</td>
<td>24.8 ± 1.7</td>
<td>7.56 ± 1.80</td>
<td>147 ± 1.7</td>
<td>147 ± 1.7</td>
<td>1.42 ± 0.16</td>
<td>1.42 ± 0.16</td>
</tr>
<tr>
<td>Oryza sativa L.</td>
<td>108</td>
<td>22.1 ± 1.0</td>
<td>7.93 ± 1.10</td>
<td>152 ± 1.9</td>
<td>152 ± 1.9</td>
<td>1.35 ± 0.17</td>
<td>1.35 ± 0.17</td>
</tr>
<tr>
<td>Oryza sativa L.</td>
<td>Wcr</td>
<td>20.5 ± 1.4</td>
<td>8.63 ± 1.65</td>
<td>158 ± 2.0</td>
<td>158 ± 2.0</td>
<td>1.38 ± 0.18</td>
<td>1.38 ± 0.18</td>
</tr>
<tr>
<td>Oryza sativa L.</td>
<td>Ruf</td>
<td>22.2 ± 1.5</td>
<td>8.70 ± 1.65</td>
<td>157 ± 1.7</td>
<td>157 ± 1.7</td>
<td>1.34 ± 0.18</td>
<td>1.34 ± 0.18</td>
</tr>
<tr>
<td>Oryza sativa L.</td>
<td>ANOVA</td>
<td>24.5 ± 5.5</td>
<td>6.24 ± 2.65</td>
<td>19.7 ± 5.1</td>
<td>19.7 ± 5.1</td>
<td>1.19 ± 0.31</td>
<td>1.19 ± 0.31</td>
</tr>
</tbody>
</table>

Kᵢ, leaf hydraulic conductance; A, leaf photosynthesis; K₁, hydraulic conductance inside xylem; K₂, hydraulic conductance outside xylem; gₘ, mesophyll conductance of CO₂; Cᵢ, intercellular CO₂ concentration.

The variable J method described in Harley et al. (1992) was used to calculate mesophyll conductance of CO₂ (gₘ) and CO₂ concentration in chloroplast (Cᵢ). Cᵢ was calculated as follows:

\[ Cᵢ = \frac{Γ*(A + R_d)}{J - 4(A + R_d)} \]

(Cᵢ, intercellular CO₂ concentration). For all the selected genotypes, gₘ was also estimated by using the A – Cᵢ curve-fitting method (Ethier & Livingston, 2004), and a tight correlation was obtained between the two estimates of gₘ considering all the data averaged per genotype (R² = 0.99, P < 0.001; Fig. S1b in Xiong et al., 2015d). As the gₘ values from both methods were very similar, we used the values obtained by the variable J method to compare with other parameters.

Leaf hydraulic conductance

Measurements were made on nonnecing and fully expanded leaves of two adjacent tillers on each plant (one for Kₑالة and another for leaf hydraulic conductance inside xylem (Kᵢ)) and nine plants for each genotype were used. The tillers were harvested under water and kept submerged until measurements were performed. Kₑالة was measured using the evaporative flux method (EFM) (Sack et al., 2002; Brodribb et al., 2007; Sack & Scoffoni, 2012; Xiong et al., 2015d). Leaves were excised under the water and placed under conditions favorable to transpiration (i.e. under PPFD of 1200 µmol m⁻² s⁻¹ and air temperature of 28°C). In order to ensure a tight seal with tubing that supplied water, first, leaf sheaths’ vertical coating surrounding the cone-shaped plastic stick (the gap between leaf sheath and stick was sealed up by using petroleum jelly and adjusting the position of the stick), and then the outside of sheathes were wrapped in thread seal tape (polytetrafluoroethylene film). The tubing system was connected to a plastic erlenmeyer flask (250 ml) with degassed water situated on analytical balance (Sartorius BP 2215, Göttingen, Germany). Before measuring, the leaks were checked by creating a high gradient (c. 60 cm) between leaves and water surface in the following Valentini et al. (1995), from the relationship between 1/4Φ₉₉ and Φ₀₋₀ obtained by varying either light intensity under nonphotorespiration conditions (O₂ < 1%). To do this, light response curves were determined (Xiong et al., 2015d).
when leaves reached steady state (the water weight lost linearly with time, typically, \(c.20\) min), the weight of water was recorded every 60 s and the water flow rate was calculated as the slope of linear regression between weight and time (15 min). The leaf area was measured using a portable leaf area meter (LI-Cor 3000C; Li-Cor Inc.) and then the liquid water flow rate was normalized by leaf area \(E\). The leaves were detached and cut into small sections and mixed immediately (<20 s), and the subsamples were used for leaf water potential \(\Psi_{\text{leaf}}\) measuring by using a WP4C Dew Point Potential Meter (Decagon, Pullman, WA, USA). \(K_{\text{leaf}}\) was calculated as follows:

\[
K_{\text{leaf}} = \frac{E}{\Psi_{\text{water}} - \Psi_{\text{leaf}}}
\]

\((\Psi_{\text{water}},\ \text{water potential of distilled water}; \quad \Psi_{\text{water}} = 0\ \text{was used in the present study}).\)

The method of ‘cutting minor veins’ was widely used for estimating \(K_{x}\) in dicotyledonous leaves (Scoffoni & Sack, 2015). This is because in dicotyledons the bulk of water in major veins influx into minor veins and, thus, the major and minor veins act approximately in series. However, veins are parallel distributed in grass leaves, and the major and minor veins are not in series. One potential risk in \(K_{x}\) estimation by using the ‘cutting minor veins’ method in grass leaves is that water flow may mainly follow the large vessels in the major veins. To avoid this risk, we estimated the \(K_{x}\) by using an intact leaf rather than the middle part of the leaf (Wei et al., 1999; Stiller et al., 2003, 2005; Maherali et al., 2008). In fact, our results showed that the size of the major vein decreases significantly from leaf base to tip in rice and that the size of veins at leaf tips tend to be uniform (similar to minor veins) (Supporting Information Fig. S1). This kind of cut is very similar with the \(K_{x}\) estimation method that is used widely in dicotyledonous species (Buckley et al., 2015; Scoffoni & Sack, 2015). Moreover, a good correlation was obtained between measured \(K_{x}\) and modeled \(K_{x}\) \((K_{x}\text{-Model})\) (Fig. 1). In this study, \(K_{x}\) was measured using the method originally described by Stiller et al. (2003). The leaf with 2–3 cm leaf sheath was cut from the tiller underwater to avoid introducing embolism, and the apical 2–4 cm of the leaf blade was removed. Such leaf segments usually lacked continuous aerenchyma. In a similar way used in \(K_{\text{leaf}}\) measurement, the leaf sheath was contacted to the tubing system that was connected to a water-filled plastic erlenmeyer flask that could be lowered \(c.60\) cm to create pressure gradient. The leaf tip was submerged in a small water-filled cup that was placed on an electronic balance. Five different pressures (i.e. different heights) were used to measure the flow rates of water off the balance and calculated \(K_{x}\) from the linear regression between flow rate and applied pressure. After the measurements, the leaves were flushed at \(c.100\) kPa with water to examine the continuous aerenchyma, with the apical end submerged under the water. If large air bubbles emerged from the cut surface during the flushing, the leaf was discarded. The genotypes of Lat and Rhi were removed from the \(K_{x}\) analysis, because the flushing measurements indicated that they have continuous aerenchyma in all the measured leaves.

In the present study, the modeled xylem hydraulic conductance \((K_{x}\text{-Model},\ \text{mmol m}^{-2} \text{s}^{-1} \text{MPa}^{-1})\) was calculated using the Hagen–Poiseuille equation (North et al., 2013):

\[
K_{x}\text{-Model} = \frac{\pi (b^{2} \times b^{2})}{128 \eta} \times n \times VLA_{\text{major}} + \frac{\pi (A^{2} \times B^{2})}{128 \eta} \times VLA_{\text{minor}}
\]

\((b\ \text{and } H,\ \text{height of conduit in minor and major veins (including midrib and large veins), respectively (for their determination see ‘Light microscopy analysis’); } b \text{ and } B,\ \text{width of conduit in minor and major veins, respectively; } n,\ \text{number of conduits per minor or major vein (sum of midrib and large veins); } \eta, \ \text{viscosity of water (8.324 \times 10^{-10} \text{ MPa s at 28°C})}.\) We note that this measurement may underestimate the real conductance, because the transverse veins were ignored. \(K_{ox}\) was calculated as follows:

\[
\frac{1}{K_{\text{leaf}}} = \frac{1}{K_{ox}} + \frac{1}{K_{x}}
\]

The \(K_{ox}\) was also simulated by using a model based on leaky cable theory as developed for roots (Landsberg & Fowkes, 1978) and modified for leaves (North et al., 2013). In this model, the axial flow along the leaf blade declines with distance from the bases to tips along the leaf blade, and the decline is equal to radial (lateral) flow into the mesophyll. This leads to a second-order ordinary differential equation for water potential with respect to distance along the blade, with the boundary conditions that axial flow is zero at the end of the leaf, and that water potential is zero.

![Fig. 1](image_url) The correlation between (a) measured leaf hydraulic conductance inside xylem \(K_{x}\) and modeled leaf hydraulic conductance inside xylem \(K_{x}\text{-Model}\) and (b) measured leaf hydraulic conductance outside xylem \(K_{ox}\) and modeled leaf hydraulic conductance outside xylem \(K_{ox}\text{-Model}\) in Oryza. Values are mean ± SD \((n = 3–9\) plants). The fitting lines are shown when \(P < 0.05.\)
at the base of the leaf. The solution can be expressed as the following relationship between $K_{\text{leaf}}$ and $K_{\text{ox}}$:

$$K_{\text{ox}} = \alpha l K_{\text{leaf}} \left( \frac{\exp(2\alpha l) + 1}{\exp(2\alpha l) - 1} \right)$$

($l$, leaf length). $\alpha$ is defined as:

$$\alpha = \sqrt{\frac{w}{K_{\text{ox}}}}$$

($w$, leaf width). Both sides of the first equation above depend on $K_{\text{ox}}$, but the equation cannot be solved analytically for $K_{\text{ox}}$; we therefore used SOLVER in Microsoft Excel to determine the value of $K_{\text{ox}}$ that satisfies the equation. Then the modeled $K_{\text{ox}}$ ($K_{\text{ox, Model}}$) was solved by input leaf width ($w$), leaf length ($l$), the modeled $K_{c}$ ($K_{c, Model}$) and $K_{\text{leaf}}$. Because $K_{c}$ and $K_{\text{ox}}$ from measured and modeled methods were quite similar (Fig. 1), the measured $K_{c}$ and $K_{\text{ox}}$ were used to estimate the correlations with other traits.

Leaf vein length per leaf area

Leaves were cleaned in 20% aqueous NaOH after their widths were recorded and then three sections of each leaf (c. 5.0 mm length) were excised from the middle portion, stained with safranin O and fast green (Sigma-Aldrich), and mounted in glycerol for the determination of vein number. Rice vascular bundles can be categorized into three types based on their size: midrib, large veins and minor veins (Scarpella et al., 2003; Smillie et al., 2012; Xiong et al., 2015d). In the present study, the numbers of major veins (sum of midrib and large veins) and minor veins were recorded using a microscope at $\times 40$ magnification.

Light microscopy analysis

After gas exchange measurement, small leaf sections of c. 4.0 $\times$ 1.2 mm were also cut from the middle of new fully expanded leaves (avoiding midribs). The leaf sections were infiltrated with fixative 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH = 7.6) at 4°C with a vacuum chamber (DZF-6050; Shanghai Hasuc Co. Ltd, Shanghai, China), and post-fixed in 2% buffered osmium tetroxide at 20°C for 2 h. The samples were embedded in Spurr’s epoxy resin. For light microscopy, semithin leaf cross-sections were cut using a fully automated rotary microtome (Leica RM2265; Leica Microsystems, Milton Keynes, UK). The leaf sections were stained with 1% (w/v) toluidine blue in 1% (w/v) Na2B4O7, and they were examined at $\times 40$ and $\times 100$ magnification with an Olympus IX71 light microscope (Olympus Optical, Tokyo, Japan). For transmission electron microscopes (TEM), H-7650 (Hitachi – Science & Technology, Tokyo, Japan) were used for observation and photography. Three leaves in each variety or treatment were analyzed. The total cross-sectional area of mesophyll tissues ($S_{\text{mes}}$) and intercellular air space area ($S_{\text{IAS}}$), the total length of the mesophyll cell wall exposed to intercellular air space ($l_{m}$), the total length of chloroplasts touching the plasma membrane appressed to intercellular air space ($l$), and the width of the analyzed leaf cross section ($D$) were measured using IMAGEJ software (National Institute of Health, Bethesda, MD, USA). The volume fraction of intercellular air space ($f_{\text{IAS}}$) calculated as:

$$f_{\text{IAS}} = \frac{S_{\text{IAS}}}{S_{\text{mes}}}$$

$S_{m}$ and $S_{c}$ were then calculated as follows:

$$S = \frac{l_{m}}{l_{c}} \times F$$

where $S$ is $S_{m}$ or $S_{c}$, $l_{m}$ is $l_{m}$ or $l_{c}$, and the $F$ is the curvature correction factor, 1.42 according to previous studies (Xiong et al., 2015b). The height ($a$ and $A$) and width ($b$ and $B$) of conduit in at least 15 major and minor veins were measured per genotype in the light microscopy images ($\times 40$) (Fig. S2).

Scanning electron microscope analysis

Five small leaf discs (c. 10 $\times$ 10 mm) were removed from the middle of each leaf sections (the same leaf used for anatomy analysis) (Xiong et al., 2015c). For each genotype, three leaves from different plants were measured. The leaf discs were infiltrated in a vacuum chamber (DZF-6050; Shanghai Hasuc Co. Ltd) with the fixative 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH = 7.6) at 4°C and then the samples were stored at 4°C until analysis. Six to 12 images of the abaxial and adaxial epidermal surfaces for each leaf were captured under vacuum with a scanning electron microscopes (JSM-6390LV, Tokyo, Japan). Stomatal number per measured area (stomatal density, SD), guard cell length (GL), width of entire stoma at center of the stoma (SW), stoma pore length (PL), pore width at center of the stoma (PW) and the guard cell width (GW) at the center of the stoma on the abaxial and adaxial lamina surface were measured using Image J software (National Institute of Health). In this study, the stomatal size was calculated based on the assumption that stoma is an ellipse with its major axis equal to GW and its minor axis equal to SW. More detail about the stomatal features measurement is provided in the legend for Fig. S3.

Calculating anatomical theoretical stomatal conductance

Maximum theoretical stomatal conductance to CO2 as defined by stomatal anatomy ($g_{\text{max}}$ mol m$^{-2}$ s$^{-1}$) was estimated for each genotype using a double end-correction version of the equation by (Franks & Farquhar, 2001; Dow et al., 2014):

$$g_{\text{max}} = \frac{d \cdot \text{SD} \cdot a_{\text{max}}}{1.6 \pi (PD + \frac{4}{3} \sqrt{2a_{\text{max}}})}$$

($d$, diffusivity of water in air (24.9 $\times$ 10$^{-6}$ m$^{-2}$ s$^{-1}$, at 25°C); $a_{\text{max}}$ mean maximum stomatal pore area, defined as an ellipse with major axis equal to PL and minor axis equal to PW; $v$, molar
volume of air (22.4 × 10⁻³ m³ mol⁻¹, at 25°C, 101.3 kPa); PD, stomatal pore depth, which is equal to GW; π, mathematical constant). The \( g_{\text{max}} \) for each leaf was calculated as the sum of \( g_{\text{max}} \) abaxial and adaxial. In the present study, PD and \( a_{\text{max}} \) values were genotype averages.

Statistical analysis

One-way ANOVA was used to test the differences in measured traits (Tables 1, 2) among genotypes. Regression analyses were performed with mean values to test the correlations between parameters. All regressions were fitted by both linear and power models, and the model with higher regression coefficient was selected. Regression lines were shown for \( P < 0.05 \). All analyses were performed in R v.3.2.2 (https://cran.r-project.org).

Results

Variation of gas exchange and \( K_{\text{leaf}} \) across genotypes

The *Oryza* genotypes used in the present study exhibited a substantial variation in photosynthetic physiology as well as leaf hydraulic physiology (Table 1). A very large range of \( A \) extending from 17.6 mmol m⁻² s⁻¹ in the Wcr to 35.9 mmol m⁻² s⁻¹ in the Lat was observed. Genotypes varied 3.1-fold in \( g_{\text{st}} \), from 0.19 mmol m⁻² s⁻¹ in the Rhi to 0.58 mmol m⁻² s⁻¹ in the Lat. The \( g_{\text{st}} \) varied 4.7-fold across genotypes, from 0.10 mmol m⁻² s⁻¹ in the Wcr to 0.47 mm in the Lat. The \( K_{\text{leaf}} \) varied from 3.31 mmol m⁻² s⁻¹ MPa⁻¹ in the Rhi to 12.2 mmol m⁻² s⁻¹ MPa⁻¹ in the Lat. Genotypes also differed significantly in \( K_{\text{g}} \) and \( K_{\text{ox}} \). The \( K_{\text{g}} \) varied from 13.8 mmol m⁻² s⁻¹ MPa⁻¹ in Nipponbare to 24.0 mmol m⁻² s⁻¹ MPa⁻¹ in the Wcr, \( K_{\text{ox}} \) (inferred as \( 1/(1/K_{\text{leaf}}-1/K_{\text{g}}) \)) varied from 5.0 mmol m⁻² s⁻¹ MPa⁻¹ in the I90 to 18.1 mmol m⁻² s⁻¹ MPa⁻¹ in Huanghuazhan, the modeled \( K_{\text{g}} \) \( K_{\text{Model}} \) varied from 13.3 mmol m⁻² s⁻¹ MPa⁻¹ in the Nipponbare to 29.2 mmol m⁻² s⁻¹ MPa⁻¹ in Lat, and the modeled \( K_{\text{ox}} \) \( K_{\text{ox-Model}} \) varied from 3.97 mmol m⁻² s⁻¹ MPa⁻¹ in the I90 to 15.2 mmol m⁻² s⁻¹ MPa⁻¹ in Lat.

Leaf venation and stomatal features on \( K_{\text{leaf}} \)

In order to determine whether the \( K_{\text{g}} \) can be predicted from leaf vein features, the relationship between \( K_{\text{g}} \) and \( K_{\text{Model}} \) was investigated. A linear relationship between \( K_{\text{g}} \) and \( K_{\text{Model}} \) was observed \( (R^2 = 0.78, P < 0.001; n = 3–9; \text{Fig. 1a}) \). Likewise, there was a tight correlation between \( K_{\text{ox}} \) and \( K_{\text{Model}} \) \( (R^2 = 0.92, P < 0.001; \text{Fig. 1b}) \). Across the selected genotypes, the percentage of leaf hydraulic resistance inside xylem \( (R_{\text{L}}/R_{\text{leaf}} = K_{\text{leaf}}/K_{\text{g}}) \) and outside xylem \( (R_{\text{ox}}/R_{\text{leaf}} = K_{\text{leaf}}/K_{\text{ox}}) \) varied widely (Fig. 2). Across genotypes VLA showed a large variation, and a highly significant linear relationship was observed between \( K_{\text{g}} \) and VLA \( (R^2 = 0.77, P < 0.001) \). However, there was no significant positive relationship between \( K_{\text{ox}} \) and VLA \( (R^2 = 0.27, P = 0.151; \text{Fig. 3}) \). The relationships between leaf hydraulic traits and \( g_{\text{st}} \) are shown in Fig. S4. An earlier study found that \( K_{\text{leaf}} \) was linearly correlated with \( g_{\text{st}} \) across the selected genotypes \( (R^2 = 0.57, P = 0.007; \text{Xiong et al., 2015c}) \), but the present study found no relationship between \( g_{\text{st}} \) and either \( K_{\text{g}} \) or \( K_{\text{ox}} \) individually (Fig. S4). Moreover, no coordination between \( K_{\text{g}} \) and \( g_{\text{max}} \), nor between \( K_{\text{ox}} \) and \( g_{\text{max}} \) was found across the selected genotypes. Stomatal density and size varied considerably among genotypes in the present study (Figs 4, 5, S5, S6). A negative correlation between stomatal density and stomatal size was observed (Fig. 4). However, neither \( g_{\text{st}} \) nor \( K_{\text{leaf}} \) was correlated with stomatal density and stomatal length when data were pooled across all genotypes (Figs S5, S6).

Correlations of \( K_{\text{ox}}, g_{\text{m}} \) and leaf structural features

Across all genotypes, \( K_{\text{ox}} \) was linearly correlated with \( g_{\text{m}} \) \( (R^2 = 0.70, P < 0.005; \text{Fig. 5}) \). To explore a potential structural basis for this correlation, we investigated leaf anatomy and

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Species</th>
<th>( f_{\text{AS}} ) (%)</th>
<th>( T_{\text{cell-wall}} ) (μm)</th>
<th>( S_{\text{m}} ) (μm² μm⁻²)</th>
<th>( S_{\text{c}} ) (μm² μm⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shanyou 63</td>
<td>Oryza sativa L.</td>
<td>19.6 ± 2.9</td>
<td>0.157 ± 0.009</td>
<td>17.9 ± 1.3</td>
<td>17.4 ± 2.3</td>
</tr>
<tr>
<td>Huanghuazhan</td>
<td>Oryza sativa L.</td>
<td>21.7 ± 2.9</td>
<td>0.164 ± 0.014</td>
<td>20.8 ± 2.0</td>
<td>19.6 ± 0.9</td>
</tr>
<tr>
<td>N22</td>
<td>Oryza sativa L.</td>
<td>18.3 ± 0.9</td>
<td>0.180 ± 0.003</td>
<td>16.8 ± 2.3</td>
<td>12.1 ± 2.5</td>
</tr>
<tr>
<td>Nipponbare</td>
<td>Oryza sativa L.</td>
<td>21.2 ± 3.1</td>
<td>0.165 ± 0.019</td>
<td>20.3 ± 2.5</td>
<td>17.9 ± 4.5</td>
</tr>
<tr>
<td>Lat</td>
<td>Oryza latifolia L.</td>
<td>23.4 ± 3.0</td>
<td>0.142 ± 0.017</td>
<td>25.4 ± 2.0</td>
<td>23.4 ± 2.0</td>
</tr>
<tr>
<td>Aus</td>
<td>Oryza australisiansis L</td>
<td>16.5 ± 1.5</td>
<td>0.183 ± 0.004</td>
<td>14.1 ± 1.3</td>
<td>10.7 ± 1.7</td>
</tr>
<tr>
<td>I90</td>
<td>Oryza rufipogon L.</td>
<td>19.9 ± 0.2</td>
<td>0.168 ± 0.009</td>
<td>18.3 ± 1.8</td>
<td>16.4 ± 3.2</td>
</tr>
<tr>
<td>I08</td>
<td>Oryza punctata L.</td>
<td>14.9 ± 1.7</td>
<td>0.187 ± 0.015</td>
<td>9.6 ± 1.8</td>
<td>6.6 ± 0.7</td>
</tr>
<tr>
<td>Wcr</td>
<td>Oryza granulata L.</td>
<td>16.3 ± 1.2</td>
<td>0.185 ± 0.016</td>
<td>11.3 ± 0.3</td>
<td>6.4 ± 0.7</td>
</tr>
<tr>
<td>Ruf</td>
<td>Oryza rufipogon L.</td>
<td>17.2 ± 2.2</td>
<td>0.180 ± 0.014</td>
<td>12.6 ± 1.7</td>
<td>9.2 ± 5.9</td>
</tr>
<tr>
<td>Rhi</td>
<td>Oryza rufipogon L.</td>
<td>16.5 ± 1.7</td>
<td>0.154 ± 0.023</td>
<td>16.1 ± 1.8</td>
<td>15.0 ± 1.6</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Average</td>
<td>18.7 ± 2.6</td>
<td>0.169 ± 0.015</td>
<td>16.7 ± 4.6</td>
<td>14.1 ± 5.5</td>
</tr>
</tbody>
</table>

* \( P < 0.05; **, P < 0.01; ***, P < 0.001.\)
structure features. For all examined anatomical traits, there were very large differences among genotypes. Across all genotypes, fraction of leaf mesophyll volume occupied by intercellular air space ($f_{IAS}$) varied from 15% to 23% and mesophyll cell wall thickness ($T_{cw}$) varied from 0.142 to 0.187 μm (Table 2). Genotypes varied by 2.6-fold in mesophyll cell surface area exposed to intercellular air space per leaf area ($S_m$) and 3.7-fold in chloroplast cell surface area exposed to intercellular air space per leaf area ($S_c$). Across all genotypes, $S_m$ increased with $f_{IAS}$ ($R^2 = 0.56, P<0.001$), $S_m$ ($R^2 = 0.82, P<0.001$) and $S_c$ ($R^2 = 0.77, P<0.001$); however, $g_m$ decreased with $T_{cw}$ ($R^2 = 0.80, P<0.001$) across all genotypes. Similar correlations were observed between $K_{ox}$ and leaf anatomical traits (Fig. 6).

**Discussion**

Water transport and CO₂ diffusion inside leaves are two important functional traits that determine the CO₂ assimilation efficiency (Sack & Holbrook, 2006; Flexas et al., 2013b). In our previous study (Xiong et al., 2015c), we found that both leaf
hydraulic conductance \( (K_{\text{leaf}}) \) and mesophyll conductance \( (g_m) \) varied widely among Oryza genotypes, and showed a large degree of correlation with one another. In the current study, our detailed examination of leaf functional and anatomical features provides new insight into the variation of \( K_{\text{leaf}} \) and \( g_m \) across related genotypes and their correlations.

Partitioning leaf hydraulic resistance \( (R_{\text{leaf}} = 1/K_{\text{leaf}}) \) into xylem and outside-xylem components is a key step to understanding leaf hydraulic design. Our results presented here show that the percentage of outside xylem hydraulic resistance varied from 39.1% to 81.8%, and averaged c. 60% in rice and rice relatives leaves (Table 1), implying that outside-xylem pathways exert slightly more control over \( K_{\text{leaf}} \) than xylem pathways, on average across genotypes. This finding is consistent with previous studies on partitioning hydraulic resistances inside leaves which showed that hydraulic resistances within the xylem are on the same order as those outside of the xylem and vary significantly across species (Sack et al., 2004, 2005; Nardini et al., 2005, 2010). For instance, Sack et al. (2005) found that percentage of outside xylem hydraulic resistance varied from 11% to 74% across 10 tropical rainforest tree species. The variation of partitioning leaf hydraulic resistance across genotypes may relate to leaf anatomy.

The correlations between \( K_{\text{leaf}} \) and leaf vein traits have been widely studied and the best supported vein anatomical correlate of \( K_{\text{leaf}} \) is vein length per area (VLA; Caringella et al., 2015). In the present study, we found that leaf hydraulic conductance inside xylem \( (K_x) \) was linearly correlated with VLA, but no correlation was found between leaf hydraulic conductance outside xylem \( (K_{ox}) \) and VLA. This finding indicates that VLA mainly impacts \( K_x \) rather than \( K_{ox} \) (Cochard et al., 2004). Moreover, a tight relationship between \( K_x \) and modeled \( K_{g} \) (\( K_{g,\text{Model}} \)), which is calculated from vein anatomical traits from Poiseuille’s Law, indicates that \( K_x \) is related to leaf vein anatomy. Several previous studies observed that the species with high VLA tends to increase \( K_{ox} \) due to the shorter water transport pathway from veins to stoma (Sack & Frole, 2006; Brodribb et al., 2007; Buckley et al., 2015). In the rice leaves the stoma clustered around leaf veins (Fig. S7), which indicates that although the distance between veins varied across genotypes the water transport pathways are relatively consistent. \( K_{\text{leaf}} \) but also anatomical traits like stomatal density and size are often shown to determine stomatal conductance \( (g_s) \) (Ocheltree et al., 2012). However, in the present study, stomatal anatomical traits showed no relationship with \( g_s \) across Oryza genotypes (Fig. S5), contrary to previous reports suggesting that differences of \( g_s \) across genotypes are related to the variation of stomatal anatomical traits under drought conditions (Xu & Zhou, 2008). Although a wide variation of stomatal size and density were found across the selected genotypes, the \( g_{\text{max}} \) value calculated from stomatal anatomy showed a narrow variation across genotypes (from 0.83 to 1.07 mol m\(^{-2}\) s\(^{-1}\)). Our result suggests that Oryza genotypes used in this study have a similar potential maximum \( g_s \) and that the differences of \( g_s \) between genotypes might be determined by regulatory mechanisms and/or leaf water status. Indeed, \( g_s \) was found to be significantly correlated to \( K_{\text{leaf}} \) (Xiong et al., 2015d) but not to either of its two components, \( K_x \) or \( K_{ox} \) (Fig. S4).

In summary, across the selected Oryza genotypes, \( g_s \) is mostly dependent on \( K_{\text{leaf}} \) but not on stomatal features that determine maximum theoretical \( g_s \), and \( K_{\text{leaf}} \) in turn is limited more by \( K_{ox} \) than by \( K_x \), although the contributions of \( K_x \) and \( K_{ox} \) varied widely across genotypes (Cochard et al., 2004; North et al., 2013). In addition, the relationship between \( g_s \) and \( g_m \) pooling all genotypes, although significant, was relatively scattered. The ratio \( g_{\text{max}}/g_s \), which is important in leaf water-use efficiency (Flexas et al., 2013a; Buckley & Warren, 2014), was highly variable, ranging from 0.48 in Wcr to 1.47 in Rhi. Altogether, these characteristics make this collection of Oryza genotypes suitable for studying the relationships between \( K_{\text{leaf}} \) and \( g_m \) without a strong interference of indirect relationships caused by the interdependency of \( g_s \) and \( g_m \). Indeed, \( g_m \) was strongly correlated with \( K_{ox} \) (Fig. 5), whereas \( g_s \) was not (Fig. 2).

The tight relationship between \( g_{\text{max}} \) and \( K_{ox} \) suggested that water and CO\(_2\) share a significant fraction of their respective flow pathways inside leaves, so they are also expected to share some anatomical determinants – mostly likely involving the mesophyll and intercellular air spaces (Flexas et al., 2013a,b). In the present study, across all genotypes, \( K_{ox} \) increased with volume fraction of intercellular air space (\( f_{\text{IAS}} \)) (Fig. 6b). This is consistent with simulations by Buckley et al. (2015), who attributed the role of \( f_{\text{IAS}} \) to the contribution of vapor transport to \( K_{ox} \). The possibility that vapor diffusion may contribute substantially to water transport within leaf was also suggested by Rockwell et al. (2014) and Buckley (2015). This suggests that gas-phase transport may play an important role in outside-xylem water transport and that the liquid water evaporates deep within the leaf and/or along the transport pathway. In this study, \( g_{\text{max}} \) also was correlated with \( f_{\text{IAS}} \), contrary to observations in nongrass species (Tomás et al., 2013). \( f_{\text{IAS}} \) can affect \( g_m \) in two ways: by increasing gas-phase CO\(_2\) diffusion, and by increasing the number of parallel diffusion pathways from outer surfaces of cell walls to chloroplasts (intercellular air
space per unit leaf area (S_m) and mesophyll cell surface area occupied by chloroplasts exposed to intercellular air space per leaf area (S_c)). Although it is likely that gaseous diffusion plays a role in g_m variation across genotypes, it is not the major factor because the diffusion of CO₂ in the gaseous phase is 10⁴ times faster than that in the liquid phase (Evans et al., 2009). Thus, the tight correlation between fIAS and g_m would be by increasing parallel diffusion pathways, which was also supported by the correlation between fIAS and S_m (Fig. S8).

The apoplastic transport pathway, which should be closely related to cell wall thickness, has been suggested as the major water transport pathway outside xylem. For instance, Buckley et al. (2015) found that K_ox was increased by 370% when cell wall thicknesses used in simulations were increased five-fold. By contrast, we found that K_ox decreased with cell wall thickness across the selected Oryza genotypes. However, in the present study, the cell wall thickness only ranged from 0.15 to 0.19 µm across genotypes, whereas K_ox varied over three-fold across the same range, which suggests that the observed negative correlation between cell wall thickness and K_leaf might be due to the influence of other traits. For example, cell wall thickness significantly decreased with increasing nitrogen (N) supplements in rice.

Fig. 6 Effects of (a, b) fraction of leaf mesophyll volume occupied by intercellular air space (fIAS), (c, d) mesophyll cell wall thickness (T_cell-wall), (e, f) mesophyll cell surface area exposed to intercellular air space per leaf area (S_m) and (g, h) mesophyll cell surface area occupied by chloroplasts exposed to intercellular air space per leaf area (S_c) on mesophyll conductance to CO₂ (g_m) and leaf hydraulic conductance (K_leaf) in Oryza. Values are mean ± SD (n = 3–9 plants).

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Author contributions

D.X. planned and designed the research; D.X. and T.Y. performed the experiments; D.X. and J.F. analyzed the data; D.X., J.F., J.H. and S.P. wrote the manuscript.

References


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**Supporting Information**

Additional Supporting Information may be found online in the Supporting Information tab for this article:

**Fig. S1** The vein size at the tip and at the middle of *Wcr* leaf.

**Fig. S2** Diagram illustrating details of leaf anatomy (*Shanyou 63*).

**Fig. S3** Diagram illustrating details of stomatal features (*Shanyou 63*).

**Fig. S4** The relationships between: stomatal conductance to CO₂ (*gₛ*) and leaf hydraulic conductance inside xylem (*Kₓ*); *gₛ* and leaf hydraulic conductance outside xylem (*Kₒx*); maximum theoretical stomatal conductance to CO₂ (*gₛₘₐₓ*) and *Kₓ* and (d) *gₛₘₐₓ* and *Kₒx*.

**Fig. S5** Effects of stomatal size and stomatal density of abaxial and adaxial lamina on stomatal conductance to CO₂ (*gₛ*) across the *Oryza* genotypes.

**Fig. S6** Effects of stomatal size and stomatal density of abaxial and adaxial lamina on leaf hydraulic conductance (*Kₗₑᵃ🇫*) across the *Oryza* genotypes.

**Fig. S7** The distribution of stoma on adaxial lamina in two genotypes.

**Fig. S8** Relationship between mesophyll cell wall surface area exposed to intercellular air space per leaf area (*Sₘ*) and fraction of leaf mesophyll volume occupied by intercellular air space (*fᵢ₃₄₈*) in rice.

**Fig. S9** Relationship between mesophyll cell wall surface area exposed to intercellular air space per leaf area (*Sₘ*) and mesophyll cell surface area occupied by chloroplasts exposed to intercellular air space per leaf area (*Sₜ*) in rice, transmission electron microscope image of Aus and I08.

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