RESEARCH PAPER

Stomatal conductance, mesophyll conductance, and transpiration efficiency in relation to leaf anatomy in rice and wheat genotypes under drought

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Abstract

Increasing leaf transpiration efficiency (TE) may provide leads for growing rice like dryland cereals such as wheat (Triticum aestivum). To explore avenues for improving TE in rice, variations in stomatal conductance (gs) and mesophyll conductance (gm) and their anatomical determinants were evaluated in two cultivars from each of lowland, aerobic, and upland groups of Oryza sativa, one cultivar of O. glaberrima, and two cultivars of T. aestivum, under three water regimes. The TE of upland rice, O. glaberrima, and wheat was more responsive to the gm/gs ratio than that of lowland and aerobic rice. Overall, the explanatory power of the particular anatomical trait varied among species. Low stomatal density mostly explained the low gs in drought-tolerant rice, whereas rice genotypes with smaller stomata generally responded more strongly to drought. Compared with rice, wheat had a higher gm, which was associated with thicker mesophyll tissue, mesophyll and chloroplasts more exposed to intercellular spaces, and thinner cell walls. Upland rice, O. glaberrima, and wheat cultivars minimized the decrease in gm under drought by maintaining high ratios of chloroplasts to exposed mesophyll cell walls. Rice TE could be improved by increasing the gm/gs ratio via modifying anatomical traits.

Key words: Drought, leaf anatomy, mesophyll conductance, rice, stomatal conductance, transpiration efficiency, wheat.

Introduction

Rice (Oryza sativa L.) is a major staple crop that adapts strongly to fully inundated conditions. Compared with other cereal crops, such as wheat (Triticum aestivum L.) grown on dry uplands, rice cultivation requires massive amounts of fresh water for irrigation. To minimize the total water requirement for rice cultivation, several water-saving regimes have already been introduced, such as alternate wetting and drying irrigation (Bouman and Tuong, 2001; Zhang et al., 2008) and controlled soil drying during grain filling (Yang et al., 2003; Yang and Zhang, 2006). However, these regimes are largely management measures to reduce water use for rice production. Solving the problem of the intrinsically high water requirement of rice would need breeding or genetic engineering approaches to develop new drought-tolerant rice genotypes with increased water use efficiency (WUE).

Genetic diversity in drought tolerance has long been explored to develop cultivars of O. sativa for diverse growing conditions: irrigated lowland, rain-fed lowland, and upland
environments. More recently, genotypes suitable for aerobic environments (moderately dry conditions, not inundated lowland or dry upland conditions), i.e. aerobic rice (Bouman et al., 2005; Singh et al., 2008), have been developed. However, aerobic rice cannot replace lowland (‘paddy’) rice in most of the rice-growing areas because of its lower grain yield and is an option only for farmers in rain-fed lowlands with limited or erratic rainfall (Atlin et al., 2006). In addition, African rice (Oryza glaberrima Steud.) has evolved as a cultivated species in parallel with O. sativa, and is more drought-resistant (Sarla and Swamy, 2005). Natural interspecific hybrids between O. sativa and O. glaberrima are cultivated in West Africa (Nuijten et al., 2009). The adaptation of rice genotypes to a wider range of edaphic conditions indicates the possibility of growing rice in the same way as other dryland cereal crops such as wheat. Also belonging to the C₄-crop type, wheat has a relatively higher WUE than rice (Kemanian et al., 2005; Haefele et al., 2009). Compared with rice, wheat shows higher plasticity in root morphological and anatomical adaptation to water-deficit conditions (Kadam et al., 2015). Further comparative analysis of the physiology and anatomy of rice and wheat in response to drought will facilitate the identification of the general traits and mechanisms required for breeding drought-tolerant yet high-yielding rice varieties (Praba et al., 2009).

Photosynthesis is the key process of primary metabolism, and its capacity can influence plant performance and productivity (Lawlor and Tezara, 2009; Pinheiro and Chaves, 2011). Photosynthesis under drought, despite being affected by Rubisco velocity, is often limited by the CO₂ concentration at carboxylation sites (C₅) inside the chloroplast, which is determined by CO₂ diffusion components, i.e. stomatal conductance (gₛ) and mesophyll conductance (gₘ) (Evans et al., 2009). Stomata regulate CO₂ diffusion into, and water diffusion out of, plant leaves (Chaves et al., 2002). Under water-deficit conditions, plants close stomata to prevent major water loss; this, consequently, reduces photosynthesis via decreased influx of CO₂ (Pinheiro and Chaves, 2011). In the long-term response to water deficit, gₛ can be influenced by leaf anatomical traits such as stomatal density and size, which can vary to accommodate to the environment (Xu and Zhou, 2008; Franks and Beerling, 2009). As the inevitable consequence of CO₂ entry through leaf stomata is water loss through transpiration, the stomata-related environmental adaptation may also affect plant instantaneous transpiration efficiency (TE), i.e. the ratio of net photosynthesis rate (Aₙ) to transpiration rate. In general, higher gₛ results in a lower TE (Flexas et al., 2008; Gu et al., 2012).

Mesophyll conductance (gₘ) has been viewed as the diffusion of CO₂ from sub-stomatal cavities to the sites of carboxylation in the chloroplasts (Flexas et al., 2008). In contrast to gₛ, increasing gₘ increases Aₙ at no cost of increased transpiration, because the CO₂ diffusion pathway involving gₘ is not shared with the diffusion pathway of transpired H₂O. Therefore, increasing gₘ not only increases Aₙ but also increases TE (Barbour et al., 2010; Flexas et al., 2013). However, long-term drought stress reduces gₘ (Scartazzia et al., 1998; Gu et al., 2012; Han et al., 2016). Along the CO₂ diffusion pathway inside leaves, the conductance through the liquid phase (gₙₐₙ) is the most limiting factor for CO₂ diffusion in the mesophyll in many species (Gorton et al., 2003; Flexas et al., 2008; Flexas et al., 2012). Growing evidence indicates that (i) genotypic differences in gₘ exist within a given species (Evans and Vellen, 1996; Gu et al., 2012; Jahan et al., 2014), and (ii) leaf mesophyll structure and anatomical properties are important determinants of gₘ. Mesophyll thickness (Tₘ), surface area of chloroplasts exposed to the intercellular airspace (Sₘ), and mesophyll cell wall thickness (Tₚ) are suggested to be the most important structural components determining gₘ (Evans et al., 1994; Evans et al., 2009; Scafaro et al., 2011; Tosens et al., 2012b; Tomás et al., 2013), as these parameters determine pathways for CO₂ permeation into chloroplasts (Terashima et al., 2011). Therefore, investigating relationships between leaf anatomy and photosynthetic features could improve understanding of leaf structural features required to enhance drought tolerance in rice.

In this study, the photosynthetic diffusion components (gₛ and gₘ) and possibly related leaf anatomical properties were examined in three species, O. sativa, O. glaberrima and T. aestivum. gₛ was measured from gas exchange, and combined gas exchange and chlorophyll fluorescence measurements were used to estimate gₘ. Leaf anatomical properties were examined through light and transmission electron microscopy. The objectives of this study were: (i) to evaluate the variation in photosynthetic capacity among the contrasting types of rice species as well as between rice and wheat in response to a long-term drought, and (ii) to assess the leaf anatomical determinants of gₛ and gₘ and how those determinants underpin genotypic differences in the response of gₛ, gₘ, and TE under drought.

Materials and methods

Plant materials and treatments

Six cultivars from O. sativa, one cultivar from O. glaberrima, and two cultivars from T. aestivum were used for the study of photosynthetic and leaf anatomical properties. The O. sativa cultivars represented rice types bred for lowland, aerobic or upland cultivation systems, respectively, whereas wheat cultivars were selected based on their drought tolerance (Table 1). The tolerance and susceptibility classifications were based on previous reports (Thanh et al., 1999; George et al., 2002; Sarla and Swamy, 2005; Atlin et al., 2006; Liu et al., 2006; Praba et al., 2009). Seeds of rice and wheat cultivars were obtained from the International Rice Research Institute (IRRI) and from the International Maize and Wheat Improvement Center (CIMMYT), respectively.

Pot experiments were conducted at Yangzhou University, Jiangsu Province, China (32°30′ N, 119°25′ E). Rice seeds were sown in saturated soil after pre-germination, and wheat seeds were sown directly in moist soil. The pots were placed in an open field sheltered from rain by a mobile transparent polyethylene shelter. Each pot (30 cm in height, 25 cm in diameter, 14.72 liters in volume) contained 20 kg of sandy loam soil from the Yangzhou University rice/wheat rotated experimental field. One day before sowing, 1.1 g CO(NH₄)₂ (urea) and 0.5 g KH₂PO₄ were pre-mixed through the soil per pot. Extra nitrogen (2.5 g urea for rice and 1.5 g urea for wheat per pot) were split-applied during different plant growth stages.

We imposed three levels of soil moisture, i.e. control (CT), mild drought stress (MD), and more severe drought stress (SD). Across all species and treatments, three replications were maintained and pots
were placed in a randomized design. Soil water potential was monitored by inserting a tension meter (Institute of Soil Sciences, Chinese Academy of Sciences, Nanjing, China) at 15 cm soil depth throughout the experiments. Because rice and wheat have different growth durations and are naturally adapted to different moisture environments during domestication histories (Praba et al. 2009), different timings and intensity of stress impositions were applied (Table 2). When tension-meter readings reached the lower limit in each stress level, tap water was added until the upper limit of the target stress was reached. Once stress was imposed, the target stress levels were maintained to ensure that leaves for measurements (see below) were initiated and developed under stress, until all the measurements were completed.

### Gas exchange and chlorophyll fluorescence measurements

An open gas exchange system integrated with a fluorescence chamber head (LI-COR 6400XT; LI-COR Inc., Lincoln, NE, USA) was used to simultaneously measure gas exchange (GE) and chlorophyll fluorescence (CF) parameters. During flowering, the penultimate leaf on the main shoot from each replication per treatment was used for measurements. To avoid the effect of fluctuation in outdoor environment on GE measurement, all measurements were taken in a climate chamber with air temperature at 25°C (23°C for wheat), 65% relative humidity and a photosynthetic photon flux density at the leaf surface of 1200 μmol m⁻² s⁻¹ (artificial light source). All measurements were made at a leaf temperature of 25°C and the leaf-to-air vapor pressure difference (VPD) was kept between 1.0 and 1.6 kPa. Light and CO₂ response curves were measured under both ambient (21%) and low (2%) O₂ conditions. The low O₂ condition was created by using a gas mixture of 2% O₂ and 98% N₂, and the infrared gas analyser calibration was adjusted for O₂ composition of the gas mixture according to the manufacturer’s instructions.

For the CO₂ response curve, under both O₂ conditions, the leaf was consecutively exposed to an incident irradiance (Iinc) of 1000 μmol m⁻² s⁻¹ with different levels of CO₂: 50, 90, 150, 250, 400, 700, 1000, 1500 μmol mol⁻¹. For the light response curve, under the 21% O₂ condition, the CO₂ was kept constant at 400 μmol mol⁻¹ and Iinc was increased in the order of 30, 50, 80, 120, 200, 500, 1000, and 1600 μmol m⁻² s⁻¹. Under 2% O₂, in order to ensure non-photorespiratory conditions, the CO₂ was kept constant at 1000 μmol mol⁻¹ with Iinc levels of 30, 50, 80, 120, and 200 μmol m⁻² s⁻¹. The measurement flow rate was 400 μmol s⁻¹. CO₂ exchange rates were corrected for CO₂ leakage into and out of the leaf cuvette, based on measurements using the same flow rate on boiled leaves across a range of CO₂ levels, and intercellular CO₂ levels (Cᵢ) were then recalculated (Flexas et al. 2007).

The steady-state fluorescence (Fₛ) was measured at each light or CO₂ step. Then a multiphase flash method (Loriaux et al., 2013) was applied to determine Fₘ (the maximum fluorescence during the saturating light pulse). The apparent photosystem II electron (e⁻) transport efficiency for each irradiance or CO₂ step was calculated as Φₑ= (Fₛ−F₀)/Fₘ (Genty et al., 1989).

### Estimation of diffusion components

Total conductance to CO₂ (gᵣₗ) was calculated based on stomatal and mesophyll conductance according to 1/Φₑ=1/gᵣₗ+1/gₘ. For the analysis, stomatal conductance for CO₂ (gᵣₗ) was obtained as stomatal conductance for water vapour divided by 1.6, from the data points measured under ambient condition (400 μmol mol⁻¹ CO₂, 1000–1500 μmol m⁻² s⁻¹ irradiance, and 25°C) of the light response curve under 21% O₂.

In order to compare gᵣₗ across species, genotypes, and treatments, the value of gᵣₗ assumed as constant was estimated using the NRH-A variant method (Yin and Struik, 2009):

\[
Aₐ = 0.5J/4 − Rₚ + gᵣₗ(C_i + 2Γ) \left( J/4 − Rₚ + gᵣₗ(C_i + 2Γ) \right) − \left( J/4 − Rₚ + gᵣₗ(C_i + 2Γ) \right) − \left( J/4 − Rₚ + gᵣₗ(C_i + 2Γ) \right)
\]

\[(1)\]

where 1⁻ is the CO₂ compensation point in the absence of day respiration (Rₚ) and J is the linear electron transport rate used for CO₂ transport efficiency for each irradiance or CO₂ step was calculated as Φₑ= (Fₛ−F₀)/Fₘ (Genty et al., 1989).
fixation and photorespiration. Equation (1) provides a model to estimate $S_{\text{g}}$ by a curve fitting that minimizes the difference between measured and estimated $A_{\text{n}}$ (Yin and Struik, 2009). In the model, $C$ is calculated from the Rubisco specificity factor ($S_{\text{inc}}$) as $C = 0.5S_{\text{inc}}$, where $O$ is O$_2$ partial pressure (mbar). $S_{\text{inc}}$ at a given temperature is expected to be constant for a specific species; however, the value at 25°C did not differ much between rice and wheat (Makino et al., 1988; Yin et al., 2009; Gu et al., 2012), so a single value for $S_{\text{inc}}$ at 25°C (3.13 mbar µbar$^{-1}$) was adopted from Makino et al. (1988) and Yin et al. (2009). Data obtained from low $I_{\text{inc}}$ levels of the light response curve and high $C$ levels of the CO$_2$ response curve measured at 21% O$_2$ were used for the curve fitting. Model inputs ($J$ and $R_d$) were estimated as described by Yin et al. (2009). In brief, using data of light-limited range under non-photorespiratory conditions (i.e. the light response curve under 2% O$_2$ with 1000 µmol mol$^{-1}$ CO$_2$ plus points from >500 µmol mol$^{-1}$ CO$_2$ response curve under 2% O$_2$), a linear regression can be performed for the observed $A_{\text{n}}$ against $I_{\text{inc}}D/J4$. The day respiration ($R_d$) is the intercept of the linear regression, and the slope of the regression yields the estimated lumped parameter $s$ (Yin et al., 2009). Then $J$ at each $C$ can be calculated using $J = sI_{\text{inc}}D_4$.

### Light and transmission electron microscopy

Stomatal density and size were determined using the silicon rubber impression technique (Smith et al., 1989). Stomatal features from this method did not differ from those based on glancing sections cut from fresh leaf sample. The method and impression material are described by Giday et al. (2013). Imprints were taken from both adaxial and abaxial surfaces of the area where GE and CF were measured, and were later on smeared with nail polish in the mid-area between the central vein and the leaf edge, for approximately 20 min. The thin film was carefully peeled off the imprint with no stretching and mounted on a glass slide with a drop of water to improve contrast. Stomatal density ($D$) was determined by counting the maximum numbers of stomata from ten randomly selected non-overlapping rectangular fields of view per leaf under the light microscope (Axio Imager D2, Carl Zeiss, Germany). Stomatal size ($S$) was calculated by multiplying guard cell length ($L'$) by width ($W$).

All parameters were determined as the average of both sides of the leaf. Specific stomatal conductance ($S_{\text{g}}$) was determined by dividing $g_{\text{s}}$ by $D$ (Ohsumi et al., 2007). An integrative parameter stomatal area index (SAI) was calculated by multiplying density by size and expressed in mm$^2$ stomata mm$^{-2}$ leaf. Another integrative parameter, so-called maximum stomatal diffusive conductance ($S_{\text{max}}$), was also calculated, according to Franks and Beeling (2009):

\[
S_{\text{max}} = \frac{d - D - a_{\text{max}}}{1.69} \left( \frac{l + \pi}{2\sqrt{d_{\text{max}}/l}} \right)
\]  

where $d$ is the diffusivity of water vapour in air, $v$ is the molar volume of air, $a_{\text{max}}$ is the maximum area of the open stomatal pore, and $l$ is the stomatal pore depth for fully open stomata. The values for standard gas constants $d$ and $v$ at 25°C are $2.82 \times 10^{-5}$ m$^2$ s$^{-1}$ and 24.5 m$^3$ mol$^{-1}$, respectively, $a_{\text{max}}$ was calculated as $\pi(p/2)^2$, where $p$ is the stomatal pore length, $p$ was calculated as $L'/2$ according to Franks and Beeling (2009), $l$ was taken as equal to $W/2$, assuming guard cells inflate to circular cross section.

For the anatomical measurement of mesophyll cells, leaflet samples were collected from the leaves where GE and CF were measured. Half of the leaves were fixed and further used for anatomical study. Tissue fixation and preparation for light and transmission electron microscopy (TEM) studies followed the method of Scafaro et al. (2011) with small modification. Small leaf samples ($4 \times 1.5$ mm$^2$) from each cultivar×water treatment combination were cut parallel to the main vein avoiding large veins. The samples were infiltrated in a solution of 3% glutaraldehyde, 2% paraformaldehyde, and 0.1 M phosphate buffer (pH 7.2) for at least 48 h. Samples were further post-fixed in 1% osmium tetroxide for 2 h, followed by dehydration in an ethanol series (30, 50, 70, 80, 90, 95, and 100%). Ethanol was replaced by 1,2-epoxypropane, and the samples were further embeded with Spurr’s resin (London Resin Company, London, UK) and cured in an oven at 70°C for 12 h. Transverse sections for light and TEM studies were cut using an ultra-micromtome (Ultra-cut R, Leica, Germany). Leaf cross-sections 1 µm thick were cut for the light microscopy study, and sections 70 nm thick were cut for the TEM study. Light sections were stained with safranin (1%) for 20 s and followed with methyl purple (1%) for 20 s, then viewed and photographed under a light microscope (Axio Imager D2, Carl Zeiss, Germany). Electron sections were doubly stained with uranyl acetate for 30 min and lead citrate for 15 min, and then photographed under a transmission electron microscope (Technai 12, Philips, The Netherlands).

Analyses of images were performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA) (Abramoff et al., 2004). All mesophyll anatomical characteristics were determined in multiple sections, with 12–30 complete mesophyll cells per replication per treatment depending on the image quality. The analysis protocol followed the method of Evans et al. (1994). Mesophyll thickness ($L_m$) was measured as the length between the two epidermises of the leaf from light microscopy images. The surface area of mesophyll cells exposed to the intercellular airspace per leaf area ($S_m$) was calculated from light microscopy images as:

\[
S_m = \frac{L_m}{w} F
\]  

where $w$ is the width of the section measured, $L_m$ is the length of mesophyll exposed to the intercellular airspace, and $F$ is the curvature correction factor (Thain, 1983; Evans et al., 1994). Although the mesophyll size differed between rice and wheat, mesophyll cells in both species are presented as lobed spheroids with a different orientation of the long axes to the veins (Chonan, 1970, 1972; Sage and Sage, 2009), and thus a difference of curvature correction factors between species was not taken into account in this study, and we adopted the value 1.55 from a previous rice study (Scafaro et al., 2011). Uncertainties were also analysed given that Barbour et al. (2016) recently assumed a value of 1.25 for wheat.

The chloroplast surface area exposed to the intercellular airspace per leaf area ($S_c$) was calculated as:

\[
S_c = \frac{L'}{L_m} S_m
\]  

where $L'$ is the length of chloroplast exposed to the intercellular airspace and $L_m$ is the corresponding length of mesophyll exposed to the intercellular airspace. $L_m$ and $L'_m$ were determined from TEM images based on average values measured for 12–30 mesophyll cells (parameters obtained from TEM are marked by a prime (′) to distinguish them from $L_m$ measured from light microscopy images). The ratio of the exposed surface area of chloroplast to the exposed surface area of mesophyll cell walls ($S_c/S_m$) was set equal to $L'/L_m$ (Tosens et al., 2012a,b).

In addition, thickness of the mesophyll cell wall ($T_m$) was also determined from TEM images. Of the thickness values measured for different sections of each mesophyll cell, only the lower half of the values were averaged as the $T_m$ of that cell to minimize the artefact of inaccurate orientation of ultra-micromtome sectioning. The average value of $T_m$ from all mesophyll cells in each cultivar and treatment was used for further analysis.

### N content measurements

The other half of the collected leaflets after GE and CF measurements were used to measure the leaf area and then oven dried at 70°C to constant weight. Leaflets were weighed to determine leaf mass per unit area (LMA), and then ground to fine powder. Samples were

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analysed for N content by using an elementary analyser (Vario Macro cube, Elementar, Germany). Leaf nitrogen per unit area (Na) was calculated from these data as a measure of leaf physiological status.

**Statistical analyses**

A one-way analysis of variance (ANOVA) was used to reveal the differences between cultivars in the studied characteristics. A two-way ANOVA was used to study the effect of genotypes, drought treatment, and their interaction on photosynthetic and anatomical parameters. Linear regression analyses were also conducted. These analyses were performed using the R programming language (http://www.R-project.org/). Non-linear fitting for Eq. (1) was carried out using the GAUSS method in PROC NLIN of SAS (SAS Institute Inc., Cary, NC, USA).

**Results**

**Variation in photosynthesis**

The net photosynthesis rate (An) varied almost threefold among species, with *T. aestivum* having a significantly higher An than *O. sativa* and *O. glaberrima* (cv. CG14) (Fig. 1A). Under the CT condition, An varied between 6.9 µmol m⁻² s⁻¹ for CG14 and 22.7 µmol m⁻² s⁻¹ for S82. Overall, drought decreased An strongly with no significant interaction between cultivars and water treatments (Supplementary Table S1 at JXB online). However, for CG14, drought increased An (Fig. 1A), and this was probably associated with an increase in Na by drought in this cultivar (Supplementary Fig. S1A). Across all cultivars and treatments, An was positively correlated with Na among wheat cultivars, but not among cultivars of *O. sativa* (Supplementary Fig. S1B).

**Variation in gs and gm**

Significant variation in stomatal conductance (gs) was observed both among species and within *O. sativa* (Supplementary Table S1). Notably, lowland rice and wheat cultivars had a much higher gs than other cultivars (Fig. 1B). gs decreased under drought in all cultivars, with mild interaction between cultivars and water treatments (0.05<P<0.1). The decrease in gs was stronger in IR64, Sal, CG14, and S82 than in other cultivars (Fig. 1B). Mesophyll conductance (gm) varied significantly among species, with wheat having a higher gm than *O. sativa*, and CG14 having the lowest value (Fig. 1C). In wheat, the drought effect on gm was only significant in We4 under SD condition (28% reduction). The differences in gm among *O. sativa* cultivars increased with an increase in stress level (P>0.05 under CT, P<0.05 under MD, and P<0.01 under SD). Only lowland rice cultivars had significant decreases in gm under the MD condition. Compared with gm in CT, a significant decrease in gm by SD was observed in lowland rice and aerobic rice cv. Apo, but not in other cultivars. The lowest decrease in gm was observed in Sal and CG14 (ca 14%), and the highest decrease was recorded in IR64 (59%).

![Fig. 1. Photosynthetic parameters obtained and estimated from GE and CF data under three treatments: control (CT), mild drought (MD), and more severe drought (SD). Values of (A) net photosynthetic rate (An), (B) stomatal conductance (gs), and (D) transpiration efficiency (TE) were obtained under the condition of 400 µmol mol⁻¹ CO₂, 1000–1500 µmol m⁻² s⁻¹ irradiance, and 25 °C. (C) Mesophyll conductance (gm) was calculated based on the NRH-A method (Yin and Struik, 2009). Bars represent standard errors of the mean for An, gs, and TE, and standard error of the estimate for gm.](https://academic.oup.com/jxb/article-abstract/68/18/5191/4158541)
Influences of $g_s$ and $g_m$ on $A_n$ and transpiration efficiency

Significant positive correlations between $A_n$ and total conductance ($g_{tot}$) were observed among species and within each group (Fig. 2A), indicating the presence of major diffusion limitations to CO$_2$ assimilation. Different individual effects of $g_s$ and $g_m$ on $A_n$ were observed (Fig. 2B, C). The correlation between $A_n$ and $g_s$ was highly significant in lowland rice, in the wheat group, and for all data combined ($P<0.001$), and weaker in aerobic and upland rice ($P<0.05$) and in O. glaberrima ($P>0.05$) (Fig. 2B). On the other hand, the correlation between $A_n$ and $g_m$ was highly significant for all data combined as well as in most of the subgroups (Fig. 2C). Multiple regression analysis indicated that $g_s$ contributed more to the variation in $A_n$ in lowland rice cultivars, whereas $g_m$ contributed more to the variation in $A_n$ in aerobic rice, upland rice, and wheat cultivars (Supplementary Table S2).

Aerobic rice, upland rice, and wheat cultivars had higher TE than lowland rice cultivars (Fig. 1D). Under the CT condition, TE varied between 2.09 µmol CO$_2$ (mmol H$_2$O)$^{-1}$ for IR64 and 4.02 µmol CO$_2$ (mmol H$_2$O)$^{-1}$ for Sal. TE increased under drought, and the highest increase in TE was recorded for CG14 under the SD condition, with a 70% increase over the CT condition. A weak negative correlation between TE and $g_s$ was observed within each genotype group (Supplementary Fig. S2A), but the correlation between TE and $g_m$ was not significant in most groups (Supplementary Fig. S2B). However, TE was well explained by combined $g_s$ and $g_m$ (Supplementary Table S3) and was actually highly correlated to the $g_m/g_s$ ratio (Fig. 2D), especially in upland rice, CG14, and wheat cultivars, although this relationship was less clear in lowland and aerobic rice cultivars. Both the $g_m/g_s$ and the TE of aerobic rice, upland rice, and wheat cultivars were higher than those of lowland rice cultivars.

Anatomical determinants of $g_s$

Wheat cultivars had significantly lower density and larger stomata than rice cultivars (Fig. 3 and Table 3). Stomatal density ($D$) varied significantly among rice cultivars, with lowland rice having higher $D$ than aerobic and upland rice, and CG14 having the lowest value. In rice, $D$ on the adaxial side ($D_{adaxial}$) was lower than that on the abaxial side ($D_{abaxial}$), and $D_{adaxial}$ varied significantly among cultivars, whereas less variation was observed for $D_{abaxial}$ (Table 3). Interestingly, the drought effect was significant only for $D_{adaxial}$ but not for $D_{abaxial}$ in...
both rice and wheat (Supplementary Table S1). Drought increased $D_{adaxial}$ in most cultivars (Supplementary Fig. S3A).

Aerobic rice cultivars had clearly larger stomatal size ($S$) than other rice cultivars (Table 3). This high $S$ resulted more from wider stomatal width ($W$) than from stomatal length ($L$) (data not shown). Drought decreased $S$, and the effect was similar on both leaf surfaces in all cultivars (Supplementary Fig. S3C, D). Cultivars CG14 and S82 had the strongest decrease in $S$ in rice and wheat, respectively.

The sum of the stomatal area index on both sides of the leaf (SAIs) differed among the three species, with CG14 having the lowest SAIs and lowland, aerobic rice having higher SAIs than the other cultivars. The variation in SAIs within rice species was more associated with $D$ ($R^2=0.58$) than with $S$ ($R^2=0.33$). SAIs decreased under drought in most cultivars, except in 148, CG14, and We4 (Fig. 4A). Specific stomatal conductance ($sg_s$) was much higher in wheat than in rice cultivars, and higher in lowland rice than in other rice cultivars under the CT condition (Fig. 4B). Drought decreased $sg_s$ in all cultivars, with IR64, CG14, and S82 showing the highest decrease among rice and wheat cultivars, respectively.

The variation in stomatal properties can partially explain the variation in $g_s$ among species and within species. $g_s$ was partially correlated with $S$ or $D$, depending on genotype groups (Supplementary Fig. S4). A significant positive correlation was found between $g_s$ and SAIs for lowland and upland *O. sativa* as well as for wheat genotypes (Fig. 5A). The theoretical maximum stomatal conductance ($g_{max}$) was higher than the measured $g_s$ in all cultivars and treatments, and was higher in rice than in wheat (Fig. 5B). The correlation between $g_s$ and $g_{max}$ was significant only for *O. sativa*, and not in wheat and each genotype group (Fig. 5B).

### Anatomical determinants of $g_m$

Estimation of the transverse sections of leaves by light microscopy revealed a similar general leaf structure among the three species (Fig. 6 left panels). Significant variations in some anatomical traits were observed among species and among cultivars within the *O. sativa* species (Table 3). Wheat mesophyll tissue was about twice as thick as that of *O. sativa* cultivars and almost three times as thick as that of CG14 (Fig. 6). No strong drought effect was observed on $T_m$ (Supplementary Table S1). The variation observed in the surface area of mesophyll cells exposed to the intercellular airspace per leaf area ($S_m$) was mainly between rice and wheat (Table 3). The variation observed in the surface area of mesophyll cells exposed to the intercellular airspace per leaf area ($S_m$) was mainly between rice and wheat (Table 3), with wheat having a higher $S_m$ than all rice cultivars even when the curvature factor 1.25 was used for wheat (Supplementary Fig. S5B). $S_m$ was significantly increased in wheat cultivars under drought ($P<0.05$), whereas no clear trend was observed in rice cultivars (Supplementary Fig. S5B).

In both rice and wheat, more than 60% of the exposed mesophyll cell surface area was covered by chloroplasts ($S_c$) (Table 3). Wheat cultivars and upland rice cultivar Sal had the highest $S_c/S_m$ ratio, and CG14 had the lowest. A significant decrease in $S_c/S_m$ was observed in IR64 and aerobic rice cultivars, whereas an increase was recorded in CG14 under the SD condition (Supplementary Fig. S5C). $S_c$ varied significantly among species and among rice cultivars, with Sal having a higher $S_c$ value than other rice cultivars (Table 3). Wheat cultivars showed a clear increase in $S_c$ under drought, whereas no clear $S_c$ response was observed in rice cultivars (Supplementary Fig. S5D). A thinner
Mesophyll thicknesses ($T_m$), mesophyll surface area exposed to intercellular airspace ($S_m$), and stomatal size ($S$) and density ($D$) were determined from light microscope images. The ratio of the exposed chloroplast surface area to the exposed surface area of mesophyll cell walls ($S_c/S_m$) and mesophyll cell wall thickness ($T_w$) were determined from transmission electron microscope images. Chloroplast surface area exposed to intercellular airspace ($S_c$) was obtained by multiplying $S_m$ by $L'/L'_m$. Data are means±standard error of three replicates. The significance of differences between cultivars was based on a one-way analysis of variance. The significance level of overall data/within $O$. sativa is shown by asterisks: *$P<0.05$, **$P<0.01$, ***$P<0.001$.

### Table 3. Leaf anatomical properties of $O$. sativa, $O$. glaberrima, and $T$. aestivum under the control (CT) condition

| Species     | Genotype group | Cultivar | Mesophyll | Stomata |
|-------------|----------------|---------|-----------|---------|
|             |                |         | $T_m$ (µm) | $S_m$ (m²) | $S_c/S_m$ | $S_c$ (m²) | $T_w$ (nm) | $S$ (µm²) | $D$ (No. mm⁻²) |
| $O$. sativa | Lowland rice   | IR64    | 70.9 ± 4.2 | 11.2 ± 0.2 | 0.77 ± 0.01 | 8.7 ± 0.3 | 126 ± 1 | 172 ± 2.1 | 186 ± 2.5 | 621.0 ± 7.6 | 732.7 ± 15.1 |
|             | II             |         | 91.6 ± 0.6 | 10.8 ± 0.2 | 0.71 ± 0.02 | 7.6 ± 0.1 | 151 ± 2 | 194 ± 10.3 | 203.5 ± 4.0 | 547.1 ± 5.8 | 680.9 ± 15.1 |
| $O$. glaberrima | African rice | CG14 | 59.8 ± 1.8 | 11.9 ± 0.4 | 0.62 ± 0.03 | 7.4 ± 0.3 | 161 ± 4 | 183.5 ± 8.1 | 174.9 ± 11.6 | 361.5 ± 34.3 | 498.0 ± 52.7 |
| $T$. aestivum | Wheat        | S82     | 164.7 ± 1.2 | 14.3 ± 1.1 | 0.81 ± 0.03 | 11.6 ± 1.0 | 110 ± 2 | 1592.4 ± 42.9 | 1755.5 ± 46.0 | 67.8 ± 0.6 | 58.7 ± 1.1 |
|             |               | We4     | 147.2 ± 2.2 | 14.9 ± 0.9 | 0.87 ± 0.01 | 13.0 ± 0.9 | 116 ± 2 | 1600.2 ± 32.9 | 1692.5 ± 72.7 | 61.7 ± 2.0 | 45.1 ± 1.8 |

**P-value**

- ***$P<0.01$***
- **$P<0.05$***
- *$P<0.01$***
- ns

Discussion

**Importance of $g_s$ and $g_m$ in determining $A_n$ and TE**

The photosynthetic capacity of leaves is closely related to their nitrogen content (Evans, 1989). In our study, the large variations in $A_n$ cannot be explained by the small difference in leaf $N_a$ among and within species (Supplementary Fig. S1B). The close correlation between $A_n$ and total leaf conductance ($g_{tot}$) among species as well as within each group (Fig. 2A) suggests that the variation in CO₂ diffusion limitation could explain the genetic variation in $A_n$. Further, $A_n$ was correlated with CO₂ diffusion conductance ($C_b$, Fig. 2B, C), especially with $g_m$, in agreement with previous reports (Flexas et al., 2008; Warren, 2008). It is of note that aerobic rice, upland rice, and wheat showed higher dependences of $A_n$ on $g_m$ than lowland rice (Supplementary Table S2).

TE was influenced mostly by $g_s$ within each group (Supplementary Table S3); and this is not surprising given that $g_s$ directly controls the transpirational water flow from leaf to the ambient air. However, $g_m$ can influence $A_n$ with no direct impact on transpiration and therefore can influence TE (Barbour et al., 2010; Jahan et al., 2014). For this reason, it has previously been suggested that the $g_m/g_s$ ratio is a potential target for improving TE under drought in several crops (Flexas et al., 2010; Galmés et al., 2011; Gu et al., 2012; Flexas et al., 2013) and that the ideal plants for arid environment have a high $g_m/g_s$ ratio (Condon et al., 2004; Giuliani et al., 2013). The observed relationship between TE and $g_m/g_s$ supports this (Fig. 2D). In the literature, a relationship between an intrinsic TE ($A_s/g_s$) and $g_m/g_s$ has often been shown to exclude any influence of VPD on the relationship, especially when data are collected from a wide range of sources (Flexas et al., 2013). Mathematically, this is equivalent to the relationship between $A_n$ and $g_m$ (Fig. 2C). The somewhat more scattered relation in Fig. 2D than in Fig. 2C may reflect the influence of VPD, which varied between 1.0 and 1.6 kPa during our measurements. However, we found no systematic effect of such a small range of VPD on transpiration and TE (results not shown), and therefore we preferred to use TE for our illustration instead of intrinsic TE, which...
Fig. 4. Response of (A) the summed stomatal area index on both sides of the leaf surface (SAIs) and (B) specific stomatal conductance ($s_g$) of rice and wheat cultivars to water treatments: control (CT), mild drought (MD), and more severe drought (SD).

Fig. 5. The relationships (A) between stomatal conductance ($g_s$) and the summed stomatal area index (SAIs), and (B) between $g_s$ and the maximum stomatal diffusive conductance ($g_{s_{max}}$). Values of $g_s$ were obtained and calculated under 400 µmol mol$^{-1}$ CO$_2$, 1000–1500 µmol m$^{-2}$ s$^{-1}$ light intensity, and 25 °C. AR, aerobic rice; CG14, O. glaberrima; LR, lowland rice; UR, upland rice; W, wheat. Linear regressions were fitted for overall data and for each genotype group. The significance of each correlation is shown by asterisks: *$P<0.05$, **$P<0.01$, ***$P<0.001$. 

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has a different biological unit and is more difficult to interpret in agronomic terms. In our case, a higher $g_{\text{ml}}/g_s$ ratio in aerobic rice, upland rice, and wheat was found to be associated with higher TE, whereas lowland rice cultivars had a low $g_{\text{ml}}/g_s$ ratio and low TE (Fig. 2D). Furthermore, there was a higher slope for the linear relationship in TE versus the $g_{\text{ml}}/g_s$
ratio in upland rice, *O. glaberrima*, and wheat (Fig. 2D), indicating that the sensitivity of TE in response to the $g_m/g_s$ ratio was higher in drought-tolerant genotypes than in drought-susceptible genotypes.

Although $g_m$ is expected to have little instantaneous impact on transpiration, our data showed that like $g_s$, $g_m$ decreased simultaneously under drought in all cultivars (Fig. 1B, C). This indicates that long-term drought during growth affected $g_m$ in our experiment, in line with reports for several species (Miyazawa *et al.*, 2008; Flexas *et al.*, 2009), including rice (Gu *et al.*, 2012). When $g_s$ and $g_m$ had a rather parallel decrease, such as in lowland rice and We4 under the MD and the SD condition, or when $g_m$ showed a stronger decrease than $g_s$, such as in aerobic rice and UPL7 under the SD condition, no benefit for TE was observed. Only cultivars with a small decrease in $g_m$ combined with a strong decrease in $g_s$ under
drought showed strong increases in TE (e.g. Sal, O. glaberrima, and S82). Therefore, a strong response of $g_s$ combined with a small response of $g_m$ would be beneficial for water conservation and increasing TE under long-term drought.

Anatomical determinants of $g_s$

Stomatal conductance ($g_s$) is influenced by the structural traits and the opening of stomata (Xu and Zhou, 2008; Franks and Beerling, 2009; Galmés et al., 2013). In our study, $D$ or $S$ alone could not explain the variation in $g_s$ (Supplementary Fig. S4). $S$ and $D$ together determine the total pore area (stomatal area index, SAI) and the maximum stomatal conductance $g_{s\text{max}}$ (Franks and Beerling, 2009; Galmés et al., 2013). Our results confirmed this with marginally significant correlations ($P<0.05$) when $g_s$ was plotted versus SAI (Fig. 5A) or versus $g_{s\text{max}}$ (Fig. 5B). The variation in SAI or in $g_{s\text{max}}$ within rice species was more associated with $D$ than with $S$. Drought-tolerant types of aerobic rice, upland rice, and O. glaberrima had lower $D$ than lowland rice (Table 3), in line with reports that mutants with reduced leaf stomatal density had enhanced the drought tolerance and WUE of plants (Yu et al., 2008; Franks et al., 2015). Furthermore, the difference in $D$ was larger on the adaxial leaf surface than on the abaxial leaf surface, indicating that rice plants adapted to drier environments have developed fewer stomata on the upper surface to avoid direct water loss.

An increase in $D$ under drought has been reported in several species (Martínez et al., 2007; Hamanishi et al., 2012), but this response has not been widely observed in other species (Xu and Zhou, 2008; Galmés et al., 2013). On the other hand, a decrease in $S$ in response to drought was consistent in those studies. In our study, cultivars with a smaller $S$ under drought were accompanied by a greater reduction in $g_s$ and $s_g$ (e.g. IR64, Sal, CG14, and S82). This is in line with the idea that smaller stomata are capable of a faster response to drought, compared with larger stomata (Franks and Beerling, 2009).

The stomatal properties examined in our study concerned only the anatomical features. However, biochemical components such as abscisic acid also influence $g_s$, especially under drought (Franks and Farquhar, 2007). Such biochemical components influence the opening of stomata and may be responsible for part of the variability observed in $g_s$.

Anatomical determinants of $g_m$

Leaf mesophyll thickness ($T_m$) and $S_m$ are the critical structural components that impact upon $g_m$ (Evans et al., 1994, 2009; Terashima et al., 2011; Giuliani et al., 2013). In our study, wheat cultivars had a thicker $T_m$, a higher $S_m$, and a higher $g_m$ than rice cultivars. However, the difference in $T_m$ or $S_m$ was not associated with $g_m$ variation within each species (Supplementary Fig. S7A, B). Drought had a stronger effect on $S_m$ in wheat than in rice cultivars. This higher increase in $S_m$ in wheat cultivars might contribute to the smaller decline in $g_m$ under drought conditions (Galmés et al., 2013).

It has been suggested that mesophyll cell walls account for more than 25% of total mesophyll resistance (Evans et al., 2009; Terashima et al., 2011; Tholen and Zhu, 2011). Han et al. (2016) reported that the decreasing $g_m$ in cotton leaf under drought was associated with increased $T_w$. Variation in $g_m$ between O. sativa and wild rice species was suggested to be attributed to the thicker $T_w$ in wild rice species (Scafaro et al., 2011). In our study, wheat cultivars had a thinner $T_w$ than all rice cultivars. However, $T_w$ varied significantly among rice cultivars and the difference was not associated with observed differences in $g_m$ (Fig. 7B). Increased $T_w$ under drought was observed in our study, but this did not lead to a decreased $g_m$, particularly in cultivars Sal and CG14. This suggests that $T_w$ might not be the most dominant limiting factor for $g_m$ under drought in rice. On the other hand, it has generally been proposed that thick mesophyll cell walls are beneficial for plant drought tolerance (Scafaro et al., 2011; Giuliani et al., 2013). The thicker $T_w$ found in drought-tolerant cultivars (e.g. upland rice and O. glaberrima) than in drought-sensitive cultivars might support this idea.

$S_m$ affects $g_m$ in many species (Tholen et al., 2008; Scafaro et al., 2011; Terashima et al., 2011; Tosens et al., 2012a; Tomás et al., 2013). Barbour et al. (2016) showed that the decrease in $g_m$ in aged wheat and maize leaves was accompanied by...
a decline in $S/S_m$. In our study, the difference in $g_m$ was explained somewhat more by $S/S_m$ (Fig. 7A) than by $S_c$ (Supplementary Fig. S7D), particularly in rice cultivars. In response to drought, $g_m$ decreased more in cultivars with clear decreased $S/S_m$ (e.g. lowland and aerobic rice) and decreased less in cultivars with a relatively constant $S/S_m$ ratio (e.g. upland rice and wheat) across water treatments. The increase in $S/S_m$ observed in *O. glaberrima* (Supplementary Fig. S5C) might be a compensating strategy for the increase in $T_w$ under drought (Tosens et al., 2012a), which resulted in a small decrease in $g_m$. The high correlation between $g_m$ and $S/S_m$ within rice (Fig. 7A) indicates that $S/S_m$ might be very important in determining $g_m$ in rice.

It has been suggested that certain biochemical components such as aquaporins and carbonic anhydrase are $g_m$-related (Miyazawa et al., 2008; Flexas et al., 2012). These characteristics were not investigated in our study. They might explain the part of the variability observed in $g_m$ that was not explained by anatomical features.

### Conclusions

Stomatal conductance and mesophyll conductance explained most of the variability in $A_s$ among species and within the species *O. sativa*. The higher $g_m/g_s$ ratio in aerobic rice, upland rice, and wheat cultivars was associated with higher TE, compared with lowland rice cultivars and *O. glaberrima*. Our study revealed the genotypic variation in the TE versus $g_m/g_s$ relationship among species and within the species *O. sativa*. There was a higher TE sensitivity in response to $g_m/g_s$ in drought-tolerant groups (O. glaberrima, upland rice, and wheat), than in lowland and aerobic rice. In particular, upland rice, O. glaberrima, and wheat cultivars minimized the decrease in $g_m$; this can simultaneously sustain photosynthesis and increase TE under drought.

Stomatal development was responsible for the variation in $g_s$ among contrasting rice types and between rice and wheat. SAs and $g_{\text{max}}$ were correlated with the genetic variation of $g_s$ among three species. More specifically, stomatal density (one component affecting SAs and $g_{\text{max}}$) was more related to the genetic variation in $g_s$ among rice cultivars, suggesting that drought-tolerant rice cultivars might have developed a mechanism of stomatal acclimation to drier edaphic environments. Moreover, cultivars with smaller stomata had a stronger decrease in $g_s$, and thus a higher increase in TE, under drought stress.

Previous studies indicated that thick $T_m$, high $S_m$, high $S_c$, and thin $T_w$ were associated with high $g_m$. These correlations were observed between wheat and rice but not among rice cultivars in our study. More importantly, for rice cultivars, the adverse impact of thick $T_m$ can be neutralized by other anatomical factors such as a high $S/S_m$ ratio. In particular, under drought, the maintained or increased $S/S_m$ ratio in upland rice, O. glaberrima, and wheat cultivars resulted in a smaller decrease in $g_m$ in them than in lowland and aerobic rice cultivars.

In short, our results suggest that rice TE might be improved by modulating stomatal and mesophyll structural traits via breeding selection and/or genetic engineering.

### Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Response of leaf nitrogen per unit area ($N_s$) of rice and wheat cultivars to water treatments, relationship between photosynthetic rate ($A_s$) and $N_s$, and relationship between mesophyll conductance ($g_m$) and $N_s$.

Fig. S2. Relationship between transpiration efficiency (TE) and stomatal conductance ($g_s$), and between TE and mesophyll conductance ($g_m$).

Fig. S3. Stomatal density ($D$) and size ($S$) from adaxial side and abaxial side of rice and wheat cultivars under three treatments.

Fig. S4. The relationships between stomatal conductance ($g_s$) and stomatal density ($D$), and between $g_m$ and stomatal size ($S$).

Fig. S5. Mesophyll cell properties of wheat and rice leaves obtained from light and electron microscope images under three treatments.

Fig. S6. Relationships between mesophyll thickness ($T_m$) and $N_s$, the surface area of mesophyll cells exposed to the intercellular airspaces per leaf area ($S_m$) and $N_s$, ratio of the exposed surface area of chloroplast to the exposed surface area of mesophyll cell walls ($S/S_m$) and $N_s$, the surface area of chloroplasts exposed to intercellar airspace per leaf area ($S_c$) and $N_s$, and thickness of the mesophyll cell wall ($T_m$) and $N_s$.

Fig. S7. The relationship between mesophyll conductance ($g_m$) and mesophyll thickness ($T_m$), the surface area of mesophyll cells exposed to the intercellular airspaces per leaf area ($S_m$), and the surface area of chloroplasts exposed to intercellular airspace per leaf area ($S_c$).

Table S1. A two-way analysis of variance of genotype versus water stress for measured and estimated photosynthetic and anatomical parameters.

Table S2. Multiple regression analysis of light-saturated photosynthesis ($A_s$) as a function of $g_s$ and $g_m$, based on data of three treatments.

Table S3. Multiple regression analysis of transpiration efficiency (TE) as a function of $g_s$ and $g_m$, based on data of three treatments.

Table S4. Multiple regression analysis of mesophyll conductance ($g_m$) as a function of $T_w$, $S/S_m$ and $N_s$, based on combined data of all three water treatments.

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