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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 (g_s) and mesophyll conductance (g_m) 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 g_m/g_s 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 g_s in drought-tolerant rice, whereas rice genotypes with smaller stomata generally responded more strongly to drought. Compared with rice, wheat had a higher g_m, 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 g_m under drought by maintaining high ratios of chloroplasts to exposed mesophyll cell walls. Rice TE could be improved by increasing the g_m/g_s 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 C_3-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 CO_2 concentration at carboxylation sites (C_c) inside the chloroplast, which is determined by CO_2 diffusion components, i.e. stomatal conductance (g_s) and mesophyll conductance (g_m) (Evans et al., 2009). Stomata regulate CO_2 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 CO_2 (Pinheiro and Chaves, 2011). In the long-term response to water deficit, g_s can be influenced by leaf anatomical traits such as stomatal density and size, which can vary to acclimate to the environment (Xu and Zhou, 2008; Franks and Beerling, 2009). As the inevitable consequence of CO_2 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 (A_n) to transpiration rate. In general, higher g_s results in a lower TE (Flexas et al., 2008; Gu et al., 2012).

Mesophyll conductance (g_m) has been viewed as the diffusion of CO_2 from sub-stomatal cavities to the sites of carboxylation in the chloroplasts (Flexas et al., 2008). In contrast to g_s, increasing g_m increases A_n at no cost of increased transpiration, because the CO_2 diffusion pathway involving g_m is not shared with the diffusion pathway of transpired H_2O. Therefore, increasing g_m not only increases A_n but also increases TE (Barbour et al., 2010; Flexas et al., 2013). However, long-term drought stress reduces g_m (Scartazza et al., 1998; Gu et al., 2012; Han et al., 2016). Along the CO_2 diffusion pathway inside leaves, the conductance through the liquid phase (g_{liq}) is the most limiting factor for CO_2 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 g_m 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 g_m. Mesophyll thickness (T_m), surface area of chloroplasts exposed to the intercellular airspace (S_c), and mesophyll cell wall thickness (T_w) are suggested to be the most important structural components determining g_m (Evans et al., 1994; Evans et al., 2009; Scafaro et al., 2011; Tosens et al., 2012*b*; Tomás et al., 2013), as these parameters determine pathways for CO_2 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 (g_s and g_m) and possibly related leaf anatomical properties were examined in three species, *O. sativa*, *O. glaberrima* and *T. aestivum*. g_s was measured from gas exchange, and combined gas exchange and chlorophyll fluorescence measurements were used to estimate g_m. 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 g_s and g_m and how those determinants underpin genotypic differences in the response of g_s, g_m, 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 $CO(NH_2)_2$ (urea) and 0.5 g KH_2PO_4 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 duration 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 28 °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 CO_2 response curves were measured under both ambient (21%) and low (2%) O_2 conditions. The low O_2 condition was created by using a gas mixture of 2% O_2 and 98% N_2, and the infrared gas analyser calibration was adjusted for O_2 composition of the gas mixture according to the manufacturer's instructions.

For the CO_2 response curve, under both O_2 conditions, the leaf was consecutively exposed to an incident irradiance (I_{inc}) of 1000 µmol m^{-2} s^{-1} with different levels of CO_2: 50, 90, 150, 250, 400, 700, 1000, and 1500 µmol mol^{-1}. For the light response curve, under the 21% O_2 condition, the CO_2 was kept constant at 400 µmol mol^{-1} and I_{inc} was increased in the order of 30, 50, 80, 120, 200, 500, 1000, and 1600 µmol m^{-2} s^{-1}. Under 2% O_2, in order to ensure non-photorespiratory conditions, the CO_2 was kept constant at 1000 µmol mol^{-1} with I_{inc} levels of 30, 50, 80, 120, and 200 µmol m^{-2} s^{-1}. The measurement flow rate was 400 µmol s^{-1}. CO_2 exchange rates were corrected for CO_2 leakage into and out of the leaf cuvette, based on measurements using the same flow rate on boiled leaves across a range of CO_2 levels, and intercellular CO_2 levels (C_i) were then recalculated (Flexas *et al.*, 2007).

The steady-state fluorescence (F_s) was measured at each light or CO_2 step. Then a multiphase flash method (Loriaux *et al.*, 2013) was applied to determine F'_m (the maximum fluorescence during the saturating light pulse). The apparent photosystem II electron (e^-) transport efficiency for each irradiance or CO_2 step was calculated as $\Phi_2=(F'_m-F_s)/F'_m$ (Genty *et al.*, 1989).

Estimation of diffusion components

Total conductance to CO_2 (g_{tot}) was calculated based on stomatal and mesophyll conductance according to $1/g_{tot}=1/g_s+1/g_m$. For the analysis, stomatal conductance for CO_2 (g_s) was obtained as stomatal conductance for water vapour divided by 1.6, from the data points measured under ambient condition (400 µmol mol^{-1} CO_2, 1000–1500 µmol m^{-2} s^{-1} irradiance, and 25 °C) of the light response curve under 21% O_2.

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

$$A_n = 0.5\left\{J/4 - R_d + g_m(C_i + 2\Gamma^*) - \sqrt{\begin{matrix}[J/4 - R_d + g_m(C_i + 2\Gamma^*)]^2 - \\ 4g_m[(C_i - \Gamma^*)J/4 - R_d(C_i + 2\Gamma^*)]\end{matrix}}\right\} \quad (1)$$

where Γ^* is the CO_2 compensation point in the absence of day respiration (R_d) and J is the linear electron transport rate used for CO_2

Table 1. Description of rice and wheat cultivars used in the study

Species	Cultivar	Abbrev.	Cultivar type	Drought tolerance
Oryza sativa	IR 64-21	IR64	High-yielding lowland cultivar	Susceptible
	IR 77298-14-1-2::IRGC 117374-1	II	Lowland cultivar	Susceptible to moderately tolerant
	NSIC RC 9	Apo	High-yielding aerobic cultivar	Moderately tolerant
	NSIC RC 192	148	High-yielding aerobic cultivar	Moderately tolerant
	UPL Ri7	UPL7	Improved upland cultivar	Drought tolerant
	Salumpikit	Sal	Traditional upland cultivar	Drought tolerant
Oryza glaberrima	CG14	CG14	Cultivated African rice	Drought tolerant
Triticum aestivum	SeriM82	S82	High-yielding irrigated cultivar	Moderately susceptible
	Weebill4	We4	Dryland-adapted cultivar	Drought tolerant

Table 2. *Description of cultivation and water regimes for rice and wheat cultivars*

CT, control; MD, mild drought; SD, more severe drought.

Species	Sowing time	Density (seedlings per pot)	Soil pH	Initial soil moisture	Stress imposed time (leaves)[a]	Water stress level			Final leaf number[b]
						CT	MD	SD	
O. sativa, *O. glaberrima*	June 2013	3	5.5–6.0	Saturated	5	Inundated	0 to −5 kPa	−20 to −40 kPa	14–15
T. aestivum	November 2013	8	Not adjusted	0 to −5 kPa	4	0 to −5 kPa	−20 to −40 kPa	−50 to −70 kPa	10–11

[a] Leaf number on the main stem.

[b] The final leaf number for the flag leaf on the main stem; this number was 18 for cv. Sal of *O. sativa*.

fixation and photorespiration. Equation (1) provides a model to estimate g_m by a curve fitting that minimizes the difference between measured and estimated A_n (Yin and Struik, 2009). In the model, Γ^* is calculated from the Rubisco specificity factor ($S_{c/o}$) as $\Gamma^*=0.5O/S_{c/o}$, where O is O_2 partial pressure (mbar). $S_{c/o}$ 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 $S_{c/o}$ 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_{inc} levels of the light response curve and high C_a 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} C_a levels of CO_2 response curve under 2% O_2), a linear regression can be performed for the observed A_n against $I_{inc}\Phi_2/4$. 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_i can be calculated using $J=sI_{inc}\Phi_2$.

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 (sg_s) was determined by dividing g_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 (g_{smax}), was also calculated, according to Franks and Beerling (2009):

$$g_{s\max}=\frac{d\cdot D\cdot a_{\max}}{1.6v\left(l+\dfrac{\pi}{2}\sqrt{a_{\max}/\pi}\right)} \tag{2}$$

where d is the diffusivity of water vapour in air, v is the molar volume of air, a_{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 2.82×10^{-5} m^2 s^{-1} and 24.5 m^3 mol^{-1}, respectively. a_{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 Beerling (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 × 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 embedded 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-microtome (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 (Tecnai 12, Philips, The Netherlands).

Analyses of images were performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA) (Abràmoff *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 (T_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:

$$S_m=\frac{L_m}{w}F \tag{3}$$

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:

$$S_c=\frac{L'_c}{L'_m}S_m \tag{4}$$

where L'_c 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'_c 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'_c/L'_m (Tosens *et al.*, 2012*a*,*b*).

In addition, thickness of the mesophyll cell wall (T_w) 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_w of that cell to minimize the artefact of inaccurate orientation of ultra-microtome sectioning. The average value of T_w 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 analysed for N content by using an elementary analyser (Vario Macro cube, Elementar, Germany). Leaf nitrogen per unit area (N_a) 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 analysis 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 (A_n) varied almost threefold among species, with *T. aestivum* having a significantly higher A_n than *O. sativa* and *O. glaberrima* (cv. CG14) (Fig. 1A). Under the CT condition, A_n varied between 6.9 µmol m^{-2} s^{-1} for CG14 and 22.7 µmol m^{-2} s^{-1} for S82. Overall, drought decreased A_n strongly with no significant interaction between cultivars and water treatments (Supplementary Table S1 at *JXB* online). However, for CG14, drought increased A_n (Fig. 1A), and this was probably associated with an increase in N_a by drought in this cultivar (Supplementary Fig. S1A). Across all cultivars and treatments, A_n was positively correlated with N_a among wheat cultivars, but not among cultivars of *O. sativa* (Supplementary Fig. S1B).

Variation in g_s and g_m

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

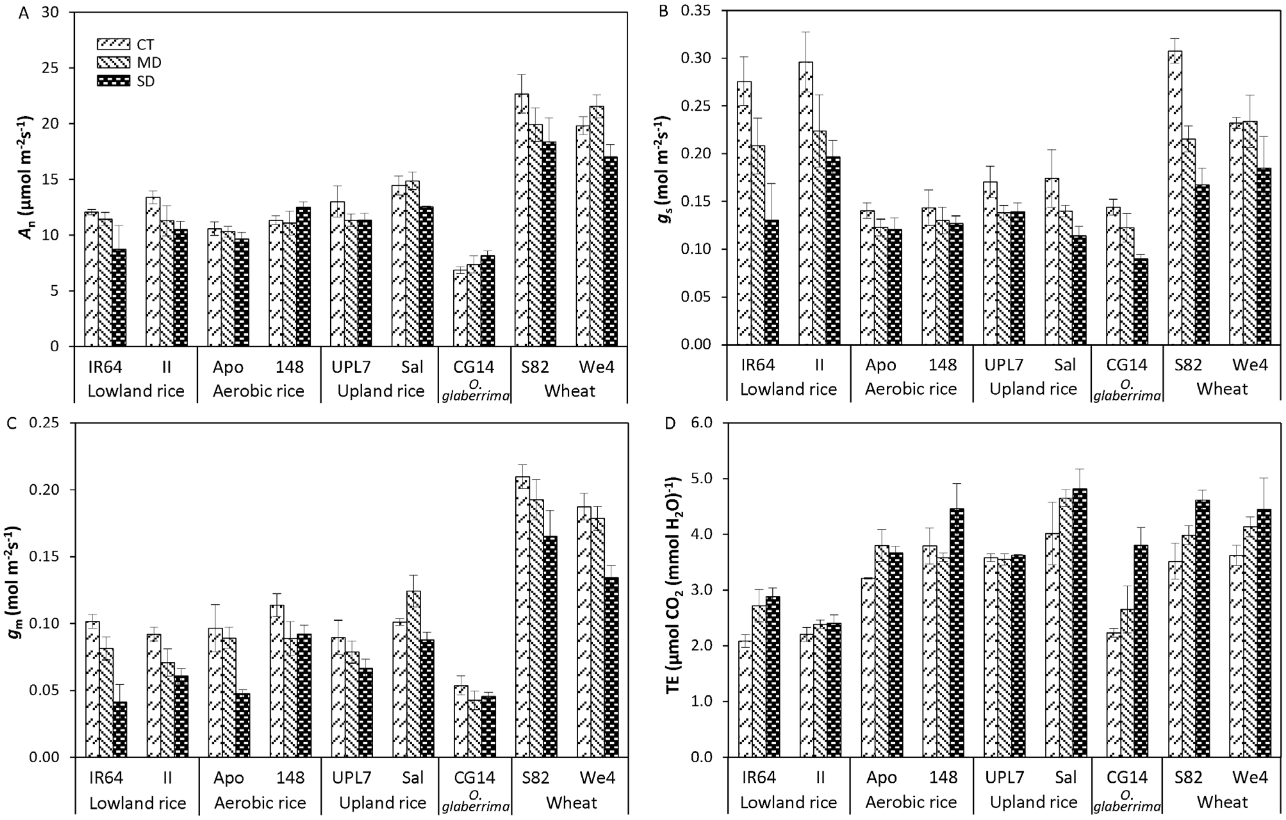

Fig. 1. Photosynthetic parameters obtained and estimated from GE and CF data under three treatments: control (CT), mild drought (MD), and more severe drought (SD). Values of (A) net photosynthetic rate (A_n), (B) stomatal conductance (g_s), and (D) transpiration efficiency (TE) were obtained under the condition of 400 µmol mol^{-1} CO$_2$, 1000–1500 µmol m^{-2} s^{-1} irradiance, and 25 °C. (C) Mesophyll conductance (g_m) was calculated based on the NRH-A method (Yin and Struik, 2009). Bars represent standard errors of the mean for A_n, g_s, and TE, and standard error of the estimate for g_m.

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 $H_2O)^{-1}$ for IR64 and 4.02 µmol CO_2 (mmol $H_2O)^{-1}$ 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

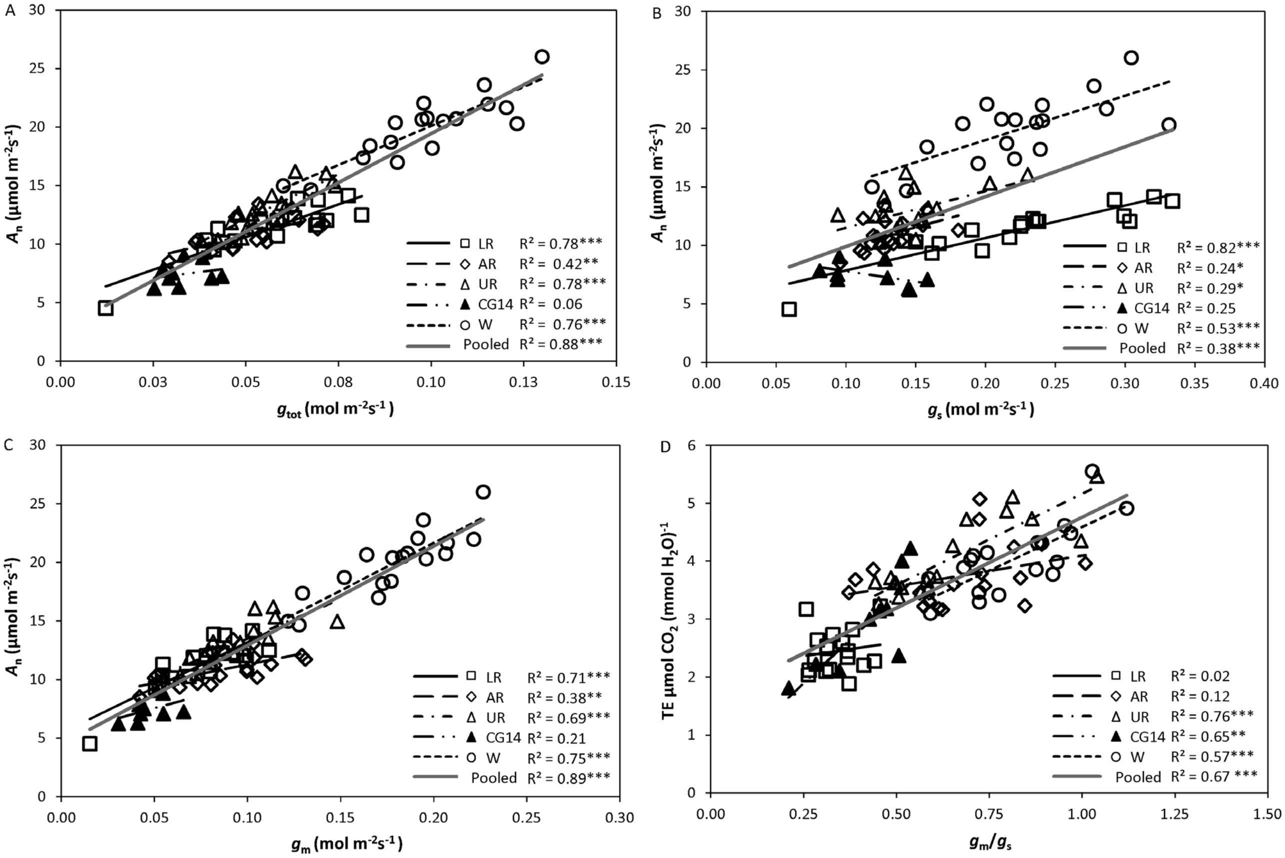

Fig. 2. The relationships (A) between photosynthetic rate (A_n) and total leaf conductance (g_{tot}), (B) between A_n and stomatal conductance (g_s), (C) between A_n and mesophyll conductance (g_m), and (D) between transpiration efficiency (TE) and the ratio of mesophyll conductance to stomatal conductance (g_m/g_s). A_n, g_s, and TE were obtained under 400 µmol mol^{-1} CO_2, 1000–1500 µmol m^{-2} s^{-1} light intensity, and 25 °C. g_m was calculated based on the NRH-A method (Yin and Struik, 2009). 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: *$P<0.05$, **$P<0.01$, ***$P<0.001$.

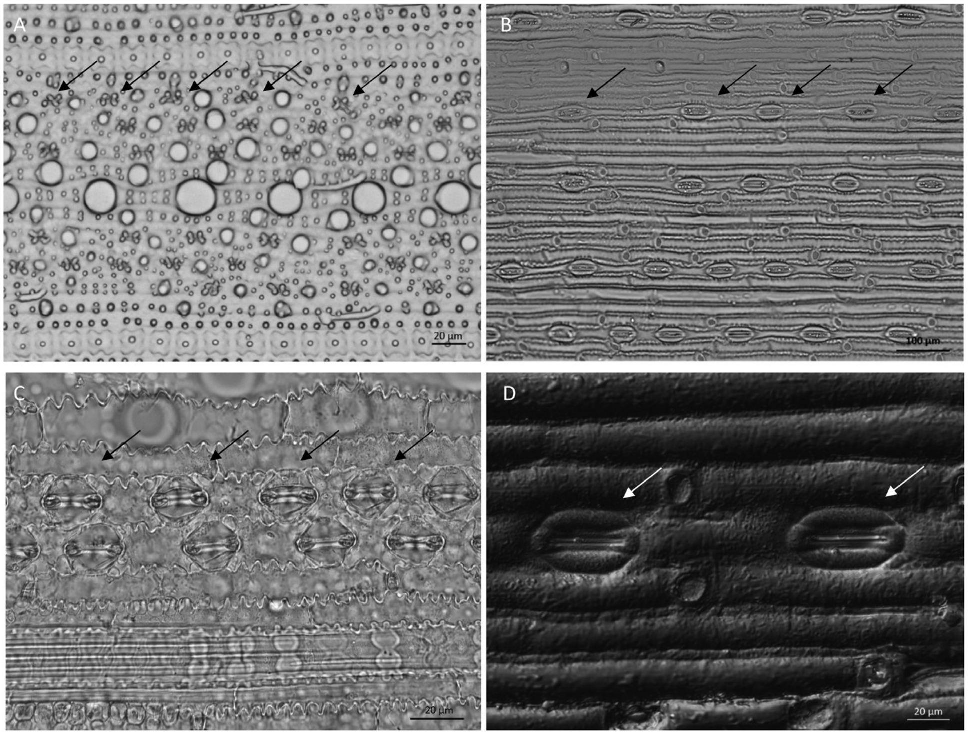

Fig. 3. Light micrographs illustrating leaf surfaces of rice (IR64; A, C) and wheat (S82; B, D) used to calculate stomatal density (A, B) and size (C, D). Arrows indicate stomata.

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_{smax}) 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_{smax} 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), 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). No strong negative effect on S_c/S_m was observed in upland rice and wheat cultivars under either drought 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

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

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'_c/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.

Species	Genotype group	Cultivar	Mesophyll					Stomata			
			T_m (µm)	S_m (m m^{-2})	S_c/S_m	S_c (m m^{-2})	T_w (nm)	S (µm^2)		D (No. mm^{-2})	
								Adaxial	Abaxial	Adaxial	Abaxial
O. sativa	Lowland rice	IR64	70.9 ± 4.2	11.2 ± 0.2	0.77 ± 0.01	8.7 ± 0.3	126 ± 1	172.1 ± 2.1	186.7 ± 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	194.7 ± 10.3	203.5 ± 4.0	547.1 ± 5.8	680.9 ± 15.1
	Aerobic rice	Apo	73.6 ± 0.9	10.6 ± 0.3	0.68 ± 0.02	7.2 ± 0.4	136 ± 3	217.8 ± 11.0	215.6 ± 11.3	460.0 ± 26.1	678.3 ± 10.2
		148	70.1 ± 1.5	9.7 ± 0.3	0.72 ± 0.02	7.0 ± 0.2	140 ± 4	211.3 ± 7.0	213.0 ± 6.1	509.1 ± 17.9	639.4 ± 22.4
	Upland rice	UPL7	82.4 ± 1.7	10.9 ± 0.2	0.71 ± 0.02	7.7 ± 0.2	174 ± 2	188.7 ± 6.3	186.8 ± 5.4	475.6 ± 36.4	640.8 ± 41.6
		Sal	79.7 ± 0.7	11.5 ± 0.1	0.87 ± 0.02	10.1 ± 0.3	182 ± 1	185.3 ± 5.6	168.7 ± 4.5	467.4 ± 15.2	685.4 ± 21.7
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			***/***	***/*	***/***	***/***	***/***	***/*	***/**	***/**	***/ns

mesophyll cell wall was observed in wheat cultivars than in all rice cultivars (Fig. 6), and drought increased T_w, with the most significant effect observed in IR64, Sal and We4 under the SD condition (Supplementary Fig. S5E). T_w was also observed to be the only anatomical parameter to correlate with N_a, a parameter related to leaf physiological maturity (Supplementary Fig. S6).

Contributions of leaf anatomical properties to the variation in g_m were different in wheat and rice. A higher g_m in wheat was associated with a thicker T_m, a higher S_m, a higher S_c and a thinner T_w compared with rice cultivars, and these associations were little altered by using a different curvature factor for calculating S_m in wheat (Supplementary Fig. S7). However, no clear relationship was observed between g_m and T_m or between g_m and S_m within *O. sativa* or within most genotype groups (Supplementary Fig. S7A, B). g_m was positively correlated with S_c (Supplementary Fig. S7C; R^2=0.14, P<0.01) and with S_c/S_m (Fig. 7A; R^2=0.33, P<0.001) among rice cultivars. g_m and T_w were negatively correlated among wheat cultivars (Fig. 7B), and this correlation only applied among rice cultivars if upland rice was excluded (R^2=0.37, P<0.001). These correlations were also confirmed by multiple regression analysis, which indicated that S_c/S_m contributed mostly to the variation in g_m within *O. sativa*, whereas T_w was the main determinant of g_m in wheat (Supplementary Table S4).

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 CO_2 diffusion limitation could explain the genetic variation in A_n. Further, A_n was correlated with CO_2 diffusional conductance (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_n/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

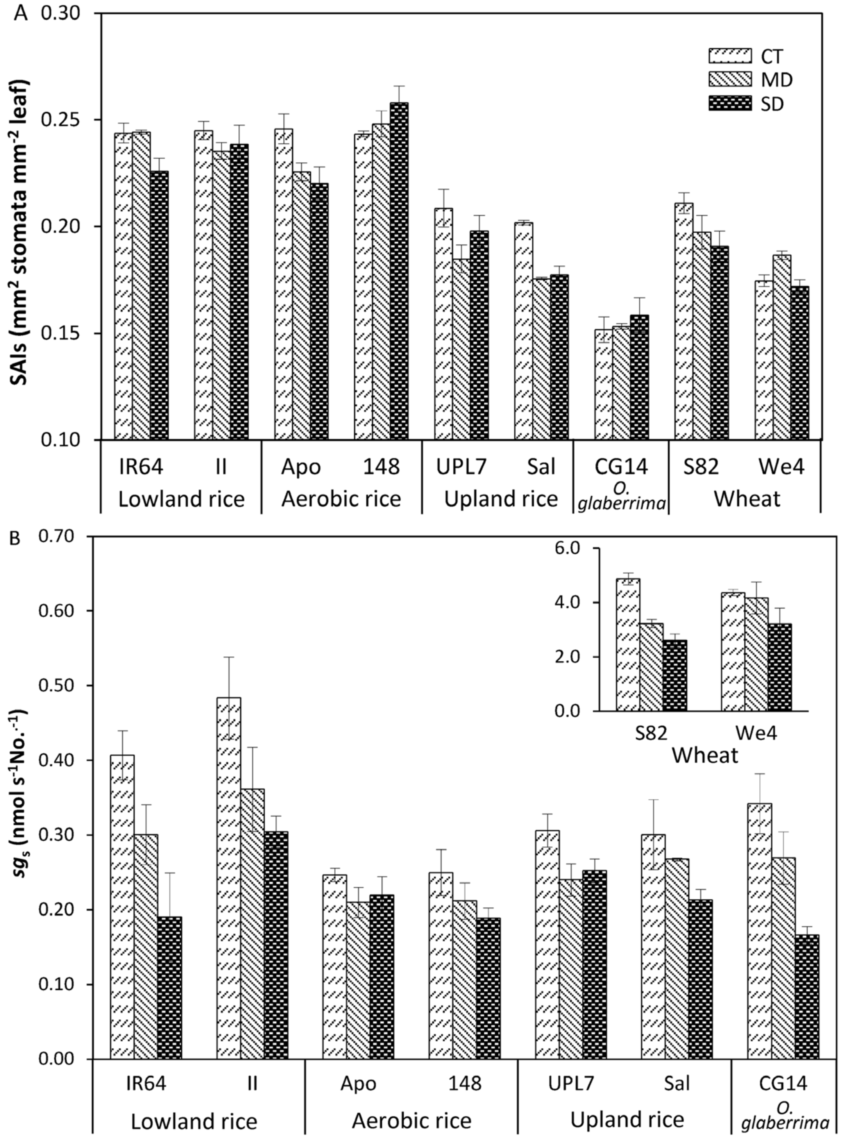

Fig. 4. Response of (A) the summed stomatal area index on both sides of the leaf surface (SAIs) and (B) specific stomatal conductance (sg_s) of rice and wheat cultivars to water treatments: control (CT), mild drought (MD), and more severe drought (SD).

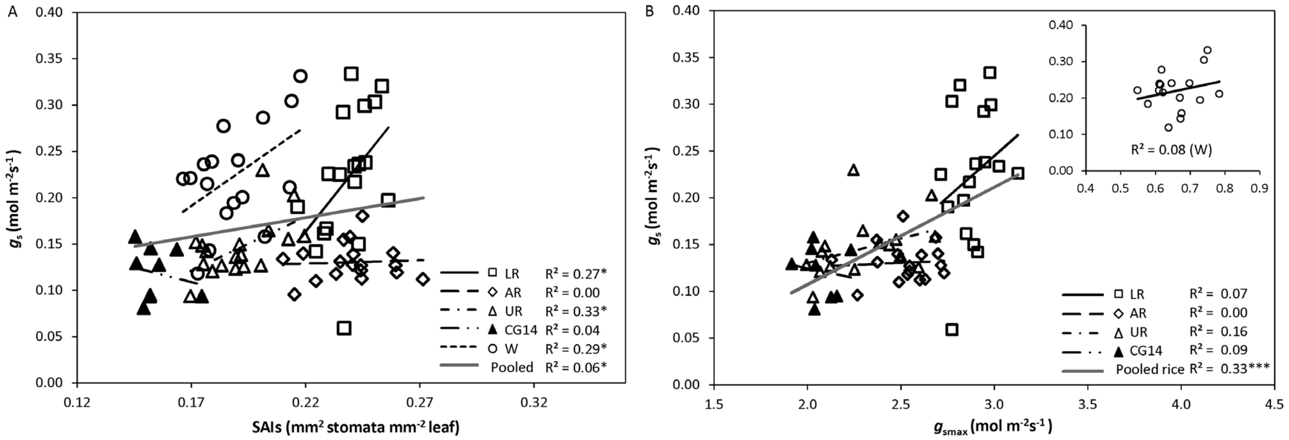

Fig. 5. The relationships (A) between stomatal conductance (g_s) and the summed stomatal area index (SAIs), and (B) between g_s and the maximum stomatal diffusive conductance (g_{smax}). Values of g_s were obtained and calculated under 400 μmol mol^{-1} CO$_2$, 1000–1500 μmol m^{-2} s^{-1} light intensity, 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.

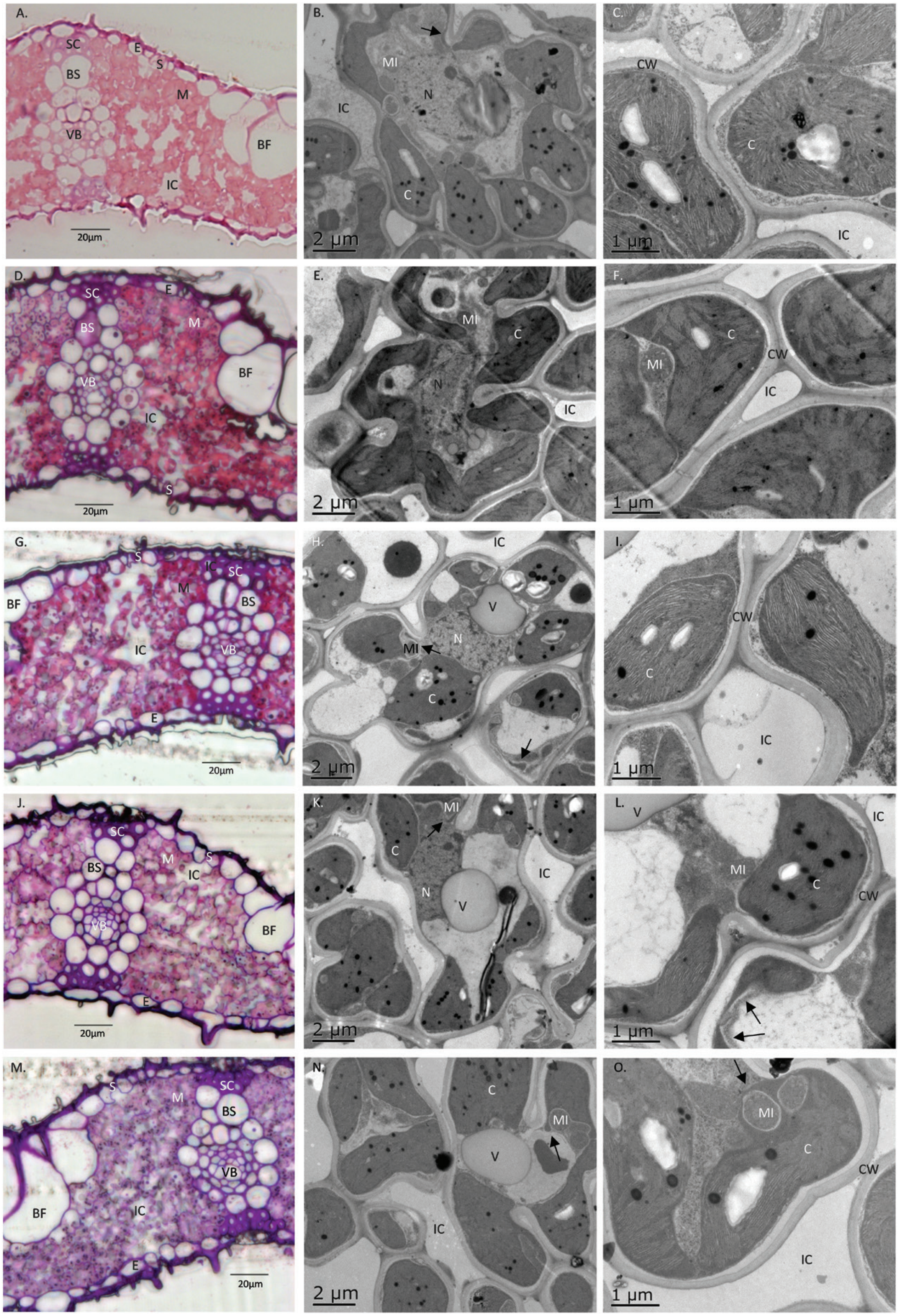

Fig. 6. Light (left panels) and electron (middle and right panels) micrographs of leaf structure and anatomy for *O. sativa* cultivars (A–C, IR64; D–F, II; G–I, Apo; J–L, 148; M–O, UPL7; P–R, Sal), *O. glaberrima* (S–U, CG14), and *T. aestivum* cultivars (V–X, S82; Y–AA, We4). The left panels show the overall leaf transverse sections; the middle panels show mesophyll cell shape, chloroplast distribution, and lobe development; and the right panels show mesophyll cell walls. Arrows mark stromules. BF, bulliform cell; BS, outer bundle-sheath cell; C, chloroplast; CW, mesophyll cell wall; E, epidermis; IC, intercellular airspace; M, mesophyll cell; MI, mitochondria; N, nucleus; S, stoma; SC, sclerenchyma strand; VB, vascular bundle; V, vacuole.

has a different biological unit and is more difficult to interpret in agronomic terms. In our case, a higher $g_\mathrm{m}/g_\mathrm{s}$ ratio in aerobic rice, upland rice, and wheat was found to be associated with higher TE, whereas lowland rice cultivars had a low $g_\mathrm{m}/g_\mathrm{s}$ ratio and low TE (Fig. 2D). Furthermore, there was a higher slope for the linear relationship in TE *versus* the $g_\mathrm{m}/g_\mathrm{s}$

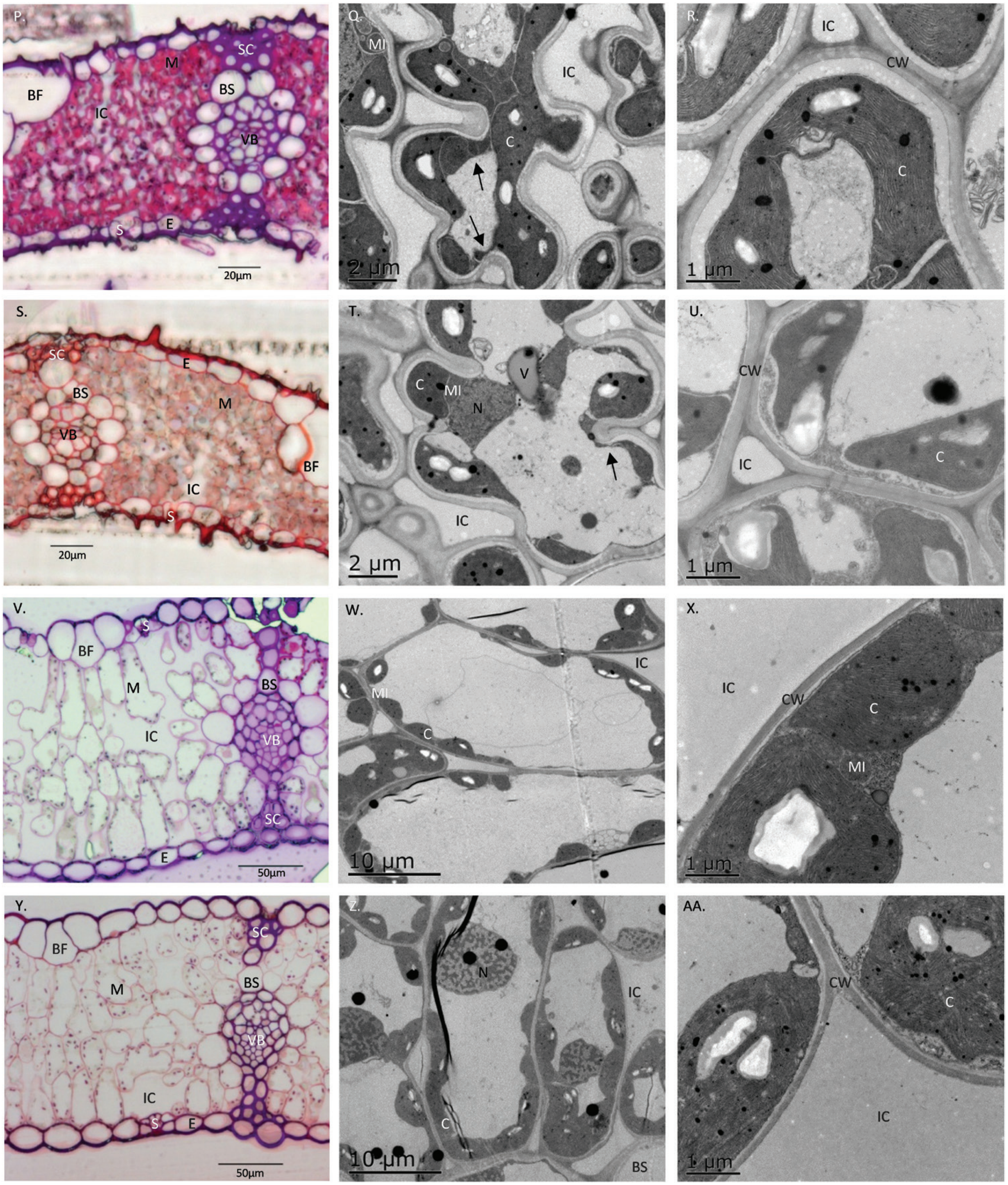

Fig.6. *Continued*

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

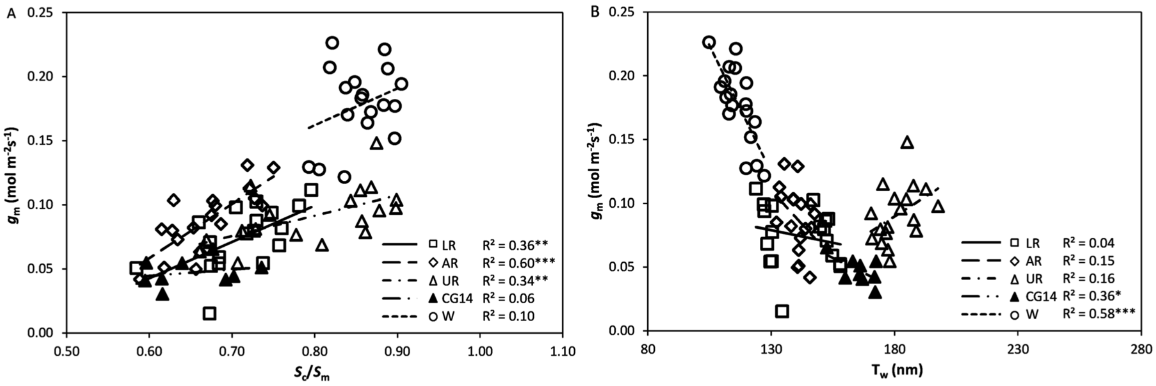

Fig. 7. The relationships (A) between mesophyll conductance (g_m) and the ratio of the exposed chloroplast surface area to the exposed surface area of mesophyll cell walls (S_c/S_m), and (B) between g_m and the thickness of the mesophyll cell wall (T_w). AR, aerobic rice; CG14, *O. glaberrima*; LR, lowland rice; UR, upland rice; W, wheat. Linear regressions were fitted for each genotype group. The significance of each correlation is shown by asterisks: *$P<0.05$, **$P<0.01$, ***$P<0.001$.

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_{smax} (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* SAIs (Fig. 5A) or *versus* g_{smax} (Fig. 5B). The variation in SAIs or in g_{smax} 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 (Martinez *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 sg_s (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_c affects g_m in many species (Tholen *et al.*, 2008; Scafaro *et al.*, 2011; Terashima *et al.*, 2011; Tosens *et al.*, 2012*a*; 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_c/S_m. In our study, the difference in g_m was explained somewhat more by S_c/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_c/S_m (e.g. lowland and aerobic rice) and decreased less in cultivars with a relatively constant S_c/S_m ratio (e.g. upland rice and wheat) across water treatments. The increase in S_c/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.*, 2012*a*), which resulted in a small decrease in g_m. The high correlation between g_m and S_c/S_m within rice (Fig. 7A) indicates that S_c/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_n 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. SAIs and g_{smax} were correlated with the genetic variation of g_s among three species. More specifically, stomatal density (one component affecting SAIs and g_{smax}) 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_w can be neutralized by other anatomical factors such as a high S_c/S_m ratio. In particular, under drought, the maintained or increased S_c/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_a) of rice and wheat cultivars to water treatments, relationship between photosynthetic rate (A_n) and N_a, and relationship between mesophyll conductance (g_m) and N_a.

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_s 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_a, the surface area of mesophyll cells exposed to the intercellular airspaces per leaf area (S_m) and N_a, ratio of the exposed surface area of chloroplast to the exposed surface area of mesophyll cell walls (S_c/S_m) and N_a, the surface area of chloroplasts exposed to intercellular airspace per leaf area (S_c) and N_a, and thickness of the mesophyll cell wall (T_w) and N_a.

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_n) 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_c/S_m and N_a, based on combined data of all three water treatments.

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