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Heterogeneity of photosynthesis within leaves is associated with alteration of leaf structural features and leaf N content per leaf area in rice

Dongliang Xiong[A], *Tingting Yu*[A], *Xi Liu*[A], *Yong Li*[A], *Shaobing Peng*[A] *and Jianliang Huang*[A,B,C]

[A]National Key Laboratory of Crop Genetic Improvement, MOA Key Laboratory of Crop Ecophysiology and Farming System in the Middle Reaches of the Yangtze River, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, Hubei 430070, China.
[B]Hubei Collaborative Innovation Center for Grain Industry, Yangtze University, Jingzhou, Hubei 434023, China.
[C]Corresponding author. Email: jhuang@mail.hzau.edu.cn

Abstract. Increasing leaf photosynthesis rate (A) is considered an important strategy to increase C_3 crop yields. Leaf A is usually represented by point measurements, but A varies within each leaf, especially within large leaves. However, little is known about the effect of heterogeneity of A within leaves on rice performance. Here we investigated the changes in gas-exchange parameters and leaf structural and chemical features along leaf blades in two rice cultivars. Stomatal and mesophyll conductance as well as leaf nitrogen (N), Rubisco and chlorophyll contents increased from base to apex; consequently, A increased along leaves in both cultivars. The variation in A, leaf N content and Rubisco content within leaves was similar to the variations among cultivars, and the extent of A heterogeneity within leaves varied between cultivars, leading to different efficiencies of biomass accumulation. Furthermore, variation of A within leaves was closely associated with leaf structural and chemical features. Our findings emphasise that functional changes along leaf blades are associated with structural and chemical trait variation and that variation of A within leaves should be considered to achieve progress in future breeding programs.

Additional keywords: biomass, CO_2 diffusion, leaf structure, leaf N content per leaf area.

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Introduction

Rice is one of the most important food crops in the world, directly feeding more people than any other crop (Mitchell and Sheehy 2006). To meet the demands of a rapidly growing population while arable land decreases, the improvement of rice productivity per area is an essential research goal. The development of modern semi-dwarf rice varieties with high harvest indices has dramatically improved rice yields since 1960s (Khush 2001). In modern agricultural systems, the rice harvest index is close to the theoretical maximum, and thus further improvement is very limited (Long *et al*. 2006). Therefore, increasing total biomass production per unit area is the key to continued increases in rice grain yield. As the fundamental process generating crop biomass and yield, the rate of photosynthesis (A) of individual leaves has become the focus of current efforts to increase rice production (Mitchell and Sheehy 2006). In most studies, leaf A is usually represented by point measurements; however, A is more likely varies with the position along leaves, especially, for large and narrow leaves.

In C_3 plants, A is mainly determined by two factors: CO_2 concentration in chloroplasts and Rubisco activity (Farquhar *et al*. 1980). The diffusion of CO_2 into leaves is restricted by the stomata, and subsequent diffusion into chloroplasts is restricted by the intercellular airspaces and mesophyll cell structure (Flexas *et al*. 2008, 2012). The diffusion process of CO_2 from outside leaf into the intercellular airspaces is quantified as 'stomatal conductance' (g_s), and the further diffusion conductance of CO_2 from the intercellular airspaces to the chloroplasts is termed 'mesophyll conductance' (g_m). In general, g_s responds physiologically within minutes to changes in the external environment (Creese *et al*. 2014), and the dynamic range of g_s plays an important role in plant production under natural conditions. Under low evaporative demand and high light conditions, the upper limit of g_s is determined by the opening and closing of stomata. When plants experience a high evaporative demand, leaves close their stomata to retain water within their highly water impermeable leaf cuticles thus minimising water loss. Greater stomatal conductance is associated with smaller stomatal sizes and higher stomatal density across broad geological time scales and diverse evolutionary lineages (Drake *et al*. 2013), which as well as within a single species across environmental gradients (Franks *et al*. 2009). Smaller stomata also open and close faster across varied environments (Drake *et al*. 2013; Lawson and Blatt 2014). Therefore, g_s is

primarily determined by stomata morphological features. Heterogeneity of g_s within dicot leaves has been the focus of several recent studies, and heterogeneity of g_s is thought to respond to local leaf water status (Mott 1995; Kamakura *et al*. 2011). However, the heterogeneity of g_s along individual monocot leaf blades, and how leaf features affect that heterogeneity are still unclear.

It is recognised that g_m is an important limiting process for photosynthesis, as it significantly affects the process of CO_2 diffusion from substomatal cavities to chloroplast stroma. Rapid changes in g_m have been documented (Flexas *et al*. 2008; Warren 2008); however, these g_m changes vary with leaf structural parameters such as cell packing, shape and wall thickness (Tosens *et al*. 2012; Tomás *et al*. 2013). The relationship between CO_2 diffusion processes and morpho-anatomical traits has been well documented, and many related trade-offs have been described (Ocheltree *et al*. 2012; Giuliani *et al*. 2013; Tanaka *et al*. 2013; Muir *et al*. 2014). However, most of these results were obtained across species, cultivars or genetic stocks. The relationship between morpho-anatomical traits and g_m within individual leaves has received less attention. Nevertheless, results from studies on maize (Sasakawa *et al*. 1989) and sugarcane (Meinzer and Saliendra 1997) leaves suggest that longitudinal variation in carbon isotope discrimination within individual leaves may equal or exceed the magnitude of genetic variable. Such results indicate that g_m is varies along individual leaves.

Leaf nitrogen (N) content per leaf area in high plants is directly related to A, because stromal enzymes and thylakoid proteins account for the majority of leaf N (Terashima and Evans 1988; Evans 1989; Tazoe *et al*. 2006). Heterogeneity of leaf N within individual leaves, is considered an adaptation to light conditions, and was observed in sugarcane leaves (Meinzer and Saliendra 1997). The relationship between leaf N content per leaf area and A is principally affected by two factors: (i) N allocation between photosynthetic and non-photosynthetic N elements, and (ii) partitioning within photosynthetic apparatuses. As a limiting factor of photosynthetic capacity in full sunlight, ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) is a particularly important photosynthetic enzyme (Brooks and Farquhar 1985), and it comprises ~50% of photosynthetic nitrogen (Evans 1989; Hikosaka and Terashima 1996). Yamori *et al*. (2011) suggested that N partitioning into Rubisco and cytochrome *f* significantly affects the limiting step of A. Previous studies suggest that leaf N content per leaf area is also correlated with both g_s and g_m (Franks and Beerling 2009; Xiong *et al*. 2015). Incorporating variables such as leaf N content per leaf area will therefore improve our understanding of functional changes along leaf blades in rice.

Revealing the relationship among structural traits, chemical properties and A in individual leaves may lead to the identification of structural and chemical features for enhancing crop productivity and as well as provide insight into plant adaptation. The aims of this study were to: (i) identify variation in gas exchange, morpho-anatomical traits and chemical properties along a leaf blade from base to apex in rice, (ii) investigate how leaf morpho-anatomical traits and chemical properties (leaf N status) influence heterogeneity of A within leaves, and (iii) to examine whether plant biomass accumulation is impacted by heterogeneity of A within leaves.

Materials and methods

Plant Materials

Two rice cultivars Aus (*Oryza. australiansis* L.) and Lat (*Oryza. latifolia* L.) were investigated in a pot experiment at Huazhong Agricultural University, Wuhan, China. Plant materials were collected from the National Key Laboratory of Crop Genetic Improvement, Wuhan, China. Rice plants were grown in 15.0 L pots that filled with 13.0 kg soil with a density of three hills per pot and one seedling per hill. N, phosphorous (P) and potassium (K) were applied as basal fertilisers in amounts of 3.0, 1.95 and 1.95 g pot^{-1} respectively. Six pots per cultivars were randomly distributed. Throughout their whole growth, plants were well watered and a water depth of at least 2 cm was maintained. Pests were controlled using chemical pesticides. Measurements were started at 50 days after planting.

Gas exchange measurements

Gas exchange was measured between 0930 and 1530 hours in an environment-controlled room with an air temperature of 28.0 ± 2.5°C, photosynthetic photon flux density (PPFD) at leaf surface of $1200 \pm 50\ \mu mol\ m^{-2}\ s^{-1}$(artificial LED light source), and RH of $78.0\% \pm 5.0\%$. Measurements were performed on newly- and fully-expanded leaves of three plants of each cultivar after the plants were acclimated to the room environment for ~1.5 h. Each leaf was divided into 10 equal sections along leaf blades, and the middle of each section was labelled with a red marker. All measurements were centred on this midpoint (the first and last sections were excluded as accurate measurements for these sections were difficult). Gas exchange and chlorophyll fluorescence were simultaneously measured using two LI-6400XT portable photosynthesis systems equipped with 6400–40 leaf chamber (Li-Cor, Lincoln, NE, USA). Photosynthesis systems were zeroed before measurements were taken. The measurements were first made on the most basal section and then progressed sequentially along the length of the leaf blade towards the apex. Leaf temperature during measurements was maintained at 28°C. In the leaf chamber, PPFD was maintained at $1500\ \mu mol\ m^{-2}\ s^{-1}$, leaf-to-air vapour pressure deficit (VPD) at 1.2–1.4 kPa, and CO_2 concentration at $400\ \mu mol\ m^{-2}\ s^{-1}$ (maintained with a supplemental CO_2 mixture). After equilibration to a steady-state (usually occurring more than 20 min after clamping the leaf), the gas exchange parameters, steady-state fluorescence (F_s) and maximum fluorescence (F'_m) were recorded. Actual photochemical efficiency of PSII (Φ_{PSII}) was calculated as follows:

$$\Phi_{PSII} = \frac{(F'_m - F_s)}{F'_m}. \quad (1)$$

Electron transport rates (ETR) were computed as follows:

$$ETR = \Phi_{PSII} \cdot PPFD \cdot \alpha \cdot \beta, \quad (2)$$

where α is the leaf absorptance and β represents the distribution of electrons between PSI and PSII.

After measurement of gas exchange and chlorophyll fluorescence, a light response curve was determined for the middle section of each leaf, under a low O_2 concentration

(<2%) by estimating α and β. After equilibration to a steady state, the gas-exchange system was immediately switched to a low O_2 concentration (<2%) without removing the leaves from the chamber. Light response curves and chlorophyll fluorescence were then simultaneously measured. During these measurements, chamber conditions were identical to those described above, except that PPFD was controlled across a gradient of 800, 600, 400, 200, 100 and 0 μmol m^{-2} s^{-1}. After reaching a steady state, the parameters of gas exchange and chlorophyll fluorescence were simultaneously recorded. Under non-respiration conditions, the relationship between ETR calculated through gas exchange and chlorophyll fluorescence is expected to be linear because electron transport flow is primarily associated with Rubisco carboxylation and the α·β values do not change as light varies. Consequently, the slope of the relationship between ¼Φ_{PSII} and the quantum efficiency of CO_2 uptake (Φ_{CO_2}) is considered to be the value of α · β (Valentini *et al*. 1995; Long and Bernacchi 2003).

The variable *J* method described by Harley *et al*. (1992) was used to calculate CO_2 concentration in chloroplast (C_c) and mesophyll conductance of CO_2 (g_m). C_c was calculated as follows:

$$C_c = \frac{\Gamma^*(\mathrm{ETR} + 8(A + R_d))}{\mathrm{ETR} - 4(A + R_d)}, \quad (3)$$

where Γ* represents the CO_2 compensation point in the absence of respiration. Γ* is related to Rubisco specific factor and is relatively conserved under a given temperature condition. In the present study, a Γ* value of 40 μmol mol^{-1} and an R_d value of 1 μmol m^{-2} s^{-1} typically for rice plants were used (Yamori *et al*. 2011; Xiong *et al*. 2015). Then, g_m was calculated as follows:

$$g_m = \frac{A}{C_i - C_c}, \quad (4)$$

where C_i represents the intercellular CO_2 concentration.

Measurements of leaf N, chlorophyll and Rubisco content per leaf area

After measurement of gas exchange, leaf samples (longitudinal sections) were photo scanned and oven-dried separately at 80°C to constant weight. Leaf area was measured by using Image J software (National Institute of Health, Bethesda, MD, USA). The dry samples were digested by the micro-Kjeldahl method, and then the N concentration was measured with a discrete wet chemistry analyser (SmartChem 200, AMS-Westco, Rome, Italy). Absolute chlorophyll concentration was measured using a spectrophotometer (UV2102, Unico, Shanghai, China) and 95% (v/v) alcohol extracts of leaf tissue. Leaf tissue for these measurements was harvested using a circular punch that yields 0.5 cm diameter leaf discs.

Rubisco concentration was measured by the SDS–PAGE method (Makino *et al*. 1985). Leaf tissue was harvested using the aforementioned circular punch, and immersed in liquid nitrogen. The samples were stored at −80°C until Rubisco concentration assays were performed. The frozen leaf samples were ground in liquid nitrogen and homogenised on ice in an extraction buffer containing 50 mM Tris-HCl buffer (pH 8.0), 5 mmol β-mercaptoethanol, and 12.5% (v/v) glycerol. After centrifugation, SDS solution, β-mercaptoethanol, and glycerol were added to the supernatant fluid yielding final concentration of 2.0% (w/v), 4% (v/v) and 10% (v/v) respectively. This preparation is immediately treated at 100°C for 1 min, and then stored at −20°C until analysed by SDS–PAGE. The samples were loaded onto SDS–PAGE containing a 12.5% (w/v) polyacrylamide gel. After electrophoresis (DYY-11, Beijing Liuyi Instrument Factory, Beijing, China), the gels were washed with deionised water several times dyed in 0.25%. Coommassie blue staining solution for 9 h and decolourised until the background was colourless. The large and small subunits were transferred into a 10 mL cuvette with 2 mL of formamide and washed in a 50°C water bath for 8 h. The absorptions of washed solutions were measured at 595 nm (Infinite M200, Tecan Inc., Männedorf, Switzerland) using the background glue as blank and bovine serum albumin (BSA) as standard protein.

Microscopic analysis

Five small leaf discs (~10 × 10 mm) were removed from the middle of each leaf sections. For each cultivar, three leaves from different plants were measured. The leaf discs were infiltrated in a vacuum chamber (DZF-6050, Shanghai Hasuc Co. Ltd, Shanghai, China) with the fixative 2.5% glutaric aldehyde in 0.1 M phosphate buffer (pH = 7.6) at 4°C and then the samples were stored at 4°C until analysis. Images of the abaxial epidermal surfaces were captured under vacuum with a scanning electron microscopes (JSM-6390 LV, Tokyo, Japan). Stomatal size, stomatal density, guard cell length and the minor distance between the minor vein and stoma (D_s) on the abaxial lamina surface were calculated for each leaf based on 6–12 measurements.

Biomass and leaf morphology

Biomass samples were collected at 60 days after planting. The newly and fully expanded leaves were cut and the leaf width and length were measured quickly using a plastic ruler. After the leaf area was measured using a leaf area meter (LI-Cor 3000C, Li-Cor). For leaf area per plant, all green leaves per plant were collected and measured using the leaf area meter. Finally, the green and dead leaf blades, leaf sheaths and culms of each plant were collected, and the biomass determined by weighing the samples after drying for 5 days in an oven at 80°C.

Statistical analysis

One-way analysis of variance was calculated using SAS9.2 (SAS Institute Inc., Carey, NC, USA). The means of three replications were compared using the least significant difference (LSD) multiple comparison test ($P < 0.05$). Regression analyses were performed with mean values to test the correlations between parameters using SigmaPlot 12.5 (SPSS Inc., Chicago, IL, USA). All regressions were fitted by both linear and power models, and the model with higher regression coefficient was selected. Regression coefficients and significance are shown for $P < 0.05$.

Results

Growth characteristics and photosynthetic performance

There were significant differences in total biomass, tiller number, and leaf area between Lat and Aus (Table 1). Average tiller number, biomass, and leaf area per plant for Lat were 27.8, 127.5 and 83.9%, higher than those for Aus respectively. Both *A* and transpiration rate (*E*) increased from leaf blade base to apex for both cultivars (Fig. 1*a*, *b*). There were increases of ~105 and 51% of *A*, and ~80 and 70% of *E* along the leaf blade for Lat and Aus respectively. Compared with Aus, Lat had a lower value of *A* at its blade base and a higher value at its blade apex. However, *E* at both the blade base and apex in Lat were significantly higher than in Aus. Both g_s and g_m also increased from leaf blade base to apex in both cultivars (Fig. 1*c*, *d*). The two cultivars exhibited similar values of g_s at their blade bases, but the g_s values of Lat blade apexes were significantly higher than those of Aus. conversely, Aus exhibited higher values of g_m at its blade bases, but similar g_m values at its blade apexes.

Leaf N, Rubisco and chlorophyll content per leaf area

Along leaf blades (from base to apex), the leaf N content per leaf area, chlorophyll content per leaf area, Rubisco content per leaf area, Rubisco to leaf N ratio, and Rubisco to chlorophyll ratio increased significantly in both Lat and Aus (Fig. 2). However, the ratio of chlorophyll to leaf N decreased from leaf base to apex. The two cultivars had similar leaf N content per leaf area and chlorophyll content per leaf area at their blade bases, but Aus had a higher leaf N content per leaf area and Lat had a higher chlorophyll content at their blade apexes. The increase of Rubisco content per leaf area along the leaf blades was greater in Lat (from 1.6 to 7.6 μmol m^{-2}) than in Aus (from 4.1 to 7.0 μmol m^{-2}), which resulted the high variability of Rubisco to leaf N ratio in Lat.

Table 1. Plants growth and leaf characteristics for the two investigated cultivars

Mean ± s.d. values for three replicates (from three pots) are shown for tiller numbers, leaf area per plant, biomass, leaf area, and leaf length and leaf width at leaf midpoint. Different lowercase letters indicated the significant differences ($P < 0.05$) between the two cultivars

Cultivars	Tiller numbers	Leaf area (cm^2 $plant^{-1}$)	Biomass (g $plant^{-1}$)	Leaf area (cm^2)	Leaf length (cm)	Leaf width (cm)
Lat	7.67 ± 1.15a	2883.3 ± 166.0a	33.67 ± 1.30a	134.98 ± 6.68a	76.83 ± 4.53a	2.24 ± 0.08a
Aus	6.00 ± 1.00b	1534.3 ± 187.5b	14.80 ± 1.07b	50.19 ± 8.04b	50.93 ± 3.76b	1.23 ± 0.11b

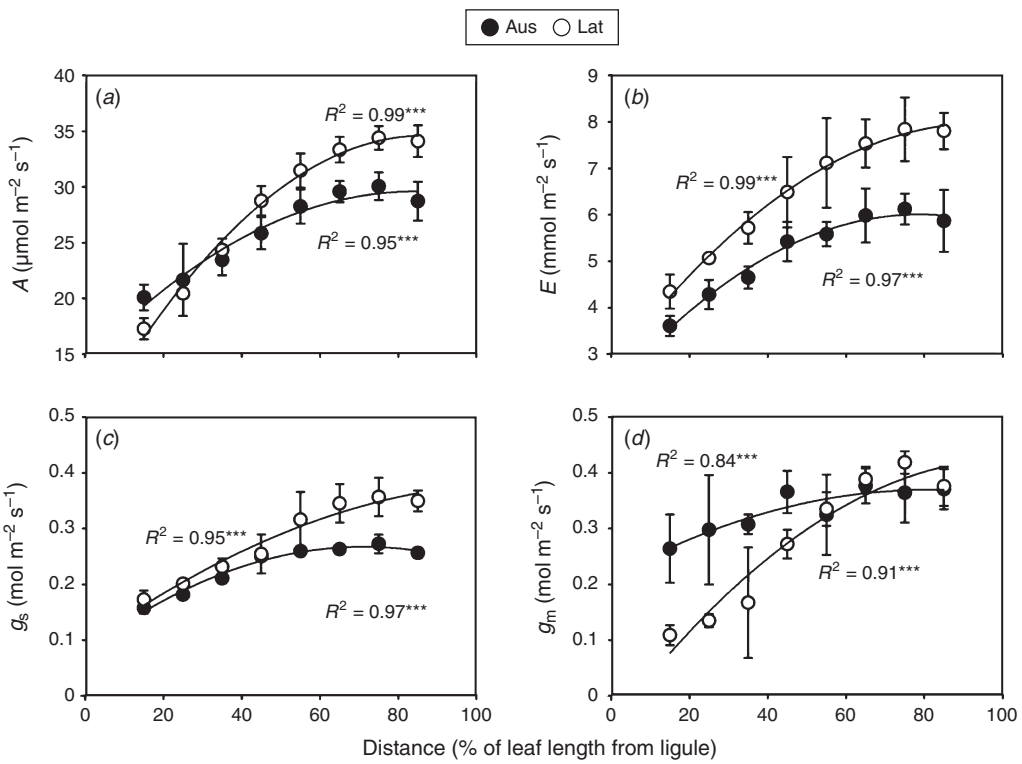

Fig. 1. Relationship of position along leaf blades (reported as the percentage of leaf length measured from ligule) with (*a*) photosynthetic rate (*A*), (*b*) transpiration (*E*), (*c*) stomatal conductance to CO_2 (g_s), (*d*) mesophyll conductance to CO_2 (g_m). The values shown are mean ± s.d. (significant differences are indicated: ***, $P < 0.001$).

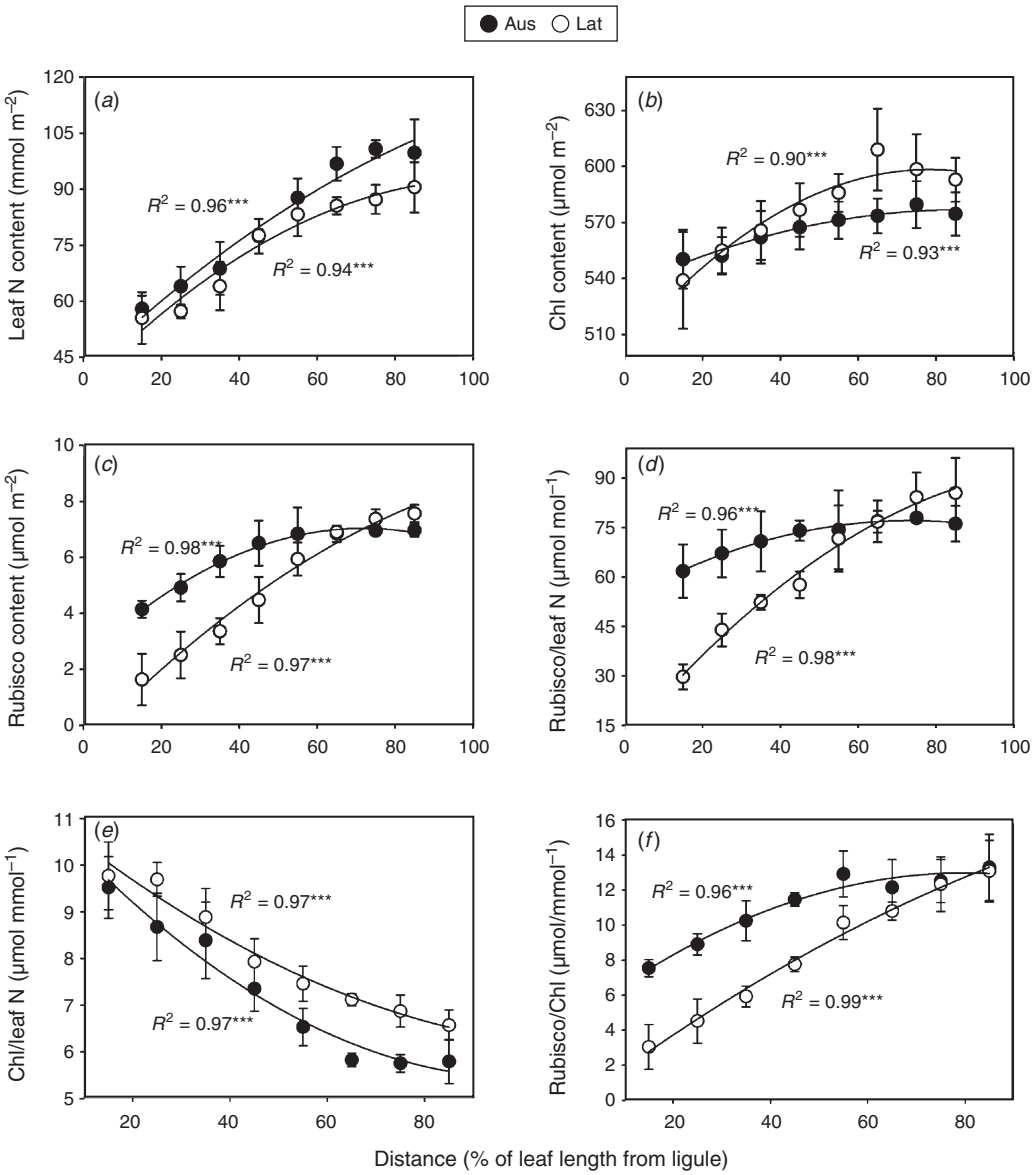

Fig. 2. Relationship of position along leaf blades (reported as the percentage of leaf length measured from ligule) with (*a*) leaf N content per leaf area, (*b*) chlorophyll content (Chl), (*c*) Rubisco content per leaf area, and the ratios of (*d*) Rubisco to leaf N, (*e*) chlorophyll to leaf N, (*f*) Rubisco to chlorophyll. The values shown are mean ± s.d. (significant differences are indicated: ***, $P < 0.001$).

Leaf anatomy

The leaf characteristics of plants analysed in this study are listed in Table 1. There were considerable differences between cultivars in leaf area, leaf length and leaf width. Leaf structure varied considerably along leaf blades (Fig. 3). The stomatal density increased considerably along leaf blades. However, the stomatal size decreased slightly, and the guard cell length was relatively constant along leaf blade in the two cultivars. The distance between stomata and the minor vein (D_s) considerably decreased within leaves of the both cultivars. At any given blade position, Lat had significantly higher stomatal densities than Aus; the stomatal densities ranged from 276 to 440 mm^2 for Lat and from 144 to 328 mm^2 for Aus. However, the stomatal sizes and guard cell lengths were significantly lower for Lat than for Aus. The two cultivars exhibited similar D_s at their blade bases; however, Aus had higher D_s than Lat at its blade apexes.

Coordination of gas exchange, structural and chemical traits

There were significant correlations between A and both g_s (Aus: $R^2 = 0.93$, $P < 0.001$; Lat: $R^2 = 0.98$, $P < 0.001$), and g_m (Aus: $R^2 = 0.98$, $P < 0.001$; Lat: $R^2 = 0.91$, $P < 0.001$) in the two rice cultivars (Fig. 4). We also observed a positive relationship between g_s and stomatal density (Aus: $R^2 = 0.97$, $P < 0.001$; Lat: $R^2 = 0.96$, $P < 0.001$), but a negative relationship between g_s and stomatal size in both cultivars (Aus: $R^2 = 0.87$, $P < 0.01$;

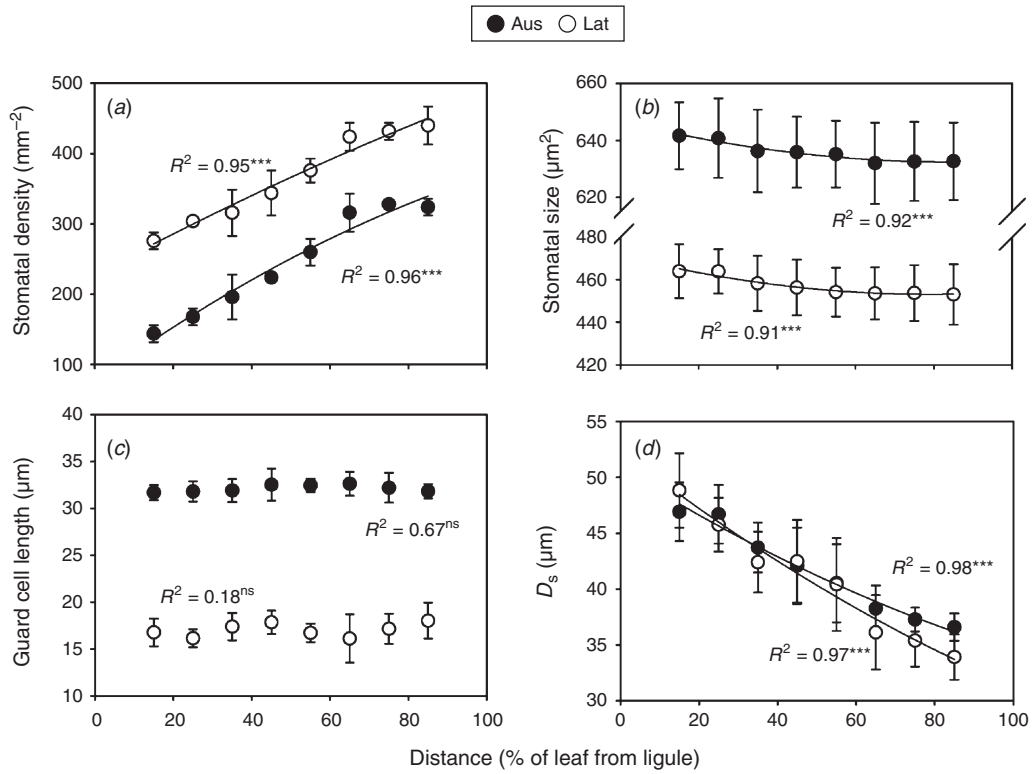

Fig. 3. Relationship of position along leaf blades (reported as the percentage of leaf length measured from ligule) with (*a*) stomatal density, (*b*) stomatal size, (*c*) guard cell length, and (*d*) distance between stomata and minor veins (D_s). The values shown are mean ± s.d. (significant differences are indicated: ns, no significant ($P > 0.05$); ***, $P < 0.001$).

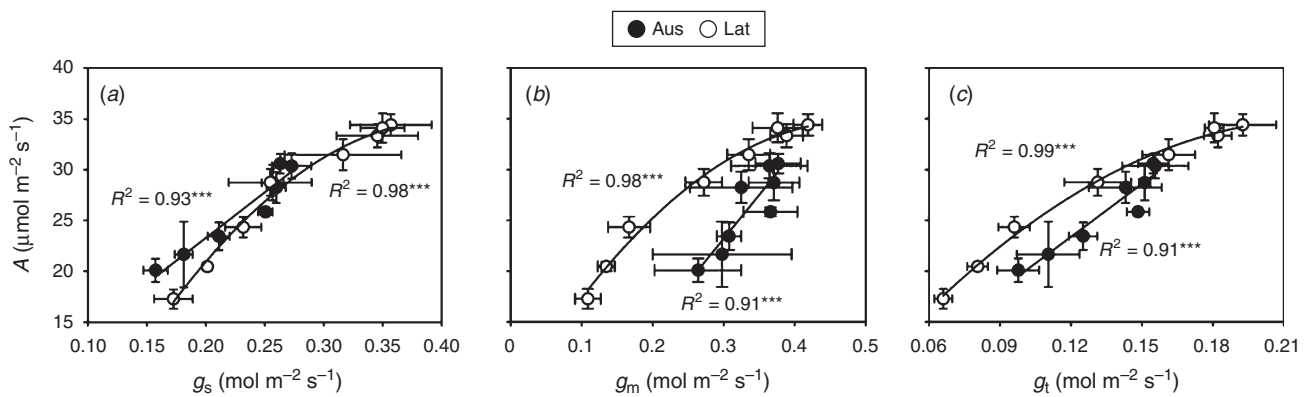

Fig. 4. Changes in photosynthesis (*A*) in relation to (*a*) stomatal CO_2 conductance (g_s), (*b*) mesophyll CO_2 conductance (g_m) and (*c*) total CO_2 conductance (g_t) along leaves. The values shown are mean ± s.d. (significant differences are indicated: ***, $P < 0.001$).

Lat: $R^2 = 0.88$, $P < 0.01$; Fig. 5). However, these correlation coefficients varied with cultivars. Furthermore, g_s was closely correlated with D_s in the both cultivars (Fig. 5*c*).

Leaf N content per leaf area was correlated with *A* in both rice cultivars (Fig. 6). The same pattern was observed between *A* and Rubisco content per leaf area. Similarly, leaf N content per leaf area was correlated with the Rubisco to leaf N ratio. In addition, the changes of g_s and g_m along rice leaf blades were closely correlated with leaf N content per leaf area (Fig. 7).

Discussion

We found substantial heterogeneity of *A* along rice leaf blades. In C_3 plants, *A* is mainly limited by CO_2 concentration in the chloroplast or carboxylation, and is often quantified with the widely used FvB model (Farquhar *et al.* 1980). For many years, the limitation of Rubisco content and activity on *A* has been confirmed, and CO_2 concentrations in chloroplasts were considered to be restricted by both g_s and g_m. The data presented here suggest that acropetal gradients of *A* are related to changes

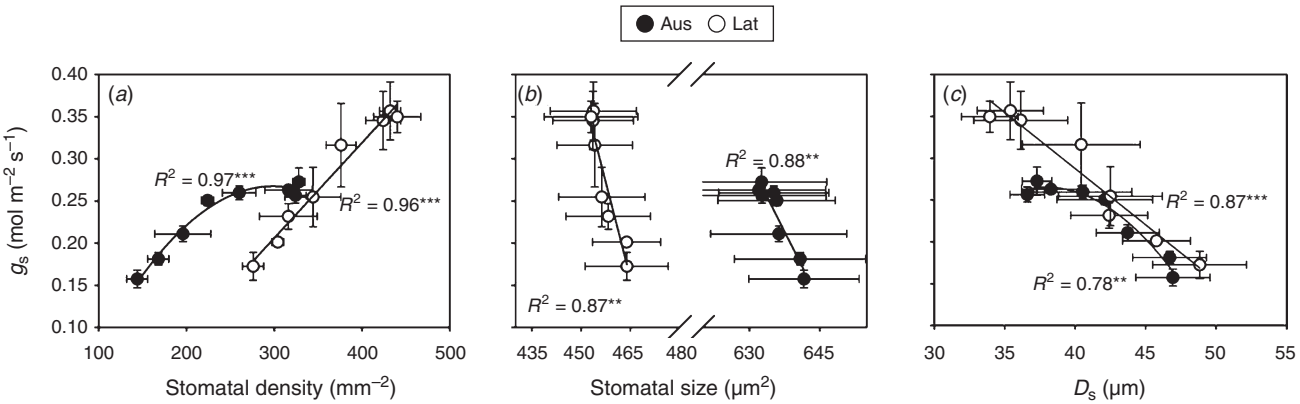

Fig. 5. Stomatal conductance (g_s) in relation to (*a*) stomatal density, (*b*) stomatal size, and (*c*) distance between stomata and minor veins (D_s). The values shown are mean ± s.d. (significant differences are indicated: **, $P < 0.01$; ***, $P < 0.001$).

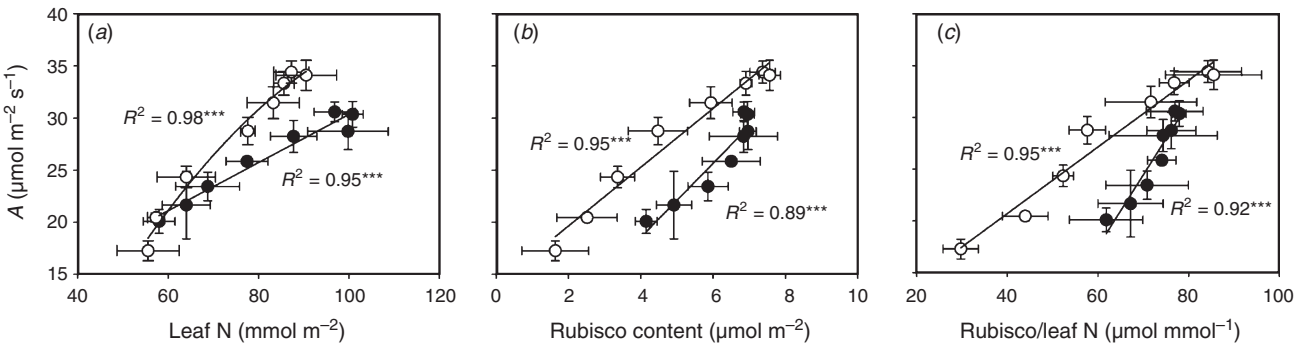

Fig. 6. Changes in photosynthesis (A) in relation to (*a*) leaf N content per leaf area, (*b*) Rubisco content per leaf area, and (*c*) the Rubisco to leaf N ratio along leaves. The values shown are mean ± s.d. (significant differences are indicated: ***, $P < 0.001$).

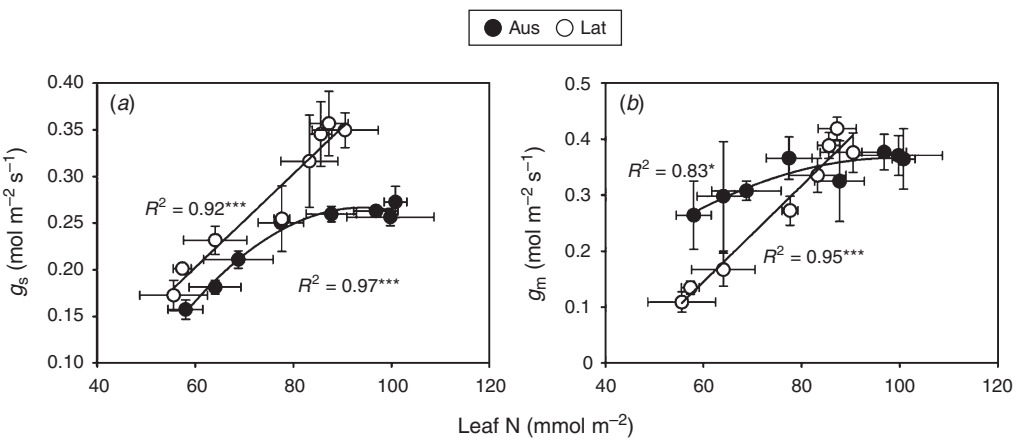

Fig. 7. Correlations of (*a*) stomatal CO_2 conductance (g_s) and (*b*) mesophyll CO_2 conductance (g_m) with changes in leaf N content along leaves. The values shown are mean ± s.d. (significant differences are indicated: *, $P < 0.05$; ***, $P < 0.001$).

in both leaf biochemistry (carboxylation), CO_2 diffusion conductance and leaf structural features.

Leaf biochemistry

It is widely acknowledged that A is closely related to leaf N content per leaf area in rice due to the close relationship between Rubisco content and leaf N content per leaf area (Evans 1989; Hikosaka and Terashima 1996). Here we observed that leaf N content, Rubisco content and chlorophyll content per leaf area are heterogeneous within individual leaf blades and the gradient of A along a leaf blade is related to the distribution of N within a leaf (Fig. 6). The mechanism by which N is heterogeneously

distributed within leaf blades is possibly related to an adaptation and the developmental dynamic (Wang *et al*. 2014) to the growth form of grasses. Under natural growth conditions, the basal portion of rice leaves may be shaded by tillers and the upper portions of leaves (Song *et al*. 2013). Resources, for example, leaf N, would be optimally allocated to the apical regions of leaves, where light availability is typically higher, to maximise A. Leaf N content per leaf area and gas change measured in the present study agree with the view that rice leaves have adapted to a growth form in which light gradients exist along leaf blades. The second piece of evidence supporting acropetal increases in A as an adaptation to the growth form of rice is the N allocation among photosynthetic apparatuses. In the present study we observed that allocated leaf N to chlorophyll decreased along the leaf blade from the base to apex; however, the allocated leaf N to Rubisco increased (Fig. 2). Rubisco is linked to carboxylation capacity, whereas chlorophyll is related to light capture. The changes of Rubisco and chlorophyll content within leaves indicate that the limitation factor of A is variable in a single leaf. This result suggests that leaves optimise resources allocation within each leaf to maximise A based on light distribution on leaf surface. Similar relationships between light and N content per leaf area have been reported in a range of plants canopy; leaves exposed to higher irradiance have higher N contents per leaf area, ratios of N to Rubisco, and A within crowns (Osada *et al*. 2014).

CO_2 diffusion conductance

Here, we found that CO_2 diffusion conductance varied greatly along leaf blade, and was related to A (Fig. 4). Along the CO_2 diffusion pathway, g_s is considered a primary factor in regulating diffusion of CO_2 from the ambient atmosphere into leaves (Hirasawa *et al*. 2010). In general, g_s is determined by both stomatal aperture characteristics (including size and density) and the opening and closing of the stomata. Within an individual leaf, water potential gradients exist between the base and the apex, especially under unfavourable conditions, for example, extremely low air humidity. Leaf apexes bear a large population of small stomata, which can respond rapidly to environmental changes (Drake *et al*. 2013) and reduce their apertures to counteract potentially high transpiration rates in dry air or high-wind-speed conditions (Drake *et al*. 2013; Aliniaeifard and van Meeteren 2014; Dow *et al*. 2014; Lawson and Blatt 2014); this, thus, minimises exposure to excessive water-potential gradients through the leaf and helps to protect plants from xylem embolisms.

The opening and closing of stomata can be regulated by water supplement (Mott and Peak 2007). Water flow within leaves can be divided into two components: flow within the xylem, and flow outside the xylem as water moves out of vascular bundle to sites of evaporation (Sack and Holbrook 2006; Scoffoni *et al*. 2008). The conductance of water within xylem remains relatively constant along the grass blades (Ocheltree *et al*. 2012), but the conductance of water outside the xylem is substantial source of resistance within leaves and is related to the internal structure of the leaves (Sack and Frole 2006; Brodribb *et al*. 2007). If path lengths for the movement of water from vascular bundles to the sites of evaporation decrease acropetally, as is consistent with the present study (Fig. 3*d*), then water movement efficiency outside the xylem would be increased therefore facilitating higher values of g_s.

After entering the stomatal pore, CO_2 must diffuse through both gas and liquid phases to reach carboxylation site. Recently, g_m, as well as g_s, has been recognised as other important limiting factors of A, especially in C_3 plants (Yamori *et al*. 2011; Flexas *et al*. 2012). Accordingly, g_m is an ecologically important limitation on photosynthetic performance and has been confirmed to vary greatly both between and within species. Here, we found that g_m varied considerably within leaf blades and significantly limited A in rice (Fig. 4*b*). The g_m gradient within leaves is likely related to leaf anatomical and structural features. Mesophyll cell wall thickness, mesophyll cell wall surface area exposed to intercellular airspace per leaf area (S_m), and surface area of chloroplasts exposed to intercellular airspaces (S_c) are the most important structural components of a leaf that related to g_m (Flexas *et al*. 2008; Tanaka *et al*. 2013). Increased S_m and S_c resulting from increased mesophyll lobes and enlarged single chloroplast volume and/or chloroplast number per mesophyll cell under high leaf N content conditions have been previously suggested (Li *et al*. 2009). In the present study, the correlated increase of g_m and leaf N content per leaf area from leaf base to apex could be related to the gradients of S_m and S_c.

Implications and conclusions

Genetic variations in leaf anatomical and functional traits have been well documented. Xiong *et al*. (2015) investigated A, g_s, g_m and several morph-anatomical traits in 11 rice cultivars, and showed that A varied from 17.6 to 35.9 $\mu mol\ m^{-2}\ s^{-1}$. The present study shows that A varied from 17.3 to 34.4 $\mu mol\ m^{-2}\ s^{-1}$ and 20.1 to 30.6 $\mu mol\ m^{-2}\ s^{-1}$ along the leaf blade in Lat and Aus respectively (Fig. 1). This gradient of A within leaves may maximise net primary productivity by optimising the use efficiency of natural resources, especially, light. Our results imply that the variation of A within individual leaves is similar to variation among cultivars, and thus variation of A within individual leaves should be more thoroughly considered in future breeding programs.

The heterogeneity of A within Lat leaves was higher than that within Aus leaves. The high biomass accumulation in Lat plants, principally due to the high leaf area. However, it is unlike that 83.9% high of leaf area can lead to 127.5% high of biomass in Lat. Our results suggest that this elevated heterogeneity of A may increase for biomass accumulation under natural conditions. Our results also highlight that functions within leaves are closely associated with leaf structural and chemical features. The increase in A along leaves demands more CO_2 and Rubisco. On one hand, increased stomatal density and decreased D_s along leaf blades allows faster transport of water, thus permitting stomata to remain open by improving the availability of water. On the other hand, carboxylation capacity also increased due to the increased N content and the ratio of N allocated to Rubisco along leaf blades. In the present study, Lat showed larger variation in A, leaf structural (i.e. D_s) and leaf chemical (i.e. Rubisco and the ratio of Rubisco to leaf N) features than Aus, and this may have resulted in its higher plant biomass accumulation. The present study provides strong

evidence that the coordination of leaf structure, chemistry, and functions within leaves substantially affects the performance of plants.

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References

Aliniaeifard S, van Meeteren U (2014) Natural variation in stomatal response to closing stimuli among *Arabidopsis thaliana* accessions after exposure to low VPD as a tool to recognise the mechanism of disturbed stomatal functioning. *Journal of Experimental Botany* **65**, 6529–6542. doi:10.1093/jxb/eru370

Brodribb TJ, Feild TS, Jordan GJ (2007) Leaf maximum photosynthetic rate and venation are linked by hydraulics. *Plant Physiology* **144**, 1890–1898. doi:10.1104/pp.107.101352

Brooks A, Farquhar G (1985) Effect of temperature on the CO_2/O_2 specificity of ribulose-1, 5-bisphosphate carboxylase/oxygenase and the rate of respiration in the light. *Planta* **165**, 397–406. doi:10.1007/BF00392238

Creese C, Oberbauer S, Rundel P, Sack L (2014) Are fern stomatal responses to different stimuli coordinated? Testing responses to light, vapor pressure deficit, and CO_2 for diverse species grown under contrasting irradiances. *New Phytologist* **204**, 92–104. doi:10.1111/nph.12922

Dow GJ, Berry JA, Bergmann DC (2014) The physiological importance of developmental mechanisms that enforce proper stomatal spacing in *Arabidopsis thaliana*. *New Phytologist* **201**, 1205–1217. doi:10.1111/nph.12586

Drake PL, Froend RH, Franks PJ (2013) Smaller, faster stomata: scaling of stomatal size, rate of response, and stomatal conductance. *Journal of Experimental Botany* **64**, 495–505. doi:10.1093/jxb/ers347

Evans JR (1989) Photosynthesis and nitrogen relationships in leaves of C_3 plants. *Oecologia* **78**, 9–19. doi:10.1007/BF00377192

Farquhar GD, von Caemmerer S, Berry JA (1980) A biochemical model of photosynthetic CO_2 assimilation in leaves of C_3 species. *Planta* **149**, 78–90. doi:10.1007/BF00386231

Flexas J, Ribas-Carbó M, Diaz-Espejo A, Galmés J, Medrano H (2008) Mesophyll conductance to CO_2: current knowledge and future prospects. *Plant, Cell & Environment* **31**, 602–621. doi:10.1111/j.1365-3040.2007.01757.x

Flexas J, Barbour MM, Brendel O, Cabrera HM, Carriquí M, Díaz-Espejo A, Douthe C, Dreyer E, Ferrio JP, Gago J, Gallé A, Galmés J, Kodama N, Medrano H, Niinemets Ü, Peguero-Pina JJ, Pou A, Ribas-Carbó M, Tomás M, Tosens T, Warren CR (2012) Mesophyll diffusion conductance to CO_2: An unappreciated central player in photosynthesis. *Plant Science* **193–194**, 70–84. doi:10.1016/j.plantsci.2012.05.009

Franks PJ, Beerling DJ (2009) Maximum leaf conductance driven by CO_2 effects on stomatal size and density over geologic time. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 10343–10347. doi:10.1073/pnas.0904209106

Franks PJ, Drake PL, Beerling DJ (2009) Plasticity in maximum stomatal conductance constrained by negative correlation between stomatal size and density: an analysis using *Eucalyptus globulus*. *Plant, Cell & Environment* **32**, 1737–1748. doi:10.1111/j.1365-3040.2009.002031.x

Giuliani R, Koteyeva N, Voznesenskaya E, Evans MA, Cousins AB, Edwards GE (2013) Coordination of leaf photosynthesis, transpiration, and structural traits in rice and wild relatives (Genus *Oryza*). *Plant Physiology* **162**, 1632–1651. doi:10.1104/pp.113.217497

Harley PC, Loreto F, Di Marco G, Sharkey TD (1992) Theoretical considerations when estimating the mesophyll conductance to CO_2 flux by analysis of the response of photosynthesis to CO_2. *Plant Physiology* **98**, 1429–1436. doi:10.1104/pp.98.4.1429

Hikosaka K, Terashima I (1996) Nitrogen partitioning among photosynthetic components and its consequence in sun and shade plants. *Functional Ecology* **10**, 335–343. doi:10.2307/2390281

Hirasawa T, Ozawa S, Taylaran RD, Ookawa T (2010) Varietal differences in photosynthetic rates in rice plants, with special reference to the nitrogen content of leaves. *Plant Production Science* **13**, 53–57. doi:10.1626/pps.13.53

Kamakura M, Kosugi Y, Takanashi S, Matsumoto K, Okumura M, Philip E (2011) Patchy stomatal behavior during midday depression of leaf CO_2 exchange in tropical trees. *Tree Physiology* **31**, 160–168. doi:10.1093/treephys/tpq102

Khush GS (2001) Green revolution: the way forward. *Nature Reviews. Genetics* **2**, 815–822. doi:10.1038/35093585

Lawson T, Blatt MR (2014) Stomatal size, speed, and responsiveness impact on photosynthesis and water use efficiency. *Plant Physiology* **164**, 1556–1570. doi:10.1104/pp.114.237107

Li Y, Gao Y, Xu X, Shen Q, Guo S (2009) Light-saturated photosynthetic rate in high-nitrogen rice (*Oryza sativa* L.) leaves is related to chloroplastic CO_2 concentration. *Journal of Experimental Botany* **60**, 2351–2360. doi:10.1093/jxb/erp127

Long SP, Bernacchi CJ (2003) Gas exchange measurements, what can they tell us about the underlying limitations to photosynthesis? Procedures and sources of error. *Journal of Experimental Botany* **54**, 2393–2401. doi:10.1093/jxb/erg262

Long SP, Zhu X-G, Naidu SL, Ort DR (2006) Can improvement in photosynthesis increase crop yields? *Plant, Cell & Environment* **29**, 315–330. doi:10.1111/j.1365-3040.2005.01493.x

Makino A, Mae T, Ohira K (1985) Enzymic properties of Ribulose-1,5-bisphosphate carboxylase/oxygenase purified from rice leaves. *Plant Physiology* **79**, 57–61. doi:10.1104/pp.79.1.57

Meinzer FC, Saliendra NZ (1997) Spatial patterns of carbon isotope discrimination and allocation of photosynthetic activity in sugarcane leaves. *Functional Plant Biology* **24**, 769–775.

Mitchell PL, Sheehy JE (2006) Supercharging rice photosynthesis to increase yield. *New Phytologist* **171**, 688–693. doi:10.1111/j.1469-8137.2006.01855.x

Mott KA (1995) Effects of patchy stomatal closure on gas exchange measurements following abscisic acid treatment. *Plant, Cell & Environment* **18**, 1291–1300. doi:10.1111/j.1365-3040.1995.tb00188.x

Mott KA, Peak D (2007) Stomatal patchiness and task-performing networks. *Annals of Botany* **99**, 219–226. doi:10.1093/aob/mcl234

Muir CD, Hangarter RP, Moyle LC, Davis PA (2014) Morphological and anatomical determinants of mesophyll conductance in wild relatives of tomato (*Solanum* sect. *Lycopersicon*, sect. *Lycopersicoides*; Solanaceae). *Plant, Cell & Environment* **37**, 1415–1426. doi:10.1111/pce.12245

Ocheltree TW, Nippert JB, Prasad PVV (2012) Changes in stomatal conductance along grass blades reflect changes in leaf structure. *Plant, Cell & Environment* **35**, 1040–1049. doi:10.1111/j.1365-3040.2011.02470.x

Osada N, Yasumura Y, Ishida A (2014) Leaf nitrogen distribution in relation to crown architecture in the tall canopy species, *Fagus crenata*. *Oecologia* **175**, 1093–1106. doi:10.1007/s00442-014-2966-y

Sack L, Frole K (2006) Leaf structural diversity is related to hydraulic capacity in tropical rain forest trees. *Ecology* **87**, 483–491. doi:10.1890/05-0710

Sack L, Holbrook NM (2006) Leaf hydraulics. *Annual Review of Plant Biology* **57**, 361–381. doi:10.1146/annurev.arplant.56.032604.144141

Sasakawa H, Sugiharto B, O'Leary MH, Sugiyama T (1989) $\delta^{13}C$ values in maize leaf correlate with phosphoenolpyruvate carboxylase levels. *Plant Physiology* **90**, 582–585. doi:10.1104/pp.90.2.582

Scoffoni C, Pou A, Aasamaa K, Sack L (2008) The rapid light response of leaf hydraulic conductance: new evidence from two experimental methods. *Plant, Cell & Environment* **31**, 1803–1812. doi:10.1111/j.1365-3040.2008.01884.x

Song Q, Zhang G, Zhu X-G (2013) Optimal crop canopy architecture to maximise canopy photosynthetic CO_2 uptake under elevated CO_2? A theoretical study using a mechanistic model of canopy photosynthesis. *Functional Plant Biology* **40**, 108–124. doi:10.1071/FP12056

Tanaka Y, Sugano SS, Shimada T, Hara-Nishimura I (2013) Enhancement of leaf photosynthetic capacity through increased stomatal density in *Arabidopsis*. *New Phytologist* **198**, 757–764. doi:10.1111/nph.12186

Tazoe Y, Noguchi K, Terashima I (2006) Effects of growth light and nitrogen nutrition on the organization of the photosynthetic apparatus in leaves of a C_4 plant, *Amaranthus cruentus*. *Plant, Cell & Environment* **29**, 691–700. doi:10.1111/j.1365-3040.2005.01453.x

Terashima I, Evans JR (1988) Effects of light and nitrogen nutrition on the organization of the photosynthetic apparatus in spinach. *Plant & Cell Physiology* **29**, 143–155.

Tomás M, Flexas J, Copolovici L, Galmés J, Hallik L, Medrano H, Ribas-Carbó M, Tosens T, Vislap V, Niinemets Ü (2013) Importance of leaf anatomy in determining mesophyll diffusion conductance to CO_2 across species: quantitative limitations and scaling up by models. *Journal of Experimental Botany* **64**, 2269–2281. doi:10.1093/jxb/ert086

Tosens T, Niinemets Ü, Westoby M, Wright IJ (2012) Anatomical basis of variation in mesophyll resistance in eastern *Australian sclerophylls*: news of a long and winding path. *Journal of Experimental Botany* **63**, 5105–5119. doi:10.1093/jxb/ers171

Valentini R, Epron D, De Angelis P, Matteucci G, Dreyer E (1995) *In situ* estimation of net CO_2 assimilation, photosynthetic electron flow and photorespiration in Turkey oak (*Q. cerris* L.) leaves: diurnal cycles under different levels of water supply. *Plant, Cell & Environment* **18**, 631–640. doi:10.1111/j.1365-3040.1995.tb00564.x

Wang L, Czedik-Eysenberg A, Mertz RA, Si Y, Tohge T, Nunes-Nesi A, Arrivault S, Dedow LK, Bryant DW, Zhou W, Xu J, Weissmann S, Studer A, Li P, Zhang C, LaRue T, Shao Y, Ding Z, Sun Q, Patel RV, Turgeon R, Zhu X, Provart NJ, Mockler TC, Fernie AR, Stitt M, Liu P, Brutnell TP (2014) Comparative analyses of C_4 and C_3 photosynthesis in developing leaves of maize and rice. *Nature Biotechnology* **32**, 1158–1165. doi:10.1038/nbt.3019

Warren CR (2008) Stand aside stomata, another actor deserves centre stage: the forgotten role of the internal conductance to CO_2 transfer. *Journal of Experimental Botany* **59**, 1475–1487. doi:10.1093/jxb/erm245

Xiong D, Yu T, Zhang T, Li Y, Peng S, Huang J (2015) Leaf hydraulic conductance is coordinated with leaf morpho-anatomical traits and nitrogen status in the genus *Oryza*. *Journal of Experimental Botany* **66**, 741–748. doi:10.1093/jxb/eru434

Yamori W, Nagai T, Makino A (2011) The rate-limiting step for CO_2 assimilation at different temperatures is influenced by the leaf nitrogen content in several C_3 crop species. *Plant, Cell & Environment* **34**, 764–777. doi:10.1111/j.1365-3040.2011.02280.x