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Original Article

Rapid responses of mesophyll conductance to changes of CO_2 concentration, temperature and irradiance are affected by N supplements in rice

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ABSTRACT

Photosynthesis in C_3 plants is significantly limited by mesophyll conductance (g_m), which can vary with leaf anatomical traits and nitrogen (N) supplements. Several studies have investigated the response of g_m to N supplements; however, none examined the implications of N supplements on the response of g_m to rapid environmental changes. Here we investigated the effect of N supplement on g_m and the response of g_m to change of CO_2, temperature and irradiance in rice. High N supplement (HN) increased mesophyll cell wall surface area and chloroplast surface area exposed to intercellular airspace per leaf area, and reduced cell wall thickness. These changes resulted in increased g_m. The g_m of leaves with HN was more sensitive to changes in CO_2 concentration, temperature and irradiance. The difference in leaf structural features between low N supplement and HN indicates that a rapid change in g_m is related to the regulation of diffusion through biological membranes rather than leaf structural features. These results will contribute to an understanding of the determinants of g_m response to rapid changes in environmental factors.

Key-words: environment change; leaf structure; nitrogen.

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important cereal crops in the world, and rice grain yield has doubled in the last 50 years. However, because of improved crop management and breeding strategies, harvest indices are approaching a threshold (Long *et al.* 2006). Therefore, enhancing the photosynthetic productivity of individual leaves within the canopy could be an efficient strategy for improving rice yield (von Caemmerer & Evans 2010; Raines 2011). Photosynthesis (A) of rice leaves is limited by CO_2 concentration at carboxylation sites (C_c) inside chloroplast (Li *et al.* 2009; Yamori *et al.* 2011; Adachi *et al.* 2013). During photosynthesis, CO_2 molecules in the air diffuse through the stomata into the substomatal cavities and then move via cell walls, plasmalemma, cytosol and chloroplast envelope membranes to the carboxylation sites in the stroma. Early studies suggested that mesophyll conductance (g_m; CO_2 diffusion conductance from substomatal cavities to carboxylation sites inside chloroplast) was infinite and that C_c was mainly limited by stomatal conductance (g_s). Subsequent studies showed that a large gradient of CO_2 concentration is present between substomatal cavities (C_i) and the chloroplasts (C_c) and that the mesophyll layers constitute an important barrier for CO_2 movement inside leaves (Evans *et al.* 2009).

The effect of leaf anatomical properties on g_m has been examined in many species (Peguero-Pina *et al.* 2012; Tosens *et al.* 2012; Giuliani *et al.* 2013; Tomas *et al.* 2013; Muir *et al.* 2014). Mesophyll cell wall thickness ($T_{cell\ wall}$), 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 anatomical parameters inside leaves that affect g_m (Evans *et al.* 2009; Scafaro *et al.* 2011; Terashima *et al.* 2011; Peguero-Pina *et al.* 2012; Muir *et al.* 2014).

In addition to these parameters, the effects of environmental factors on g_m have been widely studied. Long-term changes in g_m were observed under drought stress (Warren 2008; Flexas *et al.* 2009; Cano *et al.* 2013), salinity (Flexas *et al.* 2004) and nutrient supplement (Warren 2004; Li *et al.* 2009; Yamori *et al.* 2011). In contrast, rapid responses (within minutes) to changes in leaf temperature (Bernacchi *et al.* 2002; Warren & Dreyer 2006; Evans & von Caemmerer 2013; Walker *et al.* 2013; von Caemmerer & Evans 2014), CO_2 concentration (Flexas *et al.* 2007b, 2008, 2012; Douthe *et al.* 2011) and irradiance (Flexas *et al.* 2008, 2012; Douthe *et al.* 2011) have frequently been reported. Rapid changes of anatomical properties (i.e. S_c) and activity of aquaporins (cooporins), 'molecular channel' proteins in biological membranes, are

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considered as two possible reasons (Hanba *et al*. 2004; Flexas *et al*. 2006, 2008, 2012; Heckwolf *et al*. 2011; Mori *et al*. 2014). However, there is no consensus on the cause of such rapid responses of g_m to environmental changes. Furthermore, several other studies have reported g_m to be stable in response to changes in CO_2 concentration (von Caemmerer & Evans 1991; Tazoe *et al*. 2009) and irradiance (Tazoe *et al*. 2009).

Many researchers have investigated the uncertainties of g_m using different methods and have suggested that a rapid change in g_m with variable CO_2 concentration and irradiance might be a methodological artefact (Tholen *et al*. 2012; Gu & Sun 2014). There are two methods commonly used for estimating instantaneous g_m: online carbon isotope discrimination (Evans *et al*. 1986) and chlorophyll fluorescence (Harley *et al*. 1992). For both of these methods, one of the potential risks is that the theoretically predicted C_c is based on a measured C_i by introducing a finite g_m. Hence, methodological artefacts seem unavoidable when estimating g_m using these methods, and comparisons between different species and/or different treatments are a feasible approach to identify the responses of g_m to environmental changes.

To achieve optimum production in agricultural systems, crops are highly dependent on inputs of N fertilizer. A strong and positive correlation between A and leaf N content per leaf area has been demonstrated in many plants. Several studies have shown that N promotes A by increasing Rubisco (ribulose 1·5-bisphosphate carboxylase/oxygenase) content and CO_2 diffusion conductance (both g_s and g_m) in rice leaves (Li *et al*. 2009; Yamori *et al*. 2011). However, the mechanism of g_m increases with N supplement is unclear. Moreover, the relative importance of N supplements to the response of g_m to a rapid change in environmental factors is unknown.

In the present study, rice was grown in a pot experiment with two levels of N to investigate: (1) the effects of N supplement on rice photosynthesis; (2) the effects of N supplement on leaf structure and g_m; and (3) whether the response of g_m to CO_2, leaf temperature and irradiance is affected by N supplements.

MATERIALS AND METHODS

Plant materials

The experiment was conducted outdoors at the campus of Huazhong Agricultural University, Wuhan, China. Rice seeds (c.v. Heshengwanyou) were bought from the local market. After germination on moist filter paper for 24 h, the seeds were sown into 15 L pots filled with 13 kg of soil. The P and K were applied as basal fertilizers at a rate of 1.95 and 1.95 g per pot, respectively. Urea was applied at 0.5 g N per pot for the low N supplement (LN) treatment and at 3.0 g N per pot for the high N supplement (HN). Four replicated pots were planted for each treatment. After emergence, the plants were thinned to 3 hills (1 plant per hill) per pot. Plants were watered daily, and a 2 cm layer of water was maintained to avoid water deficit. Pests were controlled using chemical pesticides.

Gas exchange

We used an open-flow gas exchange system (LI-6400XT; Li-Cor, Lincoln, NE, USA) with an integrated fluorescence leaf chamber (LI-6400-40; Li-Cor) to simultaneously measure leaf gas exchange and chlorophyll fluorescence. To avoid the effect of fluctuating environments on gas exchange measurements, all plants were measured between 0830 and 1630 h in a controlled environment room with an air temperature of 27.8 ± 2.1 °C, a photosynthetic photon flux density (PPFD) at leaf surface of 1200 ± 47 μmol m^{-2} s^{-1}, and a relative humidity of 77 ± 5%. To minimize leaf position and age effects, measurements were taken on the newest fully expanded leaves after the plants were acclimatized in the room for approximately 1.5 h. In the Li-Cor leaf chamber, ambient CO_2 concentration was adjusted to 400 μmol mol^{-1} with a CO_2 mixture, leaf temperature was maintained at 25 °C, PPFD was 1500 μmol m^{-2} s^{-1} with a 10:90 blue : red light, leaf-to-air vapour pressure deficit (VPD) was between 1.1 and 1.4 kPa, and the flow rate was 300 μmol s^{-1}. After the leaf reached a steady state, usually after 10 min, gas exchange parameters, steady-state fluorescence (F_s) and maximum fluorescence (F_m') with a light-saturating pulse of 8000 μmol m^{-2} s^{-1} were recorded. Measurements were taken to construct an A/C_i curve by adjusting the ambient CO_2 concentration to 300, 200, 150, 100, 50, 400, 600, 800 and 1000 μmol mol^{-1}, without removing the leaf from the chamber. The aim was to estimate the response of g_m to rapidly changing C_i. Three dead rice leaves (obtained by heating the leaves until no variable chlorophyll fluorescence was observed) per treatment were used to estimate the leakage effects of the chamber (Flexas *et al*. 2007a) under different CO_2 concentrations. The leakage values between two N treatments did not differ significantly, so the average relationship was used to correct the measured A/C_i curves. To estimate the response of g_m to rapidly changing leaf temperature, simultaneous leaf gas exchange and chlorophyll fluorescence measurements were performed at five temperature levels (20, 25, 30, 35 and 40 °C). At each temperature, the CO_2 concentration and PPFD in the leaf chamber were controlled as above. For the leaf responses to light variations, measurements were conducted under saturating light (1500 μmol m^{-2} s^{-1} of PPFD) at steady-state gas exchange and fluorescence measurements were recorded, and then the light was decreased to 600 μmol m^{-2} s^{-1} PPFD.

The actual photochemical efficiency of photosystem II (Φ_{PSII}) was calculated as follows:

$$\Phi_{PSII} = \frac{(F_m' - F_s)}{F_m'}.$$

The electron transport rate (J) was then calculated as follows:

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

where α is the leaf absorptance and β is the partitioning of absorbed quanta between PSII and PSI. The product $\alpha\beta$ was

estimated from the slope of the relationship between Φ_{PSII} and $4\Phi_{CO_2}$ (i.e. the quantum efficiency of gross CO_2 fixation), which was obtained by measuring the photosynthetic light response curves under non-photorespiration conditions (i.e. O_2 <1%). There were no differences in $\alpha\beta$ between LN and HN leaves, thus eliminating out any confounding effect of different leaf optical properties as a result of N nutrition between the treatments.

The variable *J* method described in Harley *et al*. (1992) was used to calculate g_m and C_c. C_c and g_m were calculated as follows:

$$C_c = \frac{\Gamma^*(J + 8(A + R_d))}{J - 4(A + R_d)},$$

$$g_m = \frac{A}{C_i - C_c},$$

where Γ^* represents the CO_2 compensation point in the absence of respiration. For each data point generated, we checked whether it met the criterion $(10 > dC_c/dA > 50)$ (Harley *et al*. 1992).

The day respiration (R_d) and the apparent CO_2 photocompensation point (C_i^*) were determined at five leaf temperatures using the Laisk method. Briefly, the A/C_i curves were measured over the linear portion of the response curve (at 400 to 50 μmol CO_2 mol^{-1} air) under three PPFD (50, 250 and 500 μmol m^{-2} s^{-1}) with an LI 6400-02B chamber (Li-Cor), and then linear regressions to the responses for each PPFD were fitted for individual leaves. Then the intersection point of three A/C_i curves was considered as C_i^* (*x*-axis) and R_d (*y*-axis) (von Caemmerer *et al*. 1994). Γ^* was calculated as follows:

$$\Gamma^* = C_i^* + \frac{R_d}{g_m}.$$

Recently, Gu and Sun (2014), through a simulation approach, pointed out that using variable *J* to estimate the response of g_m to environmental factors can be profoundly impacted by methodological artefacts. They also identified three sets of covariable parameters: (1) R_d and Γ^* from the Laisk method; (2) a wrong assumption with respect to processes that limit the RuBP regeneration; and (3) biases in the measurements of C_i, *A* and *J*. During the measurement, we accounted for measurement biases (i.e. leakage effects of the chamber) and it seemed that C_i, *A* and *J* were reliable. To identify the effects of R_d and Γ^* on the response of g_m to C_i and PPFD in both LN and HN treatments, sensitivity analyses (Douthe *et al*. 2011) were conducted using a wide range of R_d and Γ^* from rice based on previous literature (Supporting Information Table S1). R_d and Γ^* are sensitive to temperature and their temperature responses can be modelled with an exponential equation (Bernacchi *et al*. 2002). However, there are no available scaling constants and activation energy parameters in rice, therefore we used the average values of two N treatments under each leaf temperature to analyse the effects of R_d and Γ^* on the response of g_m to leaf temperature. RuBP regeneration requires both NADPH and ATP. According to the Farquhar model, the relationship between *A* and *J* can be expressed as follows:

$$A = \frac{J(C_c - \Gamma^*)}{p_1 C_c + p_2 \Gamma^*} - R_d.$$

The values of p_1 and p_2 depend on the limited steps of RuBP regeneration. If RuBP regeneration is limited by NADPH, $p_1 = 4$ and $p_2 = 8$. If RuBP regeneration is limited by insufficient ATP, then based on two different assumptions regarding the Q cycle operation and the proton requirement for synthesizing ATP, we used $p_1 = 4.5$ and $p_2 = 10.5$ or $p_1 = 4$ and $p_2 = 9.33$, respectively. Here we analysed all three p_1 and p_2 sets on g_m response to leaf temperature, CO_2 concentration and PPFD.

Leaf N, chlorophyll and Rubisco content

After the leaf area measurements (Li-Cor 3000C; Li-Cor), each of the leaf samples were cut into small sections (~5 mm). Absolute chlorophyll concentration measurements were conducted using 95% (v/v) alcohol extracts of leaf tissue and a spectrophotometer (UV2102; Unico, Shanghai, China). The samples for leaf N measurement were oven dried at 80 °C to constant weight after leaf area measurement, and ground using a mixer mill homogenizer (MM400; Retsch, Haan, Germany). A subsample of 5.0 mg was used to measure N concentration using an NC analyser (IsoPrime100 IRMS; Isoprime Ltd, Stockport, UK).

The Rubisco content was measured using the SDS–PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) method (Makino *et al*. 1985). Leaf tissue was harvested using a 1 cm^2 circular punch, and immersed in liquid nitrogen and then stored at –80 °C before measuring. The frozen leaf sample was ground with liquid nitrogen on ice and homogenized in an extraction buffer [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 to a final concentration of 2.0% (w/v), 4% (v/v) and 10% (v/v), respectively. This preparation was immediately treated at 100 °C for 1 min, and then the samples were loaded onto SDS–PAGE containing a 12.5% (w/v) polyacrylamide gel. After electrophoresis, the gels were washed with deionized water several times and then dyed in 0.25% Coomassie blue staining solution (Sigma-Aldrich, St. Louis, USA) for 9 h and decolourized until the background was colourless. The large subunits and relevant small subunits were transferred into a 10 mL cuvette with 2 mL of formamide and then washed in a 50 °C water bath at room temperature for 8 h. The washed solutions were measured at 595 nm (Infinite M200; Tecan Männedorf, Switzerland) and bovine serum albumin (BSA) was used as a standard protein.

Leaf structure

Following the A/C_i curve measurements, the small leaf discs (4.0 mm × 1.2 mm) from within the gas exchange chamber

Table 1. Effects of N supplement on tiller number, leaf area (LA), biomass (BM), leaf N content per leaf area, chlorophyll content per leaf area and Rubisco content per leaf area in rice

Treatment	Tillers (hill)	LA (cm^2 per hill)	BM (g per hill)	Leaf N ($g\ m^{-2}$)	Chlorophyll content ($\mu mol\ m^{-2}$)	Rubisco ($\mu mol\ m^{-2}$)
LN	6.0 ± 1.0b	530 ± 51 b	4.37 ± 0.26b	0.66 ± 0.05b	469 ± 29b	3.23 ± 0.85b
HN	13.3 ± 1.5a	1273 ± 187a	12.12 ± 1.77a	1.12 ± 0.03a	570 ± 37a	5.55 ± 0.64a

Data are mean ± SE. Different letters indicate statistically significant differences ($P < 0.05$) between LN and HN. The tiller number, LA and BM were measured at 50 d after sowing.
LN, low N supplement; HN, high N supplement.

were removed and infiltrated in a syringe withthe fixative 2.5% glutaric aldehyde in 0.1 M phosphate buffer (pH = 7.6) at 4 °C, and post-fixed in 2% buffered osmium tetroxide at 20 °C for 2 h. The samples were embedded in Spurr's epoxy resin (Sigma-Aldrich, St. Louis, USA). For light microscopy, semithin leaf cross sections were stained with toluidine blue, and observed at 200× magnification with an Olympus IX71 light microscope (Olympus Optical, Tokyo, Japan). Ultrathin leaf cross sections were stained with 4% (w/v) uranyl acetate followed by 2% (w/v) lead citrate. Transmission electron microscope (H-7650; Hitachi – Science & Technology, Tokyo, Japan) and Soft Imaging System software (H-7650; Hitachi – Science & Technology, Tokyo, Japan) were used for observation and photography.

The total length of the mesophyll cell wall exposed to intercellular airspace (l_m), the total length of chloroplasts touching the plasma membrane appressed to intercellular airspace (l_c), and the width of the analysed leaf cross section (L) were measured using Image J software (National Institute of Health, Bethesda, MD, USA). S_m and S_c were then calculated as follows:

$$S = \frac{l}{L} \times F,$$

where S is S_m or S_c, l is l_m or l_c, and the F is the curvature correction factor, 1.42 according to previous studies (Scafaro *et al*. 2011; Giuliani *et al*. 2013).

Statistical analysis

One-way analysis of variance was calculated using SAS9.2 (SAS Institute Inc. Carey, NC, USA). The mean values were compared using the least significant difference (LSD) multiple comparison test ($P < 0.05$).

RESULTS

Effects of different N supplements on plant performance and leaf N content

The mean aboveground biomass in HN rice plants was nearly three times higher than in LN plants at 50 d after sowing (Table 1). At the same growth stage, tiller number and leaf area were respectively 3.8 and 2.4 times higher in HN than in LN. Leaf N content, chlorophyll content and Rubisco content per leaf area increased with increasing N supplement in rice plants. However, as the leaf N content per leaf area increased, the increase in Rubisco content per leaf area (72%) was greater than the increase in chlorophyll content per leaf area (22%).

Effects of different N supplements on leaf gas exchange

A significantly higher photosynthetic rate (A) in HN leaves was observed in rice (Table 2), and it was systematically associated with a higher dark respiration rate (R_d), stomatal conductance (g_s), mesophyll conductance (g_m), intercellular CO_2 compensation point (C_i*) and chloroplastic CO_2 compensation point (Γ*) in HN leaves compared with LN leaves. However, the intercellular CO_2 concentration (C_i) and CO_2 concentration at carboxylation sites inside chloroplasts (C_c) in HN leaves were lower than in LN leaves.

Effects of different N supplements on leaf anatomical and structural traits

Significant differences in mesophyll cell wall thickness ($T_{cell\ wall}$), mesophyll cell wall surface area exposed to

Table 2. Mean values for the photosynthetic parameters at 25 °C

Treatment	A ($\mu mol\ m^{-2}\ s^{-1}$)	R_d ($\mu mol\ m^{-2}\ s^{-1}$)	g_s ($mol\ m^{-2}\ s^{-1}$)	C_i ($\mu mol\ mol^{-1}$)	g_m ($mol\ m^{-2}\ s^{-1}$)	C_c ($\mu mol\ mol^{-1}$)	C_i* ($\mu mol\ mol^{-1}$)	Γ* ($\mu mol\ mol^{-1}$)
LN	19.3 ± 1.8 b	0.99 ± 0.07 b	0.23 ± 0.02 b	243 ± 8 a	0.19 ± 0.07 b	134 ± 7 a	41.3 ± 0.7 b	39.9 ± 0.6 b
HN	36.7 ± 2.0 a	1.45 ± 0.17 a	0.39 ± 0.04 a	219 ± 13 b	0.39 ± 0.08 a	124 ± 5 b	44.3 ± 0.8 a	43.3 ± 1.2 a

Data are mean ± SE. Different letters indicate statistically significant differences ($P < 0.05$) between LN and HN.
A, photosynthetic rate; R_d, day respiration; g_s, stomatal conductance; C_i, substomatal CO_2 concentration; g_m, mesophyll conductance to CO_2; C_c, chloroplastic CO_2 concentration; C_i*, apparent CO_2 photocompensation point; Γ*, CO_2 compensation point in the absence of respiration; LN, low N supplement; HN, high N supplement.

Table 3. Effects of N on leaf anatomical and structural traits in rice

Treatment	LMA (g m^{-2})	T_{leaf} (mm)	$T_{cell\ wall}$ (μm)	S_m ($\mu m^2\ \mu m^{-2}$)	S_c ($\mu m^2\ \mu m^{-2}$)
LN	41.3 ± 2.3	0.21 ± 0.04	0.21 ± 0.02a	12.8 ± 2.2b	7.2 ± 1.5b
HN	39.9 ± 0.4	0.24 ± 0.03	0.18 ± 0.01b	15.6 ± 0.6a	12.2 ± 2.2a

Data are mean ± SE. Different letters indicate statistically significant differences ($P < 0.05$) between LN and HN.
LMA, leaf mass per leaf area; T_{leaf}, leaf thickness; $T_{cell\ wall}$, the thickness of mesophyll cell wall; S_m, mesophyll cell wall surface area exposed to intercellular airspace per leaf area; S_c, surface area of chloroplasts exposed to intercellular airspace per leaf area; LN, low N supplement; HN, high N supplement.

intercellular airspace per leaf area (S_m), and the chloroplast surface area exposed to intercellular airspace per leaf area (S_c) between the two N treatments were observed (Table 3). S_m and S_c increased with increasing N supplement but $T_{cell\ wall}$ slightly decreased. However, there was a slight increase in leaf thickness (T_{leaf}), but a decrease (although not significant) in leaf mass per leaf area (LMA) in the HN. The chloroplasts were significantly enlarged by HN (Fig. 1).

Responses of gas exchange to rapid changes of CO_2, leaf temperature and PPFD

Fast intercellular CO_2, response (A/C_i) curves were analysed for the two N treatments (Fig. 2). The initial slope of the A/C_i response curve in HN leaves was higher than in LN leaves. The maximum light-saturated photosynthetic rates were 41.2 and 28.9 μmol m^{-2} s^{-1} in the HN and LN, respectively. Both g_s and g_m decreased with increasing C_i under two N supplement conditions. However, the g_m was more sensitive to increasing C_i in HN leaves than in LN leaves.

There were similar temperature response patterns of A in both HN and LN leaves (Fig. 3). Photosynthesis (A) increased with leaf temperature from 20 to 35 °C and then decreased. The optimum temperature at which A was maximal was independent of the N supplements. However, the response patterns of g_s and g_m to leaf temperature differed markedly between HN and LN. The g_s in LN leaves was decreased from 25 to 20 °C, and was constant from 25 to 40 °C. In HN leaves, g_s increased with increasing leaf temperature. In addition to leaf temperature, g_s was significantly affected by leaf-to-air vapour pressure difference (VPD) (Supporting Information Fig. S1). Similar to g_s, the temperature response of g_m differed markedly between the two N supplements. In HN leaves, there was more than a twofold increase in g_m between 20 and 40 °C, whereas there was only a slight increase in LN leaves (Fig. 3).

In both HN and LN leaves, A and g_s significantly decreased with decreasing irradiance (PPFD) (Fig. 4). However, the response of g_m to PPFD differed with N supplement. On average, g_m at a PPFD of 600 μmol m^{-2} s^{-1} was 40% less than that at 1500 μmol m^{-2} s^{-1} in HN leaves. The g_m in LN leaves did not respond to changes of PPFD.

Sensitivity analysis

The responses of g_m to changing environmental factors, estimated by the variable J method, were significantly affected by parameter inputs. In the present study, g_m values were significantly impacted by Γ^*, R_d, and p_1 and p_2 sets (Fig. 5; Supporting Information Figs S3 & S4). Values of g_m increased with increasing Γ^*, but decreased with increasing R_d. However, there was no change in the response pattern of g_m between LN and HN leaves when different parameters were used.

DISCUSSION

N supplement improved photosynthesis by enhancing the carboxylation and CO_2 diffusion process

In the present study, leaf area and A were enhanced by the HN which resulted in large biomass accumulation in rice. A positive relationship between leaf N content per leaf area and A was reported in numerous previous studies (Joha 1989; Li *et al*. 2009; Yamori *et al*. 2011; Xiong *et al*. 2015b). Generally, A in C_3 plants is considered to be limited by Rubisco carboxylation capacity and/or C_c, which is determined by the CO_2 diffusion efficiency (von Caemmerer & Evans 2010; Raines 2011). Here, we demonstrated that Rubisco content which determines the maximum activity of Rubisco and carboxylation efficiency (the initial slope of A/C_i curve) was increased in HN leaves. The g_s and g_m, as well as Rubisco carboxylation capacity, were higher in HN leaves than in LN leaves. However, a lower C_c in HN leaves suggests that this is the main limit to A under HNs.

N supplements on CO_2 diffusion

In the present study, we found that g_s was enhanced by the N supplement. The g_s is related to stomatal features (size and density) and stomatal opening status. Further study should address how stomatal size, density and opening are affected by leaf N status. Leaf structural features are believed to play a central role in determining g_m. $T_{cell\ wall}$, S_m and S_c have been recognized as the most important structural features limiting g_m (Evans *et al*. 2009; Scafaro *et al*. 2011; Terashima *et al*. 2011; Peguero-Pina *et al*. 2012; Muir *et al*. 2014). In the present study, a slight response of $T_{cell\ wall}$ and S_m, and a large response of S_c to N supplements were found in rice. Interestingly, the amplification of S_c from LN to HN was almost equal to the increase in g_m. This finding highlights that S_c plays a central role in determining g_m.

Aquaporins are water channels and have been demonstrated in both animals and plants, and recently, aquaporins

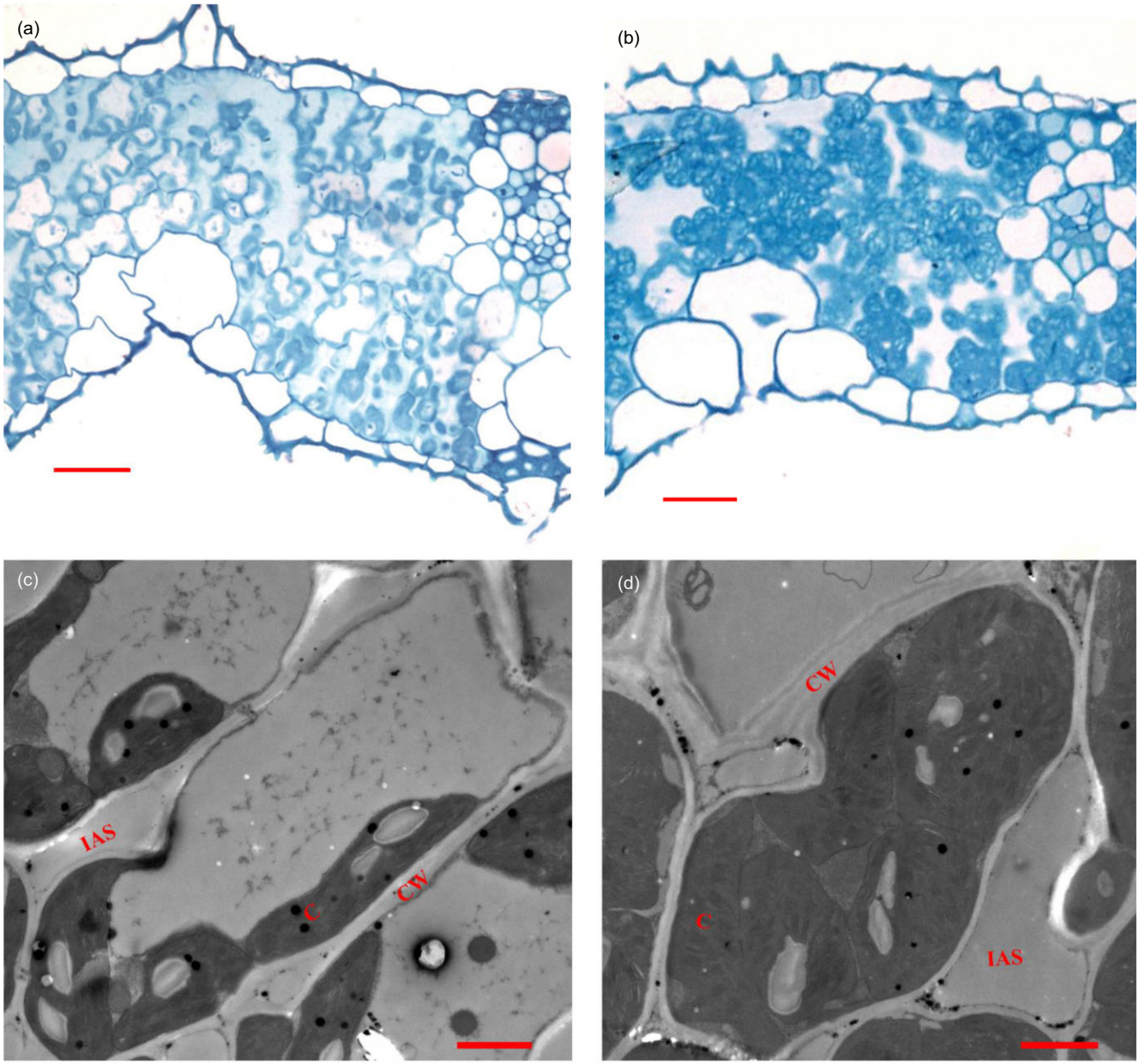

Figure 1. Light (a, b) and transmission electron (c, d) microscope images of LN (a, c) and HN (b, d) leaves in rice. C, chloroplast; CW, mesophyll cell wall; IAS, intercellular airspace. Bars represent 50 μm in (a) and (b) and 2 μm in (c) and (d).

have been found to act as CO_2 channels in many plants such as barley (Mori *et al*. 2014), rice (Hanba *et al*. 2004) and *Arabidopsis* (Heckwolf *et al*. 2011). Several studies have shown that aquaporin gene expressions are affected by nutrient conditions; for instance, expression of the PIP2 aquaporin gene family is promoted by N fertilization (Clarkson *et al*. 2000; Guo *et al*. 2007; Hacke *et al*. 2010). High expression of aquaporins would benefit CO_2 and H_2O transmembrane transport and consequently increase both g_s and g_m. Therefore, in addition to the observed covariation of anatomical traits, it is likely that other processes, such as aquaporins, influenced the increase of g_s and g_m in HN leaves.

Rapid changes of environmental factors on g_m

The rapid response of g_m to changes in ambient CO_2 concentration, leaf temperature and PPFD has been extensively studied. Negative relationships between g_m and CO_2 concentration (Flexas *et al*. 2007b, 2008, 2012; Douthe *et al*. 2011) and positively correlated with PPFD (Flexas *et al*. 2008, 2012; Douthe *et al*. 2011) and leaf temperature (Bernacchi *et al*. 2002; Warren & Dreyer 2006; Evans & von Caemmerer 2013; Walker *et al*. 2013; von Caemmerer & Evans 2014) have been reported, whereas some studies have found no relationship (von Caemmerer & Evans 1991; Tazoe *et al*. 2009). One

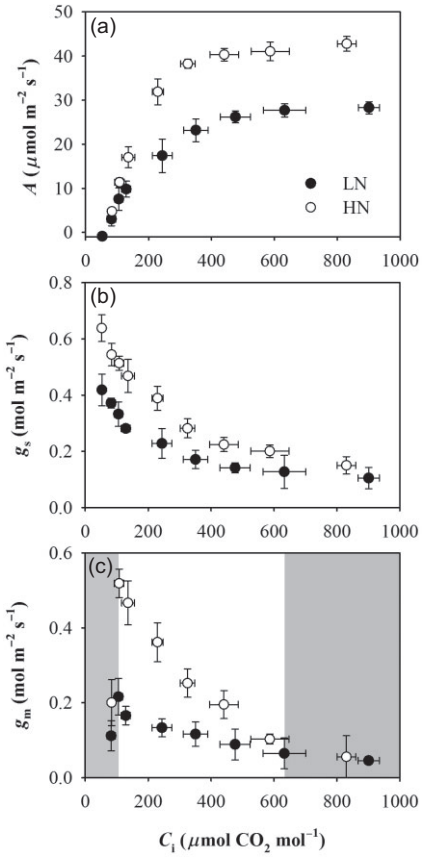

Figure 2. Effects of N supplement on photosynthesis (a), stomatal conductance (b) and mesophyll conductance (c) response to CO_2 at 25 °C. Values are mean ± SE of three replicates. Unshaded regions indicate g_m data with the dC_c/dA between 10 and 50, which are reliable according to Harley *et al.* (1992).

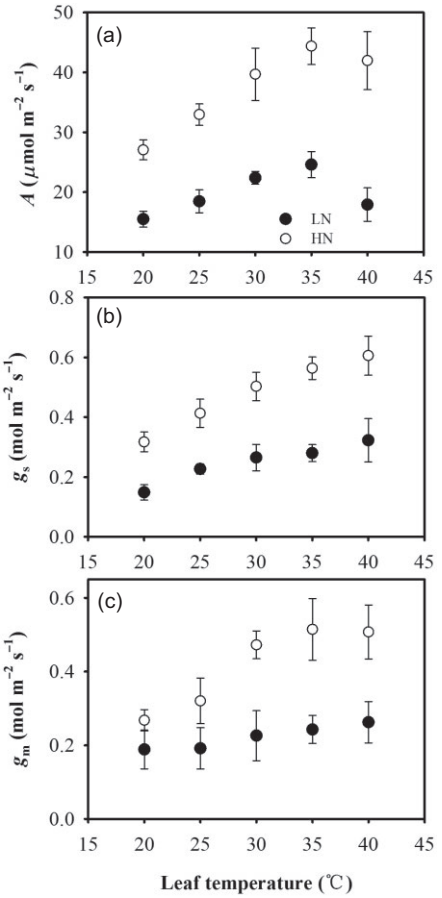

Figure 3. Effects of N supplement on photosynthesis (a), stomatal conductance (b) and mesophyll conductance (c) response to temperature. Values are mean ± SE of three replicates.

interpretation of the discrepancy among these studies is the potential error in g_m estimates from the different methods. It has been affirmed that potential errors exist in all of the currently available estimation techniques for g_m (Tholen *et al.* 2012; Gu & Sun 2014). Many efforts have been made to improve the estimation accuracy of g_m. For instance, Loriaux *et al.* (2013) showed that an improved saturating flash method significantly improved estimates of g_m. In the present study, a sensitivity analysis was conducted to analyse the impact of potential methodological artefacts on the response of g_m to CO_2 concentration and PPFD in both LN and HN treatments. Although the response of g_m to temperature, C_i and PPFD was influenced by Γ^*, R_d, and sets of p_1 and p_2 (Fig. 5, Supporting Information Figs S3 & S4), the different response of g_m to temperature, C_i and PPFD between HN and LN leaves repeated the same pattern, indicating that it was unlikely to be caused by these methodological artefacts. However, we did not estimate the potential artefacts from chlorophyll fluorescence measurement and they need to be examined in the future.

The different response patterns among studies are usually assumed to be species dependent, but no common trait seems to be shared by each group. Recently, von Caemmerer and Evans (2014) investigated the temperature response of g_m in nine species with the online isotope discrimination method under low O_2, and they found that the temperature response of g_m widely varied with species. However, the mechanisms of different responses among species are still unclear. More recently, Flexas and Diaz-Espejo (2014) summarized three potential mechanisms that regulate the g_m response to a rapid change of environment: (1) changes in cell wall properties; (2) regulation of membrane properties; and (3) reshaping and redistribution of chloroplasts.

In the present study, we found that the response of g_m to a rapid change of environment varied with N supplement in rice. The g_m of plants growing in HN was more sensitive to a change in environmental conditions, which suggests that N may play a role in g_m rapid response. Mesophyll cell wall thickness is very unlikely to change fast enough. The effect of membrane properties on g_m response to environment changes is related to aquaporins and their expression and activity are regulated by light (Cochard *et al.* 2007; Voicu *et al.* 2009), CO_2 concentration (Alguacil *et al.* 2009) and temperature (Kuwagata *et al.* 2012). More functional aquaporins (due to the large S_c) and relative high expression of aquaporins in HN plants improve g_m under standard ambient

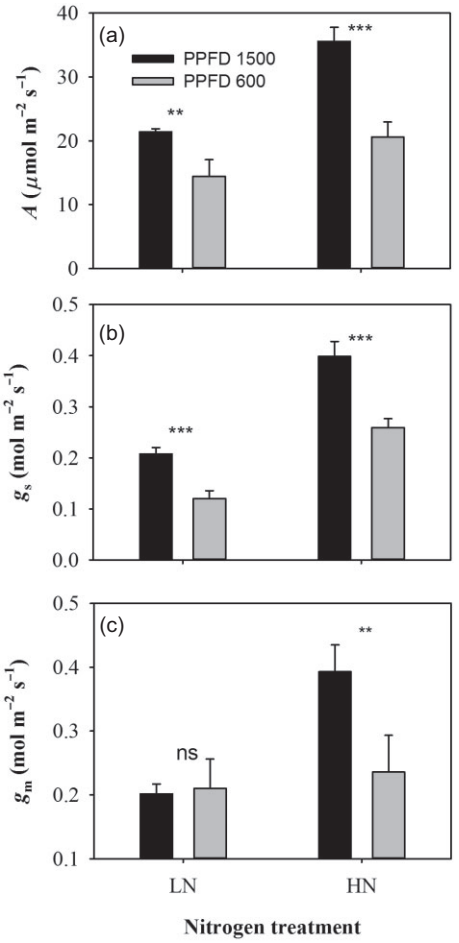

Figure 4. Effects of N supplement on photosynthesis (a), stomatal conductance (b) and mesophyll conductance (c) response to light at 25 °C. Values are mean ± SE of three replicates.

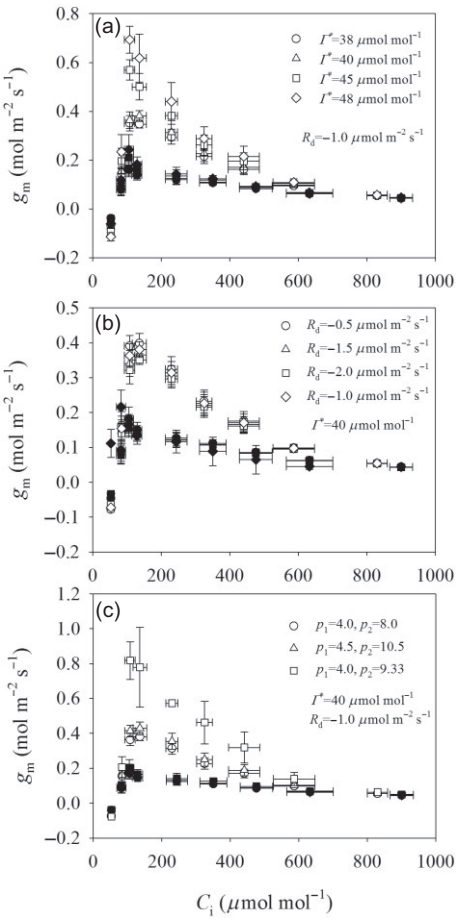

Figure 5. Sensitivity analysis of mesophyll conductance response to intercellular CO_2 concentration in LN (filled symbols) and HN (empty symbols) leaves. Effect of (a) CO_2 compensation point under the absence of respiration condition, (b) day respiration, and (c) set of p_1 and p_2 on g_m estimation. Values are mean ± SE of three replicates.

conditions. However, under varied ambient conditions, the environment-dependent expression of aquaporins could result in a greater change in the amount of aquaporins in HN than in LN leaves. In addition to the amount of aquaporins, the activation status of aquaporins also plays a role in CO_2 transport through the membrane. Assuming the response of single PIP efficiency to a varied environment is unaffected by leaf N level, the difference in gross CO_2 transport by aquaporins between HN and LN leaves is tremendous. Our study suggests that a leaf N-dependent response of g_m to a changing environment may be greatly regulated by expression and/or activity of aquaporins in rice.

On consideration of the leaf structure, the difference in g_m between high and low N leaves seems to be caused by S_c. However, the effect of a rapid change of environment on S_c is not clear. In this study, high S_c in HN leaves associated with a more sensitive g_m to changes of CO_2 concentration, temperature and PPFD. If the rapid change of g_m is related to a change of S_c, chloroplast movement could be the uppermost reason. However, enlarged chloroplasts occupy almost all of the space of the cell in HN leaves (see Fig. 1), which is very likely to prevent the rapid movement of chloroplasts. On the contrary, the reduced chloroplast area in LN leaves should be flexible to environment change. Chloroplast shrinkage, which is mainly caused by leaf dehydration, was observed in many studies and suggested as a potential mechanistic explanation for g_m response to a rapid change in environment. Indeed, cell dehydration was frequently caused by drought stress and other environmental stress. However, the positive effect of N on leaf water status, especially under high temperature stress, has been recognized (Xiong *et al.* 2015a). Analogously, the chloroplast shrinkage should first occur in LN leaves. Our results suggest that g_m response to a rapid change of environment is unlikely caused by altering of S_c in rice.

CONCLUSIONS

We confirmed that g_m increased in HN leaves, and the rapid response of g_m to the change in environmental factors was impacted by N supplements in rice. The difference in g_m between LN and HN leaves related to an alteration of anatomical features, especially the S_c. Our results suggest that the

rapid response of g_m to environmental change was related to aquaporin regulation rather than $T_{cell\ wall}$ and S_c short-term variation.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Effects of VPD on stomatal conductance under varied leaf temperatures. Data are mean ± SE with three biological replicates.

Figure S2. Temperature response of CO_2 compensation point in the absence of respiration (a) and day respiration (b) in LN and HN. Data are mean ± SE with three biological replicates.

Figure S3. Sensitivity analysis of mesophyll conductance response to leaf temperature in LN (filled symbols) and HN (empty symbols) leaves. Effect of (a) CO_2 compensation point under the absence of respiration condition, (b) day respiration, and (c) set of p_1 and p_2 on g_m estimation. Values are mean ± SE of three replicates. Γ^*_{av} represents the average values of Γ^* in LN and HN leaves. R_{dav} represents the average values of R_d in LN and HN leaves under each leaf temperature condition.

Figure S4. Sensitivity analysis of mesophyll conductance response to PPFD in LN and HN leaves. Effect of (a) CO_2 compensation point under the absence of respiration condition, (b) day respiration, and (c) set of p_1 and p_2 on g_m estimation. Values are mean ± SE of three replicates.

Table S1. The range of CO_2 compensation points under the absence of respiration condition (Γ^*) and day respiration (R_d) of rice reported in the literature.