Original Article

Rapid responses of mesophyll conductance to changes of CO₂ concentration, temperature and irradiance are affected by N supplements in rice

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ABSTRACT

Photosynthesis in C₃ plants is significantly limited by mesophyll conductance (gₑₑ), which can vary with leaf anatomical traits and nitrogen (N) supplements. Several studies have investigated the response of gₑₑ to N supplements; however, none examined the implications of N supplements on the response of gₑₑ to rapid environmental changes. Here we investigated the effect of N supplement on gₑₑ and the response of gₑₑ to change of CO₂, 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 gₑₑ. The gₑₑ of leaves with HN was more sensitive to changes in CO₂ concentration, temperature and irradiance. The difference in leaf structural features between low N supplement and HN indicates that a rapid change in gₑₑ 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 gₑₑ 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 CO₂ concentration at carboxylation sites (C₅) inside chloroplast (Li et al. 2009; Yamori et al. 2011; Adachi et al. 2013). During photosynthesis, CO₂ 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 (gₑₑ; CO₂ diffusion conductance from substomatal cavities to carboxylation sites inside chloroplast) was infinite and that C₅ was mainly limited by stomatal conductance (gₛ). Subsequent studies showed that a large gradient of CO₂ concentration is present between substomatal cavities (C₅) and the chloroplasts (C₅) and that the mesophyll layers constitute an important barrier for CO₂ movement inside leaves (Evans et al. 2009).

The effect of leaf anatomical properties on gₑₑ has been examined in many species (Peguero-Pina et al. 2012; Tönsen et al. 2012; Giuliani et al. 2013; Tomas et al. 2013; Muir et al. 2014). Mesophyll cell wall thickness (Tₑₑ wall), mesophyll cell wall surface area exposed to intercellular airspace per leaf area (Sₑₑ), and surface area of chloroplasts exposed to intercellular spaces (Sₑₑ) are the most important anatomical parameters inside leaves that affect gₑₑ (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 gₑₑ have been widely studied. Long-term changes in gₑₑ 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), CO₂ 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., Sₑₑ) and activity of aquaporins (cooporins), ‘molecular channel’ proteins in biological membranes, are
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 CO2 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 CO2 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 $C_i$ 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 (rubulose 1,5-bisphosphate carboxylase/oxygenase) content and CO2 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 CO2, 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 acclimated in the room for approximately 1.5 h. In the Li-Cor leaf chamber, ambient CO2 concentration was adjusted to 400 μmol mol$^{-1}$ with a CO2 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 CO2 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 CO2 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 CO2 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 ($\Phi_{PSII}$) was calculated as follows:

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

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

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

where $\alpha$ is the leaf absorptance and $\beta$ is the partitioning of absorbed quanta between PSII and PSI. The product $\alpha \beta$ was
estimated from the slope of the relationship between $\Phi_{\text{PSII}}$ and $40\Delta CO_2$ (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 $\Delta$P 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 $C_i$. $C_i$ and $g_m$ were calculated as follows:

$$C_i = \frac{\Gamma^*(J + 8(A + R_0))}{J - 4(A + R_3)},$$

$$g_m = \frac{A}{C_i - C_i^*},$$

where $\Gamma^*$ represents the CO$_2$ compensation point in the absence of respiration. For each data point generated, we checked whether it met the criterion ($10 > dC_i/dA > 50$) (Harley et al. 1992).

The day respiration ($R_d$) and the apparent CO$_2$ photosynthesis compensation point ($C_i^*$) were determined at five leaf temperatures using the Laik method. Briefly, the $A/C_i$ curves were measured over the linear portion of the response curve (at 400 to 50 $\mu$mol CO$_2$ mol$^{-1}$ air) under three PPFD (50, 250 and 500 $\mu$mol 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). $\Gamma^*$ 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 $\Gamma^*$ from the Laik method; (2) for 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 $\Gamma^*$ 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 $\Gamma^*$ from rice based on previous literature (Supporting Information Table S1). $R_d$ and $\Gamma^*$ 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 $\Gamma^*$ 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:

$$A = \frac{J(C_i - \Gamma^*)}{p_1C_i + p_2\Gamma^* - R_d}.$$

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 mix 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 $\beta$-mercaptoethanol and 12.5% (v/v) glycerol]. After centrifugation, SDS solution, $\beta$-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 (Infiniti 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
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 ($C^*$) and chloroplastic CO$_2$ compensation point ($P^*$) in HN leaves compared with LN leaves. However, the intercellular CO$_2$ concentration ($C_i$) and CO$_2$ concentration at carboxylation sites inside chloroplasts ($C^*$) 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 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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tillers (hill)</th>
<th>LA (cm$^2$ per hill)</th>
<th>BM (g per hill)</th>
<th>Leaf N (g m$^{-2}$)</th>
<th>Chlorophyll content (µmol m$^{-2}$)</th>
<th>Rubisco (µmol m$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LN</td>
<td>6.0 ± 1.0b</td>
<td>530 ± 51 b</td>
<td>4.37 ± 0.26b</td>
<td>0.66 ± 0.05b</td>
<td>469 ± 29b</td>
<td>3.23 ± 0.85b</td>
</tr>
<tr>
<td>HN</td>
<td>13.3 ± 1.5a</td>
<td>1273 ± 187a</td>
<td>12.12 ± 1.77a</td>
<td>1.12 ± 0.03a</td>
<td>570 ± 37a</td>
<td>5.55 ± 0.64a</td>
</tr>
</tbody>
</table>

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.

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$A$ (µmol m$^{-2}$ s$^{-1}$)</th>
<th>$R_d$ (µmol m$^{-2}$ s$^{-1}$)</th>
<th>$g_s$ (µmol m$^{-2}$ s$^{-1}$)</th>
<th>$C_i$ (µmol mol$^{-1}$)</th>
<th>$g_m$ (µmol m$^{-2}$ s$^{-1}$)</th>
<th>$C^*$ (µmol mol$^{-1}$)</th>
<th>$P^*$ (µmol m$^{-2}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LN</td>
<td>19.3 ± 1.8 b</td>
<td>0.99 ± 0.07 b</td>
<td>0.23 ± 0.02 b</td>
<td>243 ± 8 a</td>
<td>0.19 ± 0.07 b</td>
<td>134 ± 7 a</td>
<td>41.3 ± 0.7 b</td>
</tr>
<tr>
<td>HN</td>
<td>36.7 ± 2.0 a</td>
<td>1.45 ± 0.17 a</td>
<td>0.39 ± 0.04 a</td>
<td>219 ± 13 b</td>
<td>0.39 ± 0.08 a</td>
<td>124 ± 5 b</td>
<td>44.3 ± 0.8 a</td>
</tr>
</tbody>
</table>

Data are mean ± SE. Different letters indicate statistically significant differences ($P < 0.05$) between LN and HN.

A, photosynthetic rate; $R_d$, dark respiration; $g_s$, stomatal conductance; $C_i$, substomatal CO$_2$ concentration; $g_m$, mesophyll conductance to CO$_2$; $C^*$, chloroplastic CO$_2$ concentration; $P^*$, apparent CO$_2$ photocompensation point; $T_{cell\ wall}$, mesophyll cell wall thickness.
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 $T^o$, $R_e$ and $p_1$ and $p_2$ sets (Fig. 5; Supporting Information Figs S3 & S4). Values of $g_m$ increased with increasing $T^o$, but decreased with increasing $R_e$. 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 $C_i$, 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 $C_i$ 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...
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$_2$O transmembrane transport and consequently increase both $g_c$ 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 $g_c$ 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
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 conditions.
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 \( S_i \) short-term variation.

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REFERENCES


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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 CO₂ 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) CO₂ compensation point under the absence of respiration condition, (b) day respiration, and (c) set of p₁ and p₂ on gₘ estimation. Values are mean ± SE of three replicates. Γₑ av represents the average values of Γₑ 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) CO₂ compensation point under the absence of respiration condition, (b) day respiration, and (c) set of p₁ and p₂ on gₘ estimation. Values are mean ± SE of three replicates.

Table S1. The range of CO₂ compensation points under the absence of respiration condition (Γₑ) and day respiration (R_d) of rice reported in the literature.