Anatomically induced changes in rice leaf mesophyll conductance explain the variation in photosynthetic nitrogen use efficiency under contrasting nitrogen supply

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Abstract

Background: The ratio of CO₂ mesophyll conductance ($g_m$) to Ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) content has been suggested to positively affect photosynthetic nitrogen use efficiency (PNUE). The anatomical basis of $g_m$ has been quantified, but information on the relationship between cell-level anatomies and PNUE is less advanced. Here, hydroponic experiments were conducted in rice plants supplied with ammonium ($\text{NH}_4^+$) and nitrate ($\text{NO}_3^-$) under three N levels (low, 0.71 mM; intermediate, 2.86 mM; high, 7.14 mM) to investigate the gas exchange parameters, leaf anatomical structure and PNUE.

Results: The results showed a lower PNUE in plants supplied with high nitrogen and $\text{NH}_4^+$, which was positively correlated with the $g_m$/Rubisco ratio. A one-dimensional within-leaf model revealed that the resistance to CO₂ diffusion in the liquid phase ($r_{\text{liq}}$) dominated the overall mesophyll resistance ($r_m$), in which CO₂ transfer resistance in the cell wall, cytoplasm and stroma were significantly affected by nitrogen supply. The chloroplast surface area exposed to intercellular space ($S_c$) per Rubisco rather than the $g_m/S_c$ ratio was positively correlated with PNUE and was thus considered a key component influencing PNUE.

Conclusion: In conclusion, our study emphasized that $S_c$ was the most important anatomical trait in coordinating $g_m$ and PNUE with contrasting N supply.

Keywords: Leaf anatomies, $\text{NH}_4^+$, $\text{NO}_3^-$, Mesophyll conductance, PNUE, Rubisco

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Background

Photosynthetic nitrogen use efficiency (PNUE), determined as the ratio of photosynthesis rate ($P_n$) to leaf organic nitrogen content [1], is a key component of nitrogen use efficiency (NUE) and an indicator of the relationship between leaf nitrogen (N) and $P_n$. Under the present atmosphere, the unsaturated CO$_2$ concentration in C3 leaves influences the carboxylation of Ribulose-1,5-disphosphate (RuBP) and results in a finite $P_n$, which fails to match the increase in leaf N and induces a decrease in PNUE [2]. By using an “evolutionary” algorithm, the partitioning of photosynthetic enzymes was altered based on a fixed total amount of protein-nitrogen for maximizing $P_n$, and the result showed that an increase in Ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) was required to maximize $P_n$ [3]. It was also well documented that higher leaf N allocation into Rubisco was linked with an enhancement in PNUE [4].

Numerous studies have clarified that the enhancement in Rubisco activity is another favorable candidate for improving RuBP carboxylation efficiency and $P_n$ because of its poor catalytic ability under ambient conditions due to the low CO$_2$ concentration and the low affinity for CO$_2$ [1, 5]. As the substrate of Rubisco, CO$_2$ concentration in the chloroplast ($C_c$), which is determined by stomatal conductance ($g_m$) and mesophyll conductance ($g_m$), plays a dominant role in regulating Rubisco activity [6, 7]. It has been demonstrated that $g_m$ induces 40% of the total decrease in CO$_2$ concentration between the atmosphere and the carboxylation sites of Rubisco [8]. In a previous study, Li et al. [5] argued that an increase in $g_m$ was not sufficient to meet the carboxylation demand of the increased Rubisco content and eventually resulted in a decreased PNUE. Therefore, it is speculated that factors affecting $g_m$ would influence Rubisco activity and the relationship between $P_n$ and leaf N content.

Evidence is now mounting that $g_m$ is largely dependent on leaf anatomical characteristics, including leaf thickness, cell wall thickness and chloroplast morphology [9, 10]. Higher leaf density and thicker mesophyll cell walls contribute to a reduction in $g_m$ [9, 11–14], and mesophyll and/or chloroplast surface areas exposed to the intercellular space, $S_{mes}$ and $S_c$, respectively, are positively correlated with $g_m$ [15]. The overall importance of different anatomical traits in the restriction of $g_m$ varies [16]. For gymnosperms, the strongest sources of $g_m$ are cell wall and chloroplast thickness, variation in chloroplast shape and size, and $S_c$ [9]. In lycophytes and bryophytes, the highest CO$_2$ diffusive resistance is mainly driven by extremely high cell wall thickness and low $S_c$ [17]. Even though the anatomical factors influencing $g_m$ have been widely studied, the role of these anatomical factors in influencing PNUE and their relative contribution in rice plants are still largely unknown.

Leaf anatomy is remarkably influenced by N nutrition; for example, decreasing leaf thickness and smaller chloroplasts with no starch granules have been detected in nitrogen-deficient leaves, while high-N leaves have more large chloroplasts with well-developed grana, stroma lamellae and starch granules per mesophyll cell [5, 18–20]. For different nitrogen forms, increased leaf thickness and a doubling of chloroplast volume with a larger internal membrane length have been found in NH$_4^+$-fed plants compared with NO$_3^-$-fed plants [21–23]. In this study, we examine the responses of leaf anatomical characteristics, including leaf thickness, mesophyll cell size, chloroplast length and thickness, chloroplast number per mesophyll cell under NH$_4^+$ and NO$_3^-$ nutrition with different N levels; moreover, we discuss the implications for understanding leaf trait variation with changes in N nutrition along the PNUE. Our objectives of the present study were as follows: (1) to identify the response of PNUE and leaf anatomical traits upon NH$_4^+$ and NO$_3^-$ nutrition at different N levels; (2) to clarify the role of leaf anatomical factors in coordinating the $g_m$ and PNUE under NH$_4^+$ and NO$_3^-$ nutrition supply; and (3) to investigate the most limiting fraction of leaf anatomy in determining PNUE under different N supply.

Results

Effects of nitrogen supply on rice photosynthetic nitrogen use efficiency (PNUE)

Compared with those with low nitrogen supply (LAN and LNN), rice biomass and leaf area with intermediate and high nitrogen supply increased by 70–73% and 33–42% under NH$_4^+$ nutrition and by 30–48% and 40–41% under NO$_3^-$ nutrition, respectively (Table 1). There were no significant differences in rice biomass and leaf area between the intermediate and high nitrogen supply conditions. Rice biomass was less affected by nitrogen forms at the same nitrogen level, while for intermediate nitrogen supply, the leaf area was decreased by 10% in NO$_3^-$-fed plants than in NH$_4^+$-fed plants (Table 1). The leaf N content ($N_l$) was 54–62% and 66–80% higher under intermediate N supply and high N supply than under low N supply and was decreased by 9–11% under NO$_3^-$ nutrition compared with that under NH$_4^+$ nutrition (Table 1). Neither nitrogen supply levels nor nitrogen forms affected the chloroplastic CO$_2$ concentration ($C_c$). With increasing leaf N content, the light-saturated photosynthetic rate ($P_n$) increased, while the photosynthetic nitrogen use efficiency (PNUE)
Table 1 Effects of different nitrogen supply levels on rice biomass (g), leaf area (cm²), leaf nitrogen content (N₄, g m⁻²), Rubisco content (g m⁻²), stomatal conductance (gₛ, mol CO₂ m⁻² s⁻¹), mesophyll conductance (gₘ, mol CO₂ m⁻² s⁻¹), and photosynthetic nitrogen use efficiency (PNUE, μmol CO₂ mmol⁻¹ N s⁻¹) under 3 different amounts, low N (0.71 mM, LAN and LNN), intermediate N (2.86 mM, MAN and MNN), and high N (7.14 mM, HAN and HNN). Data are presented as the mean ± SD of four replications. Significant differences (P < 0.05) between treatments are indicated by different letters.

| Treatments | Biomass | Area | N₄ | Rubisco | gₛ | gₘ | Cₑ | Pₙ | PNUE |
|------------|---------|------|----|---------|----|----|-----|-----|------|
| LAN        | 3.29 b  | 658 c| 1.55 d| 2.02 d  | 0.35 b | 0.16 c | 180 ab | 24.61 b | 0.22 b |
| MAN        | 5.71 a  | 932 a| 2.38 b| 3.16 b  | 0.45 a  | 0.24 ab| 171 ab | 28.74 a | 0.17 cd |
| HAN        | 5.59 a  | 873 ab| 2.58 a| 3.67 a  | 0.49 a  | 0.28 a | 187 a  | 29.25 a | 0.16 d  |
| LNN        | 3.77 b  | 595 c | 1.30 e| 1.49 e  | 0.27 b  | 0.16 c | 165 b  | 25.00 b | 0.27 a  |
| MNN        | 4.92 a  | 837 b | 2.11 c| 2.50 c  | 0.44 a  | 0.20 bc| 176 b  | 29.86 a | 0.20 bc |
| HNN        | 5.57 a  | 834 b | 2.34 b| 2.90 b  | 0.44 a  | 0.19 bc| 176 ab | 30.30 a | 0.18 cd |

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decreased (Table 1, Fig. 1a, b). The PNUE was 21, 17 and 14% higher in LNN, MNN and HNN than in LAN, MAN and HAN, respectively (Table 1). Positive correlations existed between PNUE and both the Cₑ/Rubisco ratio and the gₘ/Rubisco ratio (Fig. 1c, d).

**Effects of nitrogen supply on leaf anatomical properties**

With increasing leaf N supply levels, leaf thickness (Tₑ) and mesophyll cell thickness (Tₘₑ) increased in NH₄⁺-fed plants but decreased in NO₃⁻-fed plants (Supplementary Fig. S1, Fig. 2a, b). Leaf dry mass per area (Mₐ), leaf density (Dₑ), and mesophyll cell wall thickness (Tₘcw) were increased by high N supply either with NH₄⁺ or NO₃⁻, and were lower under NO₃⁻ nutrition than under NH₄⁺ nutrition. Mesophyll surface area exposed to intercellular airspace (Sₘes) and the chloroplast surface area facing intercellular airspace (Sₑ) were upregulated significantly by increasing the nitrogen supply level (Fig. 2b). The Sₘes increased by 22–37 and 21% under intermediate and high N supply conditions in NH₄⁺ and NO₃⁻ nutrition, respectively, and the corresponding Sₑ increased by 22–38% and 21–24%, both compared with their respective low N supply conditions. No obvious differences in Sₑ between NH₄⁺ and NO₃⁻ under low N levels were observed, but the Sₑ decreased by 11 and 20% under MNN and HNN, respectively, compared to that under MAN and HAN (Fig. 2b).

We further analyzed the chloroplast number per mesophyll cell (Nₑ), chloroplast length (Lₑ), chloroplast thickness (Tₑ), chloroplast section area (Sₑ), chloroplast volume (Volₑ), and chloroplast and cell wall area (Secₑ). Lₑ was lower in NO₃⁻-fed plants than in NH₄⁺-fed plants under intermediate and high N levels (Supplementary Fig. S1, Fig. 2c). Compared with that in LAN and LNN, the Tₑ was increased by 13–27% in MAN and HAN and 23–29% in MNN and HNN (Supplementary Fig. S1, Fig. 2c). Higher Sₑ and Volₑ were observed under high N supply. The chloroplast size was decreased under NO₃⁻ nutrition by 10–22%, 15–33%, and 10–40% in Sₑ, Volₑ and Secₑ under low N, intermediate N and high N supply, respectively (Supplementary Fig. S1, Fig. 2d).

**Anatomical limitations of mesophyll conductance**

The values of gₘ, calculated according to the methods of Harley et al. [24] and Tomas et al. [16] were strongly positively linearly correlated (Supplementary Fig. S2, R² = 0.936). Further quantitative analysis showed that both the resistance in the gas phase (rₑgas) and proportion of gas-phase limitations (lₑgas) of gₘ had little impact on the overall mesophyll resistance (Fig. 3), and that the liquid phase resistance (rₗₘ) was responsible for the limited gₘ majority, among which stroma played a dominant role. High N supply significantly increased the resistance in the cell wall (rₑcw) and stroma (rₑst); compared with low N supply, gₘ limited by the stroma (lₑst) was increased by 9–10% under moderate N supply and by 9–13% under high N supply (Fig. 3b). Consistent with the absolute cytoplasm resistance, gₑst limited by the cytoplasm (lₑcyt) and cell wall (lₑcw) were downregulated under high N supply and NO₃⁻ nutrition, respectively (Fig. 3). Among all the components, rₑcw was the primary component affected by N forms and was 19, 23 and 16% higher under NH₄⁺ nutrition than under NO₃⁻ nutrition in low N, intermediate N and high N supply, respectively (Fig. 3a).

**Discussion**

**Effects of N supply on the gₘ/Rubisco ratio and photosynthetic nitrogen use efficiency (PNUE)**

Decreased PNUE under high N supply has been reported in previous and present studies (Table 1, Fig. 1) [5, 25–27]; referring to N forms, higher PNUE under NO₃⁻ nutrition than NH₄⁺ nutrition in the present study is consistent with results in barley (Hordeum vulgare L.) [28], pine [29], and cucumber [30]. Leaf nitrogen allocation is an important factor influencing PNUE. Onoda et al. [31] indicated that a higher fraction of photosynthetic nitrogen in electron transport and Rubisco would contribute to increased PNUE in leaves with lower dry mass per area (Mₐ), while in leaves
with higher $M_A$, the over-investment of nitrogen in photosynthetic nitrogen and/or cell walls would reduce PNUE [1]. The effect of the proportion of Rubisco in leaf N content on PNUE can be expressed based on Eq. (6):

$$\text{PNUE} = \frac{g_m}{\text{Rubisco}} (C_i - C_c) \frac{\text{Rubisco}}{N_L}$$  \hspace{1cm} (1)

Our study detected that the Rubisco allocation ratio was increased under high nitrogen supply but decreased under NO$_3^-$ nutrition compared with NH$_4^+$ nutrition; however, the portion of Rubisco in leaf N content was not associated with PNUE (Fig. 4a). These results implied that Rubisco activity, rather than its content, played a dominant role in regulating PNUE [1, 5, 32].

An increased Rubisco allocation ratio requires increased CO$_2$ partial pressure at the carboxylation site ($C_c$) to meet carboxylation demands; however, the extent of the increase in $C_c$ was less than that in Rubisco content, which resulted from the finite stomatal conductance ($g_s$) and mesophyll conductance ($g_m$). Li et al. [5] demonstrated that the smaller increases in $g_m$ relative to Rubisco content resulted in relatively lower CO$_2$ levels in chloroplasts and PNUE (Fig. 1c, d), which implied that the $g_m$/Rubisco ratio rather than the absolute value of $g_m$ was the key factor that regulates PNUE. We further compared the gap between estimated $g_m$ and $C_c$ proposed by Harley et al. [24] (Eq. 5, 6) and theoretical $C_c$ ($C_c$-Theoretical) and $g_m$ ($g_m$-Theoretical), which were calculated as follows based on Ding et al. [27], to evaluate the equilibrium state of $g_m$ and Rubisco under different N nutrition conditions:

![Fig. 1 The relationship between leaf N content ($N_L$) and the photosynthetic rate ($P_n$) (a) and photosynthetic nitrogen use efficiency (PNUE) (b) and the relationship between PNUE and the ratio of chloroplast CO$_2$ concentration to Rubisco ($C_c$/Rubisco) (c) and the ratio of mesophyll conductance to Rubisco ($g_m$/Rubisco) (d). Each point represents one replicate (four replicates per treatment). The lines represent the following regression equations: a) $y = -1.8756x^2 + 11.3310x + 13.0290, R^2 = 0.6228, P < 0.05$; b) $y = -0.0763x + 0.3565, R^2 = 0.7886, P < 0.05$; c) $y = 0.0017x + 0.0774, R^2 = 0.8295, P < 0.01$; d) $y = 1.5663x + 0.0733, R^2 = 0.4215, P < 0.01$]
\[ g_m = \text{Theoretical (intermediate or high N)} \]
\[ = \text{Rubisco (intermediate or high N)} \times \frac{g_m}{\text{Rubisco}} \quad \text{(low N)} \quad (2) \]

\[ C_c = \text{Theoretical (intermediate or high N)} \]
\[ = \text{Rubisco (intermediate or high N)} \times \frac{C_c}{\text{Rubisco}} \quad \text{(low N)} \quad (3) \]

As shown in Fig. 5, both theoretical and estimated \( C_c \) and/or \( g_m \), as well as the differences between them, increased obviously with increasing leaf N content, and the gap between theoretical and estimated \( C_c \) and/or \( g_m \) under \( \text{NH}_4^+ \) nutrition was larger than that under \( \text{NO}_3^- \) nutrition. These results confirmed that the balance between Rubisco content and \( C_c \) and/or \( g_m \) was weaker when high N and \( \text{NH}_4^+ \) were supplied, and the relatively lower \( C_c \) failed to meet the carboxylation demands of the increased Rubisco content, resulting in decreased PNUE (Fig. 1c, d).

Overall importance of leaf anatomy in determining \( g_m \) and PNUE

When leaf nitrogen content was expressed on a leaf dry mass basis, no significant differences in leaf N content between \( \text{NH}_4^+ \) and \( \text{NO}_3^- \) nutrition were obtained. Therefore, the discrepancies in PNUE between different N forms were primarily caused by the difference in \( M_A \) (Fig. 2), which resulted from leaf anatomy characteristics such as leaf density \( (D_L) \), leaf thickness \( (T_L) \), mesophyll cell wall thickness \( (T_{mc}) \), chloroplast length \( (T_c) \), and chloroplast thickness \( (T_{mc}) \). These contrasting results were explained by different ways to
enhance $M_A$, as the increases in $D_L$ and $T_L$ were associated with higher $g_m$, while the opposite conclusion would be obtained if the increase in $M_A$ was a result of a thickened cell wall [8]. Our positive correlation between $M_A$ and $g_m$ implied that the contributions of $D_L$ and/or $T_L$ compensated for the inhibitory effect of $T_{mc}$ on $g_m$.

To qualify the relative importance of each leaf anatomy trait in explaining $g_m$, a one-dimensional within-leaf model was calculated to clarify the limitation of $g_m$ in each process [16]. The results showed that more than 90% of the total limitation of $g_m$ came from $L_{liq}$, which was a consequence of limitation in the cell wall ($L_{cw}$), plasma membrane ($L_{pl}$), envelope ($L_{en}$), cytoplasm ($L_{cyt}$), and stroma ($L_{st}$) (Fig. 3) [34]. The decreased contribution of cytoplasmic resistance to $g_m$ under high N resulted from the decreased distance between adjacent chloroplasts, rather than the distance between the cell wall and chloroplasts [16], and the increase in chloroplast thickness ($T_c$) extended the transport path for CO$_2$ from the chloroplast membrane to the carboxylation site in the interior of chloroplasts and resulted in an increasing in $r_{st}$ [35, 36]. Except for the resistance of each part, the chloroplast surface area exposed to intercellular airspace ($S_c$) was a paramount factor affecting CO$_2$ liquid resistance ($r_{liq}$). However, the decreased $r_{cyt}$ and increased $S_c$ partially compensated for the increased $r_{st}$ under high N supply and resulted in an increased $g_m$, although the effects were weak and did not match the increase in Rubisco content.

Considering the dominant role of $S_c$ in determining $g_m$, the $g_m$/Rubisco in Eq. (1) was replaced by the product of $g_m$ and $S_c$ to demonstrate the effect of leaf anatomies and $g_m$ on PNUE, which can be expressed as follows:

**Fig. 3** Anatomical limitations of mesophyll conductance ($g_m$) (a) and the share of the overall $g_m$ limitation (b) by cell wall (cw), plasma membrane (pl), chloroplast envelope (en), cytoplasm (cyt) and stroma (st) in rice leaves supplied with NH$_4^+$ (AN) or NO$_3^−$ (NN) under 3 different amounts, low N (0.71 mM, LAN and LNN), intermediate N (2.86 mM, MAN and MNN), and high N (7.14 mM, HAN and HNN). The inset figure shows the anatomical limitations of $g_m$ and the share of the overall $g_m$ limitation by gas phase. The error bars indicate the standard deviation and at least 15 replicates were conducted for each parameter. Different letters indicate statistically significant differences ($P < 0.05$) between different treatments.
PNUE = \frac{g_m \cdot S_c \cdot \text{Rubisco}}{S_c \cdot \text{Rubisco}} \cdot \frac{\text{Rubisco}}{N_L} \cdot (C_i - C_c) \tag{4}

According to the formula above and Terashima et al. [37], a positive correlation would be summarized between PNUE and \( g_m/S_c \), which emphasized the potential role of leaf anatomical characteristics except for \( S_c \) as well as the activity of carbonic anhydrase (CA) in contributing to PNUE [37]. However, the weak negative relationship between \( g_m/S_c \) and PNUE and the significant positive correlation between the \( S_c/\text{Rubisco} \) ratio and PNUE suggested the dominant role of \( S_c \) in influencing PNUE. Due to the limited knowledge of the relationship between PNUE and the \( S_c/\text{Rubisco} \) ratio, Onoda et al. [14] did not take this component into consideration when they analyzed the physiological and structural tradeoffs underlying the leaf economics spectrum, and they argued that \( S_c \) per Rubisco may not correlate strongly with \( M_A \) or PNUE. However, we detected that \( S_c \) per Rubisco was a critical parameter associated with PNUE in rice plants supplied with different N nutrition levels. Similar results were well documented in a review by Terashima et al. [15] and Terashima et al. [37], in which they speculated that, from the perspective of Rubisco and nitrogen use efficiency, thicker leaves with larger \( S_c \) were advantageous because the increased ratio of \( S_c \) to Rubisco would increase chloroplast CO2 concentration. The increased \( S_c/\text{Rubisco} \) ratio in \( \text{NO}_3^- \) and high N–fed plants partially resulted from the lower leaf density, which allowed more chloroplast surface area to be exposed to intercellular airspace (Fig. 2a).

**Conclusions**

In conclusion, we demonstrated that PNUE is decreased in rice plants supplied with high N and ammonium nutrition, which results from unbalanced increases in \( g_m \) and Rubisco content. Nitrogen-induced variation in \( g_m \) is associated with leaf anatomical traits, especially chloroplast surface area exposed to intercellular airspace (\( S_c \)). We further concluded that the \( S_c/\text{Rubisco} \) ratio is directly related to the response of PNUE to N supply and that its increase is advantageous to the increase in PNUE.
Methods

Plant material and growth conditions

Rice seeds (*Oryza sativa* L., ssp. japonica inbred, cv. ‘Zhendao 11’) were purchased from Mingtian Seed Company (Nanjing, China), disinfected with 10% H$_2$O$_2$ for 30 min and germinated in 2.0 mM CaSO$_4$ at 25 °C. The rice seedlings were transferred to 6 L rectangular containers (30 × 20 × 10 cm) when the seedlings developed 2.5 visible leaves, and one quarter-strength mixture of NH$_4^+$ and NO$_3^-$ nutrient solution (for composition, see below) was supplied. Three days later, the seedlings were transferred to a one half-strength nutrient solution. After 6 days, the seedlings were supplied with full-strength nutrient solution for 1 week, after which the seedlings were supplied with either (NH$_4$)$_2$SO$_4$ (AN) or Ca (NO$_3$)$_2$ (NN) at three different N levels: low N (0.71 mM), intermediate N (2.86 mM), and high N (7.14 mM). Thus, six treatments were applied: LAN (low NH$_4^+$), MAN (intermediate NH$_4^+$), HAN (high NH$_4^+$), LNN (low NO$_3^-$), MNN (intermediate NO$_3^-$), and HNN (high NO$_3^-$). In addition, the macronutrients in the solution were as follows (mM): 0.32 P as KH$_2$PO$_4$, 1.02 K as K$_2$SO$_4$ and KH$_2$PO$_4$ and 1.65 Mg as MgSO$_4$. The micronutrients were (µM) as follows: 35.8 Fe as Fe-EDTA, 9.10 Mn as MnCl$_2$·4H$_2$O, 0.52 Mo as (NH$_4$)$_6$Mo$_7$O$_24$·4H$_2$O, 18.5 B as H$_3$BO$_3$, 0.15 Zn as ZnSO$_4$·7H$_2$O, 0.16 Cu as CuSO$_4$·5H$_2$O and 100 Si as Na$_2$SiO$_3$·9H$_2$O. CaCl$_2$ was added to the AN, LNN, and MNN treatments to adjust the Ca level to that of the HNN treatment. The nitrification inhibitor dicyandiamide (DCD) was added to each nutrient solution to prevent the oxidation of NH$_4^+$. Rice leaves supplied with NH$_4^+$ (AN) or NO$_3^-$ (NN) under 3 different amounts, low N (0.71 mM, LAN and LNN), intermediate N (2.86 mM, MAN and MNN), and high N (7.14 mM, HAN and HNN).

Fig. 5 The relationships between the leaf-N content ($N_L$) and the estimated and theoretical chloroplast CO$_2$ concentration ($C_c$) (a, b) and the estimated and theoretical mesophyll conductance ($g_m$) (c, d) under NH$_4^+$ (a, c) and NO$_3^-$ (b, d) nutrition. Each point represents one replicate (four replicates per treatment). The dashed lines represent the theoretical chloroplast CO$_2$ concentration and mesophyll conductance, which were calculated according to the ratio of $C_c$/Rubisco and $g_m$/Rubisco constant at low-N levels, and the theoretical $C_c$ and $g_m$ at intermediate and high N levels was Rubisco (intermediate or high N) × ($C_c$/Rubisco or $g_m$/Rubisco (low N)). The lines represent the estimated $C_c$ and $g_m$. Rice leaves supplied with NH$_4^+$ (AN) or NO$_3^-$ (NN) under 3 different amounts, low N (0.71 mM, LAN and LNN), intermediate N (2.86 mM, MAN and MNN), and high N (7.14 mM, HAN and HNN).
treatments were replicated 5 times with a completely randomized design. The temperature in the greenhouse was maintained at 30 °C during the day and 18 °C at night. Light was supplied by SON-T AGRO 400 W bulbs, and the distance between the light and the rice plants was approximately 60 cm. The leaf intensity was maintained at a minimum of 1000 μmol photons m⁻² s⁻¹ at the leaf level using a 14-h photoperiod.

Measurement of biomass and leaf N content
After all the measurements were completed, plant dry weight was determined after oven-drying at 105 °C for 30 min and then at 70 °C to a constant weight. Pictures of the leaves used for the measurement of Pn were taken with a camera along with a benchmark to calibrate, and the leaf area was obtained by ImageJ Pro Plus, after which the leaves were dried and digested with H₂SO₄-H₂O₂ at 260–270°C. The leaf N concentration was determined using a digital colorimeter (AutoAnalyzer 3; Bran+Luebbe).

Gas exchange measurements
Twenty days after treatments, a Li–Cor 6400 infrared gas analyzer was used for the simultaneous measurement of light-saturated photosynthesis (Pn) and chlorophyll fluorescence on the newly expanded leaves from 9:00 to 15:00. Leaf temperatures were 25 °C, the relative humidity was 45%, and photosynthetic photon flux density (PPFD) was 1500 μmol m⁻² s⁻¹ for all measurements. After equilibration to a steady state, Pn was recorded and the photosynthetic nitrogen use efficiency (PNUE) was calculated as the ratio of Pn to the leaf nitrogen content per leaf area. The fluorescence (F) was also measured simultaneously, and a 0.8 s saturating pulse of light (approx. 8000 μmol m⁻² s⁻¹) was applied to measure the maximum fluorescence (Fm). The efficiency of photosystem II (ΦPSII) was calculated as ΦPSII = 1 - F/Fm. The total electron transport rate (J) was calculated as J = ΦPSII × PPFD × αleaf × β, where αleaf and β were leaf absorption and the proportion of quanta absorbed by photosystem II, respectively. In this study, αleaf was also assumed to be 0.85, and β was assumed to be 0.5 [38].

The following equations proposed by Harley et al. [24] were used to calculate the CO₂ mesophyll conductance (gₘ) and chloroplast CO₂ concentration (Cₖ):

\[
g_m = \frac{P_n}{C_i - I^* \frac{J_T + 8 \times (P_n + R_d)}{J_T - 4 \times (P_n + R_d)}}
\]

(5)

\[
C_k = C_i - \frac{P_n}{g_m}
\]

(6)

where C is the intercellular CO₂ concentration, I* is the CO₂ compensation point and Rₐ is the mitochondrial respiration rate in the light. In the present experiment, I* and Rₐ were measured on newly expanded leaves according to the method of Li et al. [39]. PPFDs in the cuvette were controlled as the series of 150, 300, and 600 μmol m⁻² s⁻¹. At each PPFD, the ambient CO₂ concentration in the cuvette was adjusted as the series of 25, 50, 75 and 100 μmol CO₂ mol⁻¹. Thirty minutes prior to initiating measurements, leaves were placed in the cuvette with a PPFD of 600 μmol photons m⁻² s⁻¹ and a Cₚ of 100 μmol CO₂ mol⁻¹.

Anatomical analysis
For the anatomical analysis, approximately 1–2 mm² leaf sections were cut and fixed in FAA (95% ethanol: glacial acetic acid: formalin: distilled water = 10:1:2:7), dehydrated in ethanol series, and embedded in paraffin. After cutting into 6 μm transverse sections with a microtome and mounting on glass, the glass was stained with Safranin O and fast green and then mounted in DPX mounting medium. Images of each section were obtained with a light microscope (BX 53, Olympus) with a CCD camera (eXcope X3, DIX, Korea). Leaf thickness (T₁), mesophyll thickness (T₂), leaf density (D₂), and the volume fraction of intercellular air space (Fias) were measured and/or calculated from at least 5 sections from four different leaves, and at least 5 different fields of view were observed for a given section of images. D₂ and Fias were calculated as:

\[
D_2 = \frac{M_A}{T_L}
\]

(7)

\[
f_{\text{ias}} = 1 - \frac{\Sigma S_m}{T_m W}
\]

(8)

where M_A is the specific leaf weight (g m⁻²), ΣS_m is the total sectional area of mesophyll cells, and W is the width of the section.

For the transmission electron microscope (TEM) analysis, approximately 1–2 mm² leaf sections were cut from the middle of newly expanded leaves using two razor blades, fixed in 2.5% glutaraldehyde (0.1 mol L⁻¹ phosphate buffer, pH 7.0) and postfixed with 2% osmium tetroxide. Specimens were dehydrated in a graded acetone series and embedded in Epon 812. Ultrathin cross-sections of 90 nm for transmission electron microscopy (TEM) were cut with a Power Tome-XL ultramicrotome, stained with 2% uranyl acetate, and examined with an H-7650 transmission electron microscope. For each sample, 15 cross-sections were chosen to measure mesophyll cell wall thickness (Tₚ) and total length of the mesophyll cells (Lₚ) and chloroplasts (Lₖ) facing the intercellular air space. At least 40 chloroplasts from TEM were observed to measure the chloroplast traits, including chloroplast length (Lₖ), chloroplast thickness (Tₖ), chloroplast section area (Secₖ), distance between...
two neighbor chloroplasts ($\Delta L_{\text{ch}}$), and chloroplast distance from the cell wall ($\Delta L_{\text{cyt}}$). The surface area of mesophyll cells to the intercellular air-spaces ($S_{\text{mes}}$), the surface area of chloroplasts exposed to intercellular airspace ($S_{\text{c}}$), the chloroplast surface area ($S_{\text{ur}}$) and volume ($V_{\text{ol}}$) were calculated by using the following formula:

$$S_{\text{mes}} = \frac{L_{\text{mes}}}{W} F$$  \hspace{1cm} (9)

$$S_{\text{c}} = \frac{L_{\text{ch}}}{L_{\text{mes}}} S_{\text{mes}}$$  \hspace{1cm} (10)

where $W$ is the width of the measured section, and $F$ is the curvature correction factor and taken as 1.55 [40].

$$S_{\text{ur}} = 4 \times \pi \times \left( \frac{a \times b^2}{2} \right)^{2/3}$$  \hspace{1cm} (11)

$$V_{\text{ol}} = \left( \frac{4}{3} \right) \times \pi \times \left( \frac{a \times b^2}{2} \right)$$  \hspace{1cm} (12)

where $a = L_{\text{c}}/2$, and $b = T_{\text{c}}/2$.

The chloroplast number per mesophyll cell ($N_{\text{c}}$) was determined according to the method of Pyke [41]. Briefly, the leaves were cut into 1–5 mm widths with a scalpel or razor blade, submerged in 3.5% (v/v) glutaraldehyde in a tube and kept in the dark at room temperature for 1 h. The glutaraldehyde solution was then replaced with 0.1 M Na-EDTA (pH 9), and the leaf discs were heat-blocked at 60 °C for 12 h and incubated overnight in the dark at 4 °C. To view chloroplasts in individual cells, a piece of tissue was removed from the tube with fine forceps and placed on a microscope slide in a drop of water. A scalpel handle was used to tap and macerate the tissue fairly vigorously, and a Leica DM2700 M microscope with DIC/Nomarski optics was used to image and count chloroplast numbers with changing focus to avoid duplicate and uncounted chloroplasts (Fig. S3).

The qualification of the anatomical limitations of mesophyll conductance

The one-dimensional gas diffusion model of Tomas et al. [16] was applied in our present study to determine the anatomical limitations of mesophyll conductance, which was given as:

$$g_{\text{m}} = \frac{1}{\frac{1}{g_{\text{ias}}} + \frac{RT_{\text{k}}}{H g_{\text{liq}}}}$$  \hspace{1cm} (13)

where $g_{\text{ias}}$ and $g_{\text{liq}}$ are the gas phase conductance and liquid phase conductance, respectively. $R$ is the gas constant (8.31 Pa m$^3$ K$^{-1}$ mol$^{-1}$), $T_{\text{k}}$ is the absolute temperature, and $H$ is Henry’s law constant (2943.3 Pa m$^3$ K$^{-1}$ mol$^{-1}$ for CO$_2$). The $g_{\text{liq}}$ was calculated as:

$$g_{\text{liq}} = \frac{1}{r_{\text{liq}}} = \frac{D_a f_{\text{liq}}}{\Delta L_{\text{liq}} \zeta}$$  \hspace{1cm} (14)

where $r_{\text{liq}}$ is the resistance of the gas phase to CO$_2$, $D_a$ is the diffusion coefficient for CO$_2$ in the gas phase and is set to 1.51 $\times$ 10$^{-5}$ m$^2$ s$^{-1}$ at 25 °C, $f_{\text{liq}}$ is the volume fraction of intercellular air space, $\Delta L_{\text{liq}}$ was taken as half of the mesophyll thickness, and $\zeta$ is the diffusion path tortuosity (1.57 m$^{-1}$).

The $g_{\text{liq}}$ was determined by different components in the cell, including the conductance in the cell wall ($g_{\text{cw}}$), plasma membrane ($g_{\text{pl}}$), cytosol ($g_{\text{cyt}}$), chloroplast envelope ($g_{\text{env}}$), and stroma ($g_{\text{st}}$). Eventually, $g_{\text{liq}}$ was calculated as:

$$g_{\text{liq}} = \frac{S_{\text{c}}}{(r_{\text{cw}} + r_{\text{pl}} + r_{\text{cyt}} + r_{\text{env}} + r_{\text{st}})}$$  \hspace{1cm} (15)

where $r_{\text{cw}}$, $r_{\text{pl}}$, $r_{\text{cyt}}$, $r_{\text{env}}$, and $r_{\text{st}}$ are the reciprocal terms of $g_{\text{cw}}$, $g_{\text{pl}}$, $g_{\text{cyt}}$, $g_{\text{env}}$, and $g_{\text{st}}$, respectively. We used an estimate of 0.0035 m s$^{-1}$ for the $g_{\text{pl}}$ and $g_{\text{env}}$ as Tomas et al. [16] suggested. In addition, $g_{\text{cw}}$, $g_{\text{cyt}}$, and $g_{\text{st}}$ were calculated as:

$$g_i = \frac{1}{r_i} = \frac{r_i D_w p_i}{\Delta L_i}$$  \hspace{1cm} (16)

where $i$ stands for cell wall, cytosol, or stroma conductance. $r_i$ accounted for the reduction in the aqueous phase diffusion coefficient for CO$_2$ ($D_w$, 1.79 $\times$ 10$^{-9}$ m$^2$ s$^{-1}$ at 25 °C) and was taken as 1.0 for cell walls and 0.3 for cytosol and stroma, respectively. $p_i$ was the effective porosity (m$^3$ m$^{-3}$) and was taken as 1.0 for the cytosol and stroma and 0.28 for the cell walls. $\Delta L_i$ (m) is the diffusion path length in the corresponding component of the diffusion pathway.

The proportion of $g_{\text{m}}$, determined by limited gas-phase conductance ($l_{\text{m}}$) was calculated as:

$$l_{\text{m}} = \frac{g_{\text{m}}}{g_{\text{liq}}}$$  \hspace{1cm} (17)

The share of $g_{\text{m}}$ by different components of the cellular phase conductance ($l_i$) was determined as:

$$l_i = \frac{g_{\text{m}}}{g_{i} S_m}$$  \hspace{1cm} (18)

Statistical analysis

One-way ANOVA was applied to assess the differences in each parameter among the treatments with the SPSS 16.0 statistical software package. Significant differences ($P < 0.05$) among treatments are indicated by different letters using the least significant difference test.
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Abbreviations
C2: Chloroplast CO2 concentration; C3: Intercellular CO2 concentration; Dc: Leaf density; gmc: Mesophyll conductance; gsm: Stomatal conductance; HAN: High NH4+; HNN: High NO3−; LAN: Low NH4+; Lc: Chloroplast length; LNN: Low NO3−; M0: Specific leaf weight; MAN: Intermediate NH4+; MNN: Intermediate NO3−; Nc: Chloroplasts number per mesophyll cell; Nl: Leaf nitrogen content; Pn: Net photosynthetic rate; PNU: Photosynthetic nitrogen use efficiency; Sc: Chloroplast surface area facing intercellular air spaces; Sec: Chloroplasts section area; Smm: The surface area of mesophyll cells to the intercellular air-spaces; Suc: Chloroplast surface area; Vchl: Chloroplast volume; Tc: Chloroplast thickness; Tl: Leaf thickness; Tm: Mesophyll thickness; Tmm: Mesophyll cell wall thickness

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Authors’ contributions
S.W.G. and L.M.G. conceived and designed the experiments; L.M.G. and K.L.X. performed the experiments; L.M.G. and Z.F.L. analyzed the data and wrote the paper; L.M.G., Z.F.L. and L.D. helped in analysis of the results and manuscript writing; all authors discussed the results and wrote the manuscript. The author(s) read and approved the final manuscript.

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Availability of data and materials
The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Additional file 1: Figure S1. Representative light micrographs (A ~ F; scale bar = 200 μm) and transmission electron micrographs (G ~ I; scale bar = 5 μm; J ~ L; scale bar = 1 μm) of rice leaves supplied with NH4+ (mB, mC; intermediate N) or NO3− (mD, mE, mF; high N) under 3 different amounts, low N (0.71 mM, LAN and LNN), intermediate N (2.86 mM, MAN and MNN), and high N (7.14 mM, HAN and HNN). UEP, upper epidermis; LEP, lower epidermis; V, vascular bundle; CP, chloroplast; CW, cell wall; SG, starch grain; OG, osmiophilic globule. Figure S2. The relationship between mesophyll diffusion conductance (gmm) measured with the Harley et al. method and zmm modeled with anatomical parameters (Eq. 13–16). Values are means ± SD of four replicates. The data were fitted by linear regression. Broken lines correspond to the 1:1 relationship. Figure S3. Differential interference contrast image of chloroplasts in mesophyll cells separated from leaves. Leaves were cut into small pieces and fixed with 3.5% glutaraldehyde, and the mesophyll cells were individually dispersed on the glass plate and observed by microscopy. The red circles in the figure indicate individual mesophyll cells, and the chloroplast numbers therein were counted; the arrows indicate that the mesophyll cells did not separate efficiently. Bars = 20 μm.

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