Leaf nitrate accumulation influences the photorespiration of rice (*Oryza sativa* L.) seedlings

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Abstract

Aims The form of nitrogen (N) supply influences photorespiration in C3 plants, but whether nitrate (NO_3^-) regulates photorespiration and, if so, the underlying mechanisms for such regulation are still unclear.

Methods Three hydroponic experiments were conducted in a greenhouse to investigate the relationships between leaf NO_3^- concentrations and photorespiration rates in rice (*Oryza sativa* L.) genotypes *cv.* 'Shanyou 63' hybrid *indica* and 'Zhendao 11' hybrid *japonica* or using mutants that overexpress *NRT2.1* (in *cv.* 'Nipponbare' inbred japonica). We estimated photorespiratory rate from the CO_2 compensation point in the absence of daytime respiration (Γ*) using the biochemical model of photosynthesis.

Results Higher Γ* values under high N level or NO_3^- were significantly and positively correlated with leaf NO_3^- concentrations. Further elevating leaf NO_3^- concentrations by either resuming NO_3^- nutrition supply after N depletion (in *cv.* 'Shanyou 63' hybrid *indica* and 'Zhendao 11' hybrid *japonica*) or using mutants that overexpress *NRT2.1* (in *cv.* 'Nipponbare' inbred japonica) increased Γ* values. Additionally, the activities of leaf nitrate reductase (Nr) and concentrations of organic acids involving in the tricarboxylic acid (TCA) cycle synchronously changed as environmental conditions were varied.

Conclusions Photorespiration rate is related to the leaf NO_3^- concentration, and the correlation may links to the photorespiration-TCA derived reductants required for NO_3^- assimilation.

Keywords Rice (*Oryza sativa* L.) · Ammonium · Nitrate · Photorespiration rate · Tricarboxylic acid cycle · Malic acid

Abbreviations

A, net photosynthetic rate; C_i, intercellular CO_2 concentration; C_i*, apparent CO_2 compensation point in the absence of respiration; g_m, mesophyll conductance; g_s, stomatal conductance; J_T, total electron transport rate N, nitrogen; NH_4^+, ammonium; NO_3^-, Nitrate; Nr, nitrate reductase; PPFD, photosynthetic photon flux density; $\boldsymbol{R}_d$, day respiration rate; TCA, tricarboxylic acid; Γ*, CO_2 compensation point in the absence of daytime respiration.

Introduction

The rate of photosynthesis in C3 plants is related to the carboxylation capacity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), which catalyzes both the carboxylation and oxygenation of ribulose-1,5-bisphosphate (RuBP) (Long et al. 2006; Timm et al. 2016). The product of the RuBP oxygenation reaction, 2-phosphoglycolate, is further metabolized in chloroplast, mitochondria, and peroxisomes (Long et al. 2006; Somerville 2001). This process is called photorespiration and is closely linked to many physiological processes, including the carbon and nitrogen (N) cycle, cell energy metabolism and redox regulation in plants (Hodges et al. 2016). Generally, photorespiration is regarded as an energetically wasteful process (Voss et al. 2013; Walker et al. 2016), which consumes 25%–50% of the produced NADPH and 25%-30% of the fixed carbon (Bauwe et al. 2010). However, more recent studies suggested that photorespiration maybe more energy-efficient than previous assumed and this process stimulates chloroplastic malate production to provide reductants for plant energy-intensive activities, therefore have positive effects on plant physiological responses (Bloom and Lancaster 2018; Busch 2020). This aligns with observations that photorespiration is extremely important for plant normal growth, despite its general adverse effects on carbon fixation and plant productivity at normal CO_2/O_2 conditions. For example, the knock-down of the key genes encoding photorespiratory enzymes will provoke abnormal plant growth (Timm and Bauwe 2013). In water-stressed grapevine (Guan et al. 2004), high irradiated soybean (Jiang et al. 2006), and *P. syringae* pv. *tabaci* challenged *Arabidopsis* (Rojas et al. 2012), reduced photorespiration was linked to decreased plant tolerance to indicate the role of the photorespiratory cycle in countering environmental stresses in C3 plants. These findings underline the importance of understanding the physiological contribution of photorespiration in plant growth and productivity.

N nutrition is essential for photosynthesis and photorespiration (Hodges et al. 2016). Generally, leaf photosynthetic rates can be increased by N fertilization (Dordas and Sioulas 2008; Makino 2003; Makino 2011), but increasing N supply leads to a significant decrease in photosynthetic N use efficiency (PNUE, calculated as the photosynthetic rate per unit leaf organic N content) (Li et al. 2012). One reason for this, is the relative insufficient CO_2 supply at the Rubisco carboxylation sites under high N conditions (Li et al. 2012; Yamori et al. 2011), which would enhance photorespiration rate (Guilherme et al. 2019; Li et al. 2009). N concentrations in plant tissues decrease at elevated atmospheric CO_2 condition, and the magnitude of the decrease exceeds what would be expected by any dilution effect from N driving production of additional biomass (Bloom et al. 2002; Wujeska-Klause *et al.*, 2019; Dong *et al.*, 2018). Wujeska-Klause *et*

al. (2019) suggested that the decrease in N concentration may relate to the decreased activity of nitrate (NO_3^-) reductase, due to a limited supply of reductant from lower photorespiration at elevated atmospheric CO_2. Such changes are most probably connected to changes of organic acids in the tricarboxylic acid (TCA) cycle (Obata et al. 2016; Timm et al. 2015). This highlights the link between photorespiration and N metabolism.

Ammonium (NH_4^+) and NO_3^- are two forms of inorganic N and photorespiration rates are higher in NO_3^- compared to NH_4^+ fed plants (Guo et al. 2005). Moreover, Oliveira et al. (2002) described a negative relationship between leaf NH_4^+ concentrations and photorespiration rates in transgenic tobacco plants overexpressing cytosolic glutamine synthetase. This clearly suggested that NO_3^-, rather than NH_4^+, is related to photorespiration. However, the question of whether NO_3^- is involved in photorespiratory regulation and its mechanism has not been systematically studied.

In the present study, three different experiments were conducted in rice (*Oryza sativa* L.) plants to address these questions. Firstly, two rice genotypes (*cv.* 'Shanyou 63' and 'Zhendao 11') were supplied with the combinations of three different N levels (Low-N: 10 mg L^{-1}; Medium-N: 40 mg L^{-1} and High-N: 100 mg L^{-1}) and three different N forms (NH_4^+, NO_3^-, and the mixture of equal mol of NH_4^+ and NO_3^-), to study whether photorespiration rate is related to the bulk leaf N content, or related to the inorganic N of NH_4^+ or NO_3^-. Secondly, the rice plants of 'Shanyou 63' and 'Zhendao 11' were supplied with N-free nutrient solutions for one week to deplete leaf inorganic nitrogens. They were then supplied with three different concentrations of NO_3^- (20, 40 and 60 mg NO_3^- L^{-1}) for three days to assess the effect of exogenous supply of NO_3^- on photorespiration rates. Thirdly, the differences in photorespiration rate were studied in two transgenic lines of rice plants (*cv.* Nipponbare), overexpressing the *OsNRT2.1* which encodes a high-affinity NO_3^- transporter, to investigate whether photorespiration rates can be influenced through genetic manipulation. Finally, the underlying mechanisms were discussed, linking leaf NO_3^- content, leaf N metabolism, and the photorespiration process.

Material and methods

Plant material and growth conditions

Two rice cultivars 'Shanyou 63' hybrid *indica* China and 'Zhendao 11' *japonica* China were selected in this study. Rice seeds were surface sterilized in 10% H_2O_2 (V/V) for 30 min and washed thoroughly with water; then they were transferred to a mesh for germination at 37 °C. When the seedlings had developed an average of 2-3 visible leaves, they were transplanted into 6.0 L containers (30×20×10 cm) containing 1/4 strength of NH_4^+ and NO_3^- mixture nutrient solution (see compositions below) with 12 seedlings per container. Three days later, the seedlings were supplied with a 1/2 strength NH_4^+ and NO_3^- mixture nutrient solution. Another three days later, they were then supplied with full-strength NH_4^+ and NO_3^- mixture solutions. One week later, different treatments were applied to the plants as indicated by the requirements of a given experiment.

The compositions of the full-strength of NH_4^+ and NO_3^- mixture nutrients were as follows. Macronutrients: 40 mg L^{-1} (2.85 mM) N as equal mol of $(NH_4)_2SO_4$ and $Ca(NO_3)_2$, 10 mg L^{-1} phosphorus (P) as KH_2PO_4, 40 mg L^{-1} potassium (K) as K_2SO_4 and KH_2PO_4, and 40 mg L^{-1} magnesium (Mg) as $MgSO_4$. Micronutrients: 2.0 mg L^{-1} iron (Fe) as Fe- EDTA, 0.5 mg L^{-1} manganese (Mn) as $MnCl_2{\cdot}4H_2O$, 0.05 mg L^{-1} molybdenum (Mo) as $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.2 mg L^{-1} boron (B) as H_3BO_3, 0.01 mg L^{-1} zinc (Zn) as $ZnSO_4{\cdot}7H_2O$, 0.01 mg L^{-1} copper (Cu) as $CuSO_4{\cdot}5H_2O$, and 2.8 mg L^{-1} silicon (Si) as $Na_2SiO_3{\cdot}9H_2O$. A nitrification inhibitor (dicyandiamide, DCD) was added to each nutrient solution to prevent the oxidation of NH_4^+. The nutrient solution was changed every 3 days, and the pH was adjusted to 5.50 ± 0.05 by every day using 0.1 mM HCl and 0.1 mM NaOH. All of the following three experiments were conducted in an environmental-controlled growth room. The environmental conditions in the growth chamber were set to 30/20°C day/night temperature, 70% air humidity, 400 μmol mol^{-1} CO_2 concentration, 1000 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) at the leaf level, and a 12-h photoperiod.

Experiment I

After growth on full-strength of NH_4^+ and NO_3^- solution for one week, 'Shanyou 63' and 'Zhendao 11' were divided into nine groups, with the combinations of three different N levels (Low-N: 10 mg L^{-1}; Medium-N: 40 mg L^{-1} and High-N: 100 mg L^{-1}) and three different N forms (NH_4^+, NO_3^-, and the mixture of equal mol of NH_4^+ and NO_3^-). All other nutrients, except for N, were as listed above. N was supplied with different concentrations, with either NH_4^+, NO_3^-, or an equal mol of NH_4^+ and NO_3^-. The Ca content with NH_4^+ and the equal mol of NH_4^+ and NO_3^- treatments were compensated by the addition of $CaCl_2$ to the level in NO_3^- solution. Three weeks after treatments, gas-exchange and fluorescence measurements were

conducted and the fresh leaf samples were flash-frozen with liquid nitrogen, and then stored at -80 °C before further analysis.

Experiment II

After the supplement of full-strength of NH_4^+ and NO_3^- mixture solution for one week, 'Shanyou 63' and 'Zhendao 11' were supplied with N-free nutrient solutions for one week to deplete leaf inorganic nitrogens. All other nutrients were as listed above. Afterwards, the seedlings were divided into three groups and supplied with different levels of NO_3^- (20, 40 and 60 mg NO_3^- L^{-1}) for three days. Thereafter, the measurements of gas-exchange, fluorescence and biochemical parameters were conducted.

Experiment III

Two transgenic lines of rice (ssp. *Japonica cv.* 'Nipponbare') plants, overexpressing the *OsNRT2.1* gene using a ubiquitin (Ubi) promoter (*pUbi*: *OsNRT2.1*) or the OsNAR2;1 promoter (*pOsNAR2.1-NRT2.1*)., together with their wild type were supplied with full-strength NH_4^+ and NO_3^- solutions for two weeks. Thereafter, the measurements of gas-exchange, fluorescence and biochemical parameters were conducted. Detailed description of the transgenic genotypes can be found in Chen et al. (2016).

Gas exchange and fluorescence measurements

The light-saturated photosynthetic rate and chlorophyll fluorescence of newly expanded leaves were measured from 9:30 to 15:30 in the growth chamber using a Li-Cor 6400 portable photosynthesis open system (LI-COR, Lincoln, NE, USA). Leaf temperature during measurements was maintained at 28.0±0.5°C, with a photosynthetically active photon flux density (PPFD) of 1500 μmol photons m^{-2} s^{-1}. The CO_2 concentration in the chamber was adjusted to 400±10 μmol CO_2 mol^{-1}, and the relative humidity was maintained at approximately 40%. After equilibration to a steady-state (about 10 min), 0.8 s saturating pulses of saturating light (~8,000 mol m^{-2} s^{-1}) were supplied to measure the total electron transport rate (J_T), the maximum and steady-state fluorescence (F_m' and F_s, respectively), the net photosynthesis rate (A), stomatal conductance (g_s), and intercellular CO_2 concentration (C_i). The actual photochemical efficiency of photosynthetic system II (ϕ_{PSII}) was calculated as:

$$\phi_{PSII} = \frac{(Fm' - Fs)}{Fm'}$$

Then the total electron transport rate (J_T) was calculated as:

$$J_T = \phi_{PSII} \times PPFD \times \alpha_{leaf} \times \beta$$

where α_{leaf} is the leaf absorptance and β is the partitioning of absorbed quanta between PSII and PSI. The values of α_{leaf} and β were designated as 0.85 and 0.5 respectively according to Manter and Kerrigan (2004).

Measurement of day respiration rate ($\boldsymbol{R}_d$) and the CO_2 compensation point in the absence of respiration (Γ*)

The $\boldsymbol{R}_d$ and apparent CO_2 compensation point in the absence of respiration (C_i*) were measured through the *A*/C_i response curves on newly expanded leaves of rice plants. This takes advantage of the photorespiration rate being dependent on and $\boldsymbol{R}_d$ being independent of PPFDs. When *A*/C_i response curves were conducted at a various of CO_2 concentrations and PPFDs, they intersected at a single point where *A* was taken as -$\boldsymbol{R}_d$, and C_i represented C_i* (Supplementary Fig. 1). The PPFDs used in the cuvette were a series of 150, 300, and 600 μmol photons m^{-2} s^{-1}. At each PPFD, ambient CO_2 concentration (C_a) was adjusted to a series of 25, 50, 75, and 100 μmol CO_2 mol^{-1}. Thirty minutes prior to initiating measurements, leaves were placed in a cuvette at a PPFD of 600 μmol photons m^{-2} s^{-1} and a C_a of 100 μmol CO_2 mol^{-1}.

According to Pons et al. (2009) and Harley et al. (1992), Γ* was then calculated according to the following equations:

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

$$g_m = \frac{A}{\left\{ C_i - \Gamma^{*} \times \frac{\left[J_T + 8\left(A + R_d \right) \right]}{\left[J_T - 4\left(A + R_d \right) \right]} \right\}}$$

where g_m represents leaf mesophyll conductance.

Measurement of leaf total N, NH_4^+ and NO_3^- content

The total N in rice leaves was determined by the Kjeldahl H_2SO_4–H_2O_2 digestion method of Nelson and Sommers (1972). The extraction and measurement of NH_4^+ and NO_3^- were conducted following the method of Cataldo et al. (1975) and (Cataldo et al. (1975); Wang et al. (2016)), with minor modification. For the measurement of leaf NH_4^+ content, 0.5 g fresh sample was homogenized with 5 mL of 0.3 mM H_2SO_4, and NH_4^+ content was determined using the phenol–hypochlorite method after centrifugation at 15,000×g for 15 min. To measure NO_3^- content, 0.5 g leaf sample was homogenized with 5 mL distilled water, followed by the transfer to 10 mL centrifuge tubes. They were then boiled in a water bath for 30 min, cooled down to

191room temperature and then centrifuged at 5,000×*g* for 10 min. Afterwards, 0.1 mL supernatant liquid was
192taken to a new tube, with an addition of 0.4 mL 5% sulfuric acid-salicylic acid solution. Following vortexing
193for 20 min at room temperature, 9.5 mL 8% sodium hydroxide were added and the Ab_{410nm} was measured in
194a spectrophotometer.
195
196Measurement of nitrate reductase (Nr) activity
197In order to measure Nr activity, 1.0 g fresh weight of rice leaf was ground with fine sand beads in a cold
198mortar containing 4 mL of 0.1 M potassium phosphate buffer (pH 7.5), 1 mM EDTA, 3 mM cysteine, and
1993% (w/v) casein. The homogenate was centrifuged at 4,000×*g* for 15 min, and the supernatant was reacted
200with 100 mM potassium nitrate buffer (pH 8.8) and 2 mg mL^{-1} NADH at 25 °C for 30 min. The reaction was
201terminated by adding 1 % sulphanilamide. 1 % N-(1-naphthyl) ethylene-diamine hydrochloride was then
202added, centrifuged at 4,000×*g* for 5 min, and the Ab_{540nm} measured in a spectrophotometer.
203
204Organic acid measurement
205The organic acids were extracted and identified according to the method developed by Ji et al. (2005). 500
206mg frozen leaf sample was ground in a mortar with 2 mL of methanol: water (80:20, v/v). The solvent was
207collected into a microcentrifuge tube, shaken at 1200 rpm for 3 min and then centrifuged at 12,000×*g* for 5
208min. The supernatant was assessed using high-performance liquid chromatography (HPLC) analyses.
209 Standard organic acid compounds for HPLC are used, including oxalic acid, malic acid, glycolic acid,
210glyoxylic acid, 2-ketoglutarate acid and oxaloacetic acid. The compounds were identified using an HPLC
211system (Agilent 1200, USA) with an XDB-C18 column (4.6×250 mm, Agilent, USA) (Ling et al. 2011). The
212analytical conditions were as follows, chromatographic column: XDB-C18 (4.6 mm × 250 mm), the
213temperature of column: 40 °C, detector wavelength: 210 nm, and injection volume: 20 μL. The mobile phase
214consisted of 70%:30% (v/v) acetonitrile (A) and 20 mM ammonium acetate buffers (B) with gradient
215elution. The gradients were established as follows: 0 min, 95% A plus 5% B at a flow rate of 0.4 mL min^{-1}→1 min, 95% A plus 5% B at a flow rate of 0.4 mL min^{-1}→16 min, 90% A plus 10% B at a rate of 0.5 mL min^{-1}→20 min, 90% A plus 10% B at a rate of 0.5 mL min^{-1}→stop. Only high purity chemicals were used,
218and the solvents were HPLC spectral grade. Major peaks were identified by comparing the retention time
219with that of the matching standards.
220

221Statistical analysis

222One-way analysis of variance (ANOVA) was applied to assess differences using the SPSS 16.0 statistical
223software package. Each mean was based on 4 experimental replicates and calculated standard deviations
224(SD) are reported. Significance was tested at the 5% level.

Results

Effects of different N supply on rice growth and leaf gas-exchange parameters

Feeding with high N significantly increased plant height and shoot biomass ($P < 0.01$) but limited the root growth in both 'Shanyou 63' and 'Zhendao 11' (Supplementary Table 1). This resulted in a significantly lower root/shoot ratio with increasing N supply. Different N forms also have a significant effect on root growth. Root length and root biomass were both larger in NO_3^- than in NH_4^+ treatments, although shoot biomass did not significantly differ (Supplementary Table 1).

In both genotypes, A, g_s, C_i and J_T were significantly increased with N concentration ($P < 0.01$). N form had no influence on leaf A in rice seedlings growth at low-N and medium-N levels ($P = 0.56$ and $P = 0.115$, respectively). However, at high-N, N form had significant effect on leaf A values ($P = 0.03$) with the lowest value in NO_3^- treated 'Zhendao 11' seedlings (Table 1). Further, C_i was significantly higher in NO_3^- than in NH_4^+ supply, regardless of N levels and rice cultivars.

Effects of different N supply on C_i*, $\boldsymbol{R}_d$, g_m and Γ*

Γ* values were significantly different between rice cultivars, N levels and N forms (Table 1). Γ* was significantly increased with increased N levels, in both 'Shanyou 63' ($P < 0.001$) and 'Zhendao 11' ($P < 0.001$). The changes in C_i* and g_m were consistent with Γ*, while $\boldsymbol{R}_d$ was significantly reduced under high-N compared with low-N and medium-N conditions. C_i* and Γ* values were significantly higher in NO_3^- fed than in NH_4^+ fed seedlings ($P < 0.001$). No significant difference was observed in $\boldsymbol{R}_d$ and g_m between the N forms (Table 2).

Leaf total N and inorganic N concentrations in newly expanded rice leaves

In both 'Shanyou 63' and 'Zhendao 11', leaf total N concentrations increased with the increasing N levels ($P < 0.01$), regardless of N forms (NH_4^+ *vs* NO_3^-) (Fig. 1 A, B). NH_4^+ concentration in 'Zhendao 11' was remarkably higher than that in 'Shanyou 63' ($P < 0.001$), in contrast, leaf NO_3^- concentration was lower in 'Zhendao 11' than in 'Shanyou 63'. Leaf NH_4^+ concentration in rice seedlings was not significantly changed by N supply forms. However, the leaf NO_3^- concentration was dramatically higher in NO_3^- fed than in NH_4^+ fed seedlings (Fig. 1 E, F).

Correlations between leaf Γ* and N status

The linear correlation analysis was conducted between Γ* and total N, NH_4^+ or NO_3^- (Fig. 2). A significant positive correlation was observed between leaf NO_3^- concentrations and Γ* values**,** regardless of rice cultivars or treatments. In contrast, no significant relationship was observed between Γ* values and leaf total N or NH_4^+ concentrations.

Effect of short-term exogenous NO_3^- supply after N depletion on Γ*

Leaf NO_3^- concentrations and Γ* were gradually increased by increasing exogenous NO_3^- levels in both rice cultivars (Fig. 3A and 3B). There were no significant differences in the concentrations of leaf glycolic acid and glyoxylic acid, the two most important metabolites in the photorespiratory pathway, between 20 and 40 mg L^{-1} NO_3^- supply after N depletion (Fig. 3C). Compared with those under 20 mg L^{-1} NO_3^- supply, under 60 mg L^{-1} NO_3^- treatment, glycolic acid and glyoxylic acid concentrations were increased by 26.44% and 166.32%, respectively, in 'Shanyou 63'; while they were increased by 92.87% and 22.82%, respectively, in 'Zhendao 11'. In addition, leaf NO_3^- concentrations and Γ* were significantly and positively correlated in both 'Shanyou 63' ($P < 0.01$) and 'Zhendao 11' ($P < 0.05$) (Fig. 3 D).

The variation in Γ* between the wild type lines and the lines overexpressing OsNRT2.1

Leaf NO_3^- concentrations in *pOsNAR2.1:OsNRT2.1* and *pUbi:OsNRT2.1* Nipponbare leaves were 57% and 102% higher than in WT (Fig. 4A). Interestingly, leaf Γ* values also increased by 15.7% and 26.4%, respectively (Fig. 4B). A significant positive correlation between leaf NO_3^- concentration and Γ* value was also seen in different lines of Nipponbare plants (Fig. 4C). However, glycolic acid and glyoxylic acid concentrations did not significantly differ between different lines (Fig. 4D).

Leaf nitrate reductase (Nr) activity and organic acids concentrations

Nr activities increased with the exogenous NO_3^- supply in both cultivars (Fig. 5A). When comparing plant treated with 20 mg L^{-1} NO_3^- with 60 mg L^{-1} NO_3^-, Nr activity was significantly increased by 112.64% and 66.45%, respectively, in 'Shanyou 63' and 'Zhendao 11'. Nr activities in transgenic Nipponbare lines (*pOsNAR2.1:OsNRT2.1* and *pUbi:OsNRT2.1*) were also much higher than WT (Fig. 5 B).

In both 'Shanyou 63' and 'Zhendao 11', the content of organic acids involved in the TCA cycle increased with exogenous NO_3^- supply (Fig. 6). Similarly, transgenic Nipponbare lines exhibited markedly increased oxalic acid and 2-ketoglutaric acid concentrations compared to WT (Fig. 6 B, H). However, the

285concentrations of oxaloacetic acid and malic acid did not significantly changed in the transgenic lines of 286Nipponbare plants (Fig. 6 D, F).

Discussion

The estimation of photorespiration rate

Some time ago, Sharkey (1988) considered the four different methods used for the determination of leaf photorespiration rate, which are the post illumination burst of CO_2, inhibition of photorespiration by O_2, CO_2 efflux into CO_2-free air, and the ratio of $^{14}CO_2$ to $^{12}CO_2$ uptake. However, neither of them have been widely used due to their respective limitations (Busch et al. 2012; Sage and Pearcy 1987; Sharkey 1985). Busch (2013) characterized multiple newly developed techniques, including $^{12}CO_2$ efflux into a $^{13}CO_2$ atmosphere, ^{14}C-labelling of photosynthates, photorespiratory ammonia production, ^{18}O-labelling of photorespiratory metabolites and ^{13}C-labelling of phosphorylated Calvin–Benson cycle intermediates. Nevertheless, these methods may underestimate photorespiration rate as they neglect the responses of $\boldsymbol{R}_d$ to high CO_2 concentrations, mitochondrial ammonia refixation and O_2 uptake, or re-assimilation of the photorespired CO_2 (Busch et al. 2012; Cousins et al. 2008; Mattsson 1996).

Both Sharkey (1988) and Busch (2013) emphasized the applicability of the Farquhar, von Caemmerer, and Berry (FvCB) model (Farquhar et al. 1980) to indirectly estimate photorespiration rate, by measuring Γ*. This method has been used widely during the past decades (Busch 2013; Li et al. 2013; Wujeska-Klause et al. 2019). Moreover, the consistent changes seen between photorespiratory metabolites contents and the estimated photorespiration rate from Γ*, using the FvCB model, support the applicability of the latter method (Shen et al. 2019; South et al. 2019). In the present study, the responses of Γ* to N nutrition as well as rice genotypes proved to be more sensitive than that of photorespiratory metabolites (Fig. 3, Fig. 4), which again suggested the value of the FvCB model. Therefore, this method was used to evaluate the photorespiration rate.

The interactive relationship between leaf NO_3^- concentrations and Γ*

Our results clearly showed that Γ* was related to leaf NO_3^- content, rather than reflecting bulk leaf N content or leaf NH_4^+ content, and the process of N metabolism may involve in the linkage (Fig. 2, Fig. 5). Moreover, we also found that Γ* can be genetically modified by overexpressing the gene of *OsNRT2.1* (Fig. 4). These findings are of great importance to agricultural production, especially in the context of global warming, because photorespiration increases exponentially with temperature. Interestingly, the variations of g_m to N supply are much greater than that of Γ* (Table 1). The main reason for such an event is the sensitivity of g_m determinants, including cell wall thickness, chloroplast size and carbonic anhydrase activity, to

317environmental changes (Flexas et al. 2008; Xiong et al. 2015). However, the Γ* responses are relatively
318smaller due to the photorespiratory CO_2 re-assimilation and the affinity of Rubisco for CO_2 (Berghuijs et al.
3192017).

320 Our positive correlation between leaf NO_3^- content and photorespiration rate is supported by previous
321studies (Frechilla et al. 1999; Lawlor et al. 1987), where leaf photorespiration rate and glycolate oxidase
322activity were higher in NO_3^- fed wheat and pea plants. Moreover, the expressions of *PGP* (*phosphoglycolate*
323*phosphatase*) and *GDCT* (*glycine decarboxylase T-protein*) genes, which encode the enzymes involving in
324the photorespiratory processes, were upregulated by NO_3^- supply (Parker and Armbrust 2005).

325 The variation in Nr activity with different NO_3^- treatments and across different transgenic lines were
326similar to those in Γ* values (Figs. 3-5). This has also been observed in *Eucalyptus* trees (Wujeska-Klause et
327al. 2019). A positive relationship between photorespiration rate and NO_3^- assimilation was also indirectly
328suggested by the responses of plant growth to environmental CO_2 concentrations, which can significantly
329affect photorespiration rate. For instance, the adverse effect of sub-ambient CO_2 on the growth rate of
330loblolly pine was relieved when receiving NO_3^- rather than NH_4^+ nutrition (Bloom 2015). Such a
331phenomenon may be caused by increased NO_3^- assimilation under high-photorespiration condition.
332Conversely, growth promotion with enriched CO_2 concentrations was lower in NO_3^- compared to NH_4^+-fed
333California grassland, wheat, and *Arabidopsis (Bloom 2015; Bloom et al. 2010; Rachmilevitch et al. 2004)*.
334Moreover, the enriched CO_2 inhibits NO_3^- assimilation into organic nitrogen compounds. Taken together
335these data indicate the close relationship between photorespiration with NO_3^- and NO_3^- metabolic processes.
336

337The potential mechanisms linking photorespiration and nitrate assimilation

338The present study showed increases in TCA cycle organic acids with increased NO_3^- content and enhanced
339photorespiration rate (Fig. 6). Such links between leaf NO_3^- and organic acids have been previously
340documented in tobacco (Scheible 2000), tomato (Martinez-Andujar et al. 2013) and cucumber (Wang et al.
3412018) plants. The reducing power (NADH) required for NO_3^- reduction may be the key link between NO_3^-
342and such organic acids due to the derivation of NADH from the "malate shuttle" between cytoplasm and
343mitochondria (Martinoia 1994; Scheible 1997). This is relevant as photorespiration is a vital redox transport
344system which increases the ratio of cytosolic NADH/NAD^+ through malate transport, from the chloroplast
345through the cytoplasm and into the peroxisome (Bloom 2015; Bloom et al. 2010; Voss et al. 2013). Thus, the
346TCA cycle is proposed as the critical metabolic process that connecting photorespiration, respiration, and N

assimilation (Foyer et al. 2011).

The relationships between leaf NO_3^- and photorespiration is clear when all of these features are considered. When NO_3^- is transported and accumulated in leaf tissue, NADH is required for NO_3^- reduction. The required NADH is produced from mitochondrial "malate shuttle", which is tightly coupled with the photorespiratory pathway that consumes malic acid in the peroxisome. Hence, the photorespiration cycle may be driven by NO_3^- assimilation (Bauwe et al. 2010; Rachmilevitch et al. 2004). Interestingly, the NADH/NAD^+ ratio was surprisingly higher under photorespiration conditions (low CO_2), which was inhibited in the glycine decarboxylase complex-deficient mutants (Taniguchi and Miyake 2012). This provides more evidence that NADH status and photorespiration process were closely related. Schneidereit et al. (2006) reported that, after the antisense-repression of plastidic dicarboxylate translocator 1-[2-OG/malate translocator] in tobacco, leaf NO_3^- was dramatically accumulated with the inhibited Nr activity when compared with their wild types. Therefore, leaf photorespiration is tightly linked to NO_3^- reduction through the metabolisms of organic acids and the change in leaf NO_3^- status is an important factor affecting the photorespiration rate.

Conclusions

Our results suggested that the high-N or NO_3^- nutrition induced increase in photorespiration is related to the accumulated leaf NO_3^- content. Furthermore, the causal-relationship between leaf NO_3^- and photorespiration rate was demonstrated both physiologically and biochemically. We suggest that this may be caused by an association of NO_3^- assimilation, malate transportation and photorespiration.

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Author contributions

S.W.G. and Y.R.L. conceived and designed the experiment; Y.R.L., B.W. and M.W. performed the experiments; Y.R.L., Y.M.S. and L.D. analyzed the data and contributed table and figures; Y.M.S. and S.W.G. wrote the paper; X.R.F. provided the transgenic lines of rice seedlings; Y.L., L.A.J.M. and Q.R.S. proofread and polished the manuscript; all authors reviewed the manuscript and approved the final manuscript.

Supplementary data

Supplementary Table 1 Effect of different nitrogen (N) levels and forms on the height (cm), root length (cm), root and shoot biomass (g plant^{-1}) and root/shoot ratio of rice seedlings ('Shanyou 63' and 'Zhendao 11').

Supplementary Fig. 1 The measurement of apparent CO_2 compensation point in the absence of respiration (C_i*) and day respiration rate ($\boldsymbol{R}_d$) in rice plants ('Shanyou 63' and 'Zhendao 11') under medium-N conditions (40 mg N L^{-1}) in the form of ammonium (NH_4^+, A), the mixture of equal mol of NH_4^+ and NO_3^- (NH_4^+/ NO_3^-, B) or nitrate (NO_3^-, C). Squares, circles and triangles represent different light intensity (150, 300, 600 μmol m^{-2}s^{-1}, respectively). Lines were fitted by linear regression and the co-ordinates of their intersection were taken as estimates of C_i* and $\boldsymbol{R}_d$.

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538**Tables**

Cultivars	Treatments		A	g_s	g_m	C_i	J_T	C_i*	R_d	Γ*
‘Shanyou 63’	NH_4^+	Low-N	16.4±1.8d	0.19±0.04d	0.13±0.03b	256±14c	155.01±8.51d	30.22±0.99f	0.72±0.18a	35.97±0.87ef
		Medium-N	22.3±1.5b	0.29±0.06bc	0.21±0.06ab	260±4bc	175.15±10.54bc	34.74±1.14e	0.53±0.05abc	37.40±1.35cde
		High-N	21.9±1.9b	0.35±0.06ab	0.24±0.09ab	269±6abc	169.77±1.88c	37.10±1.36d	0.40±0.19bcd	38.76±1.45cd
	NH_4^+ / NO_3^-	Low-N	18.3±1.4cd	0.29±0.03bc	0.14±0.04b	279±6ab	149.28±8.11d	32.19±1.01f	0.57±0.11ab	36.69±2.89de
		Medium-N	25.0±0.7a	0.39±0.01a	0.25±0.08ab	281±4a	185.74±7.26ab	38.16±1.61cd	0.34±0.10cd	39.63±1.38bc
		High-N	25.9±1.1a	0.40±0.01a	0.30±0.11a	287±11a	189.55±6.87a	40.79±2.59ab	0.22±0.09d	41.58±2.24ab
	NO_3^-	Low-N	16.9±2.1d	0.23±0.02cd	0.21±0.09ab	277±13ab	129.28±10.63e	30.69±1.09f	0.57±0.09ab	33.81±0.94f
		Medium-N	20.3±1.0bc	0.35±0.04ab	0.22±0.07ab	280±5ab	157.79±2.60d	39.68±0.96bc	0.47±0.07bc	41.93±0.79ab
		High-N	24.5±0.5a	0.40±0.01a	0.27±0.07a	284±5a	182.73±0.80ab	42.63±1.13a	0.34±0.16cd	43.93±0.69a
‘Zhendao 11’	NH_4^+	Low-N	13.3±0.5d	0.17±0.01d	0.09±0.01ab	246±14c	153.58±16.59abc	27.17±0.64f	0.72±0.0ab	34.94±0.50e
		Medium-N	15.6±0.7bc	0.21±0.01bcd	0.09±0.01ab	257±6bc	179.42±3.32ab	31.52±1.23cd	0.40±0.09cd	35.86±0.62de
		High-N	16.7±1.4b	0.23±0.01ab	0.10±0.01a	263±6abc	188.97±8.55a	34.39±0.75b	0.29±0.03d	37.34±0.76cd
	NH_4^+ / NO_3^-	Low-N	14.2±0.8cd	0.21±0.02abcd	0.09±0.03abcd	264±6abc	145.37±8.75bc	28.02±1.49f	0.76±0.09a	37.27±1.11cd
		Medium-N	15.6±1.3bc	0.24±0.03ab	0.07±0.01bcd	265±7ab	168.22±21.53abc	30.06±1.38de	0.61±0.10b	38.39±1.61bc
		High-N	19.1±2.2a	0.26±0.03a	0.09±0.02abc	266±4ab	187.89±20.52a	34.76±1.26b	0.40±0.11cd	39.24±0.53b
	NO_3^-	Low-N	14.3±0.4cd	0.18±0.02cd	0.07±0.01cd	267±4ab	134.52±23.70c	28.94±0.85ef	0.66±0.07ab	38.22±1.04bc
		Medium-N	14.7±1.3cd	0.20±0.01bcd	0.07±0.01d	272±4ab	163.91±24.72abc	32.04±1.21c	0.49±0.08c	39.56±0.28b
		High-N	15.8±0.5bc	0.22±0.03abc	0.07±0.01cd	279±11a	174.65±13.11abc	37.46±1.93a	0.31±0.10d	41.96±1.69a
Cultivars			**	**	**	**	ns	**	ns	*
N levels			**	**	**	**	**	**	**	**
N forms			**	**	ns	**	**	**	ns	**

539 **Table 1** Effect of different nitrogen (N) levels and forms on the net photosynthetic rate (A, μmol CO_2 m^{-2} s^{-1}) , stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}), mesophyll 540conductance (g_m, mol m^{-2} s^{-1}), intercellular CO_2 concentration (C_i, μmol CO_2 mol^{-1}), electron transport rate (J_T, μmol photons m^{-2} s^{-1}), apparent CO_2 compensation point in the 541absence of respiration (C_i*,μmol CO_2 mol^{-1}), day respiration rate (R_d, μmol CO_2 m^{-2} s^{-1}) and CO_2 compensation point in the absence of daytime respiration (Γ*, μmol CO_2 mol^{-1}) of rice seedlings (‘Shanyou 63’ and ‘Zhendao 11’).

543

Rice plants (‘Shanyou 63’ and ‘Zhendao 11’) were supplied with three N levels (10 mg L^{-1} N as low-N, 40 mg L^{-1} N as medium-N and 100 mg L^{-1} N as high-N) in the form of ammonium (NH_4^+), nitrate (NO_3^-) or the mixture of equal mol of NH_4^+ and NO_3^- (NH_4^+/ NO_3^-). The data are from Experiment 1 and the values represent the means ± SD of 4 biological replicates. ANOVA results are indicated; different letters indicate significant differences in the same genotype, $P < 0.05$. * and ** indicate significant difference at 0.05 and 0.01 probability levels, respectively; ns indicates a non-significant difference.

Figure Legends

Fig. 1 Effect of different N levels and forms on the concentrations of leaf total-N (A, B), ammonium (NH_4^+, C, D) and nitrate (NO_3^-, E, F) in 'Shanyou 63' (A, C, E) and Zhandao 11 (B, D, F).

The data are from Experiment 1 and the values represent the means ± SD of four replicates. Significant differences between treatments are indicated by different letters ($P < 0.05$). * and ** indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively; ns indicates a non-significant difference at $P < 0.05$ level.

DW: dry weight, FW: fresh weight. LN: Low-N level, 10 mg L^{-1} N; MN: Medium-N level, 40 mg L^{-1} N; HN: High-N level, 100 mg L^{-1} N. NH_4^+: ammonium nutrient solution; NO_3^-: NO_3^- nutrient solution; NH_4^+/ NO_3^-: mixture nutrient solution with equal amount of NH_4^+ and NO_3^-.

Fig. 2 The linear relationships of Γ* with leaf total N and available N (ammonium and nitrate) contents under different N levels and forms in both 'Shanyou 63' (red circle) and 'Zhendao 11' (blue diamond). The data are from Experiment 1 and the values represent the means ± SD of four replicates.

DW: dry weight; FW: fresh weight; Γ*: CO_2 compensation point in the absence of respiration.

Fig. 3 Effect of exogenous supply of NO_3^- on the leaf NO_3^- concentrations (A), Γ* values (B), the relative leaf concentrations of glycolic acid and glyoxylic acid (C), and the correlation between leaf NO_3^- concentrations and Γ* values (D) in newly expended leaves of 'Shanyou 63' and 'Zhendao 11'.

The lines in panel D represent linear regressions, and the regression equation are $y = 202.920x + 12.277$, $R^2 = 0.6945$, $P < 0.01$ for 'Shanyou 63' and $y = 77.203x + 29.793$, $R^2 = 0.9844$, $P < 0.05$ for 'Zhendao 11'.

FW: fresh weight; Γ*: CO_2 compensation point in the absence of respiration.

The exogenous NO_3^- were supplied after 3 days of N depletion, and the levels of the exogenous NO_3^- were 20, 40 and 60 mg L^{-1}, respectively. The data are from Experiment 2 and the values represent the means ± SD of four replicates, and the bars indicate the SD. Significant differences between treatments are indicated by different letters ($P < 0.05$).

Fig. 4 The leaf NO_3^- content (A), Γ* values (B), the relative leaf concentrations of glycolic acid and glyoxylic acid (C) and the linear relationship between leaf NO_3^- concentrations and Γ* values (D) in newly expended leaves of WT and transgenic lines of Nipponbare.

The lines represent linear regressions and the regression equation is $y = 60.955 x + 27.223$, $R^2 = 0.998$, $P < 0.01$.

The transgenic lines of Nipponbare enhanced the expression of the *OsNRT2.1* gene that encodes a high-affinity NO_3^- transporter, using a ubiquitin (Ubi) promoter (*pUbi:OsNRT2.1*) or the NO_3^- inducible promoter (*pOsNAR2.1-NRT2.1*) of the *OsNAR2.1* to drive *OsNRT2.1* expression in transgenic rice plants.

Nipponbare plants were supplied with full-strength nutrient under medium-N level (40 mg L^{-1}). The data are from Experiment 3 and the values represent means of four replicates; bars indicate SD. Significant differences between treatments are indicated by different letters ($P < 0.05$).

Fig. 5 (A) Effect of exogenous NO_3^- supply on the leaf nitrate reductase (Nr) activities in newly expended leaves of 'Shanyou 63' and 'Zhendao 11' after N depletion; (B) Leaf Nr activities in WT and transgenic lines of Nipponbare.

The *in vitro* NO_3^- supply was conducted after 3 days of N depletion, and the levels of NO_3^- supply were 20, 40 and 60 mg L^{-1}, respectively. While different lines of Nipponbare plants were supplied with full-strength nutrient under medium-N level (40 mg L^{-1}). The data of (A) and (B) are from Experiment 2 and 3 respectively and the values represent the means ± SD of four replicates. Significant differences between treatments are indicated by different letters ($P < 0.05$). Statistical differences are compared only in a single cultivar.

FW: fresh weight

Fig. 6 The relative leaf contents of oxalic acid (A, B), malic acid (C, D), oxaloacetic acid (E, F) and 2-Ketoglutarate acid (G,H) in 'Shanyou 63' and 'Zhendao 11' plants (A, C, E, G) of exogenous NO_3^- supply after N depletion and in WT and transgenic lines of Nipponbare (B, D, F, H). The data of (A) and (B) are from Experiment 2 and 3 respectively and the values represent the means ± SD of four replicates. Significant differences between treatments are indicated by different letters ($P < 0.05$). Statistical differences are compared only in a single cultivar.