Nitrogen-mediated alleviation of photosynthetic inhibition under moderate water deficit stress in rice (Oryza sativa L.)

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ABSTRACT

High nitrogen (N) application improves the acclimation of plant photosynthesis to water deficit stress, however, the metabolic details for this are not yet fully understood. Using hydroponics, we found that moderate water deficit simulated with 10% PEG 6000 inhibited rice photosynthetic rate by 17.2% in low N condition (LN, 0.71 mM) versus 7.9% in moderate N condition (MN, 2.86 mM). Plants grown at MN had an increased photorespiratory serine metabolism and glycerate recycling under water stress, which was in accordance with the enhancement of glutathione concentration and ascorbate-glutathione cycle, indicating that the serine metabolism plays a significant role in improving antioxidant capacity. Additionally, the up-regulation of GS2 facilitated the re-assimilation of NH3 released in photorespiration. Aspartate aminotransferase (AspAT)- and glutamate:glyoxylate aminotransferase (GGAT)-mediated glutamate transamination and phosphoenolpyruvate carboxylase (PEPC)-mediated anaplerotic reaction provided a carbon skeleton, 2-oxoglutarate, for NH3 assimilation, which reduced the depletion of sugars. Sugars therefore can be stored and used for the regeneration of RuBP through the pentose phosphate pathway to maintain CO2 assimilation. In contrast, water deficit stress-induced protein degradation, down-regulation of N assimilation, and depletion of carbohydrates in LN-supplied plants, as well as the failure of the ascorbate-glutathione cycle due to reduced glutathione biosynthesis, perturbed the function of chloroplasts. We conclude that high N supply preserves the biochemistry of photosynthesis through coordinated regulation of the C and N metabolism to facilitate the acclimation of rice photosynthesis to water deficit stress.

1. Introduction

Rice is a water-intensive crop, and even moderate water deficit can reduce its photosynthesis (Dodd et al., 2015). Mild and/or moderate water deficit stress generally leads to a progressive closure of stomata, impeding CO2 diffusion through the pathway of air-to-intercellular space-to-mesophyll cell (Cornic, 2000; Galle et al., 2009, 2010). Only when the water deficit becomes severe is the photosynthetic capacity impaired by reduced photosynthetic enzyme activities (Wingler et al., 1999). However, the adverse effects of water stress on rice photosynthesis can be improved by moderatley increasing the nitrogen (N) application rate (Otoo et al., 1989; Tran and Yamauchi, 2014; Wang et al., 2016; Cao et al., 2017). The application of high N amounts may increase the sensitivity of stomata to water stress and maintain photosynthetic machinery (Otoo et al., 1989), enhance elasticity in root system development (Tran and Yamauchi, 2014), and improve antioxidant capacity (Zhong et al., 2017).

N assimilation plays an important role in the acclimation of photosynthesis to water stress (Xu and Zhou, 2006; Zhong et al., 2018). The assimilation of inorganic N requires 2-oxoglutarate (2-OG) to provide a carbon skeleton (C-skeleton), which links the C metabolism with primary N assimilation (Foyer et al., 2011). By feeding rice plants with 2-OG or metabolizable sugars, Yuan et al. (2010) revealed that 2-OG regulates the coordination of the C and N metabolisms via enzymes such as hexokinase (HXK), NADP⁺-dependent isocitrate dehydrogenase (NADP⁺-ICDH), phosphoenolpyruvate carboxylase (PEPC), glutamine synthetase (GS), and NADH-dependent glutamate synthase (NADH-GOGAT). The interaction between the C and N metabolisms is crucial to the resistance of plants to water deficit (Reguera et al., 2013). Water deficit stress affects the expression and activities of enzymes related to sucrose synthesis and N assimilation, including sucrose phosphate synthase (SPS), sucrose synthase (SS), invertases, nitrate reductase (NR), GS, glutamate dehydrogenase (GDH), and aminotransferase, thus stimulating changes in C- and N-related compounds that affect the
stress-tolerance of plants to water deficit (Mullet and Whitsett, 1996; González et al., 1998; Pinheiro et al., 2001; Bascuñán-Godoy et al., 2016). However, the response of the C and N metabolisms to water deficit stress varies greatly among species and depends on growth conditions. Therefore, the relationship between the C and N metabolism and N-mediated acclimation of photosynthesis to water deficit stress still needs to be elucidated.

Water deficit stress-induced stomatal closure raises the O2/CO2 ratio at the carboxylation site of Rubisco, inevitably leading to an increase in photorespiration (Atkin and Macherel, 2009). Photorespiration is an important component of the physiological processes that overlap in the C and N metabolism (Peterhansel and Maurino, 2011). It provides energy (ATP) and reducing equivalents [NAD(P)H] for primary N assimilation in the cytoplasm of mesophyll cells (Igamberdiev et al., 2001; Bloom et al., 2010). Inhibition of photorespiration (e.g., CO2-enriched or low O2 conditions) strongly reduces nitrate (NO3−) assimilation in the leaves of Arabidopsis and wheat (Bloom et al., 2010). In addition, photorespiration regulates the N pool in photosynthetic tissues via the metabolism of essential amino acids such as Glu, Gly, and Ser (Igarashi et al., 2006). The N application rate and water availability modulate the metabolism of amino acids in photorespiration. Low N and water deficit enhance the recycling of Ser and reduce the export pool of amino acids (Misra, 2014). Under drought stress, compared with the wild-type, barley mutants with reduced activities of photorespiratory enzymes, e.g., chloroplastic GS2, glycine decarboxylase (GDC), or serine:glyoxylate aminotransferase (SGAT), displayed lower rates of biosynthesis and accumulation of amino acids, as well as lower photoresynthetic rates (Wingler et al., 1999). This suggests that the photorespiratory N metabolism is vital to protect plant photosynthesis from water deficiency. To date, photorespiration is viewed mainly as an alternative electron sink that provides electron acceptors and phosphorus to protect photosynthesis against photoinhibition during water deficit stress (Rivero et al., 2009; Silva et al., 2015). However, how the photorespiratory N metabolism combines with other C and N metabolism pathways in protecting photosynthesis from water deficit stress is currently not fully understood.

In this study, we aimed to identify: 1) the differences in the effects of water deficit stress on the C and N metabolism in rice grown at different N levels; 2) the metabolic mechanisms through which N alleviates the inhibition of rice photosynthesis by water deficit stress; 3) the role of photorespiratory N metabolism in protecting photosynthesis from water deficit stress. The results could provide insights into the regulation of N for the acclimation of photosynthesis in rice under conditions of water deficiency.

2. Materials and methods

2.1. Materials, growth condition, and treatments

A hybrid indica rice cultivar ‘Zhongzheyou 1,’ which is widespread in South China, was used in this study. Seeds were soaked in water and put in an incubator at 30°C for 48 h to germinate. Then the sprouted seeds were sown on a floating plate and cultivated hydroponically in a climatic chamber with a day/night temperature of 28/23°C and a 12-h photoperiod at ∼600 μmol m−2 s−1 photosynthetic photon flux density (PPFD). The CO2 concentration in the climatic chamber was ∼450 μmol mol−1, with a relative humidity of about 70%. Three-leaf-staging plants were supplied with 1/4-strength nutrient medium. The seedlings were then transferred to 1/2-strength nutrient medium for one week, followed by full-strength solution for two weeks. In the following 10 days, the plants were supplied with nutrient solutions containing either low nitrogen (LN, 0.71 mM N) or moderate nitrogen (MN, 2.86 mM N). Other nutrients were supplied in full-strength. When the plants showed significant difference in photosynthetic type, e.g., smaller plant size and chlorosis in the lower leaves of LN-treated plants, half of the plants in each N treatment group were exposed to moderate water deficit stress (∼−0.33 MPa), simulated by adding 100 g L−1 poly ethylene glycol (PEG) 6000 in the growth medium for 7 days, with the control plants treated without PEG 6000. The growth medium was refreshed twice per week and the pH of the solution was maintained at 5.50 ± 0.05.

A completely randomized experimental design with five replications was used in this study. There were four plants per pot. The growth conditions in the climatic chamber were the same as described above. The position of the pots was interchanged when refreshing medium to eliminate possible marginal effects.

2.2. Gas exchange and chlorophyll fluorescence measurements

After 7 days of water deficit stress, the light-saturated (1500 μmol m−2 s−1 PPFD) gas-exchange and chlorophyll fluorescence of newly expanded leaves were measured simultaneously using LI-6400XT portable photosynthesis system with an integrated fluorescence chamber (LI−COR, USA). The CO2 concentration in the leaf cuvette was adjusted to 450 μmol mol−1 using a CO2 mixer. The temperature was maintained at 25°C, with a relative humidity of 70%–80%. The leaves were placed in the cuvette to adjust for at least 10 min. After equilibration to a steady state, gas-exchange parameters, steady-state fluorescence (Fv′/Fm′) and maximum fluorescence (Fm′) were recorded. The Fv′/Fm′ was measured using a 0.8-s saturating pulse of light (∼8000 μmol m−2 s−1). The initial fluorescence (F0) and maximum fluorescence (Fm′) in dark-adapted plants were measured at night-time before light-saturated gas-exchange measurement. The following chlorophyll fluorescence parameters were calculated: quantum efficiency of PSII photochemistry, ΦPSII = (Fm′−F0)/Fm′; photochemical quenching, qP = (Fm′−F0)/(Fm′−F0); non-photochemical quenching, NPQ = (Fm′−Fm)/Fm′; and electron transport rate, ETR = ΦPSII × 0.5 × PPFD × αmax where 0.5 is the fraction of excitation energy distributed to PSII, and αmax is the fraction of incoming light absorbed by the leaves and assumed to be 0.85 (Li et al., 2009). The measured leaves were labelled, and the following gas-exchange measurements were conducted with the same labelled leaves.

For the measurement of A–C curves, the conditions of PPFD, temperature, and relative humidity in the cuvette were maintained as mentioned above. Prior to measurement, leaves were placed in the cuvette to adjust for at least 10 min with a PPFD of 1500 μmol m−2 s−1 and a CO2 concentration (C0) of 400 μmol mol−1. Gas-exchange parameters were then recorded following a series of 400, 300, 200, 150, 100, 50, 0, 400, 600, 800, 1000 μmol mol−1 C0 values. Maximum carboxylation rate (Vc max), maximum electron transport rate (Jmax) and triose phosphate utilization (TPU) were calculated according to Sharkey et al. (2007) based on the model developed by Farquhar et al. (1980). CO2 compensation point (I) and maximum photosynthetic rate (Amax) were calculated by fitting the A–C curve (Ye and Yu, 2009).

Release of CO2 during photorespiration was measured based on the method of post-illumination CO2 burst (PIB) (Peterson, 1983). The leaves were put in the cuvette to adjust for at least 10 min with the conditions mentioned above. When the leaves reached a photosynthetic equilibrium, the PFFD was switched off and the photosynthetic rate was recorded every two seconds for 2 min.

After measurement of photosynthesis, the newly expanded leaves were sampled, frozen in liquid nitrogen immediately, and stored at −80°C for biochemical measurements.
2.3. Measurement of leaf area, biomass, leaf relative water content (RWC), leaf water potential, and chlorophyll

After measuring photosynthesis, four plants in each treatment were harvested and their leaf areas were determined using LI-3100 area meter (LI—COR Inc. Lincoln, Nebraska, USA). The plants were then oven-dried at 70°C to a constant dry weight.

The leaf relative water content (RWC) was estimated as RWC (%) = [(Fw–Dw)/(Sw–Dw)] × 100, where Fw is fresh leaf weight, Sw is water-saturated weight obtained by immersing leaf discs in distilled water for 12 h, and Dw is dry weight. The leaf water potential was determined using WP4C Dew Point PotentiaMeter (Decagon, USA) from 09:00 to 13:00 h.

Chlorophyll in the fresh leaves was extracted using an acetone:ethanol (v:v = 1:1) solution for 24 h in the dark at room temperature. The chlorophyll concentration was calculated from the absorbance of the extract at 663 nm and 645 nm (Porra et al., 1989).

2.4. Cellular redox analysis

The H2O2 concentration in the leaves was quantified according to Yang et al. (2007). Each fresh leaf sample (100 mg) was homogenized in 3 ml 10 mM 3-aminomethyl-1, 2, 4-triazole with a mortar and pestle. The homogenates were centrifuged at 10,000 × g and 4°C for 10 min. The supernatant (2.5 ml) was reacted with 1.5 ml 0.5% thiobarbituric acid in 5% TCA in a boiling water bath for 15 min. After removing any protein deposition by centrifuging, TBARS in supernatant was measured at 600 nm, 532 nm, and 450 nm and calculated using the molar extinction coefficient of 0.155 mM⁻¹ cm⁻¹.

Total glutathione (gGSH) and oxidized glutathione (gGSSG) were measured colorimetrically at 412 nm by the method of 2-PO based on coupling glutathione reductase (GR)-mediated NADPH-oxidation at 27°C for 30 min (Griffith, 1980). Reduced glutathione (gGSH) was calculated by gGSH minus gGSSG.

Reduced ascorbic acid (AsA) and dehydroascorbic acid (DHA) were determined spectrophotometrically at 534 nm with a rapid and sensitive method using 4,7-biphenyl-1,10-phenanthroline (BP), based on the AsA-mediated formation of the Fe²⁺-BP complex (Arakawa et al., 1981). Total AsA (tAsA) was measured after reducing DHA to AsA by DTT. DHA was calculated from the difference between tAsA and AsA.

2.5. NO₃⁻ and NH₄⁺ measurements

NO₃⁻ and NH₄⁺ were extracted from each frozen-dried leaf sample by homogenization with deionized water. The extract was collected by centrifuging. NO₃⁻ was quantified spectrophotometrically at 410 nm by nitration of salicylic acid (Cataldo et al., 1975). KNO₃ was used as the standard. NH₄⁺ was measured spectrophotometrically using the indophenol blue colorimetry method at 625 nm (Zanini, 2001), with (NH₄)₂SO₄ as the standard.

2.6. Soluble sugar and starch quantification

Leaf samples were collected in the early morning (07:00-08:00 h). Samples were frozen in liquid nitrogen immediately and then frozen-dried. Soluble sugar in samples was extracted with 80% (v/v) ethanol at 80°C. Sucrose was measured as described previously by Li et al. (2012). Glucose and fructose in the preparation were measured using the anthrone reagent (Bruyn et al., 1968). The ethanol-insoluble fraction was oven-dried at 80°C, and the starch in the fraction was extracted with 0.5 M HCO₃. Starch was measured by the method determining the glucose (Bruyn et al., 1968). The amount of starch was taken to be 0.9 times the amount of glucose.

2.7. Amino acid composition analysis

Each frozen-dried leaf sample (100 mg) was homogenized at 4°C in 3 ml 3% sulfosalicylic acid using a mortar and pestle. The homogenates were centrifuged at 10,000 × g and 4°C for 10 min. The supernatant was filtered with 0.45-μm aqueous films. The composition of free amino acid in the filtrate was quantified using a Hitachi i-8900 amino acid analyzer (Minato-ku, Tokyo, Japan).

The composition of total amino acid was quantified using HPLC (del Campo et al., 2009). Frozen-dried leaf samples (200 mg) were homogenized in 1.5 mL 0.1% phenol in 6 M HCl. The homogenates were hydrolyzed at 100°C for 22 h then cooled, and 1 mL hydrolysate was dried via organomation and redissolved in 1 mL 0.1 M HCl. Amino acids were derived and then incubated with hexane for 10 min with shaking. The underlayer solution was filtered with a 0.45-μm aqueous filter. The quantification of amino acid composition was performed with RP-HPLC (Rigol L3000, China) coupling ACE column (SC18-IL) (250 mm × 4.6 mm), with mobile phase A containing 25 mM acetate buffer (pH 6.5) and 70 mL acetonitrile and mobile phase B containing 80% acetonitrile aqueous solution. The flow rate was 1.0 mL·min⁻¹ and column temperature was 40°C.

2.8. Organic acids assay

Organic acids were determined according to Ji et al. (2005), with some modifications. Frozen-dried leaf samples (200 mg) were homogenized in 1.5 mL 0.65 M HCl containing 0.1 M EDTA-Na₂. The homogenates were centrifuged at 10,000 × g and 4°C for 10 min. RP-HPLC with UV detection was employed to identify and quantify the organic acids in the supernatant. Glycylcate, pyruvate, citrate, and fumarate were monitored at 214 nm with the mobile phase of 16 mL methanol in 800 mL of 10 mM NaH₂PO₄. After adjusting the supernatant to pH 7.0, the supernatant was derived with 10 mM phenylhydrazine-HCl to generate phenylhydrazone. The glyoxylic acid and 2-oxoglutarate were measured at 324 nm with the mobile phase of 100 mL methanol in 21.2 mM K₂HPO₄-KH₂PO₄ (pH 7.0).

2.9. Enzyme activity determination

Nitrate reductase (NR, EC 1.6.6.1) in frozen leaf samples was extracted with 25 mM potassium phosphate buffer (pH 7.5) containing 10 mM L-cysteine and 1 mM EDTA-Na₂. The activity was measured colorimetrically at 540 nm as in Hageman and Reed (1980), based on the reduction of nitrate to nitrite at 25°C in 30 min.

Frozen leaf samples (100 mg) were homogenized in 4 mL 50 mM Tris-HCl buffer (pH 8.0) containing 2 mM Mg²⁺, 2 mM DTT, and 0.4 M sucrose. The homogenates were centrifuged at 10,000 × g and 4°C for 10 min and the supernatants were used for the following measurements. Nitrite reductase (NiR, EC 1.7.7.1) activity was measured using the method in Lillo (1984). The activity of glutamine synthetase (GS, EC 6.3.1.2), NADH-dependent glutamate synthetase (NADH-GOGAT, EC 1.4.1.14), and amination and deamination of glutamate dehydrogenase (GDH, EC 1.4.1.2) was determined according to the methods described by Loyola-Vargas and de Jimenez (1984). The aspartate aminotransferase (AspAT, EC 2.6.1.1) and alanine aminotransferase (AlaAT, EC 2.6.1.2) activity was tested as in Wu et al. (1998). Glutamate:glyoxylate aminotransferase (GGAT EC 2.6.1.4) activity was measured according to Igarashi et al. (2006). Serine:glyoxylate aminotransferase (SGAT, EC 2.6.1.45) activity was determined.
spectrophotometrically by coupling the conversion of hydroxyypyruvate to α-glycerate (King and Waygood, 1968).

Extracts for C metabolic enzyme assays were obtained by grinding 100 mg frozen leaf samples in 4 ml 50 mM Tris–HCl (pH 7.5) containing 10 mM β-mercaptoethanol, 12.5% glycerine, 1 mM EDTA-Na2, 10 mM MgCl2, and 1% PVP-40. The homogenates were centrifuged at 4 °C and 10,000 × g for 10 min and the supernatant was collected for enzyme assays. The activities of enzymes were analyzed in a final volume of 1 ml medium, as mentioned below. The reactions were started by adding moderate enzyme extract.

Total Rubisco (EC 4.1.1.39) activity was measured according to Li et al. (2013). The reaction medium contained 50 mM HEPES-NaOH (pH 7.5), 10 mM NaHCO3, 20 mM MgCl2, 2.5 mM DTT, 5 mM EDTA, 1 IU creatine phosphokinase, 10 IU 3-phosphoglyceric phosphokinase, 10 IU glyceraldehyde-3-phosphate dehydrogenase, 5 mM ATP, 0.15 mM NADH, 5 mM phosphocreatine, and 0.6 mM RuBP. The change of the absorbance at 340 nm (A340) was monitored for 3 min. PEPC (EC 4.1.1.31) activity was measured spectrophotometrically at 340 nm by coupling the reaction of NADH-oxidation mediated by MDH at 30 °C (Quy et al., 1991). The assay medium contained 50 mM Tris—HCl, pH 7.6, 4 mM PEP, 10 mM MgSO4, 10 mM NaHCO3, 0.15 mM NADH, and 1 IU MDH. GAPDH (EC 1.2.1.13) was measured in accordance with Schuman and Gibbs (1968). The reaction mixture contained 80 mM Tris—HCl (pH 8.4), 10 mM MgCl2, 2.5 mM DTT, 5 mM ATP, 3 mM PGA, and 0.2 mM NAD(P)H. The reaction was conducted at 30 °C by recording the A340 for 3 min. ICDH (EC 1.1.1.42) was determined at 30 °C and 340 nm for 3 min with a reaction mixture (pH 8.0) containing 3.5 mM MgCl2, 0.4 mM NADP+, 0.55 mM isocitrate, and 88 mM imidazole (Bergmeyer et al., 1974). To determine G-6-PDH (EC 1.1.1.49) activity, the reduction of NADP+ was monitored at 340 nm for 3 min with a reaction mixture containing 100 mM HEPES-NaOH (pH 7.6), 138 mM MgCl2, 20 mM Glycerol-6-P, and 7.8 mM NADP+ (Bergmeyer et al., 1974). PK (EC 2.7.1.40) was determined according to Lepper et al. (2010). The reactive system contained 100 mM Tris—HCl, pH 7.5, 10 mM MgCl2, 0.16 mM NADH, 75 mM KCl, 5.0 mM ADP, 7.0 IU α-lactate dehydrogenase (LDH), 0.1% (v/v) Triton X-100, and 1.0 mM PEP. The oxidation of NADH was monitored at 340 nm for 3 min. HXK (EC 2.7.1.1) was measured spectrophotometrically at 340 nm for 3 min (Whittaker et al., 2001). The reaction system contained 100 mM KH2PO4-K2HPO4, pH 7.5, 2 mM MgCl2, 1 mM EDTA, 0.4 mM NAD, 1 mM ATP, 1 IU glucose-6-phosphate dehydrogenase (G-6-PDH), 1 IU phosphoglucone isomerase (PGI), and 5 mM glucose or 5 mM fructose. SHMT (EC 2.1.2.1) was determined spectrophotometrically at 279 nm by measuring the decomposition of D, L-β-phenylserine (Hsiao et al., 1986). A typical assay mixture contained 50 mM D, L-β-phenylserine, 50 μM pyridoxal-phosphate, and 10 μg ml−1 cetyl-pyridinium·HCl in 0.1 M potassium phosphate buffer at pH 8.0. The reaction was carried out at 30 °C for 1 h and the concentration of benzaldehyde generated in the mixture was obtained from a standard benzaldehyde absorption curve. HPR (EC 1.1.1.29) was assayed with a reaction mixture containing 40 mM KH2PO4-K2HPO4, pH 6.2, 0.2 mM NADH or 0.2 mM NADPH, 0.015% Triton X-100, and 5 mM hydroxyypyruvate (Klecikowska et al., 1988). The change in A340 was monitored for 3 min.

The activities of SS (EC 2.4.1.13) and SPS (EC 2.4.1.14) were measured by the synthesis/decomposition of sucrose in unit time (Verma et al., 2011). SS was assayed in a reaction mixture containing 0.8 mM UDPG, 1.6 mM fructose, and 200 mM Tris—HCl (pH 8.2, containing 1 mM MgCl2). To measure SPS activity, the pH of the Tris—HCl buffer was adjusted to 7.4, fructose was replaced by fructose-6-phosphate, and 2.0 mM NaF was added in the mixture as a phosphate inhibitor. The reaction mixtures were incubated at 37 °C for 30 min, and the reactions terminated by adding 200 μl 1 M NaOH. Sucrose was measured following the procedures of the resorcinol hydrochloric acid method (Li et al., 2012).

Glycolate oxidase (GO, EC 1.1.3.15) was extracted using a potassium phosphate buffer (pH 8.3), and the activity was measured by the formation of a glycoxylate-phenylhydrazone complex at 324 nm and 30 °C (Baker and Tolbert, 1966). The 3-mL reaction mixture contained 2 ml phosphate buffer, 0.1 ml 0.1 M cysteine-HCl (pH 6.0), 0.1 ml 0.1 M phenylhydrazine-HCl (pH 6.0), 0.5 ml 0.04 M sodium glycolate (pH 8.0), and 0.3 ml enzyme extract.

Enzyme activities were expressed as moles of metabolite generated/consumed per mg protein per unit time. Protein was measured by the method of Bradford (1976) at 595 nm with bovine serum albumin (BSA) as a standard.

2.10. RNA extraction and RT-qPCR assay

The newly expanded leaves were sampled and frozen in liquid nitrogen immediately, and then finely pulverized using liquid nitrogen. Total RNA was extracted with TRIZol reagent (Invitrogen, USA), followed by precipitation with an equal volume of isopropanol, rinsing with 75% ethanol, and dissolving with RNase-free water. cDNAs were synthesized using the PrimeScript™ RT Kit with gDNA Eraser (Perfect Real Time) (TAKARA, Japan). The relative expression of genes was determined by an Applied Biosystems 7500 Real-Time PCR System using SYBR Premix Ex-Taq (TAKARA). All operations were performed according to the user manuals. The primers used in the assays are listed in Table S1. The expression data were normalized to UBQ (Sweeney et al., 2007).

2.11. Statistical analyses

Samples in the same pot were mixed as a replication. Data were evaluated by ANOVA, and multiple comparisons were performed using the least significant difference (LSD) multiple range test. Differences were considered statistically significant at \( P < 0.05 \). The differences between the control and water stress treatments (e.g., amino acid) at the same N level were evaluated using the Student’s t-test. Differences were considered statistically significant at \( P < 0.05 \) and \( P < 0.01 \).

3. Results

3.1. CO2 assimilation

Water stress inhibited leaf area by 18.2% and 21.1% at low N and moderate N level, respectively (Fig. 1a). Leaf water potential was reduced similarly by water stress at both N levels (Fig. 1b), while leaf relative water content (RWC) was not affected in either N condition (Fig. S1a). Biomass and photosynthetic rate (Pn) showed the same response to water stress, which were inhibited by 13.3% and 17.2%, respectively, in low N condition, but to a lesser extent in moderate N condition, where they decreased by 5.0% and 7.9%, respectively (Fig. 1c, d). Stomatal conductance (gs) in both N conditions decreased significantly by water stress (Fig. 1e), whereas intercellular CO2 concentration (Ci) was unaffected at low N level but markedly decreased as a result of water stress at moderate N level (Fig. 1f).

Plants showed higher \( V_{cmax} \), \( J_{max} \), and TPU at moderate N level than at low N level. \( V_{cmax} \) was not significantly affected by water stress at low N level but significantly increased at moderate N level (Fig. 2a). In parallel with \( V_{cmax} \), the total Rubisco activity in moderate N condition was enhanced significantly under water stress, but was not significantly affected in low N condition (Fig. 2b). The same effects were also observed in \( J_{max} \) (Fig. 2c, d), and \( A_{max} \) (Fig. S1b). Water stress showed no significant effect on chlorophyll concentration and chlorophyll fluorescence parameters such as \( F_{v}/F_{m} \), Fv/P, qP and ETR, while NQI was markedly increased by water stress at moderate N but not at low N level (Fig. S2).

3.2. Carbohydrate metabolism and the Krebs cycle

TPU reflects the synthesis of sucrose in mesophyll cells. Water stress
significantly reduced the concentrations of sucrose and starch but showed no significant effect on fructose and glucose at low N level (Fig. 3a-d). In contrast, sucrose, fructose, and starch concentrations in moderate N were significantly increased by water stress (Fig. 3a-d). The increase in sugar and starch concentrations was well in line with the increases in the activities of SPS (sucrose synthesis), HXK, and GAPDH.

Fig. 1. Analysis of plant growth, leaf water potential and gas-exchange in rice plants grown under different N and water conditions. (a) Whole plant leaf area. (b) Leaf water potential. (c) Whole plant biomass. (d) Light-saturated (1500 μmol m$^{-2}$ s$^{-1}$ PPFD) photosynthetic rate at ambient CO$_2$ concentration (450 μmol mol$^{-1}$). The values above bars represent decreasing rate of water stress treatment relative to the corresponding control. (e) Stomatal conductance. (f) Intercellular CO$_2$ concentration. Data were means ± SE ($n=4$) and evaluated by ANOVA and LSD multiple range test. The same letters on the bars indicates no statistically significant difference at $P<0.05$. LN, low nitrogen; MN, moderate nitrogen.

Fig. 2. Analysis of maximum carboxylation rate of Rubisco ($V_{\text{cmax}}$, a), Rubisco activity (b), maximum electron transport rate ($J_{\text{max}}$, c), and triose phosphate utilization (TPU, d) in leaves of rice plants grown under different N and water conditions. Data were means ± SE ($n=4$) and evaluated by ANOVA and LSD multiple range test. The same letters on the bars indicates no statistically significant difference at $P<0.05$. LN, low nitrogen; MN, moderate nitrogen.
RuBP, which is correlated with concentrationimprovedsucroseandstarch biosynthesis under water stress.

and water stress treatments at either N level. Thus, a high N activity (sucrose decomposition) was not different between the control and low N level were not significantly altered by water stress (Table 1). However, apart from HXK, the activities of these enzymes at different N levels.

Phosphate dehydrogenase; PK, pyruvate kinase; PEPC, phosphoenolpyruvate carboxylase; ICDH, isocitrate dehydrogenase.

Note: SS, sucrose synthetase; SPS, sucrose phosphate synthetase; G-6-PDH, glucose-6-phosphate dehydrogenase; HXK, hexokinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Activities of carbon metabolic enzymes in leaves of rice plants grown under different N and water conditions. Data were means ± SE (n = 5) and evaluated by ANOVA and LSD multiple range test. The same letters on the bars indicates no statistically significant difference at P < 0.05. LN, low nitrogen; MN, moderate nitrogen.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>LN Control</th>
<th>Water stress</th>
<th>MN Control</th>
<th>Water stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS (μg mg⁻¹ Prot·min⁻¹)</td>
<td>24.44 ± 4.73 ab</td>
<td>26.72 ± 3.58 a</td>
<td>12.27 ± 0.91 c</td>
<td>15.64 ± 1.88 bc</td>
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<td>SPS (μg mg⁻¹ Prot·min⁻¹)</td>
<td>2.80 ± 0.72 c</td>
<td>2.99 ± 0.21 c</td>
<td>9.51 ± 0.58 b</td>
<td>15.64 ± 0.91 a</td>
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<tr>
<td>G-6-PDH (nmol mg⁻¹ Prot·min⁻¹)</td>
<td>17.25 ± 0.52 ab</td>
<td>17.31 ± 0.82 ab</td>
<td>15.69 ± 0.61 b</td>
<td>18.89 ± 0.73 a</td>
</tr>
<tr>
<td>HXK (nmol mg⁻¹ Prot·min⁻¹)</td>
<td>3.08 ± 0.09 bc</td>
<td>4.62 ± 0.40 a</td>
<td>2.64 ± 0.11 c</td>
<td>3.55 ± 0.32 b</td>
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<tr>
<td>GAPDH (nmol mg⁻¹ Prot·min⁻¹)</td>
<td>19.88 ± 1.01 b</td>
<td>24.43 ± 1.77 b</td>
<td>21.34 ± 2.74 b</td>
<td>154.74 ± 15.92 a</td>
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<tr>
<td>PK (nmol mg⁻¹ Prot·min⁻¹)</td>
<td>4.08 ± 0.13 c</td>
<td>5.18 ± 0.53 b</td>
<td>5.42 ± 0.16 b</td>
<td>6.95 ± 0.26 a</td>
</tr>
<tr>
<td>PEPC (nmol mg⁻¹ Prot·min⁻¹)</td>
<td>43.15 ± 4.00 ab</td>
<td>51.32 ± 2.76 c</td>
<td>31.51 ± 3.90 a</td>
<td>45.37 ± 4.43 a</td>
</tr>
<tr>
<td>ICDH (nmol mg⁻¹ Prot·min⁻¹)</td>
<td>2.72 ± 0.18 bc</td>
<td>3.36 ± 0.13 a</td>
<td>2.44 ± 0.09 a</td>
<td>3.07 ± 0.21 ab</td>
</tr>
</tbody>
</table>

Note: SS, sucrose synthetase; SPS, sucrose phosphate synthetase; G-6-PDH, glucose-6-phosphate dehydrogenase; HXK, hexokinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PK, pyruvate kinase; PEPC, phosphoenolpyruvate carboxylase; ICDH, isocitrate dehydrogenase.

(Table 1). However, apart from HXK, the activities of these enzymes at low N level were not significantly altered by water stress (Table 1). SS activity (sucrose decomposition) was not different between the control and water stress treatments at either N level. Thus, a high N concentration improved sucrose and starch biosynthesis under water stress.

The pentose phosphate pathway (PPP) regulates the regeneration of RuBP, which is correlated with Jmax. Water stress significantly increased the activity of G-6-PDH, the rate-limiting enzyme of PPP, in moderate N but not in low N condition. The concentrations of organic acids in the Krebs cycle, such as pyruvate, citrate, and fumarate, were markedly increased by water stress at low N level, but these compounds showed no significant difference between the control and water stress at moderate N level (Fig. 4a, b, d). In contrast, the concentration of 2-oxoglutarate (2-OG) was not significantly affected by water stress at low N level, but significantly increased at moderate N level (Fig. 4c).

Water stress significantly increased the activities of PK and ICDH at both N levels, while the PEPC activity was specifically increased at moderate N level (Table 1). The Krebs cycle links the C and N metabolisms by providing the C-skeleton 2-OG for N assimilation. The different responses of 2-OG and PEPC to water stress at different N levels suggests that water stress could affect N metabolism differentially at different N levels.

3.3. N metabolism

N metabolic enzymes and amino acids were analyzed to evaluate the effect of water stress on the N metabolism. Water stress led to a significant reduction in NO3⁻ in moderate N condition but a significant increase in NH4⁺ in low N condition (Fig. 5a, b), suggesting primary N assimilation was differentially affected by water stress under different N conditions. Water stress reduced NR and NIR activities at both N levels. GS activity was notably inhibited by water stress at low N level, but not significantly affected at moderate N level (Table 2). The decrease of GS activity at low N level was parallel to the down-regulation of GS₁ and GS₂ (Fig. 5c) and GS₁ (Fig. 6d). NADH-GOGAT and GDH amination/deamination activity showed no significant difference between the control and water stress at either N level. Both AspAT and AlaAT activity were significantly reduced by water stress at low N level, while AspAT activity was increased at moderate N level (Table 2).

The results in Fig. 5c–e showed that water stress affected 17 kinds of free amino acids similarly at both N levels. While water stress lowered the concentrations of Glu, GABA, Ala, and Asp (with exception of Asp in low N), Cys and Arg were not significantly affected, and the rest of amino acids were increased significantly. Nevertheless, total free amino acid was markedly increased by water stress at both N levels (Fig. 5f). Total amino acid was significantly reduced by water stress in low N condition, but not significantly affected in moderate N condition (Fig. 5g). Accordingly, water stress raised the ratio between free amino acid and total amino acid by 61.3% in low N condition, while the increment of the ratio was only 18.9% in moderate N condition, indicating that water stress facilitated a stronger degradation of protein at low N level.
Fig. 4. Analysis of organic acid concentrations in leaves of rice plants grown under different N and water conditions. (a) Pyruvate. (b) Citrate. (c) 2-oxoglutarate. (d) Fumarate. Data were means ± SE (n = 5) and evaluated by ANOVA and LSD multiple range test. The same letters on the bars indicate no statistically significant difference at P < 0.05. LN, low nitrogen; MN, moderate nitrogen.

Fig. 5. Analysis of inorganic N compounds and free amino acid compositions in leaves of rice plants grown under different N levels and water conditions. (a) Nitrate concentration. (b) Ammonium concentration. (c) and (d) Concentrations of free amino acid compositions at low N and moderate N, respectively. (e) Fold changes of the compositions of free amino acid relative to corresponding control. (f) Total free amino acid concentration. (g) Total amino acid concentration. Data were means ± SE (n = 5) and evaluated by ANOVA and LSD multiple range test. The same letters on the bars indicate no statistically significant difference at P < 0.05. The difference of free amino acid composition between control and water stress treatment was analyzed using two-tailed Student's t-test. The significance of difference was indicated: *, P < 0.05; **, P < 0.01, and ns, not significant. LN, low nitrogen; MN, moderate nitrogen.
3.4. Photorespiratory metabolism

Photorespiration is an important protective mechanism under conditions of water stress (Rivero et al., 2009; Silva et al., 2015). The marked increases in the Gly and Ser concentrations (Fig. 5c–e), the Gly/Ser ratio, CO₂ compensation point (Γ), and CO₂ release measured by the PIB method under water stress (Fig. S4) indicated a significant increase in photorespiration.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>LN Control</th>
<th>Water stress</th>
<th>MN Control</th>
<th>Water stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR (µg NO₃⁻ · mg⁻¹ Prot · h⁻¹)</td>
<td>0.85 ± 0.02 c</td>
<td>0.78 ± 0.02 c</td>
<td>2.96 ± 0.07 a</td>
<td>2.37 ± 0.03 b</td>
</tr>
<tr>
<td>NiR (mmol NO₂⁻ · mg⁻¹ Prot · h⁻¹)</td>
<td>0.19 ± 0.01 c</td>
<td>0.15 ± 0.01 d</td>
<td>0.34 ± 0.01 a</td>
<td>0.29 ± 0.02 b</td>
</tr>
<tr>
<td>NADH-GOGAT (nmol · mg⁻¹ Prot · min⁻¹)</td>
<td>22.97 ± 1.16 b</td>
<td>18.80 ± 2.78 b</td>
<td>34.11 ± 1.24 a</td>
<td>30.68 ± 1.53 a</td>
</tr>
<tr>
<td>GDH (amination, nmol · mg⁻¹ Prot · min⁻¹)</td>
<td>8.27 ± 0.26 c</td>
<td>8.58 ± 0.31 bc</td>
<td>9.02 ± 0.13 ab</td>
<td>9.67 ± 0.14 a</td>
</tr>
<tr>
<td>GDH (deamination, nmol · mg⁻¹ Prot · min⁻¹)</td>
<td>3.00 ± 0.06 a</td>
<td>2.89 ± 0.09 a</td>
<td>2.89 ± 0.18 a</td>
<td>2.80 ± 0.09 a</td>
</tr>
<tr>
<td>AspAT (µmol · mg⁻¹ Prot · 30min⁻¹)</td>
<td>5.95 ± 0.18 a</td>
<td>5.42 ± 0.09 b</td>
<td>4.73 ± 0.13 c</td>
<td>5.33 ± 0.15 b</td>
</tr>
<tr>
<td>AlaAT (µmol · mg⁻¹ Prot · 30min⁻¹)</td>
<td>13.28 ± 0.24 b</td>
<td>11.47 ± 0.46 c</td>
<td>15.09 ± 0.49 a</td>
<td>14.51 ± 0.46 ab</td>
</tr>
</tbody>
</table>

Note: NR, nitrate reductase; NiR, nitrite reductase; GS, glutamine synthetase; NADH-GOGAT, NADH-dependent glutamate synthetase; GDH, glutamate dehydrogenase; AspAT, aspartate aminotransferase; AlaAT, alanine aminotransferase.

Fig. 6. Analysis of photorespiratory metabolism in rice plants grown under different N and water conditions. (a) Schematic diagram of photorespiration cycle [Cited from Wingler et al. (2000) with some modification]. Glycolate generated in chloroplast via oxidation of RuBP is transferred to peroxisome and oxidized to glyoxylate by glycolate oxidase (GO, 1). This step is the main source of H₂O₂ generation, and catalase (CAT, 2) plays the dominant role in H₂O₂ scavenging. The synthesized glyoxylate is converted into glycine via glutamate-glyoxylate aminotransferase (GGAT, 3) in the presence of glutamate. The glycine is imported into mitochondria via transmembrane transport, and subsequently converted into serine by the catalysis of serine hydroxymethyltransferase (SHMT, 4). Simultaneously, a CO₂ and a NH₃ molecules are released. Serine is transported back to the peroxisome and transferred to hydroxypyruvate with glyoxylate via the serine-glyoxylate aminotransferase (SGAT, 5). Following, hydroxypyruvate is oxidized to glyceraldehyde hydroxypyruvate reductase (HPR, 6) in the presence of NAD(P)H, which is returned to Calvin-Benson cycle. The released NH₃ during serine synthesis is transported into chloroplast, and re-incorporated into amino acid by GS2/Fd-GOGAT cycle (7 and 8). (b) Glycolate concentration. (c) Glyoxylate concentration. (d) Relative expression of GS2 and Fd-GOGAT. The expression data were normalized to a reference gene UBQ with the method of 2⁻ΔΔCT. Data were means ± SE (n = 5) and evaluated by ANOVA and LSD multiple range test. The same letters on the bars indicates no statistically significant difference at P < 0.05. LN, low nitrogen; MN, moderate nitrogen.
Under water stress, Ser showed 5-fold higher concentration than that of the control at low N level, compared to a less than 1-fold concentration increase at moderate N level (Fig. 5e). Therefore, we further analyzed the effects of water stress on the main metabolites and enzymes in the photorespiration pathway (Fig. 6a). We found that water stress significantly increased the accumulation of glycolate and glyoxylate at low N concentration, whereas glycolate was reduced and glyoxylate was constant at moderate N concentration (Fig. 6b–c).

GO activity was not significantly affected, while GGAT and SHMT activity were markedly increased by water stress at both N levels (Table 3). SGAT and HPR activity were constant at low N concentration but significantly increased at moderate N concentration under water stress (Table 3). GS2 and Fd-GOGAT function in NH3 re-assimilation in photorespiration. Water stress repressed GS2 expression at low N level, but up-regulated its expression at moderate N level; the expression of Fd-GOGAT was not significantly affected (Fig. 6d).

### 3.5. Cellular redox homeostasis

$\text{H}_2\text{O}_2$ and MDA concentrations, CAT activity, and the ascorbate-glutathione cycle were determined to evaluate the cellular redox homeostasis. Water stress increased MDA concentration in low N but had no significant effect on leaf $\text{H}_2\text{O}_2$ concentration in either N condition (Fig. 7a, b). In contrast to MDA, water stress increased CAT activity in moderate N, but CAT activity in low N condition was varied slightly between the control and water stress treatment (Fig. 7c).

The ascorbate-glutathione cycle plays an important role in scavenging $\text{H}_2\text{O}_2$ in chloroplasts. Water stress significantly reduced GSSG and GSH concentrations at low N level, but increased GSSG concentration at moderate N level (Fig. 7d–e). The ratio of GSH/GSSG showed no significant difference between the control and water stress under either N condition (Fig. 7f). DHA was significantly reduced but AsA was significantly increased by water stress at moderate N level, whereas both were not

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**Table 3**

Activities of photorespiratory enzymes in leaves of rice plants grown under different N and water conditions. Data were means ± SE ($n = 5$) and evaluated by ANOVA and LSD multiple range test. The same letters following the data indicate no statistically significant difference at $P < 0.05$. LN, low nitrogen; MN, moderate nitrogen.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>LN Control</th>
<th>Water stress</th>
<th>MN Control</th>
<th>Water stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO (nmol·mg⁻¹ Prot·min⁻¹)</td>
<td>5.72 ± 0.08 a</td>
<td>5.87 ± 0.13 a</td>
<td>5.69 ± 0.08 a</td>
<td>5.83 ± 0.09 a</td>
</tr>
<tr>
<td>GGAT (nmol·mg⁻¹ Prot·min⁻¹)</td>
<td>4.56 ± 0.10 b</td>
<td>5.77 ± 0.09 a</td>
<td>2.68 ± 0.06 d</td>
<td>4.17 ± 0.16 c</td>
</tr>
<tr>
<td>SHMT (μmol·mg⁻¹ Prot·min⁻¹)</td>
<td>5.03 ± 0.18 b</td>
<td>5.78 ± 0.36 a</td>
<td>2.67 ± 0.08 d</td>
<td>3.05 ± 0.07 c</td>
</tr>
<tr>
<td>SGAT (nmol·mg⁻¹ Prot·min⁻¹)</td>
<td>25.45 ± 1.82 c</td>
<td>25.50 ± 1.41 c</td>
<td>32.24 ± 1.08 b</td>
<td>40.24 ± 1.38 a</td>
</tr>
<tr>
<td>HPR (nmol·mg⁻¹ Prot·min⁻¹)</td>
<td>0.65 ± 0.02 c</td>
<td>0.68 ± 0.01 c</td>
<td>0.87 ± 0.02 b</td>
<td>0.94 ± 0.02 a</td>
</tr>
</tbody>
</table>

Note: GO, glycolate oxidase; GGAT, glutamate:glyoxylate aminotransferase; SHMT, serine hydroxymethyltransferase; SGAT, serine:glyoxylate aminotransferase; HPR, hydroxypyruvate reductase.

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Fig. 7. Analysis of cellular redox homeostasis in leaves of rice plants grown under different N levels and water conditions. (a) Hydrogen peroxide concentration. (b) Malondialdehyde concentration. (c) Catalase activity. (d) Oxidized glutathione concentration. (e) Glutathione concentration. (f) Ratio of GSH/GSSG. (g) Dehydroascorbic acid concentration. (h) Ascorbic acid concentration. (i) Ratio of AsA/DHA. Data were means ± SE ($n = 5$) and evaluated by ANOVA and LSD multiple range test. The same letters on the bars indicates no statistically significant difference at $P < 0.05$. LN, low nitrogen; MN, moderate nitrogen.
significantly affected under low N condition (Fig. 7g–h). As a result, the ratio of AsA/DHA in moderate N-supplied plants was considerably increased under water stress, whereas the ratio was not significantly affected in low N condition (Fig. 7i). These results indicate that high N supply improved the antioxidant capacity of rice under water stress.

4. Discussion

4.1. High N supply preserves biochemistry of photosynthesis to increase sucrose and starch synthesis during water stress

Our results showed a significant effect of water and N interaction on rice photosynthesis, where water stress inhibited photosynthetic rate more in low N condition than in moderate N condition, which was consistent with the results reported previously (Zhong et al., 2017). Since chlorophyll concentration and chlorophyll fluorescence parameters such as Fv/Fm and ETR were negligibly affected by water stress under both N conditions (Fig. S2), the reduction of photosynthetic rate was unlikely due to the photo-inhibition of PSII and impairment of electron transport (Misra et al., 2012). g in both N conditions was reduced by water stress, while the significantly reduced C in moderate N-supplied plants was more likely resulted from the increased Rubisco activity, which partly compensated the reduction of photosynthetic rate.

The A-C3 response curves showed that plants grown at moderate N level had increased Vcmax regeneration of RuBP (Jacmax), and TPU values during water stress, suggesting that high N supply increases the acclimation of photosynthesis to water stress by preserving biochemistry of photosynthesis. Similar results have been observed in previous studies, wherein Vcmax and Jacmax were slightly increased in castor bean plants even 14 days after water stress (Saussen and Rosa, 2010). Lawlor and Cornic (2002) proposed that while the potential rate of photosynthetic CO2 assimilation is unaffected when RWC is above 75%, a decreasing g results in a smaller Fv, and a lower C3 as well as in acclimatory changes in the C and N metabolism. In this study, Amax was not affected by water stress at low N level but significantly increased at moderate N level, while RWC of water-stressed plants was maintained at 75% with no significant difference from the control (Fig. S1). Thus, the increased Vcmax, Jacmax, and TPU observed in this study could be the result of changes in the C and N metabolisms. Thermal dissipation via NPQ plays an important role in photoprotection (Silva et al., 2015). The significantly increased NPQ also contributed to the alleviation of photo-inhibition.

The storage of sucrose and starch in tissues can drive growth and stress tolerance in plants (Villar-Salvador et al., 2015). The plants grown at moderate N level showed an increased accumulation of soluble sugars and starch under water stress, accompanied with marked increase in GAPDH and SPS activity, suggesting that sucrose and starch synthesis play an important role in increasing the acclimation of photosynthesis to water stress. HXK, which catalyzes sugar phosphorylation, is involved in sensing the endogenous levels of sugar in photosynthetic tissues, and regulating plant photosynthesis and growth (Dai et al., 1999; Lejay et al., 2003). The increase in HXK activity during water stress was in parallel with an increase in G-6-PDH activity under moderate N condition. It is speculated that water stress raised the level of Glu-6-P in the leaves, and higher Glu-6-P not only supports sucrose synthesis, but also enhances RuBP regeneration in the pentose phosphate pathway, which maintains CO2 assimilation capacity (Reguera et al., 2013).

GAPDH is critical for maintaining the capacity to use ATP and NADPH in CO2 assimilation (Ruuska et al., 2000). The oxidation of Glu-6-P by G-6-PDH also provides NADPH for many physiological reactions such as ATP synthesis (Lawlor and Tezara, 2009), N assimilation (Sarkissian and Fowler, 1974), recycling of glycerate in photorespiration (Noctor and Foyer, 1998), and reduction of oxidized glutathione (Noctor et al., 1998). The results suggest that high N supply mediates NDAPH production and utilization to increase the acclimation of photosynthesis to water stress.

4.2. High N supply preserves the chloroplast function to coordinate NH4+ assimilation and C metabolism under water stress

Rice plants alter their amino acid metabolism to acclimate to water stress. A similar response of free amino acid compositions to water stress was observed at both N levels, with decreases in Glu, GABA, Ala, and Asp levels (moderate N only), and increases in levels of branched-chain amino acids (BCAAs, e.g. Met, Thr, Ile, Lys, Val, and Leu) and phenylpropanoid metabolic amino acids (e.g. Phe and Tyr). Water stress-induced accumulation of BCAAs and phenylpropanoid metabolic amino acids has also been widely observed in woody plants and Arabidopsis (Warren et al., 2011; Pires et al., 2016; Huang and Jander, 2017). This could result from protein degradation or de novo biosynthesis of those amino acids (Huang and Jander, 2017). Increased BCAA levels act as substrates in the Krebs cycle, osmolytes, and signaling molecules during dehydration (Nambara et al., 1998; Pires et al., 2016; Huang and Jander, 2017), and the build-up of phenylpropanoid amino acids increases lignin synthesis to defend against stress (dos Santos et al., 2015). Generally, water deficit increases GABA levels to operate the GABA shunt under water stress (Warren et al., 2011; Rezaei-Chianeh et al., 2018). In this study, the concentration of GABA was markedly reduced by water stress. Decreases in Glu, Ala, and Asp levels due to the conversion of these amino acids to Glycerol (Novitskaya et al., 2002) or other BCAAs (Joshi et al., 2010) could contribute to the reduction in the GABA shunt.

Although plants grown in both low and moderate N conditions displayed a significant increase in the accumulation of free amino acids under water stress, the increase in low N condition was likely to have resulted from a greater amount of protein degradation, since it showed a greater increase in the ratio between free amino acids and total amino acids than that in moderate N condition. Protein degradation is an important mechanism for N reutilization, especially under N-deficit conditions, and chloroplasts are a major source of cellular protein (Hörtenersteiner and Feller, 2002; Huang and Jander, 2017). Thus, the degradation of protein influences the abundance and activity of enzymes in chloroplasts, which involves the C and N metabolism (Wingler et al., 1999). These responses were observed in plants grown at low N supply, with reduced GS activity and expression of GSA, which encodes the chloroplastic isoform of GS, along with a larger inhibition of photosynthesis. The results suggest that high N supply preserves the function of chloroplast during water stress.

Excessive accumulation of NH4+ in plant tissues is one of the primary causes for plant injury under stress conditions (Nguyen et al., 2005). NH4+ in plants is derived from primary NH4+ uptake, NO3− reduction, protein turnover, and release during the conversion of Gly to Ser in photorespiration. GS plays an essential role in NH4+ detoxification. NH4+ from primary uptake and protein turnover is assimilated via cytosolic GS1, while that from NO3− reduction and photorespiration is assimilated by chloroplastic GS2 (Lea and Milfin, 2003; Thomsen et al., 2014). NR and NiR activity were inhibited under water stress at both N levels, which may diminish the production of NH4+ from NO3− reduction. Nevertheless, the down-regulation of GS1 together with the increase in protein degradation at low N level was responsible for NH4+ accumulation under water stress. NH3 produced in photorespiration has to be re-assimilated by chloroplastic GS2/Fd-GOGAT (Lea and Milfin, 2003). Stabilizing the level of GS2 is key for normal growth and for the balance between the C and N metabolism in rice (Bao et al., 2015). While the plants grown at low N level showed increased accumulation of NH4+ and down-regulated expression of GS2 under water stress, GS2 was up-regulated by water stress in the plants grown with moderate N. The results indicate that GS2 plays an important role in stabilizing the levels of NH4+ under water stress.

2-Oxoglutarate (2-OG), an important intermediate in the Krebs cycle, is indispensable in NH4+ assimilation and in the regulation of the C and N metabolism.
The Krebs cycle was expectedly strengthened during water stress as indicated by increases in PK and ICDH activity and/or their relevant organic acid (e.g., pyruvate, citrate, 2-OG, and fumarate) concentrations. However, the constant 2-OG concentration combined with reduced GS activity during water stress in low N condition implied an uncoupling between the N and C metabolisms. Thus, the increases in pyruvate, citrate, and fumarate levels under such conditions were likely to have resulted from increased sucrose depletion as indicated by the significant reduction in sucrose. The activity of PEPC was specifically increased by water stress in the plants grown at moderate N supply. PEPC participates in the anaplerotic reaction in the Krebs cycle, replenishing the C-skeleton to meet the demand for biosynthesis of organic acids and amino acids (Miyao and Fukayama, 2003; Nunes-Nesi et al., 2010). The increase of PEPC activity can enhance NH$_4^+$ assimilation, protecting plant cells from the toxicity of high NH$_4^+$ concentrations (Vanlerberge et al., 1990; Balkos et al., 2010). The transamination of Glu via AspAT and GGAT also provides 2-OG for the Krebs cycle and amino acid biosynthesis under stress conditions (Joshi et al., 2010). The increase in the activities of PEPC, AspAT, and GGAT at moderate N level with water stress may maintain N assimilation with a reduced consumption of stored carbohydrates. Therefore, sugars can make contributions to osmotic adjustment and be used for the pentose phosphate pathway, facilitating RuBP regeneration and CO$_2$ fixation.

4.3. High N supply improves Ser metabolism to increase antioxidant capacity under water stress

The protective roles of photorespiration are well known in facilitating dissipation of excessive excited energy and preventing photo-inhibition (Silva et al., 2015). Recent studies have revealed that the photorespiratory N metabolism affects photosynthetic CO$_2$ uptake (Busch et al., 2018). Gly and Ser are marker amino acids in photorespiration. Although Gly and Ser levels were considerably increased by water stress at both N levels, Ser showed a much greater increase at low N level than at moderate N level. These results indicate that low N supply attenuated the photorespiratory Ser metabolism during water stress. The accumulation of Ser serves as a metabolic signal for down-regulating enzymes in photorespiration (Timm et al., 2013), diminishing the recycling of amino acid and glycerate, and reducing the export pool of organic N-compounds and carbohydrates (Misra, 2014). The photorespiratory enzymes GGAT, SHMT, SGAT, and HPR are key factors in regulating the biosynthesis and metabolism of amino acids (Wingler et al., 1999; Igarashi et al., 2006; Misra, 2014). In this study, water stress increased GGAT and SHMT activity at both N levels, but SGAT and HPR activity were increased only under moderate N condition, suggesting that downstream reactions after the conversion of Gly into Ser constrained the Ser metabolism at low N level. Repression of the Ser metabolism also led to the accumulation of glycolate and glyoxyxlate, which are deleterious to plant cells (Wingler et al., 1999).
Hence, the increased Ser metabolism under high N condition is advantageous to improve the acclimation of rice photosynthesis to water stress, as it eliminates glycolate toxicity and facilitates the re-generation of glyceral, which can return to the Calvin-Benson cycle to support CO₂ fixation.

Photorespiration is recognized as a vital mechanism for mitigating the generation of H₂O₂ in plants, although it is itself a source of H₂O₂ (Voss et al., 2013). Water stress-induced up-regulation of antioxidative systems is crucial for enhancing the resistance of plants to water stress (Reddy et al., 2004). In accordance with the results reported previously (Zhong et al., 2017), moderate N supply alleviated membrane lipid peroxidation under water stress. The increases in catalase (CAT) activity, GSSG concentration, and the AsA/DHA ratio contributed to minimizing peroxidative damage in plants. Ser is a principal component of amino acids for the biosynthesis of glutathione (Zhu et al., 2006) and phospholipids (Igarashi et al., 2006). The remarkable descent of GSH concentration in water-stressed plants grown at low N level was most likely resulted from the accumulation of Ser. GSH-GSSG and AsA-DHA are coupled to control the level of H₂O₂ in chloroplasts (Noctor et al., 2000). At moderate N condition, the significantly increased GSSG concentration, combined with a reduction in DHA and an increase in AsA levels during water stress, implied that the ascorbate-glutathione cycle functioned effectively in the maintenance of chloroplast redox homeostasis. Our results reveal that high N supply-mediated increase in the photorespiratory Ser metabolism is closely related to cellular anti-oxidant capacity, thereby protecting chloroplasts against water stress and ultimately preserving the biochemistry of photosynthesis.

5. Conclusion

Our results reveal that high N supply coordinates the C and N metabolism to alleviate photosynthetic inhibition induced by water deficit stress. High N supply improved the photorespiratory Ser metabolism under water stress, thus increasing the biosynthesis of glutathione and the recycling of glyceral to the Calvin-Benson cycle. The coordination of the ascorbate-glutathione cycle together with increased CAT activity maintained redox homeostasis in chloroplasts. The re-assimilation of NH₃ during photorespiration was increased by the up-regulation of GS₂, combined with increases in AspAT- and GGAT-mediated transamination of Glu and PEPC-mediated anaplerotic reaction, which provided 2-OG without the consumption of stored carbohydrates. Therefore, sugars can be stored and used for the regeneration of RuBP via the pentose phosphate pathway (Fig. 8b). Under low N condition, water stress increased protein degradation, down-regulated N assimilation, and enhanced carbohydrate depletion in the Krebs cycle. The repressed Ser metabolism reduced glutathione biosynthesis and attenuated the ascorbate-glutathione cycle, resulting in aggravated membrane lipid peroxidation. All these factors perturbed the function of chloroplasts and uncoupled C and N metabolism, leading to a greater inhibition of photosynthesis (Fig. 8a). Our results suggest that the N metabolism plays an important role in the acclimation of rice photosynthesis to water stress conditions.

Author contributions

CZ and XCC conducted the experiments, collected and analyzed the samples, and drafted the manuscript. ZGB and CQZ participated in sample collection and analysis. LFZ and JHZ analyzed the data and revised the manuscript. CZ, JHL and QYJ conceived and designed this work. All authors read and approved the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.envexpbot.2018.10.021.

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