Plant Physiology and Biochemistry 135 (2019) 41–50

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Trade-off of within-leaf nitrogen allocation between photosynthetic nitrogen-use efficiency and water deficit stress acclimation in rice (*Oryza sativa* L.)

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ARTICLE INFO

Keywords:
Leaf N allocation
Water deficit stress
Photosynthetic N-Use efficiency
Limiting factor
Oryza sativa L

ABSTRACT

Nitrogen (N) allocation in leaves affects plant photosynthesis-N relationship and adaptation to environmental fluctuations. To reveal the role of leaf N allocation in water deficit stress acclimation in rice, the plants were grown in infertile soil supplying with low N (0.05 g N·kg^{-1} soil) and high N (0.2 g N·kg^{-1} soil), and then imposed to water deficit stress (~75% relative soil water content). We found that the proportion of leaf N allocated in the photosynthetic apparatus was significantly positive correlated with photosynthetic N-use efficiency (PNUE), and that N allocation in the carboxylation system and bioenergetics were the primary two limiting factors of PNUE under the conditions of high N and water deficit stress. PNUE was not significantly affected by water stress in low N condition, but markedly reduced in high N condition. Under low N condition, plants reduced N allocation in the light-harvesting system and increased soluble protein and free amino acids, or reduced N allocation in the cell wall to maintain PNUE under water deficit stress. Under high N, however, plants decreased N allocation in bioenergetics or carboxylation, but increased N allocation in non-photosynthetic components during water stress. Our results reveal that the coordination of leaf N allocation between photosynthetic and non-photosynthetic apparatus, and among the components of the photosynthetic apparatus is important for the trade-off between PNUE and the acclimation of water deficit stress in rice.

1. Introduction

Nitrogen (N) is the most quantity of nutrient required for plant growth, and an important factor determining the capacity of photosynthesis (Evans, 1989; Makino et al., 1997, 2003). Plants typically invest about 75% of leaf N in chloroplasts for photosynthesis (Evans, 1989). Leaf N allocation could affect CO_2 diffusion in leaves by changing mesophyll cell thickness and permeability, or affecting photosynthetic enzyme abundance and activity (Makino et al., 1997). Therefore, leaf N allocation in photosynthetic tissues is of great importance for plant photosynthetic capacity (Funk et al., 2013). Due to the considerably variation of the dependence of photosynthetic capacity on the N content among species, photosynthetic nitrogen-use efficiency (PNUE, photosynthetic capacity per unit leaf N) has been viewed as the most important factor for the interspecific differences in photosynthetic capacity (Takashima et al., 2004; Feng, 2008; Feng et al., 2008).

Crops growth in the field is frequently repressed by water deficit stress. Photosynthesis is the major source of crop yield, and is very sensitive to soil water deficit. Photosynthetic inhibition caused by water deficit stress is resulted from stomatal closure under mild/moderate stress, or the reduction of photosynthetic enzyme activities under severe stress (Wingler et al., 1999). Water deficit stress can induce the disintegration of the thylakoid membrane and degradation of photosynthetic proteins (Tian et al., 2013; Batra et al., 2014), thus resulting in a lower photosynthetic capacity and PNUE. On the other hand, water deficit stress triggers defense responses in plants. Most commonly, the up-regulation of antioxidant enzymes protects plants from water deficit

Abbreviations: CAT, catalase; C_i, intercellular CO_2 concentration; Cyt *f*, cytochrome *f*; MDA, malondialdehyde; J_{mc}, is the maximum electron transport rate per unit of Cyt *f*; J_{max}, maximum electron transport rate; P_B, fraction of leaf N in bioenergetics; P_C, fraction of leaf N in carboxylation; P_L, fraction of leaf N in light-harvesting; P_n, net photosynthetic rate; PNUE, photosynthetic nitrogen-use efficiency; SLM, specific leaf mass; SLN, specific leaf nitrogen; Vc_{max}, maximum carboxylation rate; V_{cr}, specific activity of Rubisco

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https://doi.org/10.1016/j.plaphy.2018.11.021
Received 15 August 2018; Received in revised form 6 November 2018; Accepted 19 November 2018
Available online 20 November 2018

stress-induced oxidative stress. The accumulation of soluble protein, free amino acids (particularly proline and branched-chain amino acids) and glutathione, which act as osmolytes or regulator of cell redox homeostasis under water stress, was widely observed in water-stressed plant tissues (Zhang and Kirkham, 1996; Huang and Jander, 2017; Zhong et al., 2018). Those results suggested that there is a trade-off between productivity (photosynthesis) and environmental adaptability (Takashima et al., 2004; Trouwborst et al., 2011). It is speculated that plants invest more N in non-photosynthetic fractions under stress conditions to coordinate growth and environmental adaptation at the expense of PNUE.

N partitioning between photosynthetic and non-photosynthetic proteins reflects the trade-off of productivity and environmental adaptability. More leaf N allocation in the fraction of structural proteins (e.g., cell-wall binding proteins) reduces photosynthetic capacity and PNUE but increases the persistence of leaf life (Takashima et al., 2004; Onoda et al., 2004; Feng, 2008). Besides, N allocation among the components of the photosynthetic apparatus also affects environmental adaptability and PNUE. Mu et al. (2016) reported that maize tended to allocate more N in the component of electron transport and photophosphorylation under low N condition to reduce light energy capture and maintain electron transport activity. In contrast, high N application increased N allocation in Rubisco. It is generally recognized that increased content of Rubisco is one of the most important reasons for the decrease of PNUE under high N condition (Suzuki et al., 2009; Li et al., 2012; Mu et al., 2016).

Plants can mediate leaf N allocation to improve the acclimation of photosynthesis to environmental fluctuations such as elevated atmospheric CO_2 concentrations (Niinemets et al., 1999) and irradiance changes (Niinemets et al., 1998). For example, Seneweera (2011) reported that spatial N deposition in the expanding leaf blade mediated the synthesis of Rubisco and possibly photosynthetic acclimation to elevated CO_2 concentration. In cucumber leaves grown under low irradiance (50 $\mu mol\, m^{-2}\cdot s^{-1}$) and subsequently exposed to moderate (200 $\mu mol\, m^{-2}\cdot s^{-1}$) irradiance, photosynthesis was increased due to N import, and N remained allocated in a similar ratio to Rubisco and bioenergetics, while relatively decreased allocated in light-harvesting system (Trouwborst et al., 2011). To date, the response of leaf N allocation between photosynthetic and non-photosynthetic apparatus, as well as among the components of the photosynthetic apparatus to water deficit stress and the effect on PNUE has been rarely reported.

In this study, by calculating N allocation with theoretical models and measuring N-containing compounds with biochemical methods in rice leaves, we aimed to: 1) examine the effects of soil water deficit stress on N allocation and its relation to PNUE in different N application rates; 2) identify the major limiting factor(s) of PNUE under water deficit stress; 3) test the hypothesis that leaf N allocation involves in the trade-off between PNUE and water deficit stress acclimation in rice.

2. Materials and methods

2.1. Plant cultivation and treatment

Rice cultivars 'Yongyou 538' (inter-subspecific hybrid) and 'Zhongzheyou 1' (*indica* hybrid) were used in this study. 'Yongyou 538' is an important one-season rice variety in Zhejiang Province of China, and 'Zhongzheyou 1' is a one-season rice variety widespread in South China. Seeds were germinated in an incubator at 30 °C, and then cultivated hydroponically in a climatic chamber at a day/night temperature of 28/23 °C, and a photosynthetic photon flux density (PPFD) of 600 $\mu mol\, m^{-2}\cdot s^{-1}$ at the plant tops provided by LED light source, for a photoperiod of 12 h (07:00 h-19:00 h). Three-leaf-old seedlings were transplanted to 5-L pots (20 cm in diameter) contained 5 kg infertile air-dried paddy soil. There were four plants in each pot. Plants were divided into two groups, and supplied with, respectively, 0.05 g N·kg^{-1} soil (low N, LN) and 0.2 g N·kg^{-1} soil (high N, HN) with the form of urea (46% N). Phosphate and potassium fertilizers in the two N treatments were the same and applied at the rates of 0.17 g kg^{-1} soil and 0.08 g kg^{-1} soil, respectively, with the forms of calcium superphosphate (16% P_2O_5) and potassium chloride (60% K_2O), respectively. Before transplanting, the soils were mixed thoroughly with the fertilizers and soaked thoroughly with tap water.

The plants were grown in a climatic chamber with the conditions of temperature, PPFD and photoperiod the same as described above to eliminate any effects of environmental fluctuations. The plants were well-watered by keeping a 3-cm water layer above the soil surface. Water stress was imposed to the plants at 25 days after transplanting (tillering stage). Half of the plants of each cultivar in each N treatment were well-watered as control, and the rest of plants were treated by withholding water for 6 (HN) or 8 (LN) days until the relative soil water content decreased to about 75%. There were three replications in each treatment and the experiment was conducted with a completely randomized design.

2.2. Photosynthetic measurement

Photosynthetic rate (P_n) at light-saturated (1500 $\mu mol\, m^{-2}\cdot s^{-1}$ PPFD) and ambient CO_2 was measured on fully expanded new leaves using a Li-6400XT portable photosynthesis system (Li-Cor Inc., Lincoln, NE, USA). The CO_2 concentration in the leaf chamber was adjusted to 400 $\mu mol\, mol^{-1}$ with a CO_2 mixer. The temperature was 25 °C, and the relative humidity was about 70%. Data were recorded after equilibration to a steady-state. The leaves were labelled, and leaf areas were calculated based on the labelled area. The following gas-exchange measurement was also conducted with the same labelled leaves.

Since there was no midday depression of photosynthesis, photosynthetic response to intercellular CO_2 concentration (C_i) (A-C_i curve) was measured from 08:30 h to 17:00 h, during which period the photosynthetic state of plants is fully activated. The conditions of PPFD, temperature, and relative humidity in the leaf chamber were the same as described above. The CO_2 concentration in the leaf chamber was set at 10 levels (1000, 800, 600, 400, 300, 200, 150, 100, 50, 0 $\mu mol\, mol^{-1}$). Prior to measurement, the labelled leaves were illuminated with 1500 $\mu mol\, m^{-2}\cdot s^{-1}$ PPFD provided by the light-emitting diodes light source of the equipment and 400 $\mu mol\, mol^{-1}$ of CO_2 concentration for at least 10 min. Data were recorded when the leaves reached a photosynthetic steady state. Maximum carboxylation rate (Vc_{max}) and maximum electron transport rate (J_{max}) were calculated using the A-C_i curves according to Sharkey et al. (2007) based on the model developed by Farquhar et al. (1980).

2.3. Leaf water potential

When accomplishing photosynthetic measurement, the corresponding leaves and the similar ones in the same plant were sampled. Then the leaves were punched into small discs and mixed. A part of the samples was used for measurement of leaf water potential (Ψ_{leaf}) immediately, using a WP4C Dew Point PotentiaMeter (Decagon, Pullman, WA, USA). The rest of the samples were frozen in liquid nitrogen immediately, and stored at −80 °C for the physiological and biochemical measurements.

2.4. Chlorophyll, total nitrogen, soluble protein and cell wall nitrogen analyses

After Ψ_{leaf} measurement, a known area of leaf discs was immersed with acetone and ethanol mixture (v: v = 1: 1) to extract chlorophyll for 24 h at room temperature in the dark. Chlorophyll concentration in the extract was determined using a spectrophotometer at 663 nm and 645 nm (Porra et al., 1989).

The rest of leaf discs used for Ψ_{leaf} measurement were oven-dried at 80 °C, and digested with H_2SO_4–H_2O_2 at 260 °C. N concentration was

measured using a high-resolution digital colorimeter (Autoanalyzer 3, SEAL, Germany).

Soluble protein and cell-wall N were determined according to Takashima et al. (2004) and Onoda et al. (2004), with some modifications. The leaf discs were powdered with liquid nitrogen and homogenized in 3 ml of Na-phosphate buffer (pH7.5). The homogenates were centrifuged at 10000 × *g* and 4 °C for 10 min, and the supernatant was the soluble protein fraction. The pellet was washed with the phosphate buffer contained 3% (v/v) sodium dodecyl sulfate (SDS) by centrifugation (10000 × *g* for 5 min) after heating at 90 °C for 5 min. This procedure was repeated three times. Membrane-binding proteins were removed during this procedure. To remove cytoplasmic proteins and the proteins weakly bound on cell wall, the pellet was washed twice with 0.2 M KOH, and then twice more with deionized water and finally twice with ethanol by centrifugation (10000 × *g* for 5 min). The tubes containing the pellets were oven-dried at 80 °C, and the samples were digested with H_2SO_4–H_2O_2 at 260 °C for N measurement.

Soluble proteins in the supernatant were precipitated with 10% trichloroacetic acid (TCA) by heating at 70 °C for 10 min. The precipitate was filtered with ash-free filter paper and then dried in an oven at 80 °C. N concentration was measured after digesting the samples with H_2SO_4–H_2O_2 at 260 °C. Soluble protein concentration was calculated as 6.25 times of N in this fraction.

2.5. Calculation of N allocation in the photosynthetic apparatus and photosynthetic N-use efficiency

Leaf N allocated in the photosynthetic apparatus can be divided into three categories: (1) carboxylation system including photosynthetic enzymes that involve in carbon reducing reactions; In C_3 plants, Rubisco is the main carboxylation enzyme; (2) proteins involved in bioenergetics, including Cyt *b/f* and CF1/CF0 related to electron transport and photophosphorylation; and (3) light-harvesting proteins such as photosystem I (PSI), photosystem II (PSII), and light-harvesting complex II (LHCII) proteins, that associated with light reactions of photosynthesis (Niinemets and Tenhunen, 1997; Mu et al., 2016). The fraction of leaf N allocated in carboxylation system (P_C), bioenergetics (P_B), light-harvesting system (P_L) were calculated using N content per unit leaf area (N_A, g·m^{-2}) together with other variables according to the equations as follows:

$$P_C = Vc_{max}/(6.25 \times V_{cr} \times N_A) \quad (1)$$

Where V_{cr} is the specific activity of Rubisco, which is assumed to be 32.9 μmol CO_2·g^{-1} Rubisco·s^{-1} (Jordan and Ogren, 1984; Sun et al., 2001); and the value of 6.25 converts N to protein.

$$P_B = J_{max}/(8.06 \times J_{mc} \times N_A) \quad (2)$$

Where J_{mc} is the maximum electron transport rate per unit of Cyt *f*, the value of which is 156 μmol electrons·μmol^{-1} Cyt *f*·s^{-1} (Nolan and Smille, 1976); and 8.06 is N in bioenergetics per unit Cyt *f* (μmol).

$$P_L = C_C/(C_B \times N_A) \quad (3)$$

Where C_C is the concentration of chlorophyll (mmol·m^{-2}); and C_B is the chlorophyll binding to PSI, PSII, and LHCII. C_B was estimated according to the linear relationship between C_B and specific leaf mass (SLM, leaf mass per unit leaf area) (Niinemets and Tenhunen, 1997).

The fraction of leaf N allocated in the photosynthetic apparatus (P_P) was the sum of P_C, P_B and P_L. N contents in carboxylation (N_C), bioenergetics (N_B), light-harvesting system (N_L) and all components of the photosynthetic apparatus (N_P) were calculated as the products of N_A with P_C, P_B, P_L and P_P, respectively. N_C is part of soluble protein N. Other soluble protein N was calculated as total soluble protein N minus N_C. N_B and N_L belong to membrane-binding proteins. Except for photosynthetic N, cell-wall N and soluble protein N, the remaining leaf N was defined as other N.

Photosynthetic N-use efficiency (PNUE) was calculated as P_n/N_A.

2.6. Malondialdehyde (MDA), catalase (CAT), proline, and free amino acids

Malondialdehyde (MDA) is an important product of lipid peroxidation. It was represented by the formation of thiobarbituric acid reactive substances (TBARS) (Hodges et al., 1999). Fresh leaf samples were homogenized with 4 ml 100 mM phosphate buffer (pH7.8), and then centrifuged at 10000 × *g* and 4 °C for 10 min. The supernatant (1 ml) was reacted with 2 ml 0.5% thiobarbituric acid in 5% TCA in boiling water bath for 15 min. After the mixture solutions cooled, they were centrifuged to remove any protein precipitate. The supernatant was measured at 600 nm, 532 nm and 450 nm, and MDA was calculated using the molar extinction coefficient of 0.155 mM^{-1} cm^{-1}.

Catalase (CAT) activity was measured using the extract of MDA. The reaction was carried out by adding a moderate enzyme extract to a 3-ml mixture containing 50 mM Na-phosphate buffer (pH7.8) and 20 mM H_2O_2. The absorbance at 240 nm was monitored for 300 s. CAT activity was calculated according to the slope of the curve and using the molar extinct coefficient of H_2O_2 (36 mM^{-1} cm^{-1}) and expressed as nmol H_2O_2·mg^{-1} protein·min^{-1} (Havir and Mchale, 1987).

Proline in the leaf samples was extracted with 5% sulfosalicylic acid in boiling water bath for 15 min and measured with a rapid spectrophotometric method at 520 nm reported by Bates et al. (1973). Standard curve was obtained from $_L$-proline.

Free amino acids in leaf samples was measured using a ninhydrin method (Yokoyama and Hiramatsu, 2003). Samples were homogenized with acetic acid/sodium acetate buffer (pH5.4), and centrifuged at 4 °C and 10000 × *g* for 10 min. A moderate supernatant was reacted with ninhydrin reagent in boiling water bath for 15 min in the presence of ascorbic acid. The concentration of amino acid was spectrophotometrically measured at 580 nm, and $_L$-leucine was used as the standard.

2.7. Data analysis

Boundary line analysis has been widely applied to understand crop yield gap (Fermont et al., 2009; Wairegi et al., 2010; Wang et al., 2015). In this study, we used this method to evaluate the contribution of individual factors of N allocation in the components of the photosynthetic apparatus to PNUE. The basic steps used to develop a boundary line are (Wang et al., 2015):

a) Grouping of data points (PNUE and N allocation factors).
b) Identification of the attainable value of PNUE (Y_{att}) in each treatment.
c) Identification of boundary points under the limitation of a specific factor (X).
d) Fitting of a boundary curve to the identified boundary points.

Boundary lines were fitted using a parabolic model. The most limiting factor in each treatment was identified according to the law of the minimum, and the factor that leading to the minimum (Y_{min}) of the maximum predicted value (Y_p) was considered as the most limiting factor (Fermont et al., 2009; Wairegi et al., 2010). The impact rate of a factor on PNUE was represented by the gap: gap = (Y_{att}-Y_{min})/Y_{att} × 100%.

Statistical analysis was done using SPSS (Statistic 19) for Windows. Means of data in different treatments were compared using two-way ANOVA and least significant difference (*LSD*) method. Correlation analysis of photosynthetic physiological parameters was performed based on Pearson's correlation coefficient.

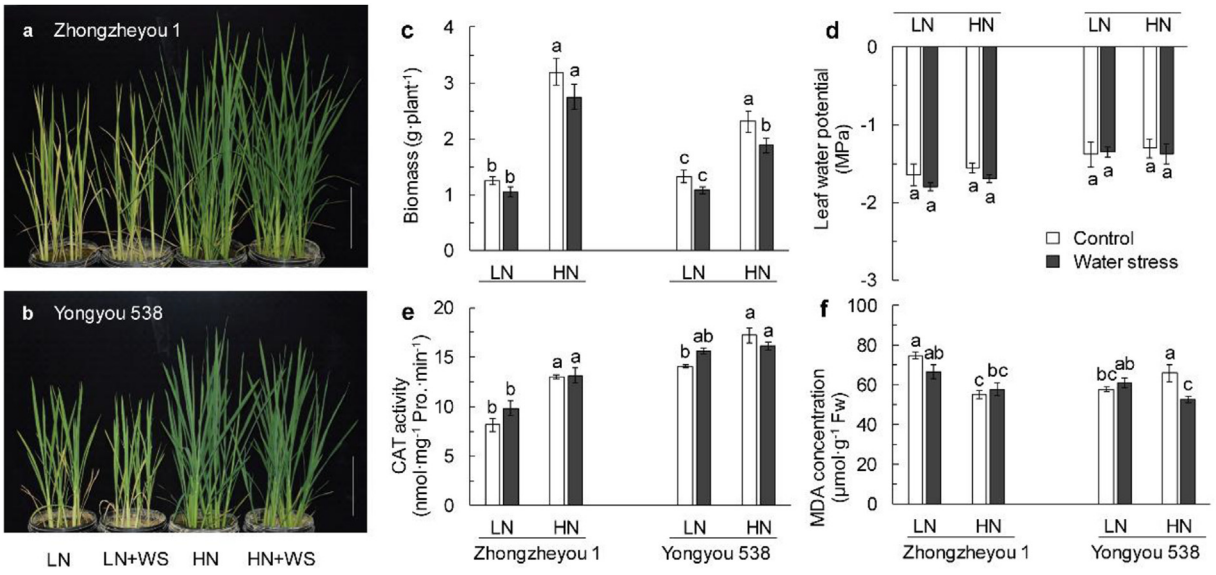

Fig. 1. Effects of water stress on rice plant growth and oxidative injury at different N levels. (**a**), (**b**) Plants grown at low N (0.05 g N·kg^{-1} soil) and high N (0.2 g N·kg^{-1} soil) were pictured after withholding water for 6 days. Scale bar = 20 cm. (**c**) Above-ground biomass of plants. (**d**) Leaf water potential. (**e**) Catalase activity in leaves. (**f**) Malondialdehyde (MDA) concentration in leaves. Data represent means ± SE (n = 4–6). The same letters on the bars indicates no statistically significant difference at $P < 0.05$ using the *LSD* method. LN, low N; HN, high N; WS, water stress.

3. Results

3.1. Plant growth and antioxidant capacity

The growth of the two rice cultivars was dramatically different between the two N levels. But at the same N level, the leaf color of 'Yongyou 538' was darker than that of 'Zhongzheyou 1', while the biomass of 'Yongyou 538' in high N was lower than that of 'Zhongzheyou 1' (Fig. 1a–c). Water stress slightly reduced the biomass of 'Zhongzheyou 1' at both N levels, but the reduction was significant in 'Yongyou 538' at high N level (Fig. 1c). Leaf water potential was not significantly affected by neither N application rate nor water stress in either cultivar (Fig. 1d). High N application increased catalase (CAT) activity in both cultivars, but it was not affected by water stress (Fig. 1e). The concentration of MDA was also not affected or even reduced by water stress (Fig. 1f). The results suggest that water deficit stress did not induce the oxidative stress in rice plants.

3.2. Leaf photosynthetic characteristics

Slopes of the A-C_i curves at $C_i < 200$ μmol mol^{-1} was significantly higher in high N condition than in low N condition (Fig. 2a and b). Water stress showed no significant effect on A in 'Zhongzheyou 1' when $C_i < 200$ μmol mol^{-1}, whereas A in 'Yongyou 538' was decreased and the slope of the curve was reduced under water stress (Fig. 2a and b). Light-saturated photosynthetic rate under $C_i < 200$ μmol mol^{-1} is mainly constrained by carboxylation (Farquhar et al., 1980). It was showed that water stress affected Vc_{max} in 'Zhongzheyou 1' marginally, while Vc_{max} in 'Yongyou 538' was significantly reduced by water stress in high N condition (Fig. 2c). J_{max} in both cultivars was not affected by water stress in low N condition, but significantly reduced in high N condition (Fig. 2d). The results suggested that water stress increased the biochemical limitation of photosynthesis in both cultivars grown in high N condition.

Specific leaf N (SLN), leaf chlorophyll, specific leaf mass (SLM) and photosynthetic rate (P_n) were markedly higher in high N condition than in low N condition (Table 1). Water stress significantly increased SLN in 'Zhongzheyou 1' at high N level, but showed no significant effect on SLN in 'Yongyou 538'. Water stress markedly decreased chlorophyll and SLM in 'Zhongzheyou 1' in low N condition, while chlorophyll in 'Yongyou 538' was significantly increased in high N condition and SLM was not different among treatments. P_n in 'Zhongzheyou 1' was not affected by water stress at either N level, but P_n in 'Yongyou 538' was significantly decreased by water stress at both N levels. Under control condition, PNUE in 'Zhongzheyou 1' was not different between low N and high N treatment, but PNUE in 'Yongyou 538' was significantly higher at low N level than at high N level. PNUE in both cultivars was not affected by water stress in low N condition, but significantly decreased in high N condition (Table 1).

The correlation analysis between those parameters showed in Table 2 that SLN was significantly positive correlated with chlorophyll concentration, LSM and P_n ($P < 0.01$), but significantly negative correlated with PNUE ($P < 0.05$). Chlorophyll was also positively correlated with SLM and P_n ($P < 0.01$), and negatively correlated with PNUE ($P < 0.05$). The correlation between SLM and P_n was positive ($P < 0.01$). However, the correlation between PNUE and SLM or P_n was not significant. The results indicate that the reduction of PNUE caused by high N or water stress is mainly related to the changes of leaf N.

3.3. Leaf nitrogen allocation in photosynthetic apparatus

To further elucidate the effect of leaf N on PNUE, N allocation in photosynthetic apparatus and non-photosynthetic tissues of leaves was analyzed under different N and water conditions. N allocated in the photosynthetic apparatus (N_P) accounted for 54.7%–62.3% and 55.9%–71.4% of total leaf N in 'Zhongzheyou 1' and 'Yongyou 538', respectively. Under the control conditions, 'Zhongzheyou 1' allocated more N to the photosynthetic apparatus in high N condition than in low N condition, with a significant increase in the carboxylation (P_C) (Fig. 3a, c). In low N condition, N allocation in the photosynthetic apparatus was not altered by water stress, but the allocation of N in the light-harvesting system was reduced by 15% (0.02 g m^{-2}) (Fig. 3b), suggesting that N allocation in the photosynthetic apparatus was adjusted among the components under such condition. Water stress reduced N allocation in the photosynthetic apparatus at high N level, with a significant decrease in the fraction of bioenergetics (P_B) (Fig. 3d).

Compared with low N treatment, high N treatment reduced N allocation in the photosynthetic apparatus of 'Yongyou 538' under the control condition, with a significant decrease in the fraction of

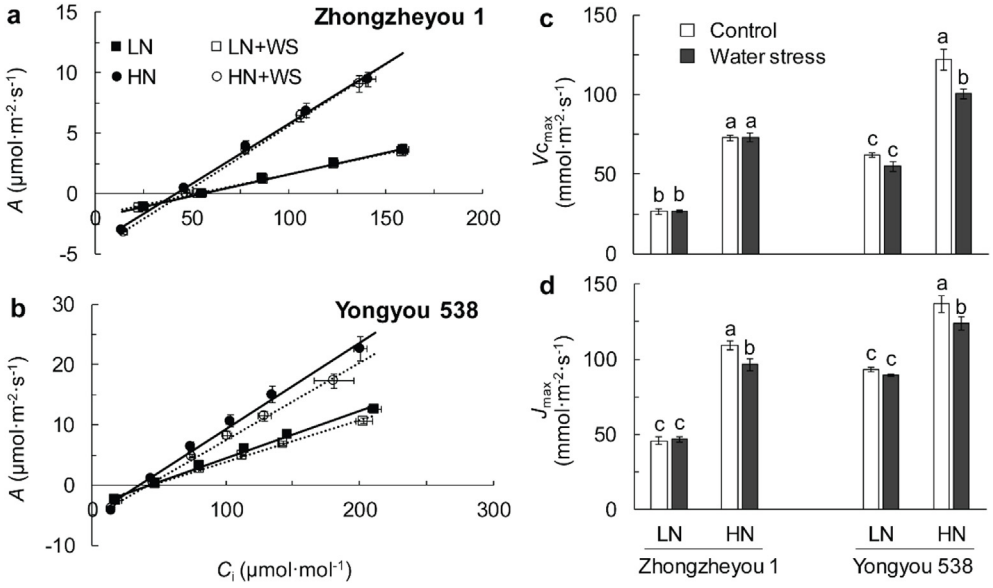

Fig. 2. Effects of water stress on photosynthetic response to intercellular CO_2 concentration (C_i) at different N levels. (**a**), (**b**) Linear regression of A-C_i curve at $C_i < 200\ \mu mol\ mol^{-1}$. (**c**) Maximum carboxylation rate. (**d**) Maximum electron transport rate. Data represent means ± SE ($n = 4$). The same letters on the bars indicates no statistically significant difference at $P < 0.05$ using the *LSD* method. LN, low N; HN, high N; WS, water stress.

bioenergetics (P_B) (Fig. 3e, g). While N allocation in the photosynthetic components was not affected by water stress in low N condition (Fig. 3f), water stress decreased N allocation in the photosynthetic components by 15% in high N condition, with a 24% (0.11 g m^{-2}) of reduction in the fraction of carboxylation (P_C) (Fig. 3h).

Among the components of the photosynthetic apparatus in both cultivars, N allocation in the light-harvesting system and carboxylation was not different between low N and high N treatments under the control condition, but N allocation in the bioenergetics was significantly lower in high N condition than in low N condition. Water stress showed different effects on the ratio of N allocated among the three components between the two cultivars. In 'Zhongzheyou 1' grown in low N condition, water stress reduced the ratio of N allocated in the light-harvesting system, but increased the ratio of N allocated in the bioenergetics (Fig. 4a). While in high N condition, water stress reduced the ratio of N allocated in the bioenergetics. In 'Yongyou 538', water stress did not affect the ratios of N allocated in the three components in low N condition. In high N condition, however, water stress increased the ratio of N allocated in the light-harvesting system, but reduced the ratio of N allocated in the carboxylation (Fig. 4b).

Table 2
Pearson's correlation coefficients between photosynthetic physiological parameters. The statistical significance was indicated:*, $P < 0.05$; and**, $P < 0.01$. SLN, specific leaf nitrogen; SLM, specific leaf mass; P_n, photosynthetic rate, and PNUE, photosynthetic nitrogen-use efficiency.

Parameters	SLN	Chlorophyll	SLM	P_n	PNUE
SLN	1	0.990**	0.644**	0.909**	−0.381*
Chlorophyll		1	0.636**	0.900**	−0.369*
SLM			1	0.723**	0.144
P_n				1	0.006
PNUE					1

3.4. Leaf nitrogen allocation in non-photosynthetic components

Under control conditions, 'Zhongzheyou 1' grown at high N level allocated more N in the fraction of other soluble protein, but allocated less N in the cell wall and other N, compared with low N treatment (Fig. 3a, c). Cell-wall N was not significantly affected by water stress at either N level, whereas water stress increased N allocation in other soluble protein but reduced N allocation in other N at low N level

Table 1
Effects of water stress on photosynthetic physiological characteristics of leaves at different N levels. Data represent means ± SE ($n = 4$). The same letters followed by the values in the same column indicates no statistically significant difference at $P < 0.05$ using the *LSD* method. *F*-values of the two-ANOVAs of nitrogen, water, and their inaction are indicated: ns, not significant;*, $P < 0.05$; and**, $P < 0.01$. SLN, specific leaf nitrogen; SLM, specific leaf mass; P_n, photosynthetic rate; PNUE, photosynthetic nitrogen-use efficiency; LN, low N; HN, high N; WS, water stress.

Treatments	SLN (g·m^{-2})	Chlorophyll (mmol·m^{-2})	SLM (g·m^{-2})	P_n (μmol·m^{-2}·s^{-1})	PNUE (μmol·g^{-1} N·s^{-1})
Zhongzheyou 1					
LN	0.47 ± 0.02 c	0.23 ± 0.01 b	28.99 ± 0.90 b	7.70 ± 0.53 b	16.22 ± 0.88 ab
LN + WS	0.45 ± 0.01 c	0.19 ± 0.01 c	25.41 ± 0.97 c	7.11 ± 0.37 b	15.70 ± 0.42 ab
HN	1.09 ± 0.05 b	0.54 ± 0.01 a	33.08 ± 1.31 a	18.71 ± 0.57 a	17.25 ± 0.62 a
HN + WS	1.20 ± 0.03 a	0.56 ± 0.01 a	34.13 ± 0.64 a	17.92 ± 0.74 a	14.98 ± 0.43 b
Nitrogen	2403.4**	866.3**	42.4**	369.9**	0.06ns
Water	112.3**	0.7ns	1.6ns	1.5ns	5.2*
N × water	19.4**	6.2*	5.5*	0.03ns	2.0ns
Yongyou 538					
LN	0.74 ± 0.01 b	0.35 ± 0.02 c	36.92 ± 0.96 a	16.53 ± 0.34 c	22.21 ± 0.43 a
LN + WS	0.70 ± 0.06 b	0.34 ± 0.02 c	38.40 ± 2.85 a	13.87 ± 0.28 d	21.64 ± 1.10 a
HN	1.56 ± 0.11 a	0.72 ± 0.04 b	39.20 ± 1.09 a	28.16 ± 1.20 a	18.12 ± 0.67 b
HN + WS	1.69 ± 0.05 a	0.81 ± 0.02 a	38.41 ± 1.77 a	21.91 ± 0.81 b	12.96 ± 0.42 c
Nitrogen	1147.3**	236.6**	0.4ns	175.4**	42.0**
Water	0.02ns	1.4ns	0.03ns	35.9**	15.8**
N × water	2.9ns	4.0ns	0.4ns	5.8*	3.4ns

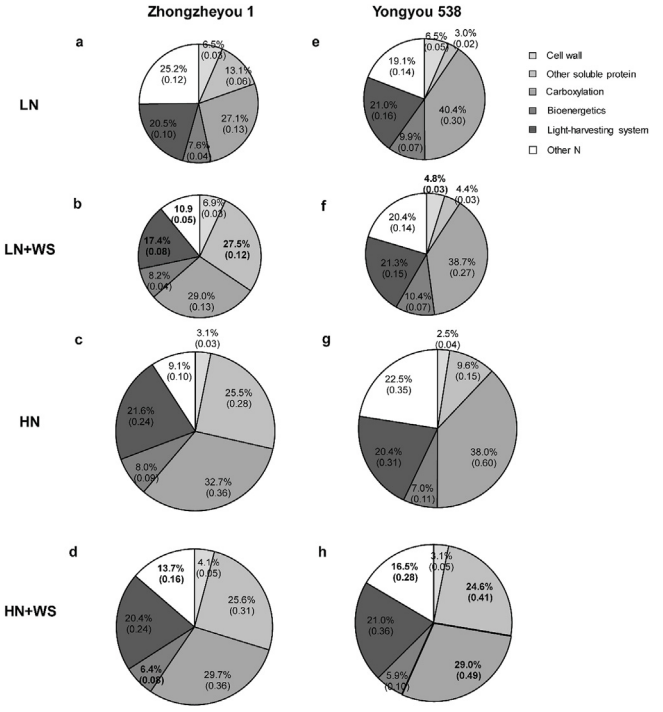

Fig. 3. Analysis of N allocation in leaves under different N and water conditions. (**a**)–(**d**) N partitioning in 'Zhongzheyou 1'. (**e**)–(**h**) N partitioning in 'Yongyou 538'. The data of percentages are the content of N in the corresponding components accounting for total leaf N content. The data in the brackets are N concentration ($g \cdot m^{-2}$). Data are averaged from four replications. The size of pie chart indicates N content. Bold data indicates significant increase/decrease compared with the control at the corresponding N level. LN, low N; HN, high N; WS, water stress.

(Fig. 3b). In contrast, N in the fraction of other N was significantly increased by water stress at high N level (Fig. 3d).

Under control conditions, 'Yongyou 538' increased the fraction of N in non-photosynthetic tissues at high N level compared to low N treatment. Although the percentage of cell-wall N was significantly reduced, N in the fractions of other soluble protein and other N was increased (Fig. 3e, g). Water stress reduced cell-wall N at low N level (Fig. 3f). While at high N level, water stress reduced the proportion of other N by 27%, but increased N in the fraction of other soluble protein by 156% (Fig. 3h).

Amino acid is one of the most abundant N pools in leaves (Funk et al., 2013). High N treatment increased proline and free amino acids in both cultivars relative to low N treatment (Fig. 5a and b), but proline showed no significant difference among treatments (Fig. 5a). However, water stress significantly increased free amino acids in 'Zhongzheyou 1' at low N level and in 'Yongyou 538' at high N level, which was consistent with the changes of total soluble protein (Fig. 5b and c), suggesting water stress induced increase in protein synthesis.

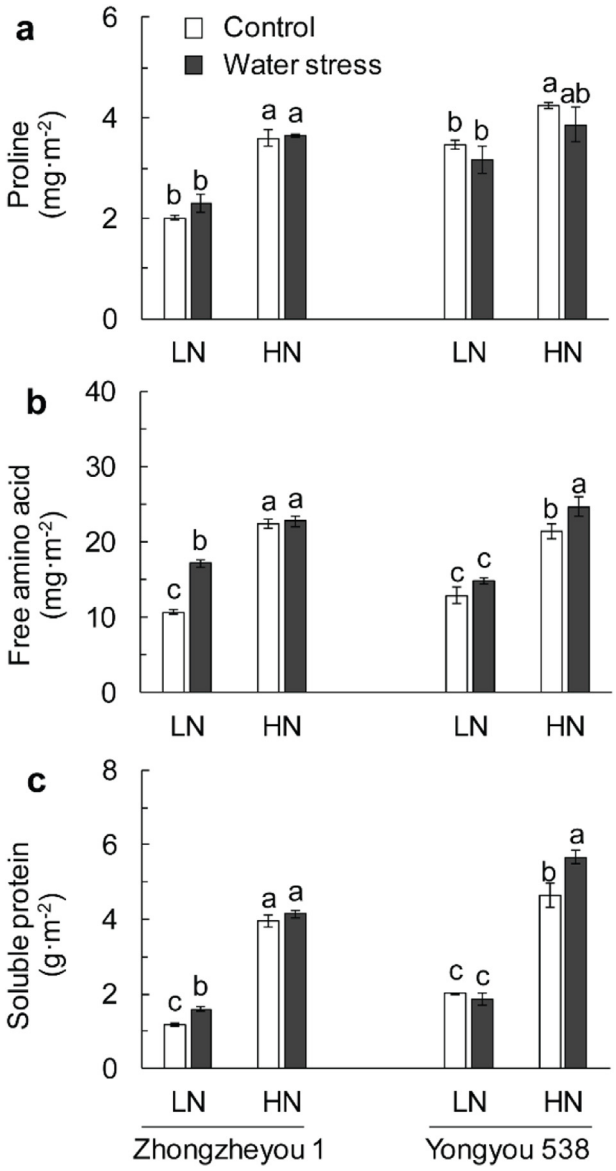

Fig. 5. Effects of water stress on proline (**a**), free amino acids (**b**), and soluble protein (**c**) concentrations under different N levels. Data represent means ± SE ($n = 4$). The same letters on the bars indicates no statistically significant difference at $P < 0.05$ using the *LSD* method. LN, low N; HN, high N.

3.5. *Effects of nitrogen allocation in photosynthetic apparatus on PNUE based on boundary line analysis*

PNUE increased linearly with the increasing N allocation in the carboxylation (P_C) and bioenergetics (P_B) (Fig. 6a–c), which averagely

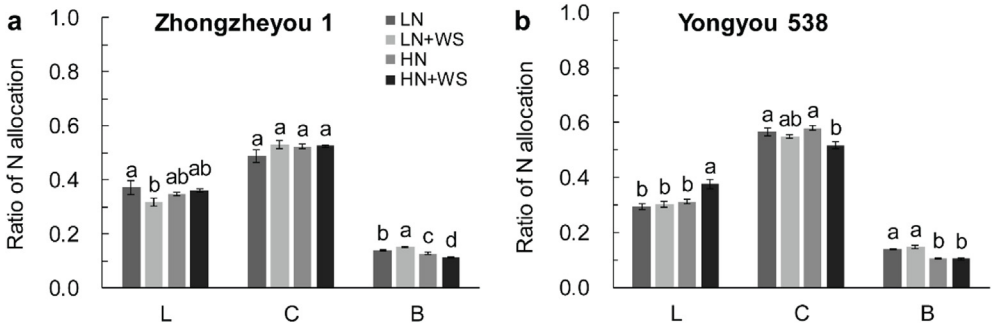

Fig. 4. Ratio of N allocation in the components of photosynthetic apparatus under different N and water conditions. L, C, and B are the ratios of N allocated to light-harvesting system, carboxylation and bioenergetics, respectively. Data represent means ± SE ($n = 4$). The same letters on the bars indicates no statistically significant difference at $P < 0.05$ using the *LSD* method. LN, low N; HN, high N; WS, water stress.

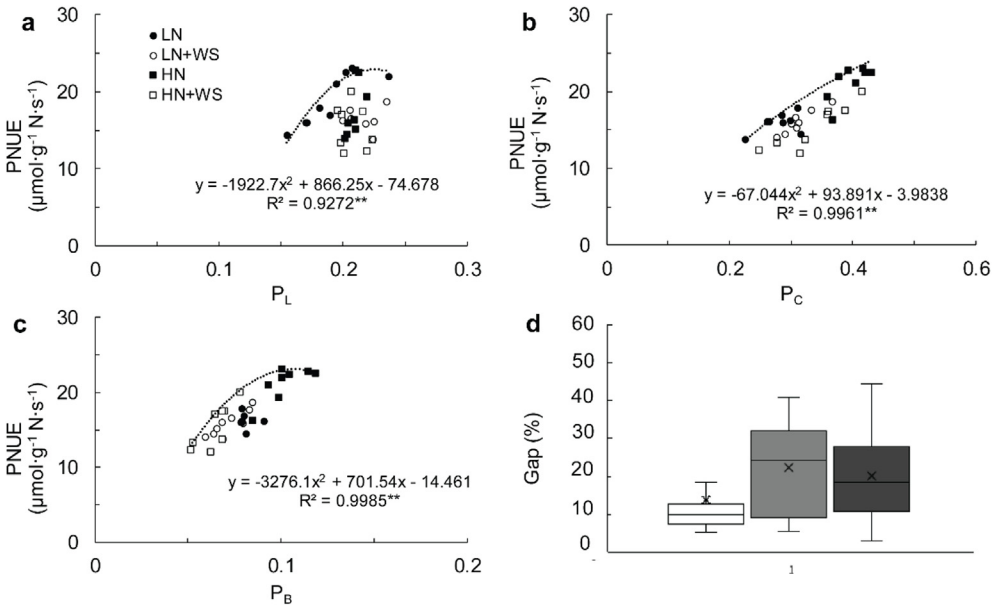

Fig. 6. Quantification of the effects of each components of the photosynthetic apparatus on PNUE based on the boundary line approach. (**a**) The relationship between PNUE and P_L. (**b**) The relationship between PNUE and P_C. (**c**) The relationship between PNUE and P_B. (**d**) Average influence of P_L, P_C and P_B on PNUE. P_L, P_C, and P_B are the ratios of N allocated to light-harvesting system, carboxylation, and bioenergetics, respectively. LN, low N; HN, high N; and WS, water stress.

contributed 22.3% and 20.3% to PNUE, respectively (Fig. 6d).

In 'Zhongzheyou 1' grown under control conditions, carboxylation and bioenergetics were predominant limiting factors of PNUE (Fig. 7a), which collectively accounted for 76.9% and 85.5%, respectively, of the total contribution of the three limiting factors at low N and high N level. Under water stress, the limiting percentage of the light-harvesting system was increased by 112.2% at low N level, and the limiting percentage of the bioenergetics was increased by 30.4% at high N level (Fig. 7a).

PNUE in 'Yongyou 538' plants grown in low N condition was limited mainly by N allocation in the light-harvesting system, which accounted for 55.7% of all the three limiting factors, but water stress increased the limitation of carboxylation and bioenergetics on PNUE by 46.6% and 111.6% (Fig. 7b). In high N condition, carboxylation and bioenergetics were the dominant limiting factors of PNUE, and water stress increased the limiting percentage of carboxylation by 100.3% (Fig. 7b).

Taken the results of N allocation and boundary line analysis together, it is indicated that the reduced PNUE of the two cultivars caused by water stress at high N level was related to the increased limitation of carboxylation and bioenergetics.

4. Discussion

It has been widely recognized that leaf morphological adjustment is a more relevant determinant of leaf photosynthetic adaptation to environment than leaf biochemical properties (Niinemets et al., 1998; Delagrange, 2011). Our results showed that although no significant oxidative injury in the leaves was observed, water deficit stress induced the alterations of within-leaf N allocation and PNUE, suggesting that leaf N allocation responds rapidly to water deficit stress and could act as a regulator of photosynthesis-N relationship. The results corroborated those reported in previous studies, where proposed that N metabolism plays a significant role in the acclimation of photosynthesis to water stress (Xu and Zhou, 2006; Zhong et al., 2018).

Photosynthetic rate increases with N concentration increasing, but reduces with soil moisture decreasing. The interactive effects of water and N on plant photosynthesis have been widely observed in many plant species (Karrou and Maranville, 1995; Tian et al., 2004; Liu and Dickmann, 2010; Li et al., 2016). In this study, the effect of water and N interaction on P_n was different in the two rice cultivars. P_n of 'Zhongzheyou 1' was mainly affected by N application rate, but 'Yongyou 538' was affected by both N application rate and water deficit stress. However, PNUE at high N was significantly reduced by water deficit stress in both rice cultivars.

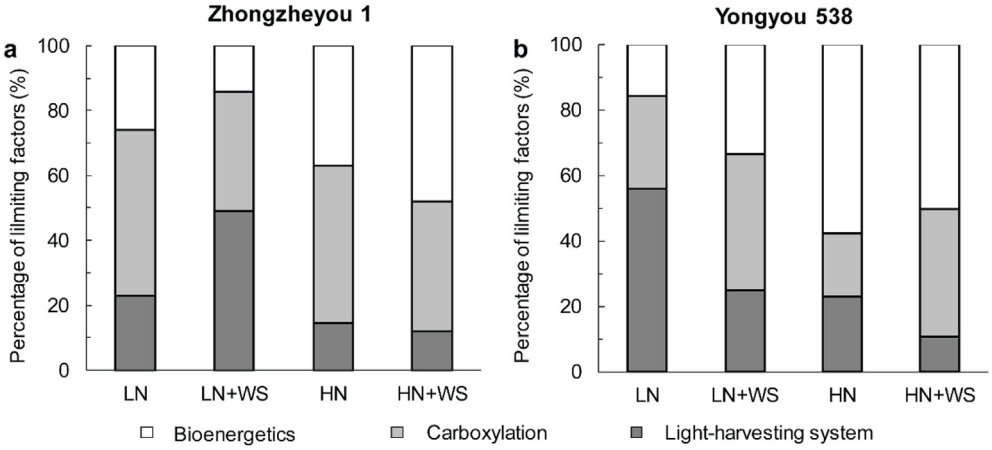

Fig. 7. Analysis of limiting factors on PNUE under different N and water conditions. PNUE was assumed to be limited by N allocation to bioenergetics, carboxylation and light-harvesting system. LN, low N; HN, high N; WS, water stress.

Plants with relatively more N partitioning in the photosynthetic machinery have a higher PNUE (Poorter and Evans, 1998). In many natural ecosystems, where N is a limiting factor of plant growth, species with greater N investment in photosynthetic proteins generally showed a higher PNUE (Feng et al., 2008; Funk et al., 2013; Moon et al., 2015). In agricultural ecosystems, even though the limitation of N on photosynthetic capacity can be eliminated via application of N fertilizer, N investment in the photosynthetic apparatus remains an important determinant of PNUE (Liu et al., 2016a, b). In this study, rice plants allocated 55.3%–71.4% of leaf N in photosynthetic apparatus, which was in accordance with the results that reported in woody plants (Feng et al., 2008) and winter oilseed rape (Liu et al., 2016a), but a little higher than that reported by Makino et al. (2003) in rice due to the different methods used for estimating N allocation (Shi et al., 2015). Further we found that the ratio of leaf N allocation in the photosynthetic apparatus was significantly positive correlated with PNUE ($r = 0.879$, $P < 0.001$, $n = 32$), although SLN was negatively correlated with PNUE. The results indicate that even though the reduced gain of photosynthetic rate with increasing SLN constrained the improvement of PNUE, the partitioning of N in the photosynthetic apparatus is more important than total leaf N for improving PNUE. Under high N application and water deficit stress conditions, to increase N allocation in the photosynthetic apparatus could be an important strategy to maintain a higher PNUE and rice photosynthetic capacity.

PNUE is not only affected by the proportion of N partitioning in the photosynthetic machinery, but also by the partitioning of photosynthetic N among light-harvesting, electron transport and Rubisco (Poorter and Evans, 1998). Mu et al. (2016) revealed that the coordination of N allocation among the components of the photosynthetic apparatus is important to maintain a higher PNUE. The boundary line analysis showed that carboxylation and bioenergetics are the major limiting factors of PNUE in 'Zhongzheyou 1' and 'Yongyou 538', respectively, grown under high N with normal water conditions. Under present atmospheric condition, Rubisco is the primary limitation of light-saturated photosynthesis (Suzuki et al., 2009). The increased N investment in carboxylation in 'Zhongzheyou 1' therefore maintained a higher PNUE under such condition. Under very high N condition, Rubisco amount becomes the major limitation of PNUE due to reduced P_n per Rubisco amount (Li et al., 2009, 2012; Suzuki et al., 2009; Mu et al., 2016). 'Yongyou 538' had a higher leaf N concentration and allocated 2-fold of N in carboxylation compared to 'Zhongzheyou 1' in the same growth condition (Fig. 3). Such a high amount of carboxylation enzymes could be higher than that required for photosynthesis (Eichelmann and Laisk, 1999; Warren et al., 2003). It is probably that the relatively lower electron transport and photophosphorylation does not well-matched with its higher carboxylation capacity, limiting the increase of photosynthetic rate, and subsequently reducing PNUE.

The boundary line analysis revealed that water deficit stress increased the bioenergetics and carboxylation limitation to PNUE in 'Zhongzheyou 1' and 'Yongyou 538', respectively, under high N condition. Drought stress generally leads to excess light energy for plants (Biehler et al., 1996). Photosynthetic electron transport system and photosynthetic enzyme activity play important roles in drought-tolerance of plants (Sanda et al., 2011; Xu et al., 2013; Ksas et al., 2015). The decreased N allocation in the bioenergetics or carboxylation breaks the balance among light energy capture, electron transport, and energy utilization, which could be a major reason of photosynthetic inhibition and PNUE decreasing under water deficit stress condition. The results verified that coordination of N allocation among the components of the photosynthetic apparatus is important for the photosynthesis-N relationship.

Coordination of N allocation also contributes to the constant of PNUE in low N with water deficit stress condition. Water deficit stress reduced N allocation in the light-harvesting proteins of 'Zhongzheyou 1', which could reduce excessive electron production to maintain a higher PNUE (Mu et al., 2016). Previous studies using chlorophyll-deficient rice mutants showed that decreased chlorophyll content did not decrease photosynthetic rate; in contrast, photosynthetic rate was increased due to higher excitation energy and electron transport (Wu et al., 2014; Gu et al., 2017). Thus, the reduced light capture in cooperation with the relative higher electron transport and carboxylation activity maintained the photosynthetic rate and PNUE in water-stressed 'Zhongzheyou 1' plant at low N. In 'Yongyou 538' grown with low N, the reduced N allocation in cell wall could contribute to the maintenance of PNUE under water deficit stress. Feng et al. (2008) has demonstrated that more N allocation in cell wall reduces PNUE. Cell wall is a major barrier limiting CO_2 diffusion into mesophyll cells (Tholen and Zhu, 2011). Reduced N partitioning in the cell wall increases its permeability for CO_2 diffusion into mesophyll cells and therefore maintains P_n and PNUE (Makino et al., 1997).

As N allocation in the photosynthetic apparatus is important for PNUE, a question arises, therefore: why rice plants grown in high N condition reduce N investment in photosynthesis under water deficit stress? A reasonable interpretation is that plants need to balance growth and stress-tolerance when subject to stress conditions (Houle, 2002; Takashima et al., 2004; Karasov et al., 2017). Transfer of N to non-photosynthetic components enhances plant resistance to stress at the cost of PNUE (Takashima et al., 2004; Feng, 2008). Soluble protein and free amino acids are two of the most abundant N pools in leaves, and they act as important osmolytes under water deficit stress (Singh et al., 2016; Zhong et al., 2018). In addition, many important N-containing compounds such as glutathione and malate dehydrogenase play key roles in alleviating water stress-induced photosynthetic inhibition (Gamble and Burke, 1984; Biehler et al., 1996). The increases in the N allocation in other N and other soluble protein, respectively, in 'Zhongzheyou 1' and 'Yongyou 538' grown with high N could be related to the acclimation of plants to water deficit stress. The increase in soluble protein in 'Yongyou 538' seems to be a result of increased Rubisco degradation. It has been reported that Rubisco degradation is occurred in response to drought stress (Aranjuelo et al., 2011; Yu et al., 2012). Thus, Rubisco degradation may also play an important role in acclimation to water deficit stress by providing Rubisco-derived N for the synthesis of other soluble proteins (Suzuki et al., 2009). The results implied that N allocation between photosynthetic and non-photosynthetic apparatus is involved in the trade-off between PNUE and water deficit stress acclimation in rice.

5. Conclusion

Our results reveal that the coordination of leaf N allocation between photosynthetic and non-photosynthetic apparatus, and among the components of the photosynthetic apparatus is of great importance for the trade-off between PNUE and the acclimation of water deficit stress in rice. N allocation in carboxylation and bioenergetics of the photosynthetic apparatus is the primary limiting factor of PNUE under the conditions of high N and water deficit stress. N allocation responses differently to water deficit stress in different N application rates. Rice plants reduced N allocation to the light-harvesting system and increased soluble protein and free amino acids, or reduced N allocation to cell wall to maintain P_n and PNUE under water deficit stress when grown in low N condition. While in high N condition, rice plants allocated more N in non-photosynthetic tissues under water deficit stress at the expense of PNUE. The results suggest that leaf N allocation regulates the acclimation of rice plants to water deficit stress. To further reveal the regulatory mechanisms of leaf N allocation in the field condition, where the lack of N and limitation of water supply is recurrent, is significant for balancing efficient N utilization and productivity of rice.

Conflicts of interest

The authors declare that they have no conflicts of interest.

CRediT authorship contribution statement

Chu Zhong: Formal analysis, Writing – original draft, Writing – original draft. **Shao-Fen Jian:** Formal analysis. **Jie Huang:** Formal analysis. **Qian-Yu Jin:** Writing – original draft. **Xiao-Chuang Cao:** Writing – original draft.

Acknowledgment

This work was funded by the Natural Science Foundation of Zhejiang Province (No. LY18C130005, LQ15C130004); the National Key Research and Development Program of China (No. 2017YFD0300100, 2016YFD0101801); and the National Basic Research Program of China (No. 2015CB150502).

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