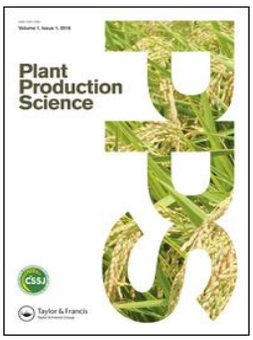

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Comparison of Susceptibility to Photoinhibition and Energy Partitioning of Absorbed Light in Photosystem II in Flag Leaves of Two Rice (*Oryza sativa* L.) Cultivars that Differ in Their Responses to Nitrogen-Deficiency

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Abstract: The energy partitioning in photosystem II (PSII) and the susceptibility to photoinhibition in PSII were investigated in flag leaves of two rice cultivars, Shirobeniya (a traditional *japonica*) and Akenohoshi (an improved *japonica-indica* intermediate) grown under standard-nitrogen (N) (SN) and low-N (LN) conditions. N-deficiency resulted in significant decreases in total dry weight, net photosynthetic rate (P_N), the energy flux via carboxylation (J_c), and content of ribulose-1,5-bisphosphate carboxylase / oxygenase (Rubisco) in flag leaves in the two cultivars, and these parameters of Shirobeniya were lower than those in Akenohoshi under the LN condition. In the two cultivars, the energy flux via alternative electron flow was significantly increased by N-deficiency, which was accompanied by enhanced activity of superoxide dismutase (SOD). Although under the LN condition no cultivar differences were found in J_a and SOD, ascorbate peroxidase activity in Shirobeniya was lower than that in Akenohoshi. N-deficiency resulted in more significant increases in the susceptibility to photoinhibition (the degree of decrease in maximum quantum yield of PSII), hydrogen peroxide (H_2O_2) content and malondialdehyde content after exposure to high irradiance in Shirobeniya than those in Akenohoshi. These results indicated that the increased susceptibility to photoinhibition in the LN plants of Shirobeniya was mainly due to oxidative damages to chloroplasts, resulting from lower carboxylation and H_2O_2-scavenging capacities. Therefore, both carboxylation and H_2O_2-scavenging capacities could be important factors in determining the cultivar difference in the productivity of rice under LN conditions.

Key words: Chlorophyll fluorescence, Energy partitioning, Hydrogen peroxide, Nitrogen-deficiency, Photoinhibition, Photoprotection, Photosynthesis, Rice (*Oryza sativa* L.).

Nitrogen (N) is a constituent of many plant cell components, such as amino acids, nucleoside bases and chlorophyll (Chl) (Lawlor et al., 2001). Therefore, the growth of plant requires a continuous supply of N. N-deficiency occurs in both wild and crop plants. The reduced growth of N-deficient plants is usually ascribed to both lower rates of leaf expansion and declines in photosynthetic rate per unit leaf area. The effects of N-deficiency on photosynthesis have been studied in the past several decades. N-deficiency caused a decrease in the light-saturated photosynthetic rate per unit leaf area, which was associated with decreases in Chl and ribulose-1,5-bisphosphate carboxylase / oxygenase (Rubisco) contents (Evans and Terashima, 1987; Terashima and Evans, 1988). The effect of N-deficiency on Rubisco is often larger than that on Chl (Evans and Terashima, 1987; Chen et al., 2003; Kumagai et al., 2007, 2009b). In the low-N (LN) leaves, a decrease in light harvesting capacity occurs, which is associated with the decrease in leaf Chl (Verhoeven et al., 1997). Light absorptance, however, is less affected than both leaf Chl content and the energy utilization capacity in photosynthesis (Chen and Cheng, 2003). It seems that

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Abbreviations: J_a, energy flux via alternative electron flow; J_c, energy flux via photosynthetic CO_2 assimilation; $J_{f,D}$, energy flux via fluorescence and light-independent constitutive thermal dissipation; J_{NPQ}, energy flux via ΔpH- and xanthophyll cycle-dependent thermal dissipation; J_o, energy flux via photorespiration; J_{PSII}, energy flux via linear electron transport; K_c, number of electron equivalents required to reduce 1 molecule of CO_2 in the Calvin cycle; K_r, number of electron equivalents required to release 1 molecule of CO_2 in photorespiration; P_G, gross photosynthetic rate; P_N, net photosynthetic rate; R_d, day respiration rate; V_c, rate of RuBP carboxylation; V_o, rate of RuBP oxygenation.

plants often absorb more light energy than they need for photosynthesis, and the excessive light energy exacerbates photoinhibition in photosystem II (PSII) under N-deficiency (Verhoeven et al. 1997; Skillman and Osmond, 1998; Bungard et al., 2000).

Previous studies suggested that several mechanisms are involved in the photoprotection against photoinhibition of PSII. Trans-thylakoid pH gradient (ΔpH)- and xanthophyll cycle-dependent thermal dissipation (Demmig-Adams and Adams, 1996), photorespiration (Kozaki and Takebe, 1996) and the consumption of reducing power via the water-water cycle (Asada, 1999) are believed to contribute to reduction and dissipation of excessive light energy. ΔpH- and xanthophyll cycle-dependent thermal dissipation within the Chl pigment bed occurs in the LN leaves (Verhoeven et al., 1997; Chen et al., 2003). The water-water cycle is also activated in the LN leaves. Both an increase in superoxide dismutase (SOD) activity and decreases in ascorbate peroxidose (APX) and glutathione reductase activities per unit leaf area were found in response to an enhanced formation of superoxide radicals in coffee plants under N-deficiency (Ramalho et al., 1998).

Quantitative analysis of the fate of absorbed light energy by PSII provides a clue to elucidate the responses of photosynthesis and photoinhibition in plants to environmental stresses. The combined use of Chl fluorescence and gas exchange techniques has been successful for evaluation of the energy partitioning of absorbed light by PSII to the various processes of photochemistry and thermal dissipations in plants exposed to abiotic stresses, such as drought (Flexas and Medrano, 2002) and salinity (Brugnoli and Björkman, 1992). Photoinhibition causes a decrease of approximately 10% in a daily carbon assimilation of a plant canopy (Long et al., 1994). Therefore, the susceptibility to photoinhibition in rice cultivars may significantly affect the grain yields under LN condition. Several studies on rice suggested that there were differences among rice cultivars in both the susceptibility to photoinhibition and the activities of antioxidant enzymes such as SOD (Jiao and Ji, 2001; Jiao et al., 2003). However, it remains unknown whether there is a difference among rice cultivars in terms of the energy partitioning in PSII and the susceptibility to photoinhibition of PSII under LN condition.

In our previous study (Kumagai et al., 2007), we investigated the effects of N-deficiency on dry matter production and flag leaf photosynthesis at heading stage by using two contrasting rice cultivars, Shirobeniya (a traditional *japonica* with a low yield) and Akenohoshi (an improved *japonica-indica* intermediate type with a high yield). We found that under the LN conditions, dry matter production and flag leaf photosynthesis in Akenohoshi were superior to those in Shirobeniya. Information obtained from analysis of the physiological basis of such differences in the response to N-deficiency would be useful to select or to create new cultivars that have increased the productivity under the LN input condition. In this study, we examined the response of photosynthetic mechanism to N-deficiency in flag leaves of the two contrasting rice cultivars at heading stage. The energy partitioning of absorbed light by PSII and the activities of antioxidant enzymes, such as SOD, APX and catalase (CAT), were also analysed in relation to photoprotection. In addition, the susceptibility to photoinhibition, hydrogen peroxide (H_2O_2) accumulation and lipid peroxidation in the flag leaves after exposure to high irradiance were investigated.

Materials and Methods

1. Plant materials and growth conditions

The imbibed seeds of two rice (*Oryza sativa* L.) cultivars, Akenohoshi and Shirobeniya, were sown in nursery boxes in a glass house at the beginning of August 2007 and at the end of July 2008. In 2007 and 2008, at three weeks after sowing, the seedlings were transplanted into 8 L pots filled with sandy loam. They were divided into standard-N (SN, Control) and LN groups which were fertilized with 1.6 g N and 0.4 g N in form of ammonium sulfate, respectively. In both groups, 1.6 g P and 1.6 g K were also applied in form of calcium superphosphate and potassium chloride, respectively. Plants were grown outdoors. Water was supplied sufficiently throughout. The N levels in this study were set based on our previous studies (Kumagai et al., 2007, 2009a, 2009c). The growth of plant was periodically surveyed, and it was evident that the LN plans were suffered from N-deficiency at the heading stage because a symptom of chlorosis was observed in the flag leaves of the LN plants. Although measurements were made for plants grown in 2007 and 2008, we confirmed that there were almost no differences in the plant growth, dry matter production and photosynthetic rate of flag leaves between them. In 2007, at one week after heading, three flag leaves from three plants were selected and used for the measurements of the energy partitioning of absorbed light by PSII, the contents of Chl and Rubisco, and the activities of antioxidant enzymes. After the measurements, the plants were sampled and dried at 80ºC for 3 d in an oven to weigh their dry mass. In 2008, at one week after heading, four flag leaves from four plants were selected and used for measurements of photoinhibition, H_2O_2 accumulation and lipid peroxidation after exposure to high irradiance.

2. Photosynthesis and Chl fluorescence measurements

Photosynthesis and Chl fluorescence were measured simultaneously by using a system that combined an open gas exchange system and a portable fluorometer (PAM-2000, Waltz, Germany). The temperature-controlled chamber of the open system was modified as follows: the

fiberoptic of the PAM-2000 was attached onto the side of the chamber at a 60º angle without significantly interfering with photosynthetic photon flux density (PPFD) distribution at the leaf surface, yet it allows for delivery of saturation pulse and measuring beam and the detection of measured signals.

All these measurements were performed at a PPFD of 200 and 1500 μmol m^{-2} s^{-1}, at leaf temperature of 29.7±0.5ºC, in the presence of CO_2 concentration of 385±12 μL L^{-1} and constant ambient oxygen concentrations of 21 and 2%, respectively. Net photosynthetic rate (P_N), day respiration rate (R_d), and transpiration rate were measured by using the assimilation chamber. R_d was the rate of day respiration other than photorespiration, which was approximated as dark respiration rate in our experiments. Gross photosynthetic rate (P_G) was obtained as a sum of P_N and R_d. The CO_2 concentration and water vapor pressure in the reference and sample air were monitored with an infrared gas analyzer (Li-6262, LI-COR, USA). Simultaneously, the steady-state Chl fluorescence (F_s) was constantly monitored to ensure that they reached a plateau before a reading was taken. A 0.8-s saturation pulse was applied to determine the maximum Chl fluorescence in the light-adapted state (F_m'). Using a leaf that was dark-adapted for 30 min, the minimum fluorescence (F_0) in non-photosynthetic conditions was determined with low intensity of a measuring beam; thereafter, the maximum fluorescence (F_m) was measured by applying a 0.8-s saturation pulse onto the leaf in order to reduce all the PSII centres. Maximum quantum yield of PSII was calculated as: $F_v/F_m = (F_m - F_0)/F_m$ (van Kooten and Snel, 1990).

3. Estimations of electron transport and energy dissipation rates

The quantum yield of PSII electron transport (Φ_{PSII}), light-dependent thermal dissipation (Φ_{NPQ}), and a combined flux of fluorescence and light-independent constitutive thermal dissipation ($\Phi_{f,D}$) were calculated from the Eqs. (1), (2) and (3) proposed by Hendrickson et al. (2004).

$$\Phi_{PSII} = 1 - F_s/F_m' \quad (1)$$

$$\Phi_{NPQ} = F_s/F_m' - F_s/F_m \quad (2)$$

$$\Phi_{f,D} = F_s/F_m \quad (3)$$

The fluxes of electron transport and energy dissipation via each process (J_{PSII}, J_{NPQ} and $J_{f,D}$) were calculated by multiplying the respective quantum yield with irradiance and coefficient α, respectively (Hendrickson et al., 2004). The coefficient, α is $I_A \times 0.5$ where 0.5 is the assumed proportion of absorbed quanta used by PSII reaction centres (Melis et al., 1987) and I_A is the absorbed irradiance assuming an average leaf absorbance of 0.84.

The rate of electron transport required to account for the photosynthetic CO_2 assimilation (J_c) and photorespiration (J_o) was calculated according to the Eq. (4).

$$J_c + J_o = K_c V_c + 0.5 K_r V_o \quad (4)$$

where V_c and V_o denote RuBP carboxylation rate and RuBP oxygenation rate, respectively, and K_c and K_r denote the number of electron equivalents required to reduce 1 molecule of CO_2 in the Calvin cycle and to release 1 molecule of CO_2 in photorespiration, respectively.

V_c and V_o were rewritten as the Eqs. (5) and (6), respectively.

$$V_c = P_{G21\%} + 0.5 V_o \quad (5)$$

$$V_o = 2 (P_{G2\%} - P_{G21\%}) \quad (6)$$

where $P_{G21\%}$ and $P_{G2\%}$ are the values measured in 21% and 2% O_2, respectively.

The theoretical minimum value of K_c is 4, but in our study its value was calculated from the Eq. (7) based on the assumption that $J_c + J_o$ is equal to J_{PSII} at 2% O_2 (under non-photorespiratory condition) and a PPFD of 200 μmol m^{-2} s^{-1} (Miyake and Yokota, 2000).

$$K_c = J_{PSII} / P_{G2\%} \quad (7)$$

K_r is estimated from the Eq. (8) that is based on the ratio of 18.5 ATP and 9 ATP, both of which are chemically equivalent energies consumed for release of 1 molecule CO_2 in the C_2 cycle and fixation of 1 molecule CO_2 in the C_3 cycle, respectively (Yoshimura et al., 2001).

$$K_r = 2.06 K_c \quad (8)$$

The rate of alternative electron flow (J_a) was calculated from the Eq. (9).

$$J_a = J_{PSII} - (J_c + J_o) \quad (9)$$

4. Determination of Chl and Rubisco contents

The contents of soluble protein, Chl and Rubisco in the flag leaves were measured according to Kumagai et al. (2007). After the gas exchange and Chl fluorescence were measured, leaf discs with a diameter of 5 mm were sampled, frozen in liquid N_2 and stored at −80ºC. Chl content was determined using a spectrophotometer (UV-1200, Shimadzu, Japan) according to the method described by Wintermans and de Mots (1965). To measure the soluble protein content, we carried out all experimental processes at 0–4ºC. Six leaf discs were powdered in liquid N_2 in a mortar. The powder was further ground with a chilled extraction buffer containing 100 mM potassium buffer (pH 7.0), 1 mM phenylmethanesulfonyl fluoride and 1% (v/v) 2-mercaptoethanol, and 1% (w/v) insoluble polyvinylpyrrolidone. Homogenates were transferred into Eppendorf tubes and centrifuged (12,000 × *g*, 4ºC, 5 min). The Bradford reagent (Bio-Rad, USA) was then added to the supernatant (Bradford, 1976), and the amount of soluble protein in the sample was spectrophotometrically determined. The amount of Rubisco in the soluble protein was quantified with SDS-polyacrylamide gel electrophoresis, according to the method described by Makino et al. (1985).

Table 1. Total dry weight, chlorophyll (Chl) content, ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) content, net photosynthetic rate (P_N), day respiration rate (R_d), maximum quantum yield of PSII (F_v/F_m), and number of electron equivalents required to reduce 1 molecule of CO_2 in the Calvin cycle (K_c) in flag leaves of two contrasting rice cultivars grown under the standard-N (SN) and low-N (LN) conditions.

Cultivar	Treatment	Total dry weight (g plant^{-1})	Chl content (g m^{-2})	Rubisco content (g m^{-2})	P_N (μmol m^{-2} s^{-1})	R_d (μmol m^{-2} s^{-1})	F_v/F_m	K_c
Shirobeniya	SN	60.7±2.9 a	0.54±0.03 a	2.95±0.04 b	18.1±0.22 ab	1.62±0.11 a	0.84±0.01 a	4.61±0.10 a
	LN	20.4±0.3 c	0.33±0.01 b	1.33±0.05 d	14.4±0.52 c	1.25±0.01 b	0.81±0.01 b	4.50±0.03 a
Akenohoshi	SN	64.4±3.0 a	0.61±0.03 a	3.79±0.13 a	20.1±0.66 a	1.63±0.02 a	0.85±0.01 a	4.75±0.04 a
	LN	28.9±0.7 b	0.37±0.01 b	1.84±0.05 c	16.9±0.17 b	1.31±0.03 b	0.84±0.01 a	4.50±0.03 a

P_N was measured at a PPFD of 1500 μmol m^{-2} s^{-1}. Values are given as the means±SE (n=3). Means followed by the same letters had no significant difference as determined by the Fisher's LSD test at 5% level. SN, standard-N; LN, low-N.

5. Antioxidant enzyme extraction and assays

All extractions were carried out at 0–4ºC. For determination of SOD activity, leaf samples were homogenized with 50 mM HEPES buffer (pH 7.6) containing 0.1 mM Na_2EDTA. Homogenates were centrifuged at 13,000×*g* for 25 min at 4ºC. SOD activity was spectrophotometrically assayed by monitoring the photochemical inhibition of nitroblue tetrazolium (NBT) reduction in 3 mL reaction mixtures at 25ºC according to the procedure of Yamane et al. (2004). The reaction mixture contained 50 mM HEPES (pH 7.6), 0.1 mM Na_2EDTA, 50 mM Na_2CO_3, 13 mM methionine, 0.025% (w/v) Triton-X 100, 75 mM NBT, 2 mM riboflavin, and 0–100 μL of enzyme extract. The riboflavin was added last, and the reaction mixture was illuminated for 10 min at a PPFD of 40 μmol m^{-2} s^{-1} provided from a fluorescent lump. One unit of SOD activity was defined as the amount of enzyme that caused 50% inhibition of NBT reduction as determined by the absorbance at 560 nm measured using a spectrophotometer (UV-1200, Shimadzu, Japan). The non-illuminated reaction mixture served as background, and its absorbance was deducted from the absorbance of the illuminated samples at 560 nm.

For the determination of APX and CAT activity, leaf samples were frozen in liquid N_2 and homogenized with 25 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA, 7 mM 2-mercaptoethanol, 0.1% (w/v) Triton X-100, 5 mM sodium ascorbate and 1% (w/v) polyvinylpyrrolidone. Homogenates were centrifuged at 12,000×*g* for 10 min at 4ºC. The activity of APX was determined following the procedure of Nakano and Asada (1981) with slight modification. The reaction mixture contained 25 mM potassium phosphate buffer (pH 7.8), 0.25 mM sodium ascorbate, 0.1 mM EDTA and 0.1 mM H_2O_2. The consumption of ascorbate was monitored at 290 nm (extinction coefficient=2.8 mM cm^{-1}) with the spectrophotometer. One unit of APX was defined as the amount of enzyme required to consume 1 μmol of ascorbate per min. The activities of CAT were determined at 25ºC following the procedure of Aebi (1974). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8) and 20 mM H_2O_2. The oxidation of H_2O_2 was monitored at 240 nm (extinction coefficient=0.0394 mM cm^{-1}) with the spectrophotometer. One unit of CAT was defined as the amount of enzyme required to consume 1 μmol of H_2O_2 per min.

6. Photoinhibitory treatment

Attached flag leaves were placed in a temperature-controlled leaf chamber at 30ºC. The leaves were exposed to a PPFD of 2000 μmol m^{-2} s^{-1} for 2 hr. Before and after exposure to the high PPFD illumination, the leaves were dark-adapted for 30 min, and F_v/F_m of the leaves was measured according to the method described above. Photoinhibition index (PI) was calculated as ($1-F_v/F_m$ after high PPFD illumination) / (F_v/F_m before high PPFD illumination) ×100 (%).

7. Determination of contents of hydrogen peroxide and malondialdehyde

After high PPFD illumination, leaf segments were sampled, frozen in liquid N_2 and stored at –80ºC. The H_2O_2 content was spectrophotometrically measured by monitoring the absorbance at 410 nm of Ti-H_2O_2 complex following the procedure of Patterson et al. (1984). Leaf samples were homogenized in cold 80% (v/v) acetone. Homogenates were centrifuged at 12,000×*g* for 10 min at 4ºC. To a known volume of supernatant, titanium reagent (2% $TiCl_4$ in conc. HCl) was added, followed by adding of 17 M ammonia solution to precipitate the Ti-H_2O_2 complex. After centrifugation at 5,000×*g* for 5 min, the supernatant was discarded and the precipitate was dissolved in 1 M H_2SO_4. The absorbance of the solution was measured at 410 nm against blanks. The content of H_2O_2 was determined using a standard curve plotted with known contents of H_2O_2.

Lipid peroxidation was estimated from the level of malondialdehyde (MDA) production following the

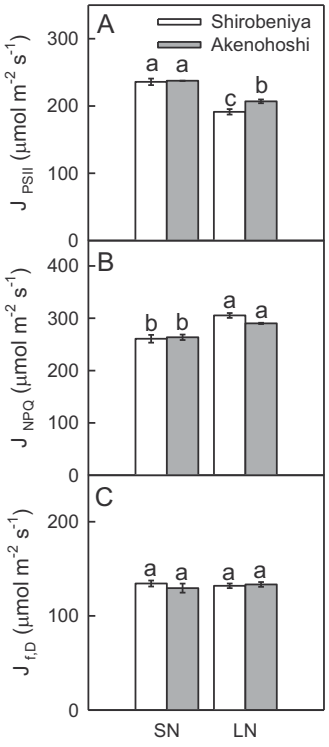

Fig. 1. Effects of N-deficiency on the energy flux via linear electron transport (J_{PSII}, A), ΔpH- and xanthophyll-regulated thermal dissipation (J_{NPQ}, B), and fluorescence and light-independent constitutive thermal dissipation ($J_{f,D}$, C) in flag leaves of two contrasting rice cultivars at heading stage. Measurements were made at a PPFD of 1500 μmol m^{-2} s^{-1}. Values are given as the means±SE (n=3). Bars followed by the same letters had no significant difference as determined by the Fisher's LSD test at 5% level. SN, standard-N; LN, low-N.

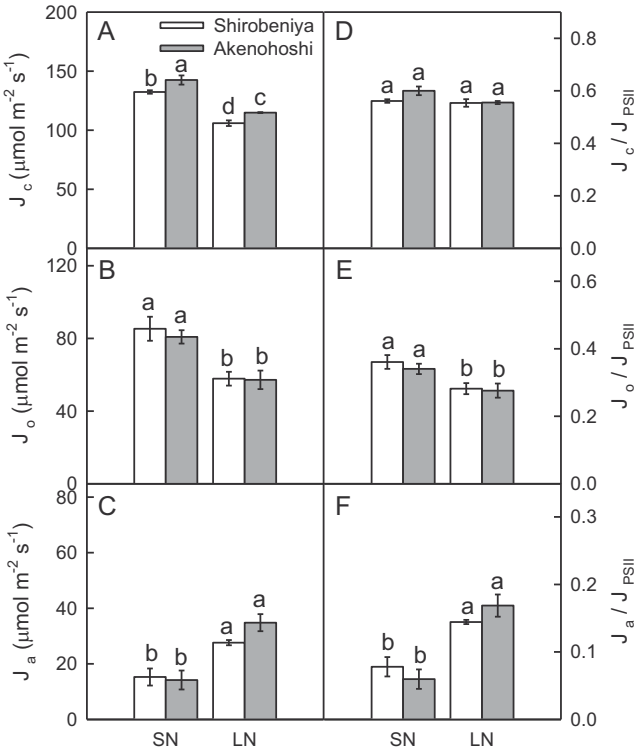

Fig. 2. Effects of N-deficiency on the energy flux via CO_2 fixation cycle (J_c, A), photorespiration cycle (J_o, B), alternative electron flow (J_a, C), J_c/J_{PSII} (D), J_o/J_{PSII} (E) and J_a/J_{PSII} (F) in flag leaves of two contrasting rice cultivars at heading stage. Measurements were made at a PPFD of 1500 μmol m^{-2} s^{-1}. Values are given as the means±SE (n=3). Bars followed by the same letters had no significant difference as determined by the Fisher's LSD test at 5% level. SN, standard-N; LN, low-N.

thiobarbituric acid (TBA) method described by Hodges et al. (1999). Leaf samples were homogenized in 2 mL of 80% (v/v) ethanol. Homogenates were centrifuged at 3,000×*g* for 10 min at 4ºC. A 0.5 mL aliquot of the supernatant was then mixed with same volume of either −TBA solution containing 20% (w/v) trichroloacetic acid and 0.01% (w/v) butylated hydroxytoluene or +TBA solution containing the above plus 0.65% (w/v) TBA. The mixture was heated at 95ºC for 25 min, cooled, and centrifuged at 3,000×*g* for 10 min. The absorbance of the supernatant was determined at 440, 532 and 600 nm. The MDA concentrations were calculated from the following formula: MDA concentrations (nmol mL^{-1}) = $(A-B/157000)\times10^6$, where $A=[(A_{532+TBA})-(A_{600+TBA})-(A_{532-TBA}-A_{600-TBA})]$, and $B=[(A_{440+TBA}-A_{600+TBA})\times0.0571]$.

8. Statistic analysis

Data were statistically analyzed using one-way ANOVA with the Fisher's LSD test (Sigmastat 3.1 for Windows, Systat Software, Inc. USA). Significant difference was analyzed based on P values<0.05.

Results

1. Characteristics of dry matter production and photosynthesis of flag leaves in two rice cultivars under the SN and the LN conditions

Total dry weight of two cultivars was significantly decreased by N-deficiency (Table 1). The degree of the decrease was higher in Shirobeniya than that in Akenohoshi. Chl and Rubisco contents in the two cultivars were significantly decreased by N-deficiency. There was no significant difference in the Chl content between the two cultivars grown under the same N condition. However, under both N conditions, the Rubisco content in Shirobeniya was significantly lower than that in Akenohoshi. P_N in the two cultivars was significantly decreased with N-deficiency. P_N in the LN plants of Shirobeniya was significantly lower than that of Akenohoshi. R_d in the two cultivars was significantly decreased under the LN condition. Under the two N conditions, no significant cultivar difference in R_d was detected. The SN plants of the two cultivars showed no significant difference in F_v/F_m. However, F_v/F_m in

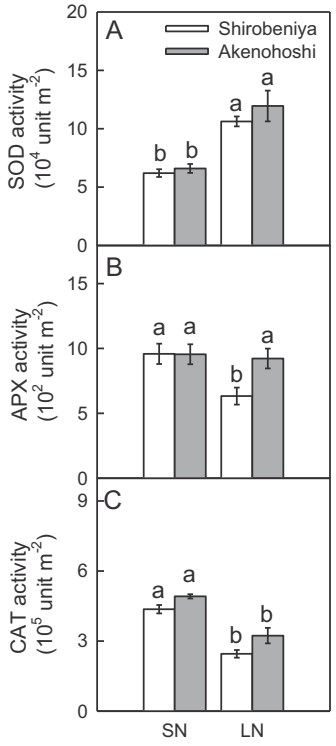

Fig. 3. Effects of N-deficiency on the activities of SOD (A), APX (B) and CAT (C) in flag leaves of two contrasting rice cultivars at heading stage. Values are given as the means±SE (n=3). Bars followed by the same letters had no significant difference as determined by the Fisher's LSD test at 5% level. SN, standard-N; LN, low-N.

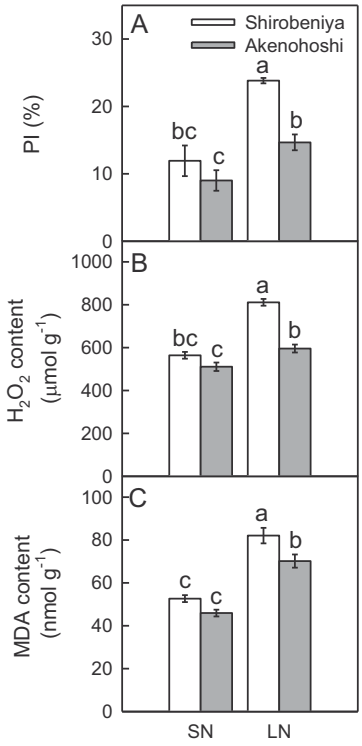

Fig. 4. Effects of N-deficiency on photoinhibition index (PI, A), and hydrogen peroxide (H_2O_2) content (B) and malondialdehyde (MDA) content (C) after high irradiance in flag leaves of two contrasting rice cultivars at heading stage. Values are given as the means±SE (n=4). Bars followed by the same letters had no significant difference as determined by the Fisher's LSD test at 5% level. SN, standard-N; LN, low-N.

Shirobeniya was significantly reduced under the LN condition, although that in Akenohoshi was not. There was no difference of K_c among the SN and the LN plants in the two cultivars, and the values obtained in our study were similar to the theoretical values of 4.5–5.0 (von Caemmerer and Farquhar, 1981).

2. Effect of N-deficiency on the energy partitioning of absorbed light by PSII in flag leaves in the two rice cultivars

N-deficiency resulted in a decrease in J_{PSII} and an increase in J_{NPQ} in the two cultivars (Fig. 1A, B). Under the LN condition, J_{PSII} in Shirobeniya was significantly lower than that in Akenohoshi. However, no difference in $J_{f,D}$ was found in either the two N conditions or the two cultivars (Fig. 1C). J_c in the two cultivars was significantly reduced under the LN condition (Fig. 2A). Under both N conditions, J_c in Shirobeniya was significantly lower than that in Akenohoshi. In the two cultivars, N-deficiency decreased J_o (Fig. 2B) and increased J_a (Fig. 2C). Under both N conditions, no cultivar differences in J_o and J_a were observed. J_c/J_{PSII} did not show any difference between the SN and the LN plants (Fig. 2D). However, J_o/J_{PSII} and J_a/J_{PSII} in the two cultivars was significantly decreased and increased by N-deficiency, respectively (Fig. 2E, F). The SN and the LN plants of the two cultivars showed no significant differences in J_c/J_{PSII}, J_o/J_{PSII} and J_a/J_{PSII}.

3. Effect of N-deficiency on antioxidant enzymes in flag leaves in the two rice cultivars

In the two cultivars, the activity of SOD in the LN plants was significantly higher than that in the SN plants (Fig. 3A). However, under each N condition, there was no significant cultivar difference in SOD activity. APX activity in Akenohoshi was almost the same in the two conditions, while the activity in Shirobeniya was significantly decreased under the LN condition (Fig. 3B). In the two cultivars, the activity of CAT in the LN plants was significantly lower than that in the SN plants (Fig. 3C). Under the same N condition, no significant difference in CAT activity was detected between the two cultivars.

4. Effect of N-deficiency on the susceptibility of photoinhibition, H_2O_2 accumulation and lipid peroxidation of flag leaves in the two rice cultivars

Under the LN condition, significant increase in PI was found in both cultivars (Fig. 4A). There was no cultivar difference in PI in the SN plants. However, PI in the LN

plant of Shirobeniya was significantly higher than that of Akenohoshi. Furthermore, the LN plants in the two cultivars showed higher H_2O_2 and MDA contents than the SN plants (Fig. 4B, C). Under the SN condition, no significant cultivar differences in H_2O_2 and MDA contents were observed, whereas under the LN condition, these parameters in Shirobeniya were significantly higher than those in Akenohoshi.

Discussion

Our study showed that N-deficiency resulted in more significant reduction of total dry weight in Shirobeniya than in Akenohoshi (Table 1). This result is in agreement with our previous study (Kumagai et al., 2007, 2009c). To improve the productivity of rice cultivars under LN input condition, the physiological factors responsible for this cultivar difference must be identified. We observed that P_N, J_{PSII} and J_c in Shirobeniya were lower than those in Akenohoshi under the LN condition (Table 1; Figs. 1A, 2A), indicating that the light utilization capacity for CO_2 assimilation in Shirobeniya was lower than that in Akenohoshi under N-deficiency. Rubisco is a key enzyme in the Calvin cycle. In rice plants, photosynthetic rate at saturating irradiance and ambient CO_2 levels is correlated with the Rubisco content (Makino et al. 1985). In this study, we also observed a linear relationship between P_N measured at 1500 μmol m^{-2} s^{-1} PPFD and the Rubisco content ($R=0.882$, $P<0.001$; data not shown). N-deficiency also resulted in a significant decrease in the Rubisco content in the two cultivars, but the Rubisco content in Shirobeniya was lower than that in Akenohoshi under the LN condition (Table 1). Therefore, it is suggested that the cultivar that maintained a higher Rubisco content in flag leaves could show a higher productivity under the LN input condition.

Excess light energy, which was not used in photosynthesis, can be dissipated as heat in the antenna pigment complexes of PSII, which involves in a ΔpH and xanthophyll cycle. ΔpH- and xanthophyll cycle-dependent thermal dissipation can safely remove excess light energy before it reaches the PSII reaction centres, thereby protecting the PSII reaction centres from the adverse effects of high light stress (Demmig-Adams and Adams, 1996). As expected, in contrast with J_{PSII}, ΔpH- and xanthophyll cycle-dependent thermal dissipation, which was measured as J_{NPQ}, in the two cultivars significantly increased by N-deficiency (Fig. 1B). Our result suggested that xanthophyll cycle-dependent thermal dissipation increased in the LN plants, as excess light energies were accumulated in the electron transport chain. Despite the compensatory changes in J_{PSII} and J_{NPQ}, $J_{f,D}$ constantly maintained about 120 μmol m^{-2} s^{-1} regardless of the N conditions and the cultivars (Fig. 1C). The majority of $J_{f,D}$ is the flux of energy dissipation via constitutive thermal dissipation process, since the flux of energy dissipation via fluorescence is minor, especially in the presence of light (Hendrickson et al., 2004). Although the light-independent constitutive thermal dissipation processes still remain unclear, our study revealed that this thermal dissipation occurs regardless of the N conditions and the cultivars.

Photosynthetic electron transport drives both Rubisco-associated CO_2 assimilation and photorespiration and also supplies electrons to other alternative electron sinks. Many studies have demonstrated that photorespiration plays a key role in the protection against photoinhibition in leaves when CO_2 assimilation is restricted under environmental stresses such as drought and high irradiance (e.g., Kozaki and Takebe, 1996; Flexas and Medrano, 2002). However, there is little information concerning the effects of N-deficiency on photorespiration. We observed that J_o in the two cultivars was decreased in response to N-deficiency (Fig. 2B). Furthermore, significant reduction in the J_o / J_{PSII} with N-deficiency was observed in the two cultivars (Fig. 2E). In C_3 plants, CAT is primarily localized in the peroxisomes (Willekens et al., 1995), where it is involved in removing the bulk of H_2O_2 generated by photorespiration. We observed that CAT activity per unit leaf area in the two cultivars significantly decreased by N-deficiency (Fig. 3C), which was associated with the decrease in J_o (Fig. 2B), indicating that photorespiration activity was down-regulated in response to N-deficiency. Furthermore, no cultivar differences in J_o and CAT activity were found under the same N condition. Therefore, we consider that under the LN condition the cultivar difference in dry matter production is not associated with difference in photorespiration activity.

Among the alternative electron sinks, nitrate reduction can consume up to 8% of J_{PSII} (Evans, 1987). In our study, however, nitrate reduction in the flag leaves is considered to be minor, because only ammonium was supplied to rice plants as a N form. Therefore, most of J_a could account for the electron flux to the water-water cycle. The water-water cycle is inevitably coupled with the generation of ROS such as O_2^- and H_2O_2 (Asada, 1999), which would potentially cause photooxidative damage on the thylakoid membrane and stroma proteins. The limiting step of the water-water cycle is the photoreduction of O_2 to O_2^- (Endo and Asada, 2006), and both SOD and APX are key enzymes involved in ROS scavenging (Weng et al., 2007). In our study, it was found that in the two cultivars the increased J_a was accompanied by enhanced activity of SOD under N-deficiency (Figs. 2C, 3A). Similarly, in rice plants the increase in J_a accompanying with enhancement of O_2^- production rate and SOD activity was observed in response to potassium deficiency (Weng et al., 2007) and phosphorus deficiency (Weng et al., 2008). Since SOD is present in mitochondria as well as chloroplasts, it can be speculated that high SOD activity under the LN condition

may be related not only to increased rate of photoreduction of oxygen in chloroplasts, but also to a rise in respiration and the subsequent scavenging of reduced ROS in the mitochondria. However, we observed that in the two cultivars R_d was significantly decreased under the LN condition (Table 1). APX activity in Akenohoshi was constant regardless of the N levels, while the activity in Shirobeniya was decreased significantly with N-deficiency (Fig. 3B). APX is mainly localized in chloroplasts (Gillham and Dodge, 1986). Hence, these results indicate that under the LN condition, despite no cultivar differences in electron flow to O_2 and subsequent disproportionation of O_2^-, there was a significant difference in H_2O_2-scavenging capacity: Shirobeniya had a lower H_2O_2-scavenging capacity than Akenohoshi. The functioning of APX is supported by a large ascorbate pool, which constitutes the largest pool of antioxidants found in plants (Chen and Gallie, 2004). However, this pool would be exhausted within a few minutes without the regeneration system consisting of the monodehydroascorbate reductase and dehydroascorbate reductase enzymes (Pignocchi et al., 2003). APX is very sensitive to H_2O_2 at very low concentrations in the absence of ascorbate (Miyake and Asada, 1996). Therefore, the decrease of APX activity in the LN plant of Shirobeniya probably results from the low concentration of reduced ascorbate. Further works would be required to elucidate the response of ascorbate pool in the rice cultivars to N-deficiency.

The extent of photoinhibition depends not only on the rate of D1 protein degradation but also on the rate of D1 protein synthesis within plastids (Kyle et al., 1984). H_2O_2 inhibits the synthesis of PSII proteins, in particular, that of the D1 protein (Takahashi and Murata, 2008). We observed that N-deficiency resulted in more significant increase of PI in Shirobeniya than that in Akenohoshi (Fig. 4A), indicating that the LN plant of Shirobeniya was more susceptible to photoinhibition when exposed to high irradiance. Furthermore, N-deficiency with high irradiance led to a more significant increase in H_2O_2 accumulation and lipid peroxidation in Shirobeniya than in Akenohoshi (Fig. 4B, C). Higher H_2O_2 levels in the LN plants of Shirobeniya could be explained by lower APX activity (Fig. 3B). Previous studies revealed that the increased accumulation of H_2O_2 in stress-sensitive plants as compared with stress-tolerant plants was associated with a lower APX activity under various stress conditions, such as salt stress (Mittova et al., 2003) and chilling stress (Zhou et al., 2006). Since the major sink for absorbed light energy is photosynthetic carbon assimilation, a higher carboxylation capacity is believed to contribute to avoiding the generation of ROS and lowering susceptibility to photoinhibition (Powles, 1984). H_2O_2 has effects on the fragmentation of large subunit of Rubisco in chloroplasts isolated from wheat leaf (Ishida et al., 1998). Zhou et al. (2007) showed that a high negative correlation was observed between H_2O_2 content and Rubisco activity in rice plants grown under severe drought stress. Hence, in our study the photosynthetic capacity in the rice cultivars would be depressed under N-deficiency with high irradiance. Previously, we found that the flag leaf in LN plants of Shirobeniya was more susceptible to midday photoinhibition than that of Akenohoshi (Kumagai et al., 2009c). The present results indicated that the increased susceptibility to photoinhibition in the LN plants of Shirobeniya is mainly due to oxidative damages to chloroplasts, resulting from lower carboxylation and H_2O_2-scavenging capacities. We therefore conclude that both carboxylation and H_2O_2-scavenging capacities could be important factors in determining the cultivar difference in the productivity of rice under LN input conditions.

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