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CHARACTERIZATION OF A WIDE LEAF MUTANT OF RICE *ORYZA SATIVA* L. WITH HIGH YIELD POTENTIAL IN FIELD

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Abstract

A new wide leaf rice mutant with high yield in the field was identified which has the leaf width about 1.5-2 folds more than that of wild-type. It possesses fewer, larger, upright leaves per plant. The chlorophyll contents of leaves were significantly higher than those of wild-type. The maximal PSII (Photosystem II) photochemistry (Fv/Fm), the electron transfer rate (ETR) and photochemical quenching (qP) were lower than those of wild-type, respectively. Because of great decrease of non-photochemical quenching (NPQ), the efficiency of excitation energy trapping by open PSII reaction centers in the light–adapted state (Fv'/Fm') was higher than that of wild-type. Low temperature fluorescence analysis showed that wide leaf mutant assigned more excited energy to PSI than to PSII. These results indicated that wide leaf mutant was better in the distribution of energy among 2 photosystems, and increase the efficiency of light utilization because of increase of chlorophyll contents. Furthermore, the SOD enzyme activities and MDA content of wide leaf mutant were nearly 44.4%, and 50.8% of those of wild-type, respectively. The low SOD and MDA contents indicated that this wide leaf mutant had high photosynthetic efficiency and its membrane structure did not probably affected(delete) by oxidation stress.

Introduction

Rice is one of the most important crops in the world as well as a model plant in the molecular biology studies of Gramineae. The increasing crop yield requires an improvement in carbohydrate production is now recognized and has become a key target in breeding program both in China and other country (Mann, 1999a,b and Hu *et al*., 2005). Canopy models have long been used to quantitatively relate with the leaf area distribution and leaf photosynthetic characteristics of plants to their net photosynthetic carbon gain. For improvement of photosynthetic ability of the canopy, traits related to plant type have been used as selection markers in most breeding programs (Rasmusson, 1991; Bibi *et al*., 2009; Rashid *et al*., 2008). To increase yield potential, researchers have tried various physical, genetic or gene-engineering methods to improve photosynthetic ability per leaf area (Tabasum *et al*., 2011; Tehrim *et al*., 2012; Zia-ul-Qamar *et al*., 2012). Among them, T-DNA insertional mutant was used in rice for identification new genes controlling rice growth and development (Zou *et al*., 2011; Kim *et al*., 2011; Kumar *et al*., 2010). An ideal super high yield rice with more erect leaf blades and longer flag leaves could enhance the capacity of source supply on the basis of significant increase of sink demand and had a higher rate of canopy photosynthesis during grain filling resulted in more dry matter accumulated in stems and sheaths before heading and transfer to grains after flowering.

The International Rice Research Institute developed a "new plant type" (NPT) under this strategy that has suitable plant height (80-100cm) and erect leaves that optimize canopy photosynthesis (Duncan, 1971). New plant type (NPT) rice plants possess fewer, larger, upright leaves (per plant) which permit a high degree of penetration of irradiance to lower leaves in the canopy (Peng *et al*., 1994; Peng *et al*., 2008). Genes related to heading date and yield potential in rice had been characterized (Xue *et al*., 2008). Although this results in a lower leaf area index, grain yields are equivalent or higher than traditional cultivars (Anon., 2000).

Furthermore, yield potential of rice also determined by light absorption and utilization rate and chlorophyll contents (Lu & Sun, 2006). The optimization of light utilization and protects against the potential stress from excess light (Anderson & Osmond, 1987) were also the main problems need to solved in rice breeding. On a chloroplast level, most plant species acclimate to irradiance over the long term by altering relative amounts of photosynthetic enzymes and pigment-protein complexes.

In this study, a wide leaf mutant was found in the T-DNA insertional pool obtained from rice *Oryza sativa* L. cv, Nipponbare (Japonica). This mutant showed characteristics of wide, large and erect leaves like NPT. Our results showed that this kind of leaf type rice could utmost use of light energy to its net photosynthetic carbon gain. The photosynthetic activities and physiological function analyses also showed that this mutant was better than wild-type in yield potential. These were resulted from high chlorophyll contents and less risk in photoinhibition.

Materials and Methods

Wild-type rice *Oryza sativa* L. cv. Nipponbare (Japonica) and wide leaf mutant rice were germinated at April, 2009. Seedlings with 5 leaves were planted in the experimental field of Chinese Academy of Tropical Agricultural Sciences (CATAS), at May 2009 in Danzhou city (19°51′51N; 109°55′63E), Hainan Province, China. In August, 2009, plants were used for followed assays.

Measurement of chlorophyll content: Chlorophyll was extracted with 80% ice cold acetone from 0.1g samples of different leaves. After centrifuged at 4000g for 10min, the supernatant was measured spectrophotometrically at 475, 645, and 663 nm with GE Ultrospec™ 2100 pro UV/Visible Spectrophotometer (USA). Specific chlorophyll contents were determined according to the method of Lichtenthaler (1987).

Thylakoid membrane preparations: Thylakoid membranes were done according to Edwards *et al*., (1979) with little modification. Thylakoids were prepared from rice leaves homogenized in 0.4 M sorbitol, 100 mM Tricine-KOH (pH 7.5), 10 mM NaCl, and 5 mM $MgClB_{2B}$. After the sample was filtered through 500, 195, 20μm nylon mesh and centrifuged for 5 min at 4,000g, the chloroplast pellet was lysed by resuspending in 5mM Hepes KOH, pH 7.5, 10mM NaCl, 5mM $MgClB_{2B}$, and the thylakoids were pelleted by centrifugation (5 min, 4,000g). Finally, thylakoids were washed in 5mM Hepes-KOH, pH 7.5, 10 mM EDTA, centrifuged, and resuspended in the same buffer with 10% glycerol added. Samples were stored at -80°C in small aliquots.

SDS-PAGE electrophoresis: Denaturing gels were composed of 15% acrylamide/0.4% bis-acrylamide with Tris-Clycine buffer pH 8.3 as a buffer, based on the system described by Wycoff *et al*., (1977) and Bury (1981). Gels were surrounded by electrophoresis buffer and typically run without cooling at a constant current of between 20 and 40 mA per gel. SDS-PAGE was performed using a Mini-protein II Electrophoresis Cell (Bio-Rad, USA), Polypeptides were stained with Coomassie Brilliant Blue R-250.

Electron microscopy: The electron microscopic analysis was done according to Wang *et al*., (2006). The samples (1 mm^3) were fixed in 3% (m/v) glutaraldehyde and in 0.1M phosphate buffer (pH 7.2) for 4h, and washed with the phosphate buffer for 1h. Then the samples were fixed with 1% OsO4 (pH 7.2) for 1 h, and rinsed with a buffer for three 10 min periods. After washing, the samples were dehydrated in a graded acetone and ethanol in series, and then embedded in Spurr's resin for 3 d. Dry sections (1-2 μm) were cut with a diamond knife using an ultramicrotome, and mounted on copper grids. Electron microscopic observation was made at 100 kV with a JEM1230 tranmission electron microscope (JEOL, Tokyo, Japan).

Chlorophyll fluorescence emission spectra and fluorescence kinetics: The fluorescence emission spectra of the thylakoid membranes were measured with a *Hitachi F-4500* fluorescence spectrophotometer (Tokyo, Japan) at 77 K in liquid nitrogen. The reaction mixture contained the isolated thylakoid membranes equivalent to 10 mg (Chl) mP^{-3P} and 50% (v/v) glycerol. Modulated Chl fluorescence measurements were made in attached leaves in the field at midday with a PAM-2000 portable fluorometer (Walz, Effeltrich, Germany) connected to a notebook computer with data acquisition software (DA-2000, Heinz, Walz). The experimental protocol of Demmig-Adams *et al*., (1996) was essentially followed. The minimal fluorescence level (F_0) in dark adapted state was measured by the measuring modulated light, which was sufficiently low (<0.1 μmol m^{-2} s^{-1}) not to induce any significant variable fluorescence. To determine the minimal fluorescence level during illumination (F_0'), a black cloth was rapidly placed around the leaf and the leaf clip holder in the presence of far-red light (7 μmol m^{-2} s^{-1}) in order to oxidize the PSII centers fully. Upon darkening of the leaf, fluorescence dropped to the F_0' level and immediately rose again within several seconds. The maximal fluorescence level in the dark-adapted state (F_m) and the maximal fluorescence level during natural illumination (F_m') were measured by a 0.8-s saturating pulse at 8,000 μmol m^{-2} s^{-1}. F_m was measured after 30 min of dark adaptation. F_m' and F_s was measured when photosynthetic photon flux densities (PPFDs) were approximately 200 and 1,400 μmol m^{-2} s^{-1}, respectively. Other parameters were calculated based on measured parameters above.

Soluble sugar analysis: By referring to Li *et al*., (2006), take 0.1g of fresh leaf samples and put it into centrifuging tubes with 5ml of 80% alcohol and heat it in water bath for 30min at 80°C. Then cool down the tube and centrifuged it at 1,000g for 10min. Transfer the upper liquid in the centrifuging tube to 25ml bottles, add 0.5ml anthrone reagent and 5ml sulfuric acid. All the reacted liquid was measured at 620nm wavelength for spectral absorption with GE Ultrospec™ 2100 pro UV/Visible Spectrophotometer (USA).The standard curve was charted according to different glucose concentrations and soluble sugar was calculated.

Free proline determination: Proline was determined following Bates *et al*., (1973). Briefly, 0.5-1g leaves was homogenized in 10ml of 3% sulfosalicylic acid and the homogenate filtered. The filtrate (2ml) was treated with 2ml acid ninhydrin and 2ml of glacial acetic acid, then with 4ml of toluene. Absorbance of the colored solutions was read at 520nm with GE Ultrospec™ 2100 pro UV/Visible Spectrophotometer (USA).

SOD and malonaldehyde determination: SOD (EC 1.15.1.1) activity was assayed by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium (NBT), according to the method of Beyer & Fridovich (1987) with some modifications. For the total SOD assay, a 5 ml reaction mixture contained 50 mM HEPES (pH 7.6), 0.1 mM EDTA, 50 mM Na_2CO_3, 13 mM methionine, 0.025% (w/v) Triton X-100, 75μm NBT, 2 μm riboflavin and an appropriate aliquot of enzyme extract. The reaction mixtures were illuminated for 15 min at a light intensity of 350μmol m^{-2} s^{-1}. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT as monitored at 560 nm. Malonaldehyde was assayed by the thiobarbituric acid method as described by Aust *et al*., (1985).

Statistical analysis: Statistical analysis was based on one-way analysis of variance (ANOVA). The result of wild-type and mutant were considered statistically significant when $p<0.01$. Data are presented as mean ± standard errors (n = 5).

Results and Discussion

Phenotype of wide leaf mutant: Wide leaf mutant which had erect leaf is characterized as wide, thick leaf with dark green color. The diameter of wide leaf mutant leaf was round 2.9-3.5cm in diameter, while the leaf of wild-type only 1.5cm in diameter. The mesophyll cells in wide leaf mutant were much more than those of wild-type because of its width. The architecture of chloroplast and thylakoid membrane of mutant was same as that of wild-type (Figs. 1 & 2).

Chl a, Chl b, and β-Car contents of wide leaf mutant were 7.34, 2.65, 1.42 mg/g fresh weight, Chl a, Chl b, and β-Car contents of wild-type were 4.32, 1.40, 0.90 mg/g fresh weight, respectively (Table 1). The Chl a, Chl b, and β-Car contents of wide leaf mutant were 69.9, 88.5, and 57.8% ($p<0.01$) higher than those of wild-type. Because of great increase of Chl b contents, the values of Chl a/b was lower than Wild-type. This was consists with the great depth of mutant.

Fig. 1. Phenotype of wide leaf mutant rice and wildtype rice.

Table 1. Chlorophyll contents of wide leaf mutant and wild-type (mg/g fresh weight).

	Chl a	Chl b	β-Car	Chl a+b	Chl a/b
Wide leaf (Wl)	7.34 ± 0.12	2.65 ± 0.07	1.42 ± 0.04	9.98 ± 0.10	2.77 ± 0.02
Wild-type (Wt)	4.32 ± 0.09	1.40 ± 0.06	0.90 ± 0.02	5.71 ± 0.11	3.08 ± 0.01
Wl/Wt%	169.9P^{**P}	188.5P^{**P}	157.8P^{**P}	174.8P^{**P}	89.9

**Means $p<0.01$. Data are presented as mean ± standard errors ($n = 5$)

Absorption peaks of wide leaf mutant and wild-type chlorophylls were 432nm at Soret band, 663nm at Q band, respectively (Fig. 3). The absorption intensity of wild-type was little higher than that of mutant chlorophyll.

The maximal efficiency of PSII photochemistry (Fv/Fm) of wide leave mutant was decrease 6.0% from that of wild-type (Table 2). The electron transport rate (ETR), photochemical quenching (qP), and the actual photochemical efficiency of PS II (ΦPS II) of wide leaf mutant were decrease 22.7, 25.6, and 22.0% compared to those of wild-type, respectively. However, the non-photochemical quenching (qN) which reflects the process competing with PS II photochemistry for absorbed excitation energy (Campbell *et al*., 1998) decreased 86.4% ($p<0.01$), this caused little increase of the efficiency of excitation energy trapping by open PSII reaction centers in the light–adapted state (Fv'/Fm') was higher than wild-type.

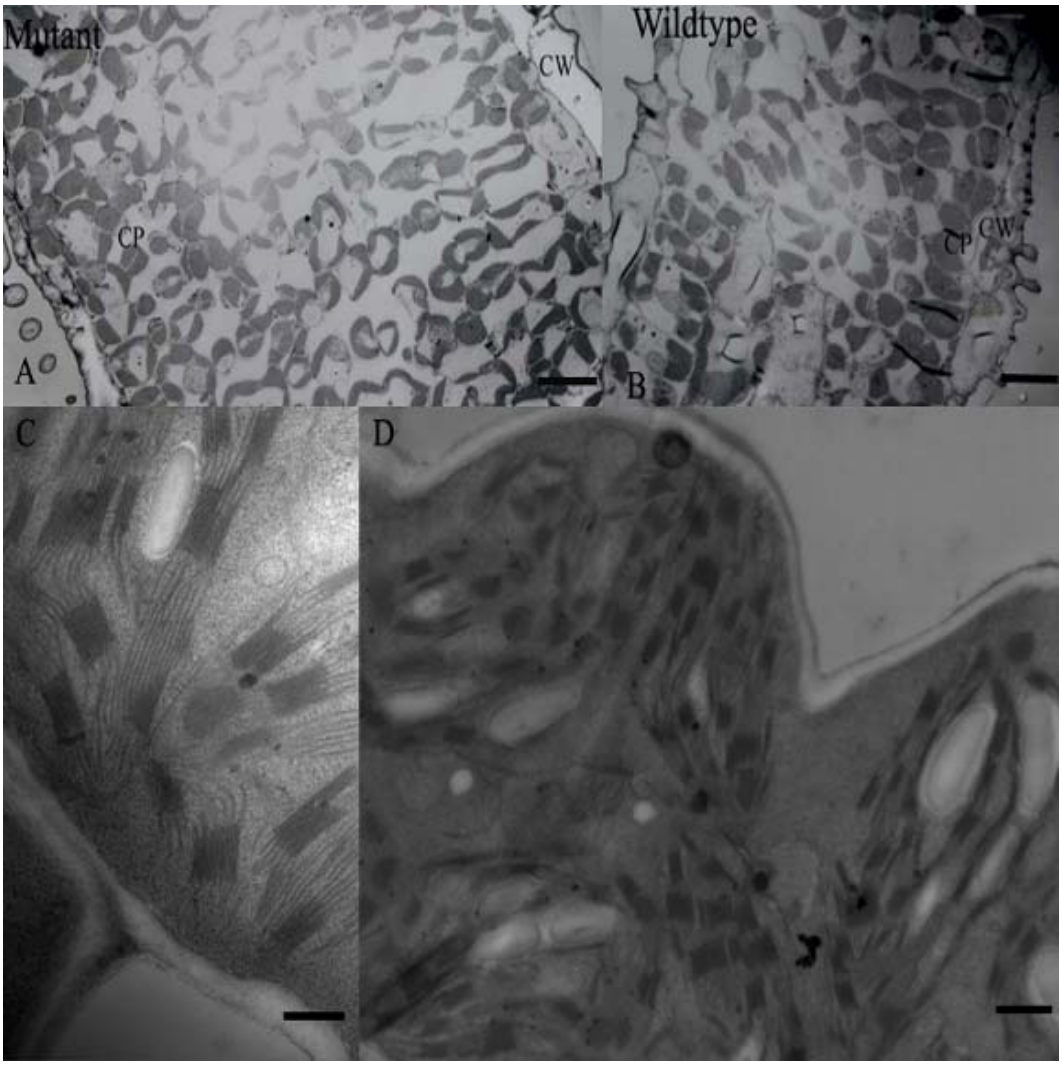

Fig. 2. Ultra structures of wide leaf mutant and wild-type. A. wide leaf mutant leaf, B. wild-type leaf, C. thylakoid membrane of mutant chloroplast, D. thylakoid membrane of wild-type chloroplast. CP, chloroplast; CW, cell wall. Bars = 10 μm in (A) and (B) and 1 μm in (C) and (D).

Table 2. PSII photochemistry parameters of wide leaf mutant and wild-type.

	Fv/Fm	ETR	qP	NPQ	Fv'/Fm'	ΦPSII
Wide leaf (Wl)	0.78 ± 0.02	19.33 ± 0.82	0.31 ± 0.05	0.40 ± 0.02	0.69 ± 0.01	0.21 ± 0.02
Wild-type (Wt)	0.83 ± 0.01	25 ± 0.64	0.41 ± 0.04	2.97 ± 0.42	0.66 ± 0.01	0.27 ± 0.01
Wl/Wt%	94.0	77.3**	74.4**	13.6**	104.5	78.0**

**Means $p<0.01$. Data are presented as mean ± standard errors ($n = 5$)

The fluorescence peaks were 683.2 and 730.4nm, respectively. Usually, 683.2nm peak representatives the fluorescence intensity of Photosystem II (PSII). 730.4nm peak representatives the fluorescence intensity of Photosystem I (PSI). The low temperature fluorescence intensity of wide leaf mutant was lower both in PSII and PSI comparing to those of wild-type, which result in the reduction of F683/F730 ratio. The lower F683/F730 ratio suggests that in wide leaf mutant, more excited energy was assigned to PSI than PSII (Fig. 4).

The CP43, CP47, D1, and D2 protein contents of PSII reaction center were same as those of widtype. The LHCII light harvesting pigments-protein complex contents was same as that of wild-type, too (Fig.5).

The analysis of enzyme activities showed that the proline content increased 14.9%; soluble content reduced 6.2%, respectively. The SOD activities and MDA content decrease 55.7% and 49.2% ($p<0.01$), respectively. These results indicated that the wide leaf mutant had a better situation in membrane structure without any sign of reactive oxygen stress, which leads to enhancement of efficiency in use light energy (Table 3).

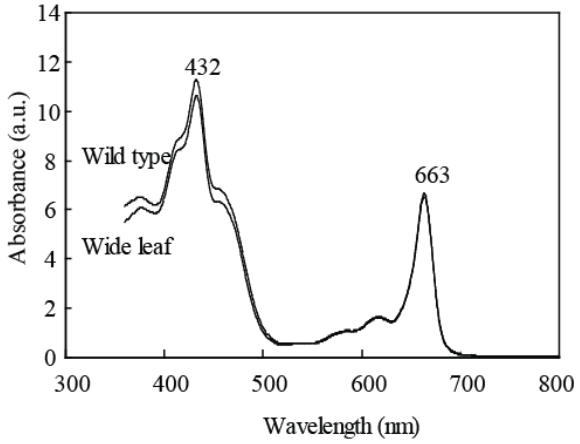

Fig. 3. The room temperature (298 K) absorption spectrum of wide leaf mutant and wild-type.

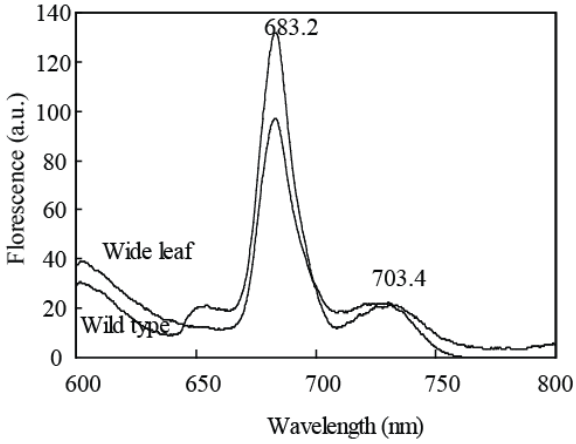

Fig. 4. Low temperature (77 K) fluorescence emission spectrum of thylakoid membrane of wide leaf mutant and wild-type. Fluorescence emission at 436nm.

Table 3. Enzyme activities of wide leaf mutant and wild-type.

	Proline (μmol g^{-1} FW)	Soluble sugar (μg/g)	SOD (U mg^{-1} Protein)	MDA (μmol g^{-1})
Wide leaf (Wl)	0.056 ± 0.02	6.18 ± 0.82	48.5 ± 2.7	1.17 ± 0.13
Wild-type (Wt)	0.047 ± 0.01	6.59 ± 0.64	106.8 ± 5.9	2.31 ± 0.42
Wl/Wt%	114.9	93.8	44.3**	50.8**

**Means $p<0.01$. Data are presented as mean ± standard errors ($n = 5$)

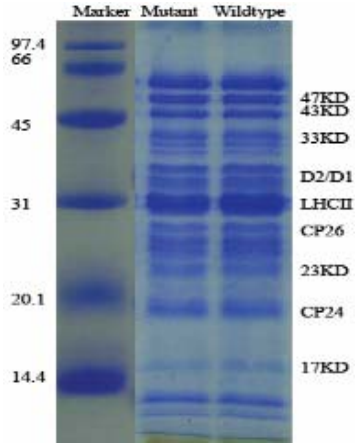

Fig. 5. Profiles of electrophoresis of thylakoid membranes of wide leaf mutant and wild-type.

The rice ideotype plant were characterizes as short, large and erect so as to absorb more light energy to increase yield potential (Cao *et al*., 2001). Especially, the upper 3 leaf (flag leaf, last 2 leafs and last 3 leafs) of rice canopy leaf are most important in final yield composition. The photosynthetic efficiency of erect leaf was higher than those of flat or bends leaf because it can absorb sunlight at both side as well as facilitated light absorption of lower leaves. Besides, the photosynthetic capacities of leaf were concerned with chlorophyll contents and enzyme activities.

In this study, although the intensities of chlorophyll absorption spectrum, the amounts of pigment-protein complex of wide leaf mutant and wild-type were same, the yield of the mutant was higher than wild-type. Murchie *et al*., (2002) found that NPT rice had more chlorophyll contents and enhance Rubsico enzyme activities. Hu *et al*., (2005) reported that although the maximal efficiency of PSII photochemistry (also called original photochemical efficiency) (Fv/Fm) was higher; the net photosynthetic rate of the NPT rice is lower than conventional *indica* rice because of practical light use efficiency was lower. The decrease of photochemical quenching (qP) and actual photochemical efficiency (ΦPSII) were found in wide leaf mutant in this study. qP indicates the oxidation-reduction state of the primary acceptor for PSII, which is determined by the rate of photoreduction of the acceptor by PSII and by its rate of reoxidation linked to CO_2 reduction via PSI (Krause & Cornic, 1987). qP values also can be used to estimate the fraction of the reduction state of Q_A, which reflects the excitation pressure on PSII (Öquist & Huner, 1993). Lower qP value in wild leaf mutant indicated a tend to photoinhibition. However, this did not occur due to on the one hand, chlorophyll contents of wild leaf mutant were higher than those of wild type, on the other; the great reduce of non photochemical quenching (NPQ). The lower actual photochemical efficiency (ΦPSII) was result form the light absorbs by this mutant was not enough for the use to all reaction centers. But the efficiency of excitation energy trapping by open PSII reaction centers in the light–adapted state (Fv'/Fm') of wide leaf mutant was higher than wild-type. This was confirmed by the enzyme activities results showed that SOD content, which reflects the anti-oxidation ability of plant, was much lower than wild-type. Again, MDA content, which reflects membrane oxidation extent, was lower than wild-type, too. These results suggest that wide leaf mutant can transfer more light energy into chemical energy without occurs of active oxygen. It can assign more energy to PSI, so make it more balance of the electron transfer between two photosystems.

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Abbreviations: Chl: chlorophyll; PS II: Photosystem II; Fo: minimal fluorescence level in dark-adapted leaves; Fm: maximal fluorescence level in dark-adapted leaves; Fv: maximum variable fluorescence level in dark-adapted leaves; Fo': minimal fluorescence level in light-adapted leaves; Fm': maximal fluorescence level in light-adapted leaves; Fv': maximum variable fluorescence level in light-adapted leaves; Fv/Fm: maximal efficiency of PS II photochemistry; NPQ: non-photochemical quenching; qP: photochemical quenching coefficient; Fv'/Fm': efficiency of excitation energy capture by open PS II reaction centers; ΦPS II: actual PS II efficiency.

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