Full Length Research Paper

Photosynthetic characterization of a rolled leaf mutant of rice (*Oryza sativa* L.)

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A new rolling leaf rice mutant was identified which showed an apparently straighter longitudinal shape normal transverse rolling characters at all developing stages. The chlorophyll contents per fresh weight of this mutant leaves were lower than those of wild-type. The electron transfer rate (ETR) and photochemical quenching (qP) were a little higher than those of wild-type. However, because of significant increase of non-photochemical quenching (NPQ), the maximal photosystem II (PSII) photochemistry (Fv/Fm) and the efficiency of excitation energy trapping by open PSII reaction centers in the light–adapted state (Fv'/Fm') were lower than those of wild-type. Low temperature fluorescence analysis showed that rolling leaf mutant assigned more excited energy to photosystem I (PSI) than to PSII. The superoxide dismutase (SOD) content, soluble sugar content, proline content and malonaldehyde (MDA) content of the rolling leaf mutant were nearly 39.4, 91.2, 96.7 and 143.7% of those of wild-type, respectively. The great increase of MDA content suggests that membrane lipid system was damaged in rolling leaf mutant leaves. These results indicate that rolling leaf mutant decrease the efficiency of light utilization compared to the wild-type. This was because of the reduction of leaf area and chlorophyll contents, and the dissipation of more excitation energy as NPQ as a result of avoiding potential damage of membrane structure.

Key words: Malonaldehyde (MDA), photosynthetic characterization, rice, rolling leaf mutant.

INTRODUCTION

The properties of leaf were focused in the high yield breeding and crop ideotypes selection in breeding program (Donald, 1968). Rolling leaves with different degree are commonly seen in crops like maize (Hay et al., 2000; Candela et al., 2008), triticale (Yang et al., 2011) and rice (Chen et al., 2005). Leaves, the collective organ produced by the shoot apical meristem (SAM), are polarized along their adaxial-abaxial axis (Yan et al., 2008). In rice, rolling leaf is classified as abaxial leaf roll (both sides of leaf roll inward along the vein) and adaxial

leaf roll (both sides of leaf roll outward along the vein) according to the leaf rolling direction. Rolling leaf with moderate rate were found helpful in keeping leaf erect, improvement of light receiving condition of rice population in the later stages of rice development, and reduce water transpiration (Chen et al., 2004; O'Toole et al., 1979). This can increase efficiency of light utilization rate, resulting in the increase of rice population yield (Lang et al., 2004 a, b; Couturier et al., 2011).

Rolling leaf material of rice were also helpful in the research of genes related with leaf growth and development as same as regulation mechanisms to osmotic stress (Hsiao et al., 1984). Rolling leaf is easily distinguished from other leaf type. As marker gene rolling leaf characterizer are widely applied in rice genetic and physical mapping research (Shi et al., 2007; Wu et al., 2010), abundance rolling leaf materials in rice which the genetic mechanisms were different have been identified

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(Hu et al., 2010; Luo et al., 2007; Rao et al., 2010). For instance, rolled leaf *RL9* encodes a GARP protein, an ortholog of Arabidopsis KANADIs (Yan et al., 2008). The *outcurved leaf1* (*oul1*) mutant showed abaxial leaf rolling phenotype. The phenotypes were caused by knockout of rice outermost cell-specific gene5 (Roc5), an ortholog of the Arabidopsis homeodomain leucine zipper class IV gene GLABRA2 (Zou et al., 2011).

However, research showed that drought or other osmotic stress caused rice leaf rolling which result in the reduction of photosynthetic activities (Saruhan et al., 2009, 2010; Terzi et al., 2009). The same results were also found in rolling leaf mutant with less chlorophyll contents and net photosynthesis rate, resulting in less yield compare to wild-type at low plant intensity (Lang et al., 2004 a, b). Therefore, whether the rolling leaf can cause insufficiency of light absorption, reduction of photosynthetic activities, and had damage effect on membrane system is still under controversy and need further study.

In this study, a rolling leaf mutant was found in the Hainan province obtained from rice (*Oryza sativa* L.) cv, Nipponbare (Japonica). It showed abaxial rolling characters in all the developing stages and had high rolling rate. The aims of this study were to determine photosynthetic activities and physiological indices of rolling leaf mutant, and also to find out the effect of rolling leaf on its light utilization.

MATERIALS AND METHODS

The rolled leaf (*rl*) mutant was identified in the T-DNA insertional lines obtained from rice, *O. sativa* L. cv, Nipponbare (Japonica). Genetic analysis showed that it was not caused by T-DNA insertion because bar gene could not be detected in this mutant. Wild-type rice cv. Nipponbare and *rl* mutant seedlings which germinated on April, 2011 were used. Seedlings with 7 leaves were planted in red clay soils at the experimental farm in Baodaoxincun, Danzhou city (19°51′51N; 109°55′63E) on May, 2011 in Hainan province, China. In the growing season, the average temperature in June, July and August were 30.3, 30.8, and 29.5°C, and precipitation were 77.7, 175.6 and 182.2 mm, respectively with around 97.5% humidity. The flag leaves of Nipponbare and *rl* mutant were used for the assays.

Electron microscopy

The electron microscopic analysis was done according to the method of Wang et al. (2006). The intact leaves were thoroughly rinsed with tap water and distilled water, and extra water on the leaf surface was sucked by filter paper. The samples (1 mm^3) were fixed in 3% (w/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 4 h, and washed with the phosphate buffer for 1 h. Then the samples were fixed with 1% (w/v) OsO$_4$ (pH 7.2) for 1 h, and rinsed with the phosphate buffer 3 times for 10 min. After washing, the samples were stepwise dehydrated in gradient acetone and ethanol, and then embedded in Spurr's resin for 3 days. Dry sections (1 to 2 μM) were cut with a diamond knife using an ultramicrotome, and mounted on copper grids. Electron microscopic observation was made at 100 kV on a JEM1230 transmission electron microscope (JEOL, Tokyo, Japan).

Detection of rice pathogenic bacteria and virus

Rice pathogenic bacteria was measured based on the method of Notteghem and Silue (1992). Briefly, 1 mm fresh leaf tissue was cultivated in 4% Lysogeny broth media for 1 day at 37°C, and 6 replicates were made. The culture media were carefully checked under the low power microscope Leica DM3000B (Leica Micro-systems GmbH, Wetzlar, Germany). For virus detection, the leaves were extracted according to the methods of Kimura (1976) and checked by GE Ultrospec™ 2100 pro UV/Visible spectrophotometer (USA).

Measurement chlorophyll

Chlorophyll was extracted with 80% acetone from 0.1 g leaf samples. The extract was measured spectrophotometrically at 475, 645 and 663 nm with GE Ultrospec™ 2100 pro UV/Visible spectro-photometer (USA). Specific chlorophyll contents were determined according to the method of Lichtenthaler (1987).

Measurements of chlorophyll fluorescence

Modulated chlorophyll (Chl) fluorescence measurements were made in attached leaves in the field at mid-day with a PAM-2500 portable fluorometer (Walz, Effeltrich, Germany) connected to a computer. The experimental protocol described by 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 sufficiency 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 8000 μmol m^{-2} s^{-1}. F_m was measured after 30 min of dark adaptation. F_m' and Fs was measured when photosynthetic photon flux densities (PPFDs) were approximately 200 and 1400 μmol m^{-2} s^{-1}, respectively. Other parameters were calculated based on the measured parameters above.

Thylakoid membrane preparations

Thylakoid membranes were prepared according to Edwards et al. (1979) with little modification. Rice leaves were homogenized in 0.4 M sorbitol, 100 mM tricine-KOH (pH 7.5), 10 mM NaCl and 5 mM MgCl$_2$. After the sample was filtered through 500, 195 and 20 μM nylon mesh and centrifuged for 5 min at 4,000 g, the chloroplast pellet was lysed by resuspending in 5 mM Hepes KOH, pH 7.5, 10 mM NaCl, 5 mM MgCl$_2$, and the thylakoid membrane were pelleted by centrifugation (5 min, 4,000 g). Finally, the thylakoid membrane was washed in 5 mM Hepes-KOH, pH 7.5, 10 mM ethylene-diaminetetraacetic acid (EDTA), centrifuged, and resuspended in the same buffer with 10% glycerol added. Samples were stored at -80°C in small aliquots.

Sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) electrophoresis

Denaturing gels were composed of 15% acrylamide/0.4% bis-acry lamide with Tris-Clycine pH 8.3, as a buffer, based on the

Figure 1. Phenotype of rolling leaf mutant in field.

system described by (Wyckoff et al., 1977; 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. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a Mini-protein II Electrophoresis Cell (Bio-Rad). Polypeptides were stained with coomassie brilliant blue R-250.

Low temperature fluorescence emission spectra

The fluorescence emission spectra of the thylakoid membranes were measured with a Hitachi F-4500 fluorescence spectro-photometer (Tokyo, Japan) at 77 K. The reaction mixture contained the isolated thylakoid membranes equivalent to 10 mg (Chl) m^{-3} and 50% (v/v) glycerol (Wang et al., 2005).

Superoxide dismutase (SOD) enzyme activities

Superoxide dismutase (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 and Fridovich (1987) with some modifications. For the total SOD assay, a 5 ml reaction mixture containing 50 mM HEPES (pH 7.6), 0.1 mM EDTA, 50 mM Na$_2$CO$_3$, 13 mM methionine, 0.025% (w/v) Triton X-100, 75

µM NBT, 2 µM riboflavin and an appropriate aliquot of enzyme extract was used. The reaction mixtures were illuminated for 15 min at a light intensity of 350 µmol 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.

Analysis of soluble sugar, proline, MDA

Soluble sugar was measured by referring to Creelman et al. (1990) and SCG4R (1956). Briefly, 0.1 g of leaf samples was added into centrifuging tubes with a volume of 10 ml, and 5 ml of 80% alcohol was added to the tube and heated in water for 30 min at 80°C. This was then cooled and the tubes were centrifuged at 1,000 g for 10 min.

Sugar content was determined by the phenol-sulfuric acid method. Malondialdehyde (MDA) was assayed by the thiobarbituric acid method as described by Aust et al. (1985). Proline was determined following the methods of Bates et al. (1973). Briefly, 0.5 to 1 g leaves was homogenized in 10 ml of 3% sulfosalicylic acid and the homogenate filtered. The filtrate (2 ml) was treated with 2 ml acid ninhydrin and 2 ml of glacial acetic acid, then with 4 ml of toluene. Absorbance of the colored solutions was read at 520 nm with DU800 Spectrophotometer (Beckman Coulter, USA).

Statistical analysis

All data were analyzed on SPSS analytical software package (version 18.0), and one-way ANOVA with Duncan's test was used to assess $P<0.05$ (probability level). Figures were drawn by Origin Data Analysis and Graphing Software, OriginPro8 (Version8E, OriginLab Corporation, Massachusetts, USA). All of the measurements were performed 6 times, and the means and calculated standard deviations (SD) are reported.

RESULTS

Phenotypes of rolling leaf mutant

As shown in Figure 1, rolling leaf mutant had normal plant height and tiller number. It showed abaxial leaf roll characters at all developing stages, even in water sufficient field. Figure 2 shows that rolling leaf mutant rolled along leaf vein in abaxial manner. The mesophyll cells of rolling leaf mutant have same number chloroplast as those of wild-type. The ultra-structure of thylakoid membrane in two kinds of rice did not change significantly.

As shown in Table 1, chlorophyll a (Chl a), chlorophyll b (Chl b), and β-carotene (β-Car) contents of rolling leaf mutants were 4.91, 1.64 and 1.03 mg/g fresh weight, respectively while Chl a, Chl b, and β-Car contents of wild-type were 7.11, 2.16 and 1.44 mg/g fresh weight, respectively. Hence, the Chl a, Chl b, and β-Car contents of rolling leaf mutant were significantly lower than those of wild-type. The great decrease of Chl a contents in rolling leaf mutant also lead to decrease of Chl a/b compared to wild-type. As shown in Figure 3, the absorption peaks of rolling leaf mutant and wild-type chlorophylls were 432 nm at Soret band, 663 nm at Q band, respectively. The absorption intensities of wild-type chlorophyll was little higher than that of the rolling leaf
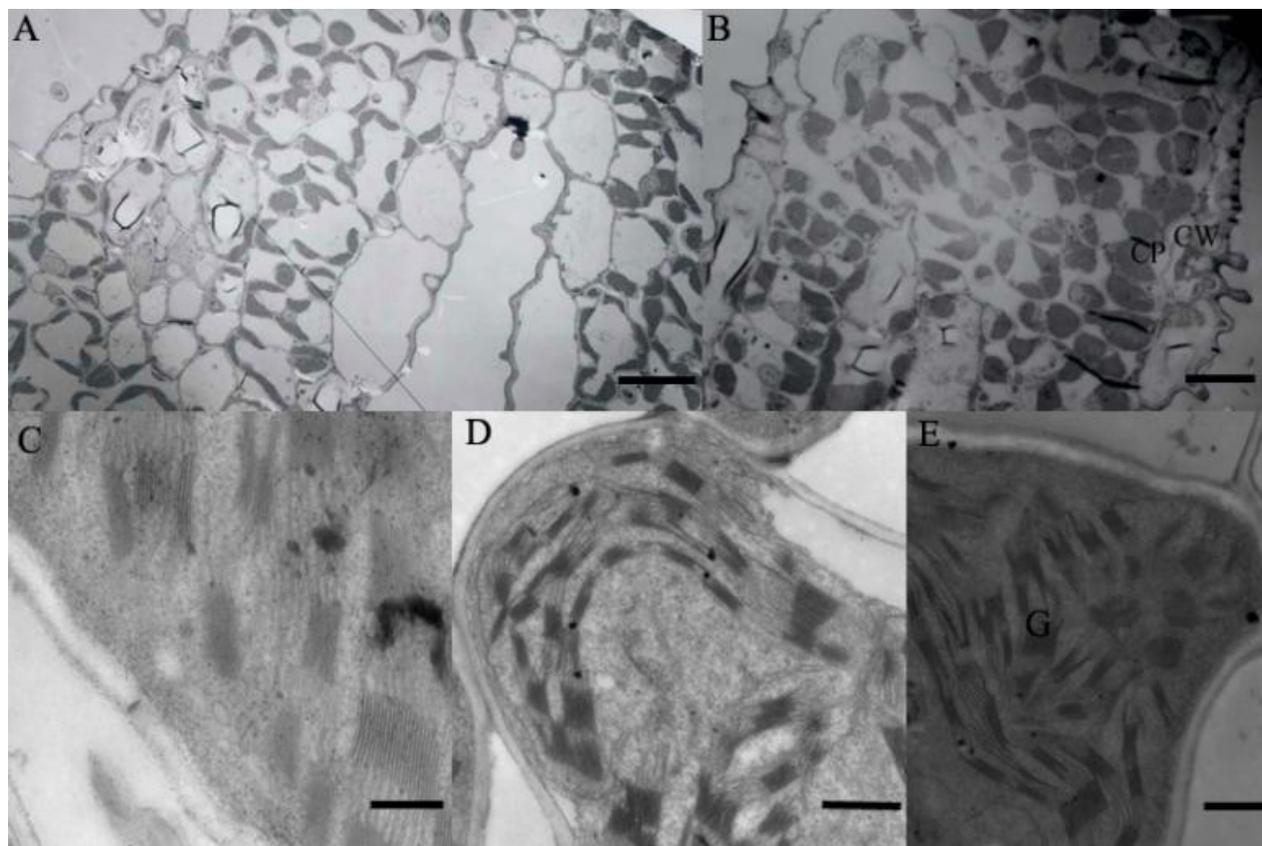
Figure 2. Ultra-structures of rolling leaf mutant and wild-type leaves. A. rolling leaf mutant leaf; B, wild-type leaf; C, D, thylakoid membrane of rolling leaf chloroplast; E, thylakoid membrane of wild-type chloroplast; CP, chloroplast; CW, cell wall; G, grana. Bars = 10 µM in A and B; 1 µM in C, D and E.

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

Parameter	Chl a	Chl b	β-Car	Chl a + b	Chl a/b
Rolling leaf(rl)	4.91 ± 0.12	1.64 ± 0.07	1.03 ± 0.04	6.54 ± 0.10	2.99 ± 0.02
Wild-type(wt)	7.11 ± 0.09	2.16 ± 0.06	1.44 ± 0.02	9.27 ± 0.11	3.29 ± 0.01
rl/wt%	69.0**	75.9**	71.8**	70.6**	91.0

Data are means ± SD, n=6, **means $P<0.01$. Chl a, Chlorophyll a; Chl b, chlorophyll b; β-Car, β-carotene.

mutant but do not significantly (Table 2).

The maximal efficiency of PSII photochemistry (Fv/Fm) of rolling leaf mutant decreased by 4.9%. The electron transport rate (ETR), photochemical quenching (qP), and the actual photochemical efficiency of PS II (ΦPS II) of rolling leaf mutant increased (8.4, 6.1 and 8.3%, respectively) compared to wild-type. However, the non-photochemical quenching (qN) which reflects the process competing with PS II photochemistry for absorbed excitation energy increased by 20% ($P<0.01$), this caused little decrease of the efficiency of excitation energy trapping by open PSII reaction centers in the light–adapted state (Fv'/Fm') lower than those of wild-type. The fluorescence emission spectra peaks of wild-type and

rolling leaf mutant were at 683.6 and 728.4 nm, respectively (Figure 4). Usually, 683.6 nm peak is a representative fluorescence intensity of photosystem II (PSII), while 728.4 nm peak is a representative fluore-scence intensity of photosystem I (PSI). The low tempe-rature fluorescence intensity of rolling leaf mutant was lower in PSII, but higher in PSI compared to those of wild-type which result in the reduction of F683/F730 ratio. The decreased F683/F730 ratio suggested that in rolling leaf mutant, more excited energy was assigned to PSI than to PSII.

The CP43, CP47, D1, and D2 protein contents of PSII reaction center of rolling leaf mutant per unit chlorophyll were the same as those of widtype (Figure 5). The light
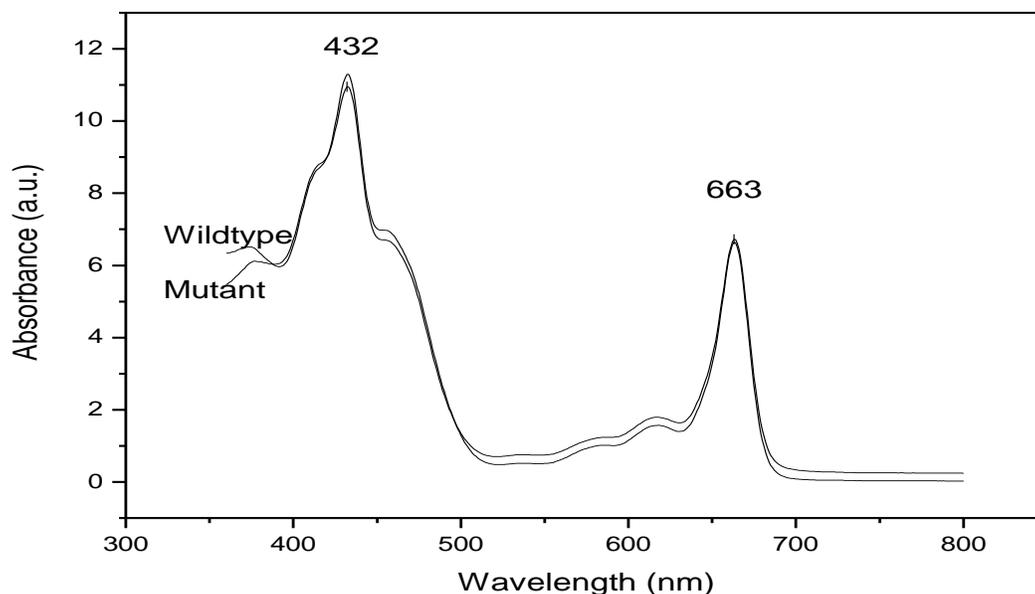
Figure 3. The room temperature (298 K) absorption spectrum of rolling leaf mutant and wild-type chlorophylls.

Table 2. Chlorophyll fluorescence parameters of rolling leaf mutant and wild-type leaves.

Parameter	Fv/Fm	ETR	qP	NPQ	Fv'/Fm'	ΦPSII
Rolling leaf(rl)	0.78 ± 0.02	23.3 ± 0.82	0.35 ± 0.05	0.57 ± 0.02	0.69 ± 0.01	0.26 ± 0.02
Wild-type(wt)	0.82 ± 0.01	21.5 ± 0.64	0.33 ± 0.04	0.47 ± 0.42	0.71 ± 0.01	0.24 ± 0.01
rl/wt%	95.1	108.4	106.1	121.3[**]	97.2	108.3

Data are means ± SD, n=6, [**]means $P<0.01$. **ETR,** Electron transfer rate; **qP,** photochemical quenching; **NPQ,** non-photochemical quenching; **PSII,** photosystem II.
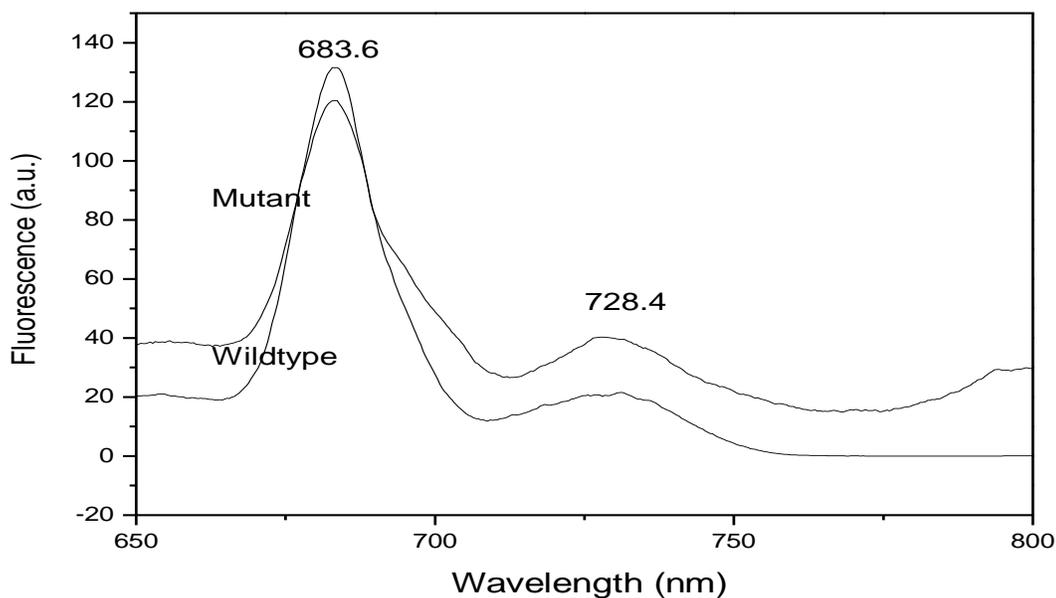
Figure 4. Low temperature (77 K) fluorescence emission spectra of thylakoid membranes of rolling leaf mutant and wild-type, emission at 436 nm.
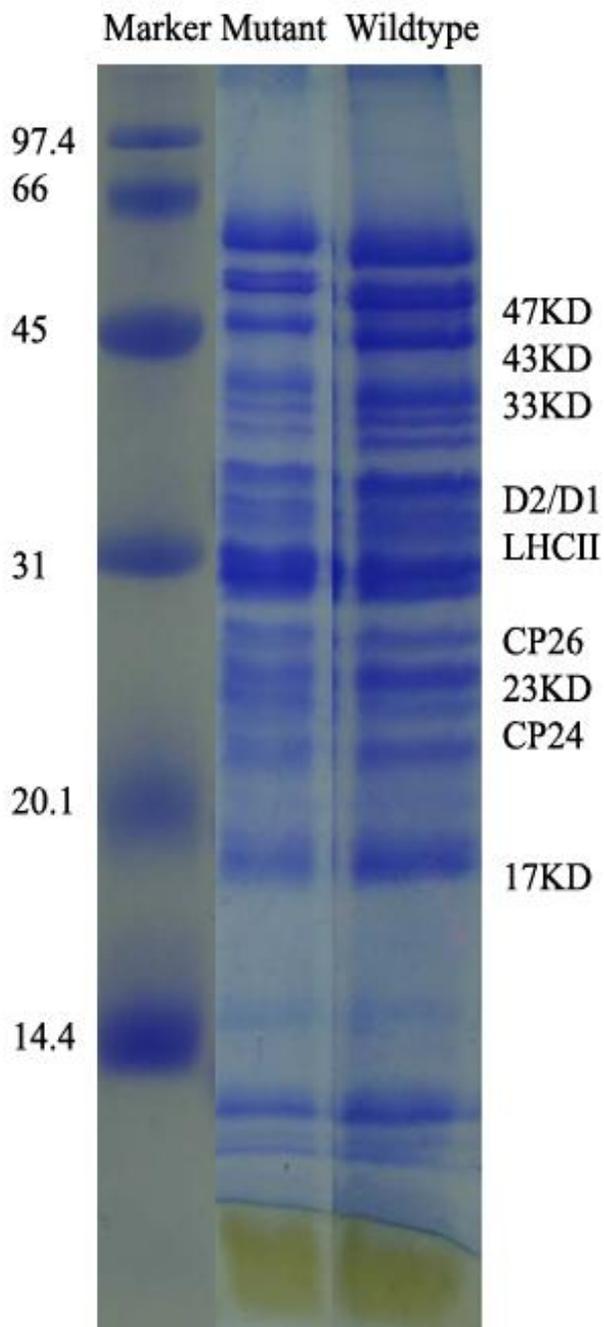
Figure 5. Profiles of SDS-PAGE electrophoresis of thylakoid membranes proteins of rolling leaf mutant and wild-type.

harvesting complexes (LHCII) light harvesting pigments-protein complex contents was the same as that of wild-type. The analysis of enzyme activities of drought related indices showed that the proline and soluble sugar content reduced by 3.3 and 8.8%, respectively (Table 3). The SOD activities decreased by 60.6% ($P<0.01$), while MDA increase by 43.7% ($P<0.01$). These results indicate that the rolling leaf cause retardation of water transport, which leads to damage on the membrane system.

DISCUSSION

Light receiving conditions at later stages of rice develop-ment is necessary in the super yield breeding and cultivation system of rice (Donald, 1968). The leaf architecture, especially upper 3 functional leaves has important influence on leaf area index and light acceptation and utilization efficiency. A big photosynthetic population needs high leaf area to accept more light energy (Duncan, 1971). However, long and wide leaves are liable to bend and shade the base leaf in the later stage. The rolling leaf character was found in the solution of both increase leaf length and width as same as avoiding leaf bends. Rolling leaf rice, which has effect on keeping leaf stand is one of the important parts in rice ideotype, improve the light accept conditions at base of population.

Most of the rolling leaf mutant increased the colony yield which depends on the decrease yield of single plant. However, the paradox was found that in some research, the net photosynthesis rate (Pn) of single plant was higher in rolling rice than that of wild-type (Chen et al., 2005). In this study, the same result was obtained in the electron transport rate (ETR), photochemical quenching (qP), and the actual photochemical efficiency of PS II (ΦPS II) of rolling leaf mutant, as these were increased compared to the wild-type, respectively. The results of low temperature fluorescence analysis showed that this mutant distributed more energy to PSI than PSII. The reason of these phenomena is that the light excitation energy was soon transferred to PSI to avoid the damage on PSII. Since qP indicates the oxidation-reduction state of the primary acceptor for PSII, which is determined by the rate of photo reduction of the acceptor by PSII and by its rate of reoxidation linked to CO_2 reduction via PSI (Krause 1988), 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 and Huner, 1993). The increase of qP and ETR values with low temperature fluorescence result suggest that this rolling mutant dissipate excitation energy more quickly than wild-type. Furthermore, the significant increase of non photo-chemical quenching (NPQ) suggests that this mutant dissipate more energy as heat rather than use for the formation of NADPH. In this study, the total chlorophyll content of rolling leaf mutant decrease significantly com-pared to those of wild-type. This was safely explained by the fact that there were no enough chlorophyll and β-car in rolling mutant to use light energy absorbed by photosystems.

Reactive oxygen species (ROS) occur when excited light energy could not be quenched in time by photo-system. ROS production triggers a network of signaling events leading to several outputs, including stress tolerance, acclimation, and cell death (Mittler et al., 2004, 2008). Malonaldehyde, one of the products of lipid per-oxidation, appears to be produced in relatively constant proportion to the degree of lipid peroxidation and is thus

Table 3. Proline, Soluble Sugar, SOD and MDA contents of rolling leaf mutant and wild-type.

Parameter	Proline (µg/g)	Soluble sugar (µg/g)	SOD (enzyme activity)	MDA (µmol/g)
Rolling leaf(rl)	6.97 ± 0.02	15.1 ± 0.82	136.2 ± 0.05	2.01 ± 0.02
Wild-type(wt)	7.2 ± 0.01	16.5 ± 0.64	345.4 ± 0.04	1.40 ± 0.42
rl/wt%	96.7	91.2	39.4[**]	143.7[**]

Data are means ± SD, n=6, [**] means P<0.01. SOD, Superoxide dimutase; MD, malonaldehyde.

a convenient indicator of the rate of lipid peroxidation. In this study, the same proline content suggests that no osmotic stress was found in rolling leaf mutant. The increase of MDA content in rolling leaf mutant reflects the high extent of membrane oxidation, resulting in high risk of damage on photosystem. To cope with this bad effect, this mutant must dissipate light excited energy as soon as possible.

Conclusion

The rolling leaf mutant had lower PSII activity than wild-type, and higher PSI activity than wild-type. This rolling leaf mutant dissipates more excited energy to PSI to cope with damages caused by rolling leaf.

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