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The effects of enhanced UV-B radiation on photosynthetic and biochemical activities in super-high-yield hybrid rice Liangyoupeijiu at the reproductive stage

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Abstract

We investigated the light reactions, CO_2 assimilation, but also the chloroplast ultrastructure in the upper three functional leaves (flag, 2nd, and 3rd leaves) of the Chinese super-high-yield hybrid rice (*Oryza sativa* L.) Liangyoupeijiu (LYPJ) with ultraviolet-B (UV-B) treatment during reproductive development. Photosynthetic parameters showed that the upper 3 functional leaves of LYPJ entered into senescence approximately 15 days after flag leaf emergence (DAE). Leaves in UV-B treatment exhibited greater efficiency in absorbing and utilizing light energy of photosystem II (PSII), characterized by higher chlorophyll (Chl) content and the whole chain electron transport rate (ETR). However, UV-B radiation reduced activities of Ca^{2+}- ATPase and photophosphorylation. The significantly decreased activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) was greatly associated with the decline in photosynthetic efficiency. The net photosynthetic rate (P_N) and stomatal conductance (g_s) suffered strong reductions before 25 DAE, and afterwards showed no significant difference between control and treatment. UV-B treatment delayed chloroplasts development of flag leaves. Chloroplast membranes later swelled and disintegrated, and more stromal thylakoids were parallel to each other and were arranged in neat rows, which might be responsible for better performance of the primary light reaction. It is likely that accumulation of starch and an increase in the number of lipid droplet and translucent plastoglobuli were results of an inhibition of carbohydrate transport. Our results suggest that long-term exposure to enhanced UV-B radiation was unlikely to have detrimental effects on the absorption flux of photons and the transport of electrons, but it resulted in the decrease of photophosphorylation and Rubisco activation of LYPJ. The extent of the damage to the chloroplast ultrastructure was consistent with the degree of the inhibition of photosynthesis.

Additional key words: chloroplast ultrastructure; gas exchange; light reaction; photosynthesis; Rubisco; UV-B radiation.

Introduction

Stratospheric ozone depletion resulting from air pollution would facilitate more UV-B radiation reflecting back to the surface of the earth, which would further contribute to climate change (Erickson *et al*. 2000). Calculations of UV radiation based on the relationship of total ozone and total irradiance suggest that the UV-B level has increased by

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Abbreviations: ABS/CS_M – the absorption flux of photons per cross section; Chl – chlorophyll; DAE – days after flag leaf emergence; DI_0/CS_M – the dissipation per cross section; ET_0/CS_M – the potential electron transport per cross section; ETR – electron transport rate; F_v/F_m – maximum photochemical efficiency of PSII; Liangyoupeijiu – LYPJ; PEP – phospho*enol*pyruvate; PEPcase – phospho*enol*pyruvate carboxylase; PI_{ABS} – the performance index is presented below on an absorption basis; PS – photosystem; Rubisco – ribulose-1,5-bisphosphate carboxylase/oxygenase; Q_A – the primary quinine acceptor of PSII; TR_0/CS_M – the phenomenological fluxes for trapping per cross section.
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6–14% at more than 10 sites distributed over middle and high latitudes of both hemispheres since the early 1980s (UNEP 2002). In this way, it is therefore likely that terrestrial plants will have to deal with enhanced UV-B levels in the next decades. This has lead to a significant research effort to better understand the acclimation strategies that could help photosynthetic organisms to cope with harmful effects.

Over the last two decades, extensive studies of the physiological, biochemical and morphological effects of UV-B in plants, as well as the mechanisms of UV-B resistance, have been carried out. The negative effects of enhanced UV-B on rice are considered to be due to effects on membrane structures (Fedina *et al*. 2010, Lidon and Ramalho 2011), the oxidative stress defense system (Mazza *et al*. 1999), proteins (Desimone *et al*. 1996, 1998), DNA (Mazza *et al*. 1999), and signal transduction (Hirouchi *et al*. 2003). Photosynthetic organisms are especially sensitive to UV-B radiation due to their requirement for light (Kim *et al*. 1996, Hada *et al*. 2003), as well as their low genome stability (Sato *et al*. 1994, 2003, Teranishi *et al*. 2004). Taken together, the results of these studies suggest that the enhanced solar UV-B radiation predicted by atmospheric models will result in reduction of growth and yield of rice in the future. However, Kumagai *et al.* (2001) found that supplemental UV-B radiation has a positive effect on the growth and grain development of rice, which may be enhanced by unusual climatic conditions such as lower temperature and less sunshine, in cool rice-growing regions. The response of plant to UV-B radiation depends on species, cultivar, developmental phase, experimental conditions, UV-B dosage, and the ratio of photosynthetically active radiation (PAR) to UV-B radiation (Hidema *et al*. 2005, Xu and Qiu 2007). Most of these studies were conducted in growth chambers and greenhouses under unrealistic conditions, where rice is variably sensitive to UV-B (Huang *et al.* 1993, Ambasht *et al.* 1997, Kakani *et al.* 2003). Under more realistic field conditions, the response of rice species to exposure to UV-B and UV-B involved in rice succession were studied only rarely.

The breeding and promotion of super-high-yielding hybrid rice Liangyoupeijiu (LYPJ-maternal PeiAi 64S and paternal WuMang 9311) is a landmark event in the history of hybrid rice, which takes a production of China's rice to a new level and it is also widely acknowledged by international communities and counterparts (Normile 2000). As a super-high-yielding hybrid rice, the average production per hectare of LYPJ is 10.5 t, which outweighs the newly promoted large-area hybrid rice Shanyou 63, with an average production per ha of 7.50–8.25 t, by 20–30% (Wang *et al*. 2002). LYPJ has been extended to 16 provinces and regions in 2009, thus the cumulative area has exceeded 7×10^6 ha. LYPJ has become one of the most important rice varieties in China. Our previous work indicated that, compared with other rice cultivars, the chloroplast structure of LYPJ has several advantages: chloroplasts appear densely occupied by thylakoids arranged in grana and in grana-interconnecting membranes, and the chloroplasts are lined close to the cell periphery (Chen *et al*. 2004, Zhang *et al*. 2010). The ability of energy conversion is as strong as the capability of chloroplast envelope transferring photosynthetic products (Zhang *et al*. 2007, Yu *et al*. 2010).

Because 60–100% of the carbon in mature rice grains originates from CO_2 assimilation during the grain-filling period (Yoshida *et al*. 1981), Jiang *et al*. (2002) indicated that the upper three functional leaves (flag, 2nd, and 3rd leaves) play a significant role in accumulating the photosynthetic products that contribute to 70–80% of rice production. Hidema and Kumagai (2006) suggested that the cyclobutane pyrimidine dimer (CPD) was a crucial factor determining the differences in UV-B sensitivity between rice cultivars, and the sensitivity of rice to UV-B was the greatest during the period between flowering and seed maturity. Leaf senescence of LYPJ occurs earlier and faster, and the photosynthetic rate is decreased significantly during the late growth. Some studies on responses of rice have reported a retardant effect of UV-B on leaf senescence, alterations in assimilate availability and assimilate partitioning (Kakani *et al*. 2003, Xu *et al*. 2006). Therefore, the present study of functional leaves was conducted in fields under relatively high level of ambient UV-B radiation during reproductive development with the objectives: (*1*) to quantify the effect on the light reaction and CO_2 assimilation responses, (*2*) to examine the alteration in the course of senescence as modified by UV-B, (*3*) to clarify the link between physiological and morphological results. The acquired information of photosynthetic characteristics could help in estimating the potential impacts on senescence and agricultural production. On the other hand, this can be taken as an important indicator for selective breeding of UV-B resistant rice cultivars.

Materials and methods

Plant material: Experiments were conducted in the experimental fields and growth rooms of the Institute of Agricultural Sciences of Jiangsu Nanjing, China (32°03'N, 118°47'E). During the period from August 1 (22 d before the first sampling) to October 8 (the last sampling date), the mean, maximal, and minimum daily temperatures were 23.39 ± 0.47°C, 28.13 ± 0.44°C, and 20.00 ± 0.59°C, respectively. In addition, the mean daily precipitation and the daily relative humidity were 9.50 ± 3.90 mm and 78.18 ± 0.86%, respectively, for the same period.

Liangyoupeijiu (LYPJ) was grown in a field in May 2009 and 2010. The total N fertilizer applied was 3.375 t ha^{-1}, with an N-P-K ratio of 1:0.6:0.6. Plants with

three leaves and a heart were transplanted from the field to the growth room and cultured with two seedlings per barrel. Each barrel contained 10 kg of a field soil. There was a distance of 0.15 m between the two seedlings. Plants were watered and fertilized regularly during the growing season.

Treatment of LYPJ: Three sets of lamp frames (0.15 m × 1.3 m) were used to provide supplemental UV-B radiation, and each lamp frame contained one UV-B-emitting fluorescent tube (*TL 40 W/12 RS*, *Huaqiang Electronics Co., Ltd.*, Nanjing, CHN). The lamp frames were oriented in the east-west direction to minimize shading. The total trial covered an area of 5 m × 10 m, and three lamps were arranged in parallel in the middle of the left half of the area. The distance between neighboring lamps was 0.60 m. Eight plants under each lamp were assigned as UV-B treated samples, and those grown under ambient UV-B at a similar position in the right half of the area were used as controls. A buffer of two rows was created around the plot to minimize border effects, and the distance between UV-B treated and control plants was 1.2 m in each row. Total daily PAR for the treated plant canopy was about 95 % that of the control.

UV emission was filtered through a 1.3×10^{-4} m thick cellulose acetate membrane (*Cadillac Products Packaging*, Chicago, USA) to eliminate the impact of UV-C. The emission spectrum of the UV-B lamps was measured with a spectroradiometer (*UV-B irradiation meter*, *Photoelectric Instrument Factory of Beijing Normal University*, Beijing, CHN). The height of the UV-B lamps was adjusted weekly to maintain a constant distance above the top of the rice canopy. UV-B lamps were turned on for 8 h per day from 8:00 to 16:00 h, beginning at booting stage (50 d) and ending on the day of harvest (150 d). The biologically weighted UV-B dosage was 5.4 kJ m^{-2} d^{-1} (normalized to 300 nm; Flint and Caldwell 2003). The supplemented UV-B radiation was similar to a flux density that would occur at a 20% stratospheric ozone reduction relative to the UV-B intensity measured on 22 June 2003 in Nanjing (6.5 kJ m^{-2} d^{-1} UV-B; Zhang *et al.* 2003) according to the mathematical model of Ruggaber *et al.* (1994). Sampling occurred from August 15 (around 5 DAE) to October 5 (close to rice grain harvesting time) on the main culm in the morning (08:30–10:30 h) on sunny days approximately every 10 d, depending on the weather.

Chlorophyll (Chl) content and gas-exchange measurements: Chl was extracted in ice-cold 80% (v/v) acetone. The extract was centrifuged at 3,000 × *g* (*SIGMA 3K-30*, Goettingen, GER) for 5 min. The absorbance of the supernatant was measured at 645 and 663 nm with a *UV-754* spectrophotometer (*Shanghai Institute of Plant Physiology*, China). The Chl content was calculated as described by Arnon (1949). P_N and g_s of the intact functional leaves were measured in the field using a portable photosynthesis system (*CIRAS-2*, *PP-Systems*, Hitchin, UK) in the morning between 09:00 and 10:00 h to avoid potential photoinhibition resulting from high-light stress at midday. Measurements were made under 360 ± 2 μmol mol^{-1} of CO_2 concentration from a fresh CO_2 cartridge, and PAR intensity at 1,500 ± 50 μmol m^{-2} s^{-1}, with cuvette relative humidity and leaf temperature corresponding to the relative humidity and leaf temperature measured outside of the cuvette. The flow rate was 197 ± 3 ml min^{-1}. Data were recorded automatically at intervals of 2 min using appropriate software (*CIRAS-2*, *PP-Systems*, Hitchin, UK). When the fluctuation in P_N was less than 0.5 μmol m^{-2} s^{-1}, 6 measurements for each leaf were recorded automatically at 3-s intervals. Two minutes was a time long enough to get stable P_N and g_s values as described above.

Fast Chl fluorescence induction dynamics analysis: We used a pocket fluorometer (*Handy PEA*, Hansatech, UK) to measure the fluorescence parameters of functional leaves by referring to methods created by Strasser *et al.* (2000). The measurement was conducted at 10:00 h, and leaf clips for dark adaptation were placed on the adaxial side of the leaves 30 min before measurement and then exposed to red light of 650 nm through LED at excitation irradiance of 3,000 μmol m^{-2} s^{-1} with a duration of 800 ms. The JIP-test analysis was performed using the professional *PEA Plus* and *Biolyzer HP3* software developed by R. Maldonado Rodriguez and freely available at www.unige.ch/sciences/biologie/bioen/ bioindex.html.

Fluorescence parameters are extracted and derived from the fast fluorescence transient O-J-I-P, according to Strasser *et al.* (2004) and Li *et al.* (2005). F_0 is the minimal and F_M is the maximal fluorescence. F_J and F_I is the fluorescence measured after 2 ms and 30 ms, respectively. Derived parameters are described in Table 1.

Thylakoid membrane isolation, photosynthetic electron transport activity, photophosphorylation and Ca^{2+}-ATPase activity: Isolation of thylakoid membranes was performed according to the method of Dunahay *et al.* (1984) with slight modifications. After removal of the midrib from 20 g of flag leaves, the specimen was kept in a refrigerator at 4°C for 30 min. Then, the leaves were cut into pieces and were homogenized with cool extracting medium B_1 [0.4 M sucrose, 2 mM $MgCl_2$, 0.2 % bovine serum albumin (BSA), 20 mM Tricine, pH 8.0]. The homogenate was filtered to remove large debris, and then centrifuged at 300 × *g* for 2 min. The supernatant was recentrifuged at 2,070 × *g* for 2 min. Chloroplasts were collected from the precipitate, diluted 3–4 times with cool extracting medium B_2 (0.15 M sucrose, 5 mM $MgCl_2$, 0.2% BSA, 20 mM Tricine, pH 8.0), and centrifuged at 4,000 × *g* for 10 min. After removing the upper solution, the precipitate was supplemented with storage buffer B_3 (15 mM NaCl, 5 mM $MgCl_2$, 20 mM MES, pH 6.5) and the tube was slightly rotated in ice blocks to make a

uniform suspension of thylakoid membranes, which was then kept in the dark on ice for use in the subsequent procedures.

Whole chain electron transport activity was measured polarographically with a Clark-type liquid-phase electrode (*Chlorolab-2*, *Hansatech*, Cambridge, UK) fitted with a circulating water jacket at 25 ± 0.5ºC according to Coombs *et al.* (1985). The electrode chamber was illuminated by actinic light from a slide project, and the light intensity was 1,000 μmol m^{-2} s^{-1}. The rate of electron transport from H_2O to methyl viologen (MV) was determined by monitoring oxygen uptake in 2 ml of a reaction buffer (pH 7.6–7.8) containing 25 mM Tricine, 5 mM NaCl, 0.2 M sucrose, 5 mM $MgCl_2$, 2 mM NaN_3, 150 μM MV, and 5 mM NH_4Cl as an uncoupler.

Photophosphorylation activity was measured with a luminescence meter (*FG-300*, *Shanghai Institute of Plant Physiology*, Shanghai, China), as described by Ketcham *et al.* (1984). About 0.1 ml of chloroplast suspension was added to 0.9 ml of a reaction buffer with 10 mM $K_3Fe(CN)_6$, 0.2 M Tricine (pH 8.0), 20 mM $MgCl_2$, 20 mM Na_2HPO_4, and 20 mM ADP, and then illuminated for 1 min, before 0.5 ml of 20% trichloroacetic acid (TCA) was added to end the reaction. The reaction solution was centrifuged at 1,000 × *g* for 5 min. 0.1 ml of the supernatant was mixed with 9.9 ml of 0.02 M Tris-HCl (pH 7.5). About 0.2 ml of this sample and 0.8 ml of luciferase were used for subsequent determinations of the photophosphorylation activity. The activity of Ca^{2+}-ATPase was measured according to Vallejos *et al.* (1983). Briefly, a 0.1 ml of the membrane suspension was added to a reaction buffer with 25 mM Tris-HCl, 2 μM EDTA, 1 μM ATP, and 5 mM DTT at pH 7.5–8.0. The mixture was activated by illuminating it for 5 min at 25ºC, and then the reaction was stopped by addition of 0.1 ml of 5 mg ml^{-1} BSA. About 0.5 ml of the activated solution was mixed with 50 mM Tris-HCl, 2 mM $CaCl_2$, and 5–10 μM ATP at pH 8.0 and 36ºC for 10 min. The reaction solution was immediately added to 0.2 ml of 20% TCA at 0ºC to end the reaction. The reaction solution was centrifuged and the supernatant was used to determine the inorganic P content, and then the Ca^{2+}-ATPase activity could be calculated.

Assays of the dark reaction key enzymes: According to the method of Yang *et al.* (2003) with some modifications, fresh leaf tissue (0.5 g) without the midrib was ground quickly in precooled mortar and pestle on ice bath with 3 cm^3 of the extraction buffer containing 100 mM Tris-H_2SO_4 (pH 8.2), 10 mM $MgCl_2$, 1 mM EDTA, 7 mM dithiothreitol, 1% (w/v) insoluble polyvinylpolypyrrolidone and 50% (w/v) glycerol. The homogenate was centrifuged at 15,000 × *g* at 4°C for 20 min, and the supernatant was used immediately to assay enzyme activities. The ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity was assayed according to Makino

Table 1. The derived fluorescence parameters.

Parameter	
Nomenclature or energy flow	
CS	excited cross section of the leaf sample
RC	PSII reaction center
ABS	absorption energy flux
TR_0	excitation energy flux trapped by the RC and utilized for the reduction of Q_A to Q_A^-
ET_0	flux of electrons from Q_A^- into the intersystem electron transport chain
$Chl_{total} = Chl_{antenna} + Chl_{RC}$	total Chl
Quantum efficiencies or flux ratios	
$F_v/F_m = \varphi P_0 = [1 - (F_0/F_M)]$	maximum quantum yield of primary photochemistry
$\psi_0 = (1 - V_J) = 1 - (F_J - F_0)/(F_M - F_0)$	probability of electron transport beyond Q_A
$\varphi E_0 = [1 - (F_0/F_M)]\ \psi_0$	quantum yield of electron transport from Q_A to the intersystem electron acceptors
$\gamma_{RC} = Chl_{RC}/Chl_{total}$	fraction of reaction center Chl relative to the total Chl
$\gamma_{RC}/(1 - \gamma_{RC}) = Chl_{RC}/Chl_{antenna} = RC/ABS$	fraction of reaction center Chl relative to antenna Chl
Phenomenological fluxes or phenomenological activities	
$ABS/CS_M \approx F_M$	absorption flux of photons per cross section
$TR_0/CS_M = \varphi P_0\ (ABS/CS_M)$	phenomenological fluxes for trapping per cross section
$ET_0/CS_M = \varphi E_0\ (ABS/CS_M)$	potential electron transport per cross section
$DI_0/CS_M = (ABS/CS_M) - (TR_0/CS_M)$	dissipation per cross section
Performance indexes	
$PI_{ABS} = [\gamma_{RC}/(1 - \gamma_{RC})]\ [\varphi_{P0}/(1 - \varphi_{P0})]\ [\psi_0/(1 - \psi_0)] = (RC/ABS)\ [\varphi_{P0}/(1 - \varphi_{P0})]\ [\psi_0/(1 - \psi_0)]$	performance index on absorbtion basis

et al. (1985). According to the protocol of Syare *et al.* (1979), the phospho*enol*pyruvate carboxylase (PEPcase) activity was assayed at room temperature in a 3-ml mixture containing 100 mM Tris-H_2SO_4 (pH 9.2), 10 mM $NaHCO_3$, 10 mM $MgSO_4$, and 1.5 units NAD^+-malate dehydrogenase, 5 mM NADH, and 4 mM phospho-*enol*pyruvate (PEP). The reaction was initiated by the addition of crude enzyme extract. The decrease in absorbance was monitored spectrophotometrically at 340 nm with a *UV-754* spectrophotometer (*Shanghai Institute of Plant Physiology*, China).

Observation of chloroplast ultrastructure in flag leaves: To avoid differential structure in different parts of flag leaves, the middle part of the flag leaves without the midrib was cut into small pieces (approximately 0.1 × 0.5 cm). These small pieces were placed in a bottle with 4% glutaraldehyde buffer solution, and the air was pumped out of the bottle with a syringe so that the leaves became fully soaked in the buffer solution, according to the method described by Zhang *et al.* (2010). Leaves were fixed at 4ºC or over 24 h. They were then rinsed in phosphate buffer (pH7.4) for 15 min and post-fixed in 5% OsO_4 at the room temperature. The fixed samples were dehydrated in an ascending series of alcohols (50%, 60%, 70%, 80%, and 90%; 15 min each) and in 100% alcohol (three times by 7–8 min), washed in 100% acetone for 15 min, and embedded in the Epon 812 resin. Thin sections were obtained with an *LKB-V* ultramicrotome (*LKB Ultrascan XL*, Bromma, Sweden) and double-stained with uranyl acetate-lead citrate before being examined with a transmission electron microscope (*Hitachi 600-A-2*, Japan) operating at 75 kV.

Statistical analysis was performed using the statistical package using *SPSS* software (*SPSS Inc.*, *version 16.0*, Chicago, USA). All measured data were expressed as means ± standard deviation, analyzed by one-way *ANOVA*. Differences between treatment and control were considered significant if $P<0.05$.

Results

Chl content and P_N: Chl content of UV-B treated leaves was slightly reduced before 15 DAE, afterwards recovered to levels above those of the control (Fig. 1*A*). The senescence occurred later than in the control. There were insignificant differences in the total Chl during the whole reproductive stage ($P>0.05$, *t*-test).

The changes in P_N and g_s of the functional leaves are shown in Fig. 1*B*,*C*. In the first, P_N in stressed plants was only 85% of that in control plants after 5 DAE, less than 92% after 15 DAE, 87% after 25 DAE, and around 89% after 35 DAE. But after that, it decreased more slowly, and the value was 14.07% higher than that of the control at 55 DAE. g_s suffered strong reductions, representing only 75.77% and 86.13% of the control value at 5 and 15 DAE, respectively, but afterwards g_s showed no significant difference between control and treatment.

Photochemical efficiency of PSII: The JIP-test represents a translation of the original data to biophysical parameters (Table 2) that quantify the energy flow through PSII. Compared with the control, ABS/CS_M in the UV-B treatment had a higher value after 5 DAE, which suggested that enhanced UV-B radiation did not damage antenna pigments in LYPJ and promoted ABS/CS_M. TR_0/CS_M and ET_0/CS_M were, respectively, 3.69 and 4.11% higher under UV-B radiation, which suggested that the flux of electrons from Q_A^- into the intersystem electron transport chain was bigger than the control. DI_0/CS_M corresponding to the increase in ABS/CS_M was 1.12% lower than control, which indicated that the energy was dissipated as heat and that the utilization of light did not change significantly as compared with those of control plants ($P>0.05$, *t*-test).

F_v/F_m and PI_{ABS}: The ratio between variable and maximal fluorescence, F_v/F_m, is widely used as an estimation of the maximum quantum yield of PSII photochemistry

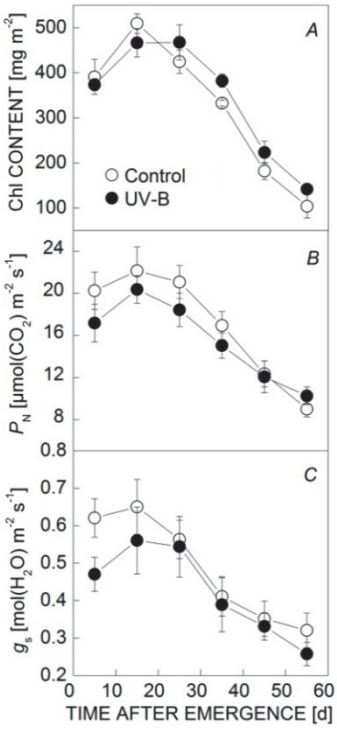

Fig. 1. Chlorophyll (Chl) content (*A*), net photosynthetic rate (P_N; *B*) and stomatal conductance (g_s; *C*) versus days after emergence of flag leaves in a super-high-yield hybrid rice LYPJ (○) and its UV-B treatment (●). Vertical bars show standard errors ($n = 3$).

Table 2. Phenomenological fluxes extracted from the fast Chl *a* fluorescence transient in the PSII reaction center of functional leaves in control and UV-B treatment. ABS/CS_M – the absorption flux of photons per cross section; TR_0/CS_M – the phenomenological fluxes for trapping per cross section; ET_0/CS_M – the potential electron transport per cross section; DI_0/CS_M – the dissipation per cross section. The number of days with UV-B treatment is shown in parentheses. The asterisk indicates significant difference between the control and UV-B treated leaves (P <0.05, t-test). Data are means ± SD (n = 25).

	ABS/CS_M	TR_0/CS_M	ET_0/CS_M	DI_0/CS_M
Control (5)	2293 ± 112	1746 ± 83	1102 ± 54	547 ± 22
UV-B treatment (5)	2260 ± 105	1720 ± 78	1071 ± 37*	540 ± 30
Control (15)	2267 ± 117*	1744 ± 72*	1108 ± 52*	523 ± 16*
UV-B treatment (15)	2487 ± 154	1963 ± 76	1298 ± 58	524 ± 28
Control (25)	2205 ± 115*	1658 ± 82*	950 ± 42*	547 ± 33
UV-B treatment (25)	2269 ± 115	1723 ± 57	1015 ± 45	546 ± 34
Control (35)	2100 ± 107*	1532 ± 43	870 ± 45	568 ± 34*
UV-B treatment (35)	2170 ± 103	1576 ± 53	891 ± 57	594 ± 27
Control (45)	2042 ± 79	1448 ± 63	780 ± 33*	594 ± 23*
UV-B treatment (45)	2096 ± 83	1472 ± 65	742 ± 34	624 ± 23
Control (55)	1811 ± 93	1172 ± 63	511 ± 24	639 ± 28
UV-B treatment (55)	1834 ± 96	1189 ± 58	523 ± 23	645 ± 23

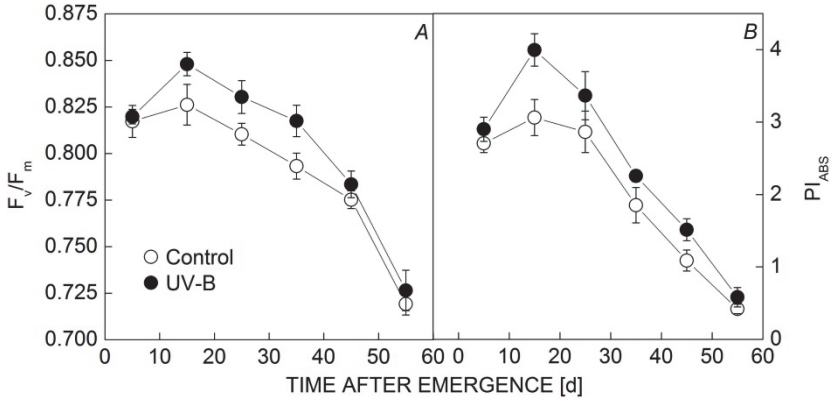

Fig. 2. Changes in the maximal quantum efficiency (F_v/F_m; *A*) and performance index of light absorption (PI_{ABS}; *B*) in the PSII reaction center for the control and UV-B treated functional leaves. Data are means ± SD (n = 25).

(Guidi and Soldatini 2002). According to Strasser *et al.* (2000), PI_{ABS} is a multiparametric expression, taking into account the independent parameters contributing to photosynthesis, namely absorption (RC/ABS), the quantum efficiency of trapping [$\varphi_{P0}/(1 - \varphi_{P0})$] and the efficiency of conversion of trapped excitation energy to electron transport [$\Psi_0/(1 - \Psi_0)$].

Fig. 2 showed that F_v/F_m and PI_{ABS} maintained relatively high values from 5 DAE to 25 DAE, and significant decreases of F_v/F_m and PI_{ABS} occurred after 35 DAE. F_v/F_m and PI_{ABS} of plants with UV-B treatment were, respectively, 1.63 and 21.83% higher than the control. PI_{ABS} decreased more than F_v/F_m at all stages of observations. Variations in F_v/F_m and PI_{ABS} between control and treatment were significant (P<0.05, t-test).

Photosynthetic electron transport and photophosphorylation: To further elucidate changes in primary photochemical reactions, the activities of photosynthetic electron transport, Ca^{2+}-ATPase and photophosphorylation were investigated using isolated thylakoids. As shown in Fig. 3*A*, although UV-B treated leaves did not have the highest electron transport values, there were later and smaller decreases in electron transport activity than in the control, which indicated that the deterioration of the electron transport chain occurred later. The mean of all ETR values measured over 55 days was 2.5% higher than the average of the control.

Ca^{2+}-ATPase and photophosphorylation activities of the control exhibited a similar trend, namely increasing till 15 DAE, after maintaining relatively high values for the same period and then decreasing significantly (Fig. 3*B*,*C*). Ca^{2+}-ATPase and photophosphorylation activities in UV-B treatment decreased, respectively, 12.5 and 10.1% than the control before 35 DAE. But UV-B treated leaves showed later and slower decreases in both Ca^{2+}-ATPase and photophosphorylation activities than control.

Key enzyme activities of the dark reaction: Fig. 4*A* shows that Rubisco activity reached the highest value at approximately 15 DAE and declined sharply afterwards. Enhanced UV-B radiation reduced Rubisco activity by 19.78%, 24.07%, 25.38%, 18.85%, 10.66%, and 10.38%, respectively, at 5, 15, 25, 35, 45, and 55 DAE. There was

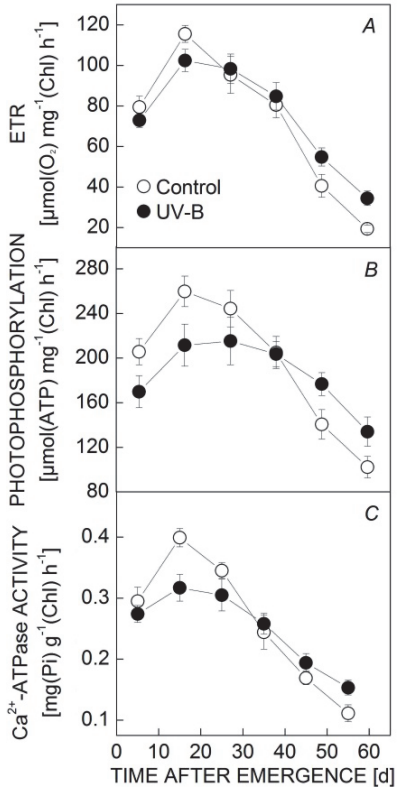

Fig. 3. Photochemical activities *vs.* time after emergence of flag leaves for: whole chain electron transport rate (ETR) (*A*; $H_2O \rightarrow MV$), photophosphorylation (*B*), and Ca^{2+}-ATPase activity (*C*). Vertical bars represent the standard errors for each mean ($n = 3$).

a significant difference ($P<0.05$, *t*-test) in Rubisco activity between UV-B treatment and the control from the emergence of flag leaves to 35 DAE. The activity of PEPcase, a key carboxylase in the C_4 photosynthetic pathway, increased after the emergence of flag leaves and peaked at around 25 DAE for LYPJ. UV-B treated leaves displayed a lower activity of PEPcase, and there was a significant difference before 35 DAE ($P<0.05$, *t*-test), but afterwards the plants began to recover from UV-B induced decline (Fig. 4*B*).

Chloroplast ultrastructure of flag leaves: At 5 DAE, chloroplasts of the flag leaves of the control appeared mostly as shuttle shapes, and the chloroplasts were lined up close to the cell periphery. A typical membrane structure was also recognized as a double membrane in the chloroplast envelope. Stromal thylakoids were parallel to each other, and grana were tightly connected by intergranal or stroma thylakoids (Fig. 5*A*). However, the development of chloroplasts in UV-B treated flag leaves was delayed. Chloroplasts in these leaves were usually shuttle-shaped but they were more rounded, the number of granal thylakoid was relatively lower, and the structure of layers was in a looser arrangement. We observed the appearance of plastoglobuli at this stage (Fig. 5*B*).

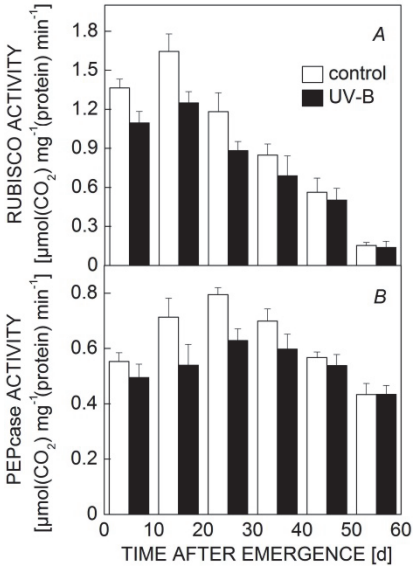

Fig. 4. Activities of ribulose-1,5-bisphosphate carboxylase (Rubisco; *A*) and phospho*enol*pyruvate carboxylase (PEPcase; *B*) between control and UV-B treatment are shown from leaf emergence to senescence. Vertical bars indicate SE of the mean value for 3 independent samples.

At 25 DAE, more and larger chloroplasts occurred in cells and chloroplasts were almost oblong in shape. Chloroplasts of the control contained granal thylakoids and were full of medium-density media. Although plastoglobuli developed further, chloroplasts displayed a typical thylakoid organization, with grana and stroma lamellae arranged in neat rows, all embedded in a stromal matrix and bounded by a double-membrane envelope in UV-B treated flag leaves (Fig. 5*C*,*D*).

At 45 DAE, the ultrastructure of chloroplasts in the control had highly changed morphology. The control chloroplasts changed from a longer to a round shape. A parallel pattern of disorganized lamellae and the orientation of the grana were considerably disrupted. Due to the large accumulation of starch grains, these chloroplasts had a considerably reduced system of granal and stromal thylakoids. Large plastoglobuli were identified, and starch grains became larger (Fig. 5*E*). In the UV-B treated flag leaves, chloroplasts were morphologically deformed and shorter, almost lens-like. Besides, more lipid droplets were observed in chloroplasts. Chloroplasts had smaller and fewer plastoglobuli than the control, while more stromal thylakoids were parallel to each other and were arranged in neat rows (Fig. 5*F*).

At 55 DAE, close to harvesting time, the ultrastructure of the chloroplasts in the two groups changed significantly. For the control, the chloroplasts were swollen and the structure was deformed, the chloroplast matrix zone expanded and the lamellae structure became loose. Thylakoids were severely swollen, and grana disappeared, forming more and larger intrathylakoid fragments (Fig. 5*G*). Although the peripheral double membrane of the chloroplasts and the internal system of granal and intergranal thylakoids became disorganized, and their stratiform structure became inconspicuous, but there were still complete chloroplasts in the cells, smaller plastoglobuli and lipid droplets were identified, and more stromal thylakoids were closely arranged in the UV-B treated leaves. (Fig. 5*H*).

Discussion

The effects of low-intensity UV-B treatment on photosynthetic and biochemical activities were systematically evaluated under natural condition, mainly focusing on the light reactions, CO_2 assimilation, and chloroplast ultrastructure.

Impact on gas exchange and PSII-related parameters: Because proteins in the plants can absorb UV light, they are easily damaged (Wu *et al.* 2011). UV-B radiation might affect the photosynthetic pigments, either through inhibition of their synthesis or effects on the enzymes involved in the Chl biosynthetic pathway (Ranjbarfordoei *et al.* 2011). However, some studies have shown that UV-B radiation has no effect on Chl, and even increased pigment content (Rech *et al*. 2005, Ruhland *et al*. 2005, Liu *et al*. 2007). Fig. 1*A* showed that the Chl content was greater after UV-B treatment than the control during the late reproductive stage between 25 DAE and 55 DAE, showing that the process of leaf senescence was delayed. Gas exchange was severely affected at the beginning of UV-B exposure (Fig. 1*B,C*). In the irradiated leaves, P_N was reduced to 84.8% and 92.0% of the control, respectively, at 5 and 15 DAE. That was accompanied by strong g_s reductions of 24.2% and 13.8%, respectively for the same days. Reflecting clear performance reductions on the photosynthetic machinery resulted in lower biomass and yield in most crop plants. However, in the leaves developed entirely at the end of the UV-B treatment, two much lower impacts on P_N and g_s were observed.

It has been widely accepted that the main target site of UV-B radiation in the photosynthesis system is PSII (Allen *et al*. 1999, Albert *et al.* 2005). Recent research reported that solar UV-B radiation effects on PSII are small and transitory (Kolb *et al.* 2001, Xiong *et al*. 2001, Xu *et al*. 2007). In the present study, the increased trapping probability of primary photochemistry, TR_0/ABS, in combination with the increased quantum yield of electron transport (ET_0/ABS) led to lower dissipation of the absorbed energy (DI_0/ABS) under UV-B radiation (Table 2). Higher Chl content, increased ABS/CS_M, TR_0/CS_M, ET_0/CS_M and decreased DI_0/CS_M in the later growth stage, might contribute to higher light-saturated photosynthetic activity of UV-B treated leaves. F_v/F_m is often used to characterize the degree of photoinhibition. Higher F_v/F_m implies that UV-B treated leaves of LYPJ were more tolerant to photoinhibition of photosynthesis in the field. Compared with F_v/F_m, PI_{ABS} is a more sensitive parameter for some stresses and suitable as an indicator to distinguish the photosynthetic performance (Appenroth *et al.* 2001, Van Heerden *et al.* 2004).

Impact on thylakoid whole chain ETR and photophosphorylation: Photosynthetic electron transport links not only primary photophysical and photochemical reactions with biochemical reactions but also oxygenic photosynthesis with photophosphorylation. Activity of electron transport can reveal photosynthetic efficiency of crops. Wilson and Greenberg (1993) and Babu *et al.* (1999) proposed that damaged D1 and D2 might be the immediate reason for a reduction of electron transfer capacity. In this study, the electron transport flux in UV-B treatment was higher than control, although the values of electron transport rate were not significantly different (Fig. 3*A*).

Photophosphorylation uses light energy to transform ADP into ATP. Photophosphorylation activity represents the capacity of chloroplasts to produce ATP and transfer light energy to chemical energy (Wei *et al*. 2003). Photosynthetic electron transport and transmembrane proton electromotive force coupled with phosphorylation play a broad regulatory role for the operation of the photosynthetic apparatus. The reaction of photophosphorylation was catalyzed by ATP-synthase in chloroplast thylakoid membranes. Lower Ca^{2+}-ATPase and photophosphorylation activities were obtained in the present study, suggesting modification in the transformation efficiency of electric energy into active chemical energy, hence leading to a decrease in photochemical capacity and CO_2 assimilation. On the contrary, a decrease in photochemical capacity and CO_2 assimilation may reduce the rate of NADPH and ATP utilization, hence altering the synthetic efficiency of photophosphorylation.

Impact on key enzymes in carbon fixation: Rubisco, accounting for more than 50% of the total stromal protein of chloroplasts in C_3 plants, is a key enzyme in carbon fixation and is also involved in metabolic pathways of photorespiration, in which organisms consume products synthesized during photosynthesis. Because of this, the loss of photosynthetic efficiency was more than 50% (Ashida *et al*. 2005). Jordan *et al.* (1992), Desimone *et al.*

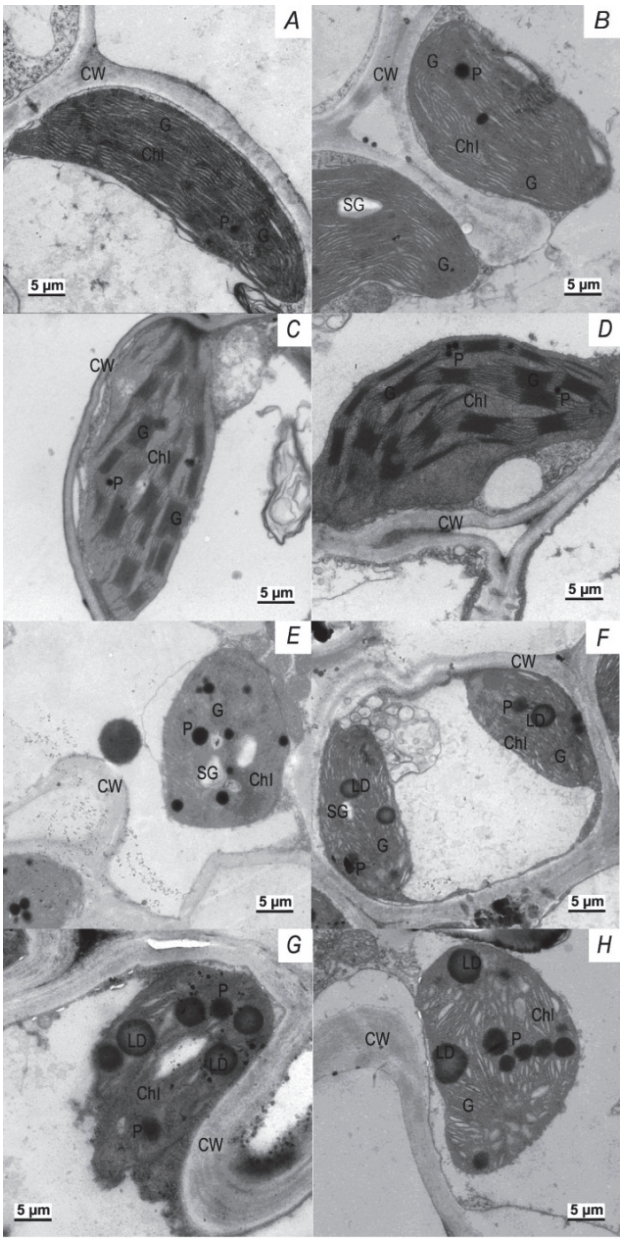

Fig. 5. Changes in the ultrastructure of chloroplasts from flag leaves in the control and UV-B treatment from flag emergence to senescence. Chl – chloroplast, CW – cell wall, G – granum thylakoids, LD – lipid droplet, P – plastoglobulus, SG –starch grain at 5, 25, 45, and 55 DAE, respectively. *A*, *C*, *E*, *G*: Chloroplast ultrastructure of flag leaves in the control at 5, 25, 45, and 55 DAE, respectively; *B*, *D*, *F*, *H*: Chloroplast ultrastructure of flag leaves in UV-B treatment at 5, 25, 45, and 55 DAE, respectively. Bars = 5 μm.

(1996, 1998) and Gerhardt *et al.* (1999) confirmed that prolonged UV-B radiation treatment could cause Rubisco content or activity to decline, parallel to a significant reduction in the mRNA level of Rubisco subunits, and the large subunit (LSU, 54 KDa) of Rubisco could change into a 66 kDa protein by oxidative modification under UV-B radiation. UV-B radiation sharply decreased the activity of Rubisco in LYPJ (Fig. 4*A*). Takeuchi *et al.* (2002) and Fedina *et al.* (2010) reported that loss of Rubisco was a primary factor in UV-B inhibition of CO_2 assimilation.

PEPcase play an important role in the metabolism of assimilates during the process of leaf senescence (Famiani *et al.* 2000). The ratio of PEPcase to Rubisco activity is an index showing the proportion of PEPcase in C_3 plant photosynthetic metabolism. This study showed that the PEPcase/Rubisco ratio of UV-B treated leaves increased to a greater extent than in the control during leaf aging. Why did the PEPcase/Rubisco ratio with UV-B treatment rise sharply at the end of the functional life of the leaves (Fig. 4*B*)? This might be attributed to the solubilization and degradation of Rubisco, while PEPcase was maintained at relatively high levels. In addition, this would mean that Rubisco is sensitive to UV-B treatment and susceptible to the inhibition.

Impact on chloroplasts ultrastructure: At the stage of full expansion of flag leaves under normal growth conditions, chloroplasts exhibited normal ultrastructure; most were of lens-like oblong shapes, with a typical close arrangement of granal and stromal thylakoids (Fig. 5*C*). With leaf aging, chloroplasts underwent significant alterations in their structure as well as in their biochemical properties. Chloroplast senescence first changed the chloroplast shape from elliptical to more spherical. Afterwards, an accumulation of starch and a gradual disturbance of thylakoid organization, including a distortion of granal arrangement, were accompanied by an increase in the number and size of translucent plastoglobuli. The lipid components of membranes that are synthesized but not utilized in thylakoid biosynthesis could be partially conserved as plastoglobuli (Ladygin 2004). Thus, plastoglobuli in senescing chloroplasts are thought to contain components from disintegrated thylakoid membranes.

Under long-term UV-B radiation, chloroplast development of flag leaves was significantly delayed. At 5 DAE, chloroplasts in these leaves were more rounded, the number of granal thylakoids was relatively smaller, and structure of layers was in a looser arrangement (Fig. 5*A,B*). However, after 5 DAE, especially in the late senescence, chloroplast membranes in UV-B treated flag leaves swelled and disintegrated, and more stromal thylakoids were parallel to each other and were arranged in neat rows, which indicated that the structure of chloroplasts was more complete and compact (Fig. 5*B,C,E–H*). Altered thylakoid membrane structure may directly affect membrane functionality and could have deleterious effects on photosynthetic activities of chloroplasts (Chen *et al.* 2004). Delayed Chl breakdown and maintenance of photosynthesis were accompanied by a less rapid breakdown of chloroplast grana, which may be responsible for a better manifestation of the primary light reaction in UV-B treatment.

Beside that, an accumulation of starch and an increase in the number of lipid droplets and translucent plastoglobuli showed that chloroplasts underwent some damage in the treated leaves. (Fig. 5*A–F*). It may be suggested that the excess accumulation of starch in chloroplasts was a result of the inhibition of carbohydrate transport. Carbohydrate accumulation in leaves when there is an imbalance between source and sink at the whole plant level can lead to a decrease in the rate of CO_2 assimilation. This result confirmed that the extent of damage of the chloroplast structure was consistent with the degree of photosynthetic inhibition.

In conclusion: Previous studies documented that plant populations from high UV-B habitats were more tolerant to enhanced UV-B (Hofmann *et al*. 2001, Ren *et al*. 2006). Teramura *et al*. (1991) also found that rice cultivars originating from regions either near the equator or at higher elevations exhibited a greater tolerance to UV-B radiation and showed an increase in total biomass and maximal photosynthetic rate due to an elevation in photosynthetic CO_2 uptake and photosynthetic O_2 evolution rate when exposed to supplemental UV-B radiation. LYPJ originated from a cross between Peiai 64S and 9311, and its parents were from low elevation areas or far from the equator. It seems that enhanced UV-B radiation tends to postpone its growth and decrease its photosynthesis. Low intensity UV-B treatment postponed the ageing, strengthened the absorption flux of photons and improved the electron transport activity at the reproductive stage. The damage of the chloroplast structure, the decreased activities of photophosphorylation and key enzymes in carbon fixation indicated the effects of UV-B radiation on photosynthetic efficiency may rely on changes in the cell structure and the completion of gene regulation of photosynthetic proteins. More definitive results could be attained by studying the acclimation response under a UV-B enhanced system.

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