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Characteristics of chlorophyll fluorescence and membrane-lipid peroxidation during senescence of flag leaf in different cultivars of rice

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Abstract

With *japonica* rice 98-08, *indica* hybrids Shanyou 63, Gangyou 881, and X07S/Zihui 100, and sub-species hybrid Peiai 64S/9311 as materials, chlorophyll (Chl) content, Chl *a* fluorescence parameters, and membrane lipid peroxidation in flag leaf were measured at late developmental stages under natural conditions. F_v/F_m, q_P, Φ_{PS2}, and electron transport rate gradually decreased while q_N increased conversely. Excessive photon energy led to the accumulation of active oxygen ($O_2^{\cdot-}$), H_2O, malonyldialdehyde, and products of membrane lipid peroxidation, and resulted in reduced Chl content and early ageing subsequent to the photooxidation during flag leaf senescence. There was obvious diversification of these parameters among rice cultivars. In comparison with *japonica* cv. 98-08 (tolerant to photooxidation), F_v/F_m decreased in *indica* cv. Shanyou 63 (susceptible to photooxidation) with greater accumulation of active oxygen and a sharp drop in Chl content, which resulted in "yellowish" early ageing, and affected the filling and setting of rice grains. The mechanism for premature ageing in *indica* rice was related to irradiance and temperature at filling stages. On a sunny day at above 25 °C, the reaction centre of photosystem 2 (PS2) exhibited a dynamic change on reversible inactivation. Under the intense irradiance at noon, PS2 function in *indica* rice exhibited obvious down-regulation and photoinhibition. Under intense irradiance with lowered temperatures, PS2 resulted in photo-damage and early ageing, related to the degradation of PS2-D1 protein and the inhibition of endogenous protection systems such as the xanthophyll cycle and enzymes scavenging active oxygen. Hence for high-yield breeding, based on a good plant-type and utilising heterosis and tolerance of photooxidation, the selection of *japonica* rice or a sterile line with the *japonica* genotype as female is a strategy worthy of consideration.

Additional key words: active oxygen; catalase; malonyldialdehyde; *Oryza*; peroxidase; photoinhibition; photooxidation; photosystem 2; premature ageing; superoxide dismutase; xanthophyll cycle.

Introduction

Indica hybrid rice is grown on more than 1 003 000 000 hectares [ha], making about 55 % of the total area of rice in China. Its yield is almost 20 % higher than that of routine rice cultivars in China being up to 9-10 t/ha, *i.e.* 0.9-1.0 kg m^{-2} (Cheng and Min 2000, Cheng and Zhai 2000). In the future, super-high yields may be achieved by using *indica-japonica* heterotic vigour (Yuan 1999). *Indica* and *indica-japonica* hybrids of rice may suffer from early ageing during late developmental stages according to observations recorded over many years. Thus early ageing which seriously restricts the potential for heterotic vigour remains a significant physiological problem.

Crop leaf senescence and premature ageing have systematically been studied and reviewed (Thomas and Stoddard 1980). After full expansion of crop leaves, the photosynthetic function weakens and Chl content gradu-

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Abbreviations: A – antheraxanthin; CA – carbonic anhydrase; CAT – catalase; Chl – chlorophyll; ETR – electron transfer rate; F_m – maximum fluorescence yield; F_0 – initial fluorescence yield; F_v – variable chlorophyll fluorescence; F_v/F_m – PS2 electron transport efficiency; MDA – malonyldialdehyde; $O_2^{\cdot-}$ – superoxygen anion; PEPC – phosphoenolpyruvate carboxylase; PPFD – photosynthetic photon flux density; POD – peroxidase; Φ_{PS2} – PS2 photochemical quantum yield; PS – photosystem; q_N – non-photochemical quenching; q_P – photochemical quenching; SOD – superoxide dismutase; V – violaxanthin; Z – zeaxanthin.
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ally decreases while photosynthetic components such as cytochrome *f*, ATPase, *etc.* and the key enzymes of carbon metabolism degrade (Ono and Watanabe 1997, Okada and Katoh 1998). The content of endogenous hormones such as cytokinin (Gan and Amasino 1988) drops and the content of abscisic acid (Millborrow 1974) is enhanced. Simultaneously, Chl degrading enzymes are activated, synthesis of δ-aminolevulinic acid (Castelfranco *et al.* 1991), a precursor of Chl, is low, and free radicals and membrane-lipid peroxidation products are accumulated (Lin *et al.* 1984, 1988). Under stress, the above physiological processes accelerate and result in early ageing of leaves (Sharkey *et al.* 1990, Lu and Zheng 1996). Moreover, the cultivar differences in early ageing are apparent (Murchie *et al.* 1999).

In this paper, six rice cultivars from different ecological areas in China were used to study the Chl fluorescence parameters and the performance of membrane lipid peroxidation in flag leaves under natural conditions. A discussion is included on the relationships between photoinhibition, photooxidation, and early ageing during flag leaf senescence in the late developmental stages. We have focused on the physiological basis for the mechanisms of early ageing and on the photosynthetic aspect of genetic approaches to crop improvement.

Materials and methods

Plants: Since 1990, more than 400 *indica* and *japonica* rice cultivars and 2 000 hybrid rice germplasms were identified according to their photooxidation and premature ageing. We found that most of *japonica* rice cultivars are more tolerant to photooxidation and have increased delay of leaf senescence as compared with the *indica* rice cultivars (Jiao *et al.* 1999). On this basis, *japonica* rice 98-08, *indica* rice X07S/Zihui 100, Ganyou 881, and Shanyou 63, and subspecies hybrid rice Peiai64s/9311 for the female parent with 50 % *japonica* element (Cheng and Zhai 2000) were selected as materials. The plants were grown in Nanjing City of China. Seeds were sterilised in 5 % H_2O_2 for 5 min, then soaked in water for 48 h at 32 °C, and sown. Plants of similar development stage were selected for transplanting into pots (5 hills per pot and 1 seedling per hill) and grown outdoors in a net room of Jiangsu Academy of Agricultural Sciences. Plants were watered and fertilised regularly. Grain yields were measured after harvesting all plants in pots. Physiological indexes related to photoinhibition and photooxidation and early ageing were measured during the late development stage.

Experimental methods: The Chl content, Chl fluorescence parameters, active oxygen generation, and the activity of scavenging enzymes in six cultivars were measured every 10 d under natural conditions at the late development stage. In the early filling stage, diurnal change of photochemical efficiency of PS2 (F_v/F_m) and physiological index related to photoinhibition were measured at noon of a clear day. In order to elucidate the influence of chilling temperature and strong irradiance at the late development stage, artificial treatment of chilling temperature and strong irradiance was established in the laboratory.

Treatment with chilling temperature and strong irradiance: Meteorology values of many years show every year a sharp drop of air temperature during the filling stage. Because the range and time of temperature drop was hard to be forecasted, the potted rice plants were put in the artificial climate room at the filling stage. The plants were divided into two groups and each group replicated three times. The first group (control) was irradiated by a PPFD of 1 050 μmol m^{-2} s^{-1} at day temperature of 26±1 °C for 12 h, night temperature was 22±1 °C, and relative humility 78-82 %. The second group (treatment) was irradiated for 12 h by a PPFD of 1 400 μmol m^{-2} s^{-1}, night temperature was 12±1 °C, and relative humility 78-82 %. Physiological measurements were carried out at 18:00 h after treatment of one day.

Chl content was determined according to Wellburn and Lichtenthaler (1984), after extraction in 16.8 M ethanol.

Chl fluorescence parameters were measured using an *FMS-2* fluorescence meter (*Hansatech*, UK) and calculated according to Genty *et al.* (1989). The rice leaves were dark-adapted for 10 min and then irradiated by weak modulated measuring beam (0.12 μmol m^{-2} s^{-1}) to determine the initial fluorescence yield (F_0). Maximum fluorescence yield (F_m) was determined during a saturating photon pulse (4 000 μmol m^{-2} s^{-1}). Variable Chl fluorescence (F_v) was calculated as $F_v = F_m - F_0$. Primary PS2 photochemical efficiency was expressed as F_v/F_m and photochemical and non-photochemical quenching coefficients were calculated as $q_P = (F_m' - F_s)/(F_m' - F_0')$, $q_N = (F_m - F_m')/(F_m - F_0)$, respectively. PS2 photochemical quantum yield $\Phi_{PS2} = (F_m' - F_s)/F_m'$. Electron transfer rate (ETR) was calculated from the fluorescence data according to the formula of Krall and Edwards (1992): $ETR = \Phi_{PS2} I$ a f, where I is irradiance, a is the estimated proportion of incident photons absorbed by leaf (usually 80 %), and f indicates an estimated value of the distribution proportion of energy in PS2, usually 50 % in C_3 plants.

Xanthophyll cycle components: According to the method of Zhao *et al.* (1995), 15 leaf discs of 0.5 cm diameter were harvested and then ground under low PPFD and 0-4 °C in a buffer containing 3.5 cm^3 of 100 % acetone, small amounts of quartz, and $CaCO_3$. The homogenate was centrifuged for 15 min at 10 000×*g*. The super-

natant was collected. Xanthophyll cycle components (produced by *Sigma*), antheraxanthin (A), violaxanthin (V), and zeaxanthin (Z), were assayed by an HPLC system. (A + Z)/(A + Z + V) represented the de-epoxidation of xanthophyll.

O_2^- generation rate: According to the method of Wang and Luo (1990), leaf segments (about 5 g fresh mass) were immediately homogenised using a chilled pestle and mortar with acid-washed quartz sand in 65 mM phosphate buffer (pH 7.8). The homogenate was filtered through 4 layers of *Miracloth*. The filtrate was centrifuged at 5 000×*g* for 10 min at 0-4 °C. 0.9 cm^3 phosphate buffer and 0.1 cm^3 10 mM hydroxylamine hydrochloride was added to 1 cm^3 of supernatant. This mixture was incubated at 25 °C for 20 min. 0.5 cm^3 of the incubated mixture was injected into 0.5 cm^3 17 mM *p*-aminobenzoic acid and 0.5 cm^3 17 mM α-napthaleneamine at 25 °C for 20 min. Afterwards the solution was shaken with equal volume of *n*-butanol and the mixture was centrifuged at 1 500×*g* for 5 min. Absorbance of the water phase was then measured at 530 nm. O_2^- production rate = O_2^- production/reaction time × the amount of protein [mM(O_2^-) kg^{-1}(protein) s^{-1}].

H_2O_2 content: According to the method of Patterson *et al.* (1984), 1 g leaf blades were homogenised in 3 cm^3 cold acetone. The homogenate was centrifuged for 10 min at 16 000×*g*. The reagents contained 0.1 cm^3 of 20 % $TiCl_2$ in concentrated HCl, 0.2 cm^3 in concentrated ammonia solution, and 1 cm^3 of supernatant. Peroxidation product, the Ti component, was washed five times with acetone, drained, and dissolved in 3 cm^3 of H_2SO_4 (1 M). Absorbance of the solution was measured at 410 nm. The standardisation curve of H_2O_2 was produced by a similar procedure.

Malonyldialdehyde (MDA): According to the method of Heath and Packer (1968), 0.5 g leaf blades were ground in a solution containing 5 cm^3 of 10 % trichloroacetic acid and a little quartz sand. The homogenate was centrifuged for 10 min at 3 000×*g*. 2 cm^3 of supernatant was collected and mixed with 2 cm^3 of 0.67 % TBA (m/v). After keeping in boiling water for 20 min and cooling fully, the mixture was centrifuged again for 10 min at 3 000×*g*. The absorbance of supernatant was measured at 532 and 600 nm with a spectrophotometer. The content of MDA was calculated by the following equation: $\varepsilon_{(532\ nm\ -\ 600\ nm)} = 1.55\times10^5\ (M)^{-1}\ cm^{-1}$.

Superoxide dismutase (SOD) activity was assayed by the method of Giannopolitis and Ries (1977). Leaf tissue was thoroughly ground with a mortar and pestle in an ice bath, until no fibrous residue could be seen. The grinding medium [4 000-6 000 cm^3 kg^{-1}(f.m.)] consisted of 0.1 M K_2HPO_4 and 0.1 mM EDTA (pH 7.8), plus homogenising glass beads. The homogenate was centrifuged twice at 13 000×*g* for 10 min in a *Sorvall RC2-B* refrigerated centrifuge at 0-5 °C. The supernatant is referred to as the crude SOD extract. SOD activity was assayed by its ability to inhibit the reduction of nitro-blue tetrazolium (NBT) by the O_2^T generation system xanthine oxidase producing an increase in absorbance at 560 nm of about 0.02 units per min at 25 °C in the absence of enzyme. One unit of SOD activity is defined as the amount of enzyme that causes 50 % inhibition of the initial rate of NBT reduction.

Catalase (CAT) activity: According to the method of Jiang and Wang (1982), 0.2 g leaf blades were put in precold mortar and homogenised in 1 cm^3 of 50 mM phosphate buffer (pH 7.8). The homogenate was centrifuged for 20 min at 10 500×*g* under 4 °C. The supernatant was crude enzyme. The 3 cm^3 of reagent contained 10 mM H_2O_2, 10 mm^3 of crude enzyme, and 50 mM phosphate buffer (pH 7.8). The change of optical density (OD) was measured at 240 nm. The enzyme activity was defined as the decrease of 0.1 ΔOD per minute.

Peroxidase (POD) activity: According to the method of Kochba *et al.* (1977), 0.2 g leaf blades were put in a precold mortar and homogenised in 1 cm^3 of 50 mM phosphate buffer (pH 7.8). The homogenate was centrifuged for 20 min at 10 500×*g* under 4 °C. The supernatant was crude enzyme. The 3 cm^3 of reagent [100 cm^3 of 50 mM phosphate buffer (pH 7.8) with 28 mm^3 guaiacol and 19 mm^3 of 30 % H_2O_2] was mixed with 10 mm^3 crude enzyme and the change of OD was recorded at 470 nm. The enzyme activity was defined as the increase of 0.1 ΔOD per min.

Fatty acids were analysed according to Yu and Su (1996). Lipids were methyl-esterified in solution of 0.4 M KOH and benzene-petroleum ether (1 : 1, v/v). The fatty acid methyl esters were separated by gas chromatography (*Shimadzu GC-17A*, Japan) supplied with a hydrogen flame detector and a capillary column *SP-2330* (15 m; i.d. 0.32 mm). The column was iso-thermally run at 165 °C and the detector was held at 250 °C. The standard reagents of fatty acids were purchased from *Sigma*.

D1 protein content: According to the method of Wu (1991), a stock solution of ^{14}C-atrazine [9.25×10^{11} Bq mol^{-1}] was diluted with buffer to 30, 60, 90, 150, 210, and 300 μmol m^{-3}. A 10-mm^3 aliquot of each of the above solutions was mixed with 1 cm^3 of chloroplast suspension. The mixture was heat-preserved for 5 min and centrifuged for 5 min at 16 000×*g*. Supernatant (0.5 cm^3) was added to 4.5 cm^3 of scintillation solution, 12 mM 2,5-diphenylozol (PPO) + 0.1 mM 1,4-di-[2'-(5'-phenyloxazolyl)]-benzene (POPOP) methylbenzene : *Triton X-100* (2 : 1, v/v). The final solution mixture was measured with a scintillation counter. The content of D1 protein was expressed as the reciprocal of Chl to bound ^{14}C-atrazine.

Measurement of electrolyte leakage: Leaves 2-cm-long were cut and submerged into 10 cm^3 of water in a cuvette, vibrated for 30 min, and then electrolyte leakage [$m\Omega\ cm^{-1}\ kg^{-1}$(f.m.)] of leaf plasmalemma was tested at 25 °C with an electrolytic meter (*DDS-307*, Shanghai, China).

Results

Changes in Chl content in flag leaf during senescence of six rice cultivars: Chl attenuation is consistent with the appearance of leaf colour and, therefore, the change in Chl content is usually considered a good index expressing leaf senescence (Thomas and Stoddart 1980). Flag leaf Chl content of six rice cultivars during natural senescence indicated differences among various rice genotypes (Fig. 1). In the heading stage, the Chl content in leaves of *japonica* rice 98-08 had only decreased by 25 %. In the mature grain stage, the Chl content remained at 4.5 g m^{-2} and the leaf blades remained green. In contrast, the Chl contents of *indica* rice Shanyou 63, Ganyou 881, and X07S/Zihui 100 were only about 1 g m^{-2} and decreased by 73 %; their leaf blades were yellowish. The Chl content changes in hybrid rice containing 50 % *japonica* element were situated between those of *indica* and *japonica* rice cultivars. Chl content at the mature stage was 2.0-2.5 g m^{-2} and had decreased by 67 %. We observed the Chl content attenuation in the six different cultivars. As compared with *japonica* rice, the Chl content in *indica* rice decreased quickly at temperatures above 25 °C. *Indica* rice exhibited obvious early ageing when the temperature dropped to 20 °C. These findings demonstrated that early ageing of different cultivars was related not only to the endogenous senescence process in genotypes, but was also influenced by sudden drops in temperature. The sudden decrease in temperature accelerated the early ageing of *indica* rice.

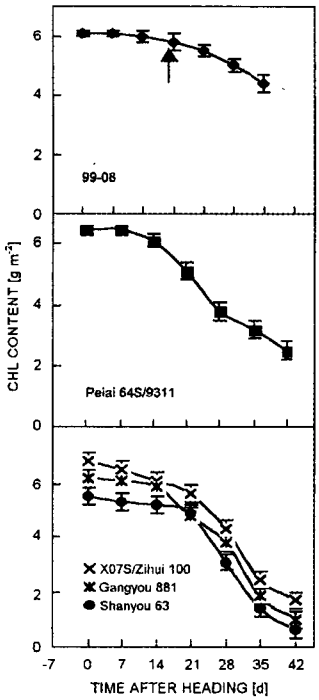

Fig. 1. Changes in chlorophyll content in different rice cultivars during senescence of flag leaves. ↑ means start of temperature below 20 °C.

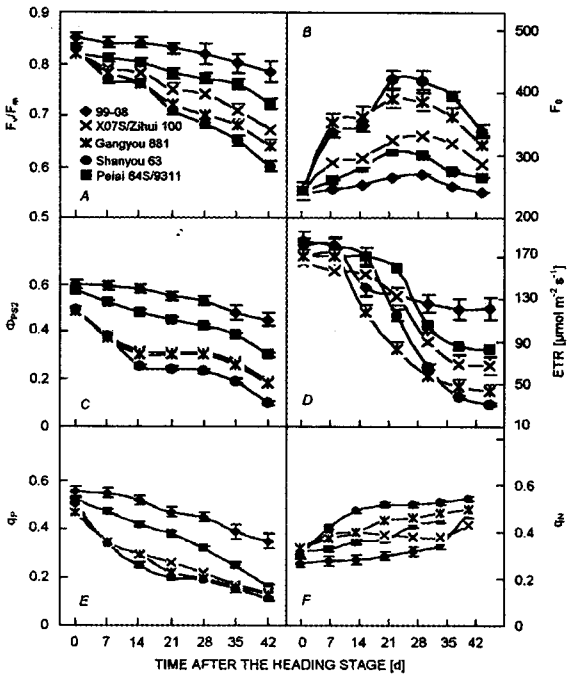

Fig. 2. Changes in chlorophyll fluorescence parameters in different rice cultivars during senescence of flag leaves.

Changes of Chl fluorescence parameters in six rice cultivars during senescence: The Chl fluorescence parameters are good indexes for assessment of the PS2 physiological state. Fig. 2 presents the Chl fluorescence parameters for six rice cultivars extending from heading to mature grain stage. F_v/F_m was consistent with Chl content during the senescence of flag leaves under natural conditions (Fig. 2*A*). F_v/F_m of *indica* rice experienced a greater decrease. F_v/F_m of hybrid rice containing 50 % *japonica* was situated between values for the *indica* and *japonica* rice cultivars. Values for F_v/F_m for the six rice cultivars were inversely proportional to the F_0 values (Fig. 2*B*). The enhancement of F_0 reflected the damage level to the PS2 reaction centre. PS2 photochemical quantum yields (Φ_{PS2}) expressed as the efficiency of captured PS2 excited energy multiplied proportionally in the open reaction centre (q_P), which reflects the actual photon energy transport efficiency when PS2 reaction centres are partially shut down. ETR indicates total elec-

tron transport rate (Fig. 2*D*). q_P indicates the photon fraction absorbed by antenna pigment used by PS2 or photochemical electron transport energy (Fig. 2*E*). In Fig. 2, the gradually decreasing changes in Φ_{PS2}, q_P, and ETR were consistent with the changes in F_v/F_m and Chl content during flag leaf senescence under natural conditions. The drop of PS2 photochemical efficiency and photon energy transport efficiency was one of the intrinsic physiological features of flag leaf senescence. Non-photochemical quenching (q_N) reflecting the photon energy absorbed by antenna pigment in PS2 was used for heat dissipation but not for ETR (Fig. 2*F*). Values for q_N and q_P in the six rice cultivars had opposite tendencies. The values of q_N for hybrid rice Shanyou 63, X07S/Zihui 100, and Ganyou 881 were high. The above-mentioned results indicate that there was greater efficiency of photon energy absorbed by the *japonica* leaf and converted into chemical energy. Greater electron flows were produced and transmitted to photosynthetic carbon metabolism as energy through a linear electron transmission chain with rapid speed under natural conditions. The efficiency of photon energy conversion for *indica* rice was low; the excessive photon energy, absorbed but not fully utilised for photosynthesis, was dissipated to protect the photosynthetic apparatus by increases in q_N. When subjected to stress under natural conditions, different rice genotypes had adapted by different means (Jiao and Ji 2001).

Metabolism of active oxygen in flag leaves in six rice cultivars during senescence: Excessive electrons react with molecular O_2 producing active oxygen during long-term photoinhibition at the reducing side of PS1 (Kochba *et al.* 1977). Since the assault of active oxygen resulted in membrane lipid peroxidation, photooxidative damage was apparent. As shown in Fig. 3*A,B,C*, the contents of $O_2^{\cdot-}$, H_2O_2, and MDA, the products of membrane-lipid peroxidation, obviously increased. Comparisons during flag leaf senescence and prior to yellowing revealed that the generation and accumulation of active oxygen in leaves of *japonica* 98-08 were less than those in *indica* hybrids Shanyou 63, Gangyou 881, and X07S/Zihui 100. Those in the hybrid rice such as Peiai 64S/9311 (containing *japonica* gene in their female lines) were in-between values for the *japonica* and *indica*. Internal protective enzymes and antioxidant materials scavenged the active oxygen in plants. The protective enzymes were SOD, CAT, POD, *etc.* SOD catalysed $O_2^{\cdot-}$ into less toxic H_2O_2, then H_2O_2 was decomposed into toxicity-free H_2O and O_2. As shown in Fig. 3*D*, during flag leaf senescence the SOD activity increased over the first seven days, then gradually decreased after the fifteenth days. Despite an apparent decreasing trend in activity by CAT, it continued to remain active to some extent in the leaves (Fig. 3*E*). The POD activity continuously decreased during leaf senescence (Fig. 3*F*). In comparison with *indica* rice, the scavenging system of active oxygen in *japonica* rice exhibited greater activity and maintained this level for a longer period of time. This would explain why the Chl content in *japonica* decreased more slowly, and the leaves were still green at maturation.

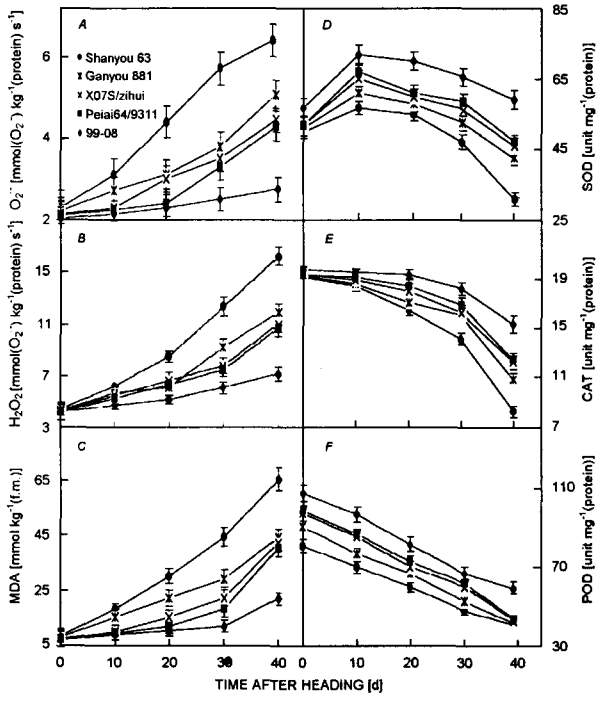

Fig. 3. Changes in active oxygen generation and scavenging enzyme activities in different rice cultivars during senescence of flag leaves.

Correlation among parameters of Chl fluorescence, the indexes of active oxygen metabolism, and Chl content in six rice cultivars during the late developmental stages: The correlations among parameters of Chl fluorescence, the contents of active oxygen and Chl in six rice cultivars were analysed (Table 1). The accumulation of active oxygen and the products of membrane lipid peroxidation were less and the Chl content was higher, exhibiting tolerance to premature ageing. Furthermore, the Chl content in rice leaves in the late developmental stages significantly correlated with the seed set, $r^2 = 0.81^{**}$ ($n = 18$). Hence there were close relationships between the degradation of Chl, photoinhibition, and photooxidation in rice; the early degradation of Chl (premature ageing) was an important effect on the seed set.

Diurnal change of F_v/F_m in six rice cultivars during the late developmental stages: The diurnal changes in F_v/F_m for flag leaves of *japonica* cultivar 99-08 and *indica* cultivar Shanyou 63 in the early filling stage showed a decrease of PS2 photochemical efficiency with increasing irradiance (Fig. 4). At noon on a clear day, the irradiance usually exceeded 1 400 μmol m^{-2} s^{-1} while the value of F_v/F_m was at its lowest. But at 17:00, there was a return to morning values, which indicated a dynamic diurnal change in PS2 photochemical efficiency and re-

versible inactivation. Obviously, the photoinhibitory extent in *japonica* 99-08 was more alleviated than that in Shanyou 63. In comparison with the morning values, there was no evidence of degradation of the D1 protein in the two cultivars at noon, demonstrating that the dynamic diurnal change in F_v/F_m resulted from the reversible inactivation of PS2 reaction centre (Fig. 4*C*). In comparison with the morning values, the generation rates of $O_2^{\cdot-}$ and MDA content in *japonica* cultivar 99-08 did not increase to the extent of *indica* Shanyou 63 cultivar at noon (Fig. 4*G,H*), demonstrating that *indica* rice not only suffered

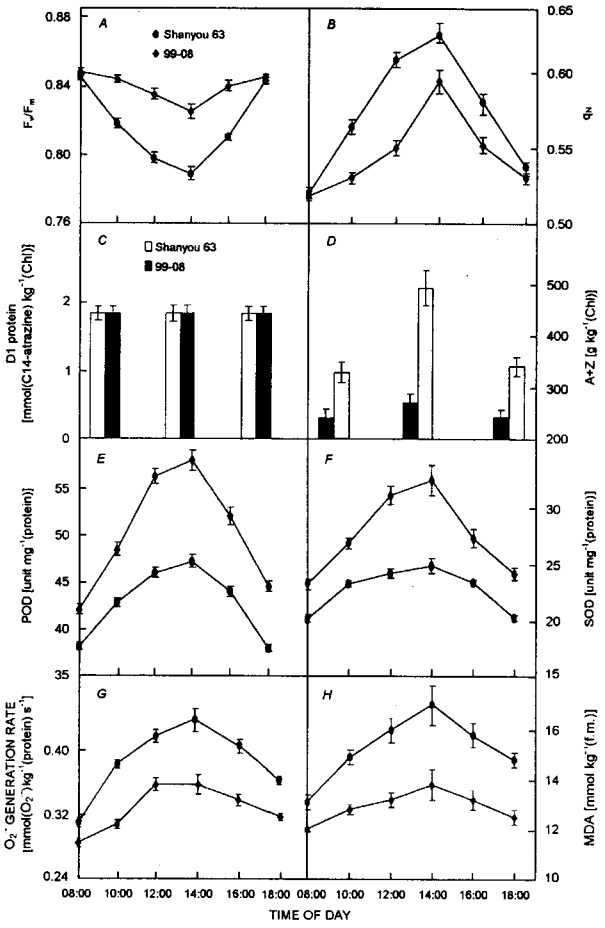

Fig. 4. Diurnal changes in fluorescence parameters and metabolism of active oxygen in different rice cultivars at heading stage.

from obvious photoinhibition, but also displayed slight photooxidation. The value of q_N in *indica* rice was higher at noon, which may account for a greater decrease in F_v/F_m. The change of q_N was consistent with that of A + Z of the xanthophyll cycle (Fig. 4*D*). Although the xanthophyll cycle in *indica* rice functions to dissipate excessive photon energy, due to decreased SOD activity the accumulation of $O_2^{\cdot-}$ and MDA in *indica* was greater. This was consistent with the greater decrease in F_v/F_m and the greater accumulation of $O_2^{\cdot-}$ and MDA (Fig. 3) under natural conditions. The long-term continual accumulation of $O_2^{\cdot-}$ before onset of low temperatures results in a certain decrease in Chl content in *indica* rice.

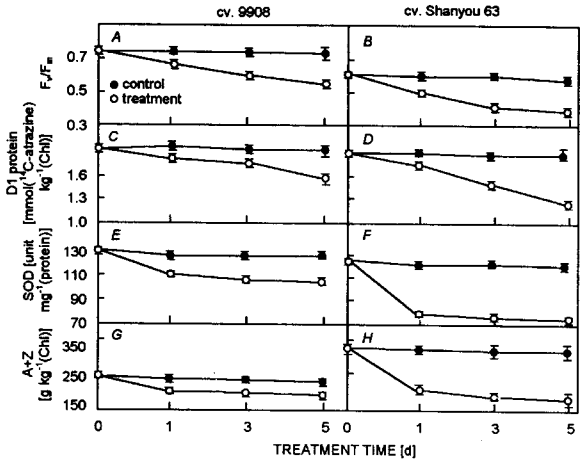

Fig. 5. Changes in physiological indexes associated with photoinhibition and photooxidation in leaves of *japonica* cultivar 9908 and *indica* cultivar Shanyou 63 at filling stage under strong irradiance and low temperature.

The photochemical efficiency and metabolism of active oxygen in leaves of *indica* and *japonica* under stress of low temperature accompanied by intense irradiance: After one-day treatment with low temperature accompanied by intense irradiance, the photochemical efficiency (F_v/F_m) in leaves of *japonica* 99-08 decreased by 17 % *vs.* 37 % in Shanyou 63 (Fig. 5*A*). This was a manifestation of the lower conversion efficiency of photon energy in *indica*. With decreases in F_v/F_m, the generation

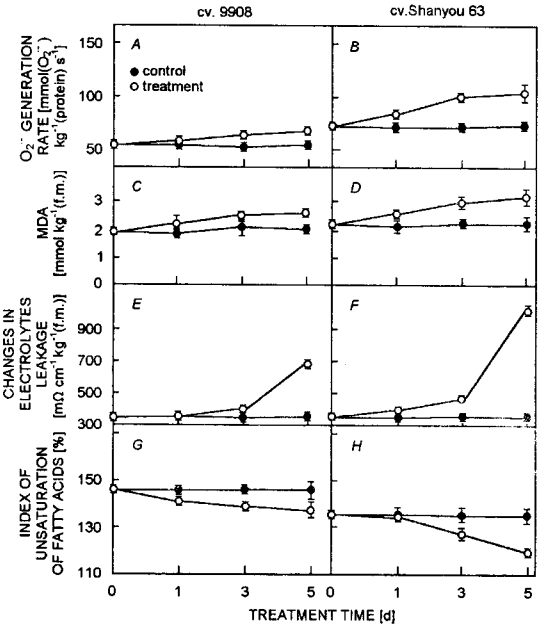

Fig. 6. Membrane lipid peroxidation and change of unsaturation degree of fatty acids in *japonica* cultivar 9908 and *indica* cultivar Shanyou 63 under strong irradiance and low temperature.

rate of $O^{\cdot-}$ in leaves of both cultivars increased (Fig. 6*A,B*), however, there was a smaller increase in *japonica* cv. 99-08 than in *indica* cv. Shanyou 63. The changes of MDA content followed correspondingly to changes in $O^{\cdot-}$ generation rate. Hence in the *indica* cv. Shanyou 63 membrane lipid peroxidation easily occurred. In connection with the correlation analysis in Table 1, these results demonstrate that an impediment to conversion of photon energy in leaves may be the major reason for membrane lipid peroxidation and early leaf ageing if the stress conditions are encountered by the rice in late developmental stages.

To investigate the mechanism for differences in photon conversion efficiency and membrane lipid peroxidation in rice subspecies under the same stress conditions, we further observed the functions of D1 protein in the PS2 reaction centre, xanthophyll cycle, and SOD. In Fig. 5*A,B,C,D*, changes in D1 protein in the photosynthetic apparatus of the two rice cultivars were consistent with the pattern of changes in F_v/F_m. Comparing the two rice cultivars after one day of stress treatment, the extent of the decrease of D1 protein content in Shanyou 63 was significant, but at the same time, Shanyou 63 suffered the effects of more serious photoinhibition. It seemed that the change in D1 protein content in the PS2 reaction centre in rice might account for the physiological basis of change in F_v/F_m under this stress. Fig. 5*G,H* presents the change of the components of the xanthophyll cycle under these stress conditions. After one day of treatment, A + Z in *indica* and *japonica* rice decreased by 37.2 and 16.4 %, respectively. Previous research discovered that the D1 protein content and F_v/F_m decreased further when the xanthophyll cycle was inhibited (Jiao *et al.* 2001). This may be one reason why both the content of D1 protein and F_v/F_m in *indica* cultivar Shanyou 63 were lower than those in *japonica* cv. 99-08 under the stress. Additionally, during the stress there was an obvious decrease of SOD activity in rice leaves, but the decrease of SOD activity in *indica* Shanyou 63 was greater than that in *japonica* 99-08 (Fig. 5*F*). The above results demonstrated that under the stress the change of D1 protein content in the photosynthetic apparatus and A + Z content in component of xanthophyll cycle decreased more in *indica* Shanyou 63. And thus the conversion efficiency of photon energy and capability for thermal dissipation diminished along with the decreased activity of SOD and the decreased capability of scavenging active oxygen, which resulted in increased exacerbation of membrane lipid peroxidation.

In order to clarify the rationale for the easy degradation of D1 protein, xanthophyll cycle, and SOD, we observed the diminished content of unsaturated fatty acids and intolerance to low temperature in *indica* rice (Fig. 6*G,H*), consequently leading to increased electrolyte leakage from membranes. Therefore, the above-mentioned functional proteins were easily degraded and/or their activities decreased.

Table 1. Correlation analysis for the parameters of chlorophyll (Chl) fluorescence, indexes of active oxygen, and Chl content for six rice cultivars at late developmental stages. The correlation coefficient significance is expressed as $^{**}p < 0.01$, $^{*}p < 0.05$. In the correlation analysis between the parameters of Chl fluorescence and Chl content, $n = 12$. In the correlation analysis between the indexes of active oxygen and the parameters of Chl fluorescence and Chl content, $n = 30$.

	Φ_{PS2}	ETR	q_P	q_N	$O_2^{\cdot-}$	H_2O_2	MDA	SOD	CAT	POD	Chl
F_v/F_m	0.913**	0.802**	0.934**	-0.951**	-0.897**	-0.872**	-0.942**	0.839**	0.894**	-0.376**	0.864**
Φ_{PS2}		0.724**	0.962**	-0.884**	-0.724**	-0.737**	-0.764**	0.749**	0.735**	-0.129**	0.703**
ETR			0.814**	-0.618**	-0.921**	-0.916**	-0.910**	0.752**	0.858**	-0.612**	0.874**
q_P				-0.847**	-0.812**	-0.803**	-0.838**	0.736**	0.781**	-0.041	0.780**
q_N					0.703**	0.697**	0.714**	-0.638**	-0.665**	0.037	-0.627**
$O_2^{\cdot-}$						0.991**	0.956**	-0.951**	-0.910**	0.502**	-0.907**
H_2O_2							0.970**	-0.858**	-0.969**	0.536**	-0.908**
MDA								-0.932**	-0.858**	0.533**	-0.900**
SOD									0.892**	0.249	0.838**
CAT										0.531**	0.879**
POD											0.519**

Discussion

Leaf senescence is the last phase during the leaf growth process. During leaf senescence, the decline of the metabolic level and the degradation of mass molecular materials such as proteins, mRNA, and rRNA occur. At the same time, the transformation from mobile substance to grain occurs. Thus normal physiological processes in each plant must complete their life cycles (Thomas and Stoddart 1980). This phase is not only controlled by the interaction of nucleus-plastid genomes, but may also be accelerated by inappropriate environmental factors. Many reports have confirmed that leaf senescence usually correlates with early ageing of the root system, in coordination of source/sink during physical (Sharkey *et al.* 1990) and chemical (Duan *et al.* 1997) stresses. As for the mechanism of early ageing, researchers observed that ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO)

is the first degraded enzyme in the Calvin cycle when comparing light and dark reactions during early senescence in leaves of wheat, speculating that the reason for premature ageing is a "light-carbon imbalance" (Zhang *et al.* 1999). Active oxygen accumulates and membrane lipid peroxidation occurs in leaves during premature ageing (Lin *et al.* 1984, 1988); this supports the "free radical damage" theory. Even the above-mentioned degradation of RuBPCO also results from oxygenation (Peng *et al.* 2000). Recent studies of the relationship between Chl fluorescence characteristics and membrane lipid peroxidation in leaves during photooxydation (Lin *et al.* 1999) lead to a proposal that photon energy conversion in PS2 and metabolism of active oxygen are closely related to premature ageing. Aligned with this theory, we attempted to disclose from the viewpoint of photon energy conversion under natural conditions the rationale for premature ageing in rice. We suggest that while photon energy conversion and photochemical activity gradually decline in flag leaves, the metabolism of active oxygen caused by excessive photon energy and membrane lipid peroxidation gradually strengthens; these are important physiological mechanisms resulting in leaf senescence. But there exists a genotype difference in the process of senescence in the flag leaf. In comparison with *indica* rice, *japonica* rice is tolerant to photoinhibition/photooxidation and resistant to early ageing. Under low temperature and strong irradiance, the higher content of unsaturated fatty acids in thylakoids and the decreased degradation of D1 protein, higher activity and longer duration induced of SOD, and the active xanthophyll components exhibited less inhibition at late development stage.

Examining the correlation among the indexes of photoinhibition/photooxidation in *japonica* or *indica* rice (Table 1), measurement of the photon energy conversion efficiency is a key index that may forecast earlier photooxidation in the cultivars. Also, the physiological bases determining photon energy conversion efficiency in rice are the D1 protein encoded by plastid gene and the protective function of xanthophyll cycle and SOD, both controlled by the nuclear gene (Jiao *et al.* 2001). The capacity for D1 protein synthesis is most important among various protective strategies in photon energy conversion (Anderson *et al.* 1995); this may explain why *japonica* rice did not easily suffer effects from photoinhibition, photooxidation, and premature ageing. Photon energy conversion and active oxygen metabolism represent only one aspect of premature ageing according to other research reports. Leaf senescence and early ageing are complex genetic and physiological phenomena in which the molecular mechanism and genetic regulation deserve thorough study from many aspects such as metabolism, hormone influences, and genetic basis. Researchers attempted to delay leaf senescence through genetic engineering by introducing the IPT gene control in the biosynthesis of cytokine (Gan and Amasino 1995). In the future, with the development of molecular biology, more research attention will be devoted to the delay of early ageing through more advanced biotechnology.

On the basis of current good plant-type, utilising hetaerists and preventing premature ageing, our strategy was to determine which *japonica* sterility line or sterility line with *japonica* gene as maternal line should be used in rice breeding. In order to select the sterility line tolerant to photooxidation and resistant to premature ageing, we adopted a simple technique for selection of germplasm (Jiao 1992, Jiao and Li 2001). Additionally, we decided to introduce our selection of transgenetic PEPC rice germplasm (Jiao *et al.* 2001, 2002) with higher photosynthetic efficiency and tolerant to photooxidation by introducing PEPC gene from maize into rice by *Agrobacterium*-mediated transformation. Using this excellent germplasm, we incorporated the traits of tolerance to photooxidation and higher photosynthetic efficiency into a sterility line and/or for restored line (Li *et al.* 2001). Thus the physiological breeding combines conventional breeding techniques with genetic engineering.

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