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The photosynthetic function analysis for leaf photooxidation in rice

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Abstract

Photooxidative damage causes early leaf senescence and plant cell death. In this study, a light-sensitive rice cultivar, 812HS, and a non-light-sensitive cultivar, 812S, were used to investigate early leaf photooxidation. Leaf tips of 812HS exhibited yellowing under a light intensity of 720 μmol(photon) m^{-2} s^{-1}, accompanied by a decrease in chlorophyll and carotenoids, but 812S was unaffected. The photosynthetic performance of 812HS was also poorer than that of 812S. The H_2O_2, $O_2^{\bullet-}$, and malondialdehyde content increased sharply in 812HS, and associated antioxidant enzymes were inhibited. The degradation of core proteins in both PSI and PSII, as well as other photosynthesis-related proteins, was accelerated in 812HS. When shaded [180 μmol(photon) m^{-2} s^{-1}], 812HS recovered to normal. Therefore, our findings suggested excess light disturbed the balance of ROS metabolism, leading to the destruction of the antioxidant system and photosynthetic organs, and thus triggering the senescence of rice leaves.

Keywords: photooxidation; reactive oxygen species; rice; shading; thylakoid membrane proteins.

Introduction

Photosynthesis is the only mechanism by which organisms convert light energy into a form that can be used directly (Zubik *et al.* 2011). However, the key abiotic factor limiting photosynthesis is also 'light' (Li *et al.* 2018). When experiencing a switch from normal light to high light intensity, plants can rapidly become photoinhibited, and their photosynthetic capacity will decrease (Kandler and Sironval 1959). When plants are exposed to strong light for a long period and photosynthesis is completely inhibited, a large amount of ROS and other substances induced by light will accumulate, which would seriously affect the metabolism of chloroplasts, resulting in the degradation of photosynthetic pigments. As a result, it transforms the initial photoinhibition into photooxidation (Kandler and Sironval 1959).

The damage of photooxidation to plants is mainly expressed in the destruction of membrane systems, enzyme activity, nucleic acid, and other substances in cells by numerous ROS, which destroys cell components and eventually may lead to plant death (Hernández and Munné-Bosch 2015, Choudhury *et al.* 2017, D'Alessandro *et al.* 2020). Although the exact mechanism of photooxidation damage is still unclear, it has been confirmed that potentially damaging ROS are produced in three parts of the photosynthetic apparatus: the LHCs, PSII reaction center, and PSI receptors associated with

Highlights

- High light caused rice leaf senescence
- The balance of ROS metabolism in rice was perturbed by high light
- Lower photosynthetic performance was demonstrated in rice when exposed to high light

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Abbreviations: CAT – catalase; C_i – intercellular CO_2 concentration; E – transpiration rate; FM – fresh mass; g_s – stomatal conductance; LHC – light-harvesting pigment complex; P_N – net photosynthetic rate; POD – peroxidase; ROS – reactive oxygen species; SOD – superoxide dismutase.

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PSII. PSII is usually considered to be the main site of photooxidation damage (Karpinski *et al*. 2001). Plants have evolved defense and repair systems to minimize damage to photosynthetic organs and enhance their tolerance to strong light (Konert *et al.* 2013). An example is the repair and protection mechanism of the D1 protein, which can be resynthesized to form a functional PSII under suitable conditions (Wang *et al*. 2016). The xanthophyll cycle can also alleviate photooxidation damage of chloroplasts through the dissipation of excess light energy. The most important is the protective effects of ROS-scavenging systems, including enzyme-induced and non-enzyme-induced systems. The main components of the enzyme-induced ROS scavenger systems are superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), which can synergistically remove $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$ in cells and prevent membrane lipid peroxidation. The non-enzymatic-induced systems are mainly composed of small antioxidant molecules, such as carotenoids (Car), ascorbic acid (AsA), and other antioxidants (Wu *et al*. 2007, Kreslavski *et al*. 2013, Sujatha *et al*. 2019).

The light-sensitive (812HS) and non-light-sensitive (812S) rice varieties were supplied by the Jiangsu Academy of Agricultural Sciences. The leaves of 812HS exhibited normal color under weak light or severe shade, while the leaf tips showed 'yellowing' when light intensity increased to 720 µmol(photon) m^{-2} s^{-1}; the non-light-sensitive cultivar, 812S, can grow normally. In recent years, the utilization of rice leaf color mutants has attracted much attention, and they have become important for the study of photosynthesis, chlorophyll biosynthesis, chloroplast structure and function, genetic development regulation mechanisms, and crop marker characters (Yu *et al*. 2016, Nguyen *et al*. 2020, 2021). The objective of our study was to improve our understanding of rice photooxidation by exploiting the photosensitivity of 812HS to investigate the early performance and changes to the thylakoid membrane proteins in the field under high light and shading treatments.

Materials and methods

Plant materials and growth conditions: Four-week-old rice seedlings (the third leaf at the fully expanded stage) of 812HS and 812S were transplanted to experimental fields on the Xianlin campus, Nanjing Normal University, Nanjing, China (32°03'N, 118°47'E), and grown under natural light. The first measurement and sampling were carried out on 25 June 2019; the light intensity reached 450 µmol(photon) m^{-2} s^{-1}. The rice plants were divided into shaded (black shading net) and non-shaded treatments, keeping water and fertilizer management, temperature, and other growth conditions the same. The second measurement and sampling were conducted on 10 July when light intensity reached 720 µmol(photon) m^{-2} s^{-1} in the natural light treatment and 180 µmol(photon) m^{-2} s^{-1} in the shaded treatment. Leaf samples were taken within 1/3 of the distance from the leaf tip and were quickly frozen and stored at –80°C for subsequent measurement. All measurements were replicated at least three times.

Phenotype investigation, pigment determination, and photosynthetic activity measurement: Observations and photographs of phenotypes were taken on 25 June and 10 July in both shaded and natural light treatments. Fresh leaves (0.1 g) were cut into pieces and soaked in 5 mL of acetone:ethanol:water mixture (4.5:4.5:1) for 12–16 h until the pigments were completely dissolved. Then the absorbance of the mixture was measured at 470, 645, and 663 nm on a spectrophotometer (*Genesys 10*, *Thermo Electron*, USA) (Lichtenthaler 1987).

A portable photosynthesis system (*CIRAS-3*, *PP System*, Hitchin, UK) was used to measure the photosynthetic parameters of 821HS and 812S. The net photosynthetic rate (P_N), stomatal conductance (g_s), transpiration rate (E), and intercellular CO_2 concentration (C_i) were measured on flag leaves exposed to sunlight from 9:00 to 11:00 h with 60% relative humidity, 400 µmol(CO_2) mol^{-1}, and PPFD of 1,200 µmol m^{-2} s^{-1}. Each treatment selected ten plants at approximately the same growth stage for measurement.

$O_2^{\bullet-}$ and malondialdehyde content: The $O_2^{\bullet-}$ content was determined using the method of Elstner and Heupel (1976) with minor modifications. Fresh leaves (1.0 g) were homogenized with 0.05 mol L^{-1} PBS (pH 7.8) at a constant volume of 8 mL and centrifuged at 4°C for 20 min. The supernatant (crude enzyme) was mixed with 0.05 mL of PBS buffer (0.05 M, pH 7.8) and 0.1 mL of hydroxylamine chloride (10 mM) in a 15-mL centrifuge tube and incubated at 25°C for 10 min. One mL of *p*-aminobenzene sulfonic acid (58 mM) and α-naphthylamine (7 mM) were added, and the mixture was incubated for 20 min. Trichloromethane (3 mL) was then added and the mixture was centrifuged at 10,000 × *g* for 3 min; the pink aqueous phase absorbance at 530 nm was measured (*Genesys 10*, *Thermo Electron*, USA). The $O_2^{\bullet-}$ production rates were calculated from the standard curve of $NaNO_2$. The results were expressed in µmol(nitrite) g^{-1}(fresh mass, FM).

Lipid peroxidation was estimated by measuring the malondialdehyde (MDA) content produced by the thiobarbituric acid (TBA) reaction (Draper and Hadley 1990). TCA–TBA solution of 1.25 mL (20% TCA + 0.5% TBA) was mixed with 0.75 mL of crude enzyme solution; the mixture was transferred to boiling water for 10 min and rapidly cooled on ice and then centrifuged at 1,800 × *g* for 10 min. The absorbance of the supernatant was measured at 450, 532, and 600 nm (*Genesys 10*, *Thermo Electron*, USA). The concentration of MDA was expressed in nmol g^{-1}(FM).

Diaminobenzidine staining: Following the staining method of Thordal-Christensen *et al.* (1997), 3-diaminobenzidine (DAB) was dissolved in water and acidified with HCl to pH 3.8 to prepare a 0.1 mg mL^{-1} DAB solution. Fresh rice leaves were cut about 6 cm from the leaf tip and completely immersed in the prepared

DAB solution, and then held at 25°C in darkness for 6 h. After dyeing, the leaves were immersed in boiling 95% ethanol to decolorize for about 10 min and then rinsed with pure water. The amount and location of red-brown precipitate produced in the leaves were measured and photographed on a cold light plate (*LED Side Look*, *Zhuhai Hema Medical Instrument*, China).

Enzyme assays: SOD (EC 1.15.1.1) activity was measured by its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) (Jin *et al*. 2008). The reaction mixture was comprised of 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 0.1 mM EDTA-Na_2, 50 mM sodium carbonate, 25 mM nitroblue tetrazolium chloride, and 0.1 mL of enzyme extract. The blank group was placed in darkness, and both the control group and the treatment group were placed in an illumination incubator with 720 μmol(photon) m^{-2} s^{-1} for 10–15 min. Absorbance at 560 nm was measured in the dark (*Genesys 10*, *Thermo Electron*, USA). The absorbance of the control group was taken as a maximum, and the amount of enzyme required to inhibit 50% photochemical reduction of NBT was calculated as one enzyme activity unit.

POD (EC 1.11.1.7) activity was determined by measuring the increase in absorbance at 470 nm (*Genesys 10*, *Thermo Electron*, USA) in a 3-mL reaction system consisting of PBS (100 mmol L^{-1}, pH 6.0), guaiacol (2-methoxyl phenol), 30% H_2O_2, and 50 μL of enzyme extract every 10 s for a total duration of 120 s (Chen *et al*. 2010). One unit of POD activity was defined as 1 μg of substrate catalyzed per min per mg of fresh mass.

CAT (EC 1.11.1.6) activity was estimated by measuring the decrease in absorbance at 240 nm (*Genesys 10*, *Thermo Electron*, USA) in a 3-mL reaction system with 1 mL of 0.3% H_2O_2, 1.9 mL of water, and 0.1 mL of crude enzyme, counting every 10 s for a total of 2 min (Beer and Seizer 1952). One unit of CAT activity was defined as 1 mg of H_2O_2 catalyzed per min per mg of fresh mass.

Immunoblotting analysis: SDS-PAGE and immunoblot analysis were performed following the method of Sonawane *et al*. (2018). Protein samples were extracted from 1.0 g of sword leaves using the TCA-acetone/phenol extraction method. The thylakoid membrane proteins were detected using 12% (w/v) polyacrylamide slab gels. For the Western blot analysis, the proteins or peptides separated on the gel were immediately transferred onto polyvinylidene fluoride (PVDF) membranes. Subsequently, the PVDF membranes were incubated with antibodies (*Beijing Qiwei Yicheng Biological Company*) against ATP-β, OEC, RbcL, PsaA, PsaB, D1 (PsbA), PsbB, PsbO, Lhca2, and then incubated with alkaline phosphatase-conjugated antibody. The amounts of photosynthesis-associated proteins were analyzed by an enhanced chemiluminescence detection reagent (*High-sig ECL*, Tanon, Shanghai, China).

Statistical analysis: *Excel 2010*, *PEP Plus*, *Biolyzer HP3*, *GraphPad Prism 6*, *Origin 96*, and *Adobe Illustrator CS6* were used for date post-processing and mapping analysis. *SPSS 22.0* was used to conduct a one-way analysis of variance (*ANOVA*), and LSD was used to conduct a difference analysis (with significant differences at $P<0.05$).

Results

Phenotype, pigment determination, and photosynthetic performance of 812HS and 812S: Both cultivars (812HS and 812S) grew normally under natural light after transplanting on 25 June [450 μmol(photon) m^{-2} s^{-1}]. When the natural light intensity reached 720 μmol(photon) m^{-2} s^{-1} (on 10 July), the leaves of 812S retained their normal green color (Fig. 1*A*,*B*). In contrast, the leaf tips of 812HS were noticeably yellowing by 10 July (Fig. 1*A*,*B*). Meanwhile, a comparison of leaf chlorosis in the shaded and unshaded treatments, which differed only in light intensity, revealed that the chlorosis of 812HS leaves was due to the high-intensity light rather than high temperatures (Fig. 1*C*,*D*). These findings suggested that 812HS suffered more easily from photooxidative damage under natural light, leading to leaf senescence and yellowing.

The content of chlorophyll (Chl) *a* and *b*, carotenoids, and the ratio of Chl *a*/*b* were comparable in the two cultivars on 25 June, but the same measurements from 812HS showed lower contents on 10 July except for the ratio of Chl *a*/*b*. Specifically, the content of Chl *a* and *b* and carotenoids in 812HS were 67.2, 74.8, and 31.1% lower than those in 812S, respectively. In contrast, the ratio of Chl *a*/*b* was about 25.1% higher in 812HS than that in 812S (Fig. 2*A–D*). However, there were no significant differences in pigment contents between the shading and natural light groups, and the Chl *a*/*b* ratio of the two cultivars stayed at the same level (Fig. 2*E–H*). Taken together, Chl and carotenoid degradation were accelerated, as observed in yellowing phenotypes of 812HS under excessive light.

The photosynthetic performance of 812HS under natural light was not different from 812S on 25 June. However, as the light intensified, there was a minor decrease in 812S. It showed a sharp decrease in P_N, E, and g_s and a clear increase in C_i for 812HS on 10 July (Fig. 3*A–D*). Moreover, there were no apparent differences in P_N, E, C_i, and g_s between the two rice varieties in the shading treatment. These observations suggest that the photosynthetic organs were protected in the shading treatment (Fig. 3*E–H*).

ROS production, MDA concentration, and antioxidant enzyme activities: The presence of H_2O_2 detected with DAB was indicated by the reddish-brown coloration in the leaves. With increased intensity of natural light, the reddish brown color gradually deepened on the leaves of both cultivars. Moreover, the reddish brown color in 812HS was darker than that in 812S on 10 July (Fig. 4*A*). The $O_2^{\bullet-}$ content in 812HS leaves was also higher than that in 812S by 145.4% on 10 July (Fig. 4*B*). However,

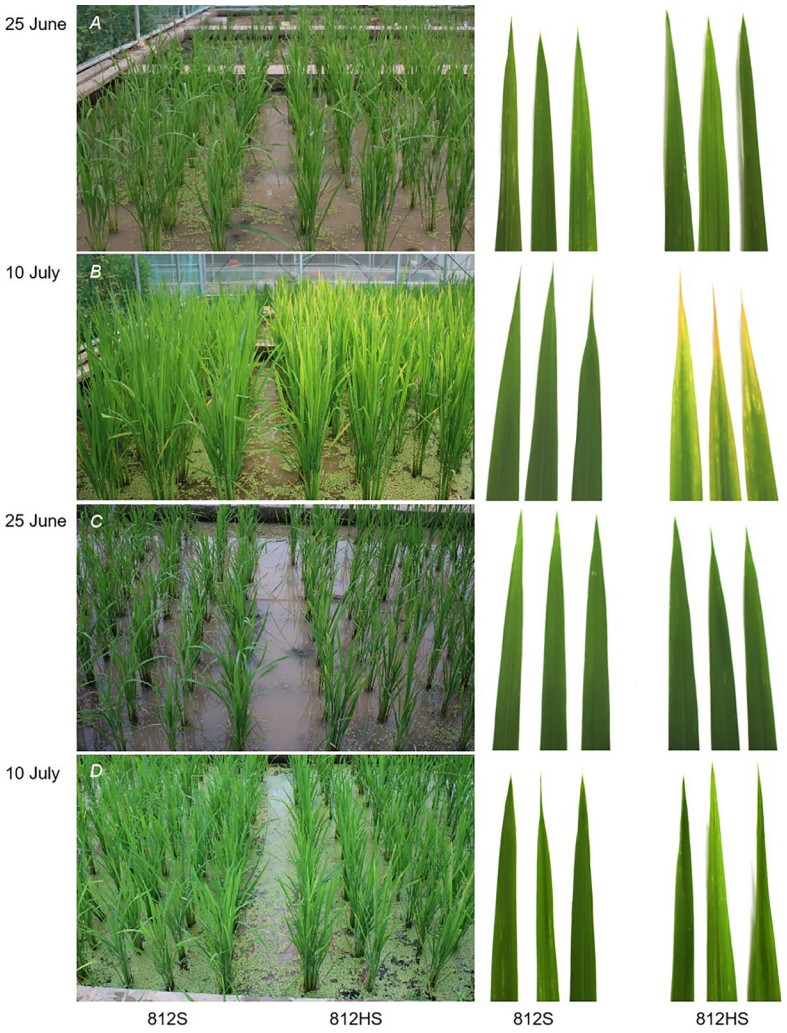

Fig. 1. Photographs showing phenotypic changes in the 812HS and 812S rice cultivars 14 d after a shade treatment was applied to the plants. (*A*,*B*) The natural light group; (*C*,*D*) the shading group. Four-week-old rice seedlings of 812HS and 812S were transplanted for some time to the same experimental plots, after which half of the plants were shaded [black shading net, 180 μmol(photon) m^{-2} s^{-1}], and the other half remained unshaded [720 μmol(photon) m^{-2} s^{-1}] for 14 d.

both the H_2O_2 accumulation and the $O_2^{\bullet-}$ content of 812S and 812HS declined with the maturation of leaves in the shading treatment (Fig. 4*D*,*E*). These results indicated that the dynamic balance of ROS in 812HS was broken owing to excessive light exposure, allowing oxidation to dominate.

MDA is a biological indicator of lipid peroxidation under various abiotic stresses. When light intensity increased, MDA in 812HS increased significantly (Fig. 4*C*). Similarly, no significant difference was found in 812HS MDA content in the shading treatment compared to 812S (Fig. 4*F*). These results showed that excessive light exposure caused oxidative degradation of lipids in 812HS.

Antioxidant enzyme activities in 812HS, such as SOD, POD, and CAT, decreased notably under excessive light on 10 July (Fig. 5*A–C*), while the shading treatment protected the antioxidant systems of 812HS, suggesting that the damage to antioxidative systems correlated with the degree of light stress (Fig. 5*D–F*).

Changes in thylakoid membrane protein complexes and thylakoid membrane proteins: The dynamic changes of the thylakoid membrane protein complex reflect the adjustment of plants to light. When light intensity increased to 720 μmol(photon) m^{-2} s^{-1}, 812HS thylakoid membrane protein complexes degraded compared to 812S, including the PSI and PSII monomer, PSII trimers, F1-ATPase, Cyt b_6f, and LHCII monomer, dimer, and other membrane complexes. However, the thylakoid membrane protein complexes of 812HS in the shaded treatment effectively avoided the damage caused by the excessive light intensity in the natural light treatment (Fig. 6).

Western blot analysis further explained changes to photosynthesis-related proteins in PSI and PSII. These results showed that high light-induced photooxidation in 812HS resulted in a large amount of degradation of ATP-β subunit, OEC, RbcL, PsaA, PsbB, PsbO, Lhca2, and other photosynthesis-related proteins. While the shading treatment reduced light intensity and effectively prevented degradation of photosynthesis-related proteins (Fig. 7). Taken together, both PSII and PSI were destroyed in 812HS plants when light intensity increased, and they showed poor repair and protection capacity compared to 812S.

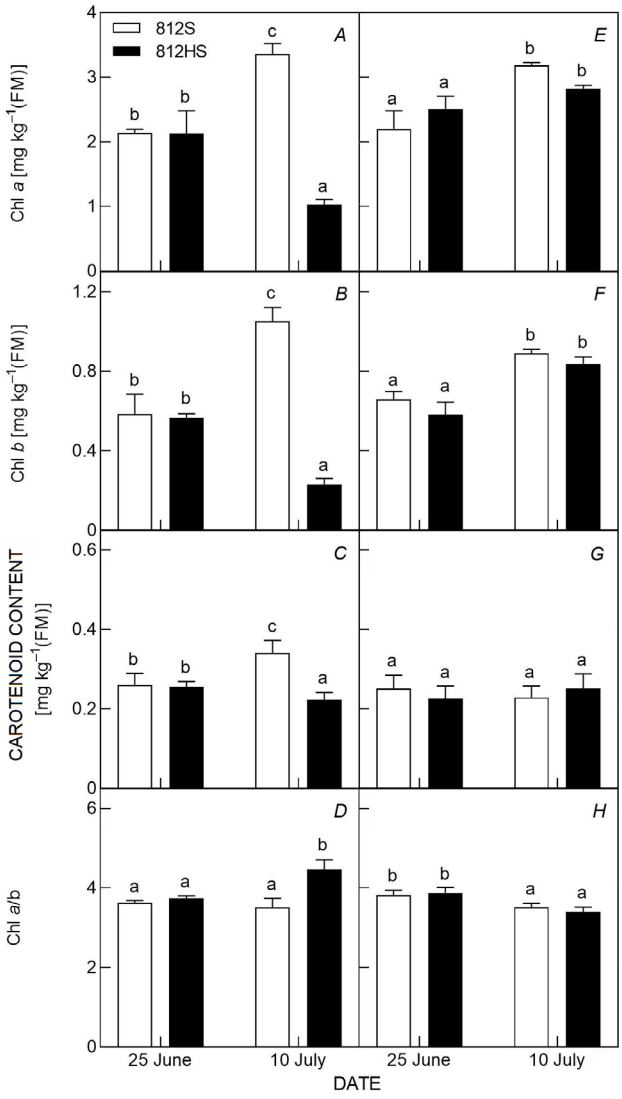

Fig. 2. Changes in photosynthetic pigment content. (*A–D*) The pigment content in 812S and 812HS in the natural light treatment on 25 June and 10 July. Chlorophyll *a* (*A*), chlorophyll *b* (*B*), carotenoids (*C*), chlorophyll *a*/*b* (*D*). (*E–H*) The pigment content of 812S and 812HS in the shaded treatment. Chlorophyll *a* (*E*), chlorophyll *b* (*F*), carotenoids (*G*), chlorophyll *a*/*b* (*H*). Data are mean ± SD, n = 9. Means with *different letters* are significantly different (P<0.05).

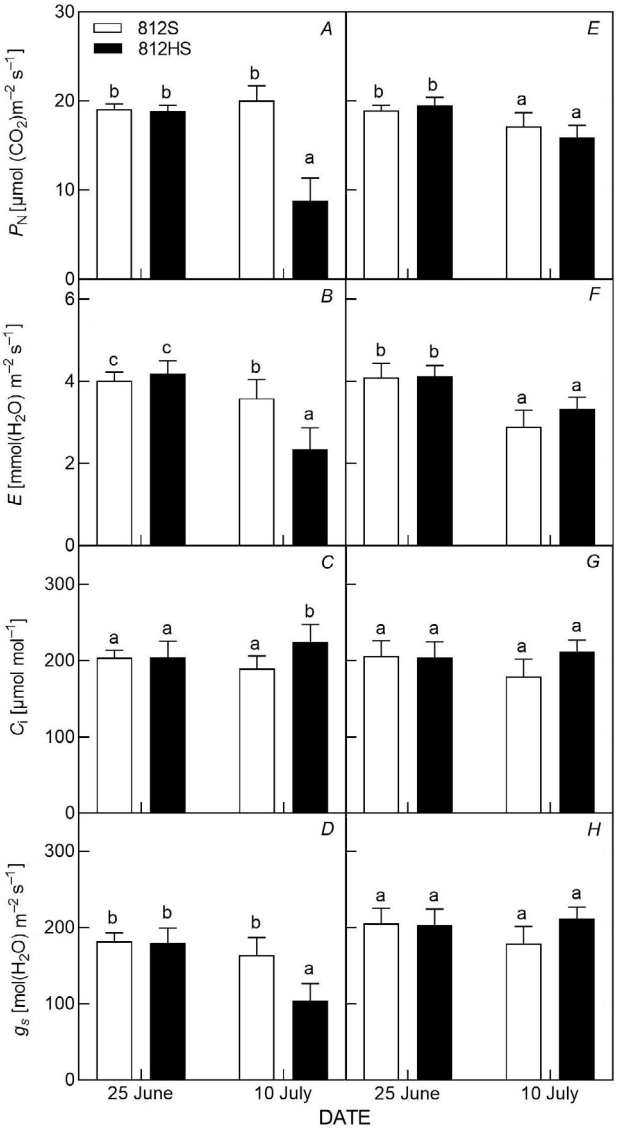

Fig. 3. The photosynthetic performance of 812S and 812HS. (*A–D*) The photosynthetic performance of 812S and 812HS in the natural light treatment on 25 June and 10 July. The net photosynthetic rate (P_N) (*A*), transpiration rate (*E*) (*B*), intercellular CO_2 concentration (C_i) (*C*), stomatal conductance (g_s) (*D*). (*E–H*) The photosynthetic performance of 812S and 812HS in the shading treatment. The net photosynthetic rate (P_N) (*E*), transpiration rate (*E*) (*F*), intercellular CO_2 concentration (C_i) (*G*), stomatal conductance (g_s) (*H*). Data are mean ± SD, n = 10. Means with *different letters* are significantly different (P<0.05).

Discussion

Excess excitation energy produced under high-light conditions cannot be used or dissipated in time and usually causes photooxidation, damaging the photosynthetic reaction centers, photosynthetic pigments, and photosynthetic membranes of the plant. Leaf senescence is one of the most obvious characteristics of biochemical changes (Powles 1984). Chl breakdown is initiated by conversion from Chl *b* to Chl *a*, which is finally hydrolyzed to produce pheophorbide *a* and phytol. When pheophorbide *a* is cleaved, the green color of Chl catabolites is completely lost, resulting in oxidized red Chl catabolite (Woo *et al.* 2019). Meanwhile, Chl is fragile and easily destroyed by ROS (Wang *et al.* 2021). In our study, leaves of 812S remained green in the natural light treatment [720 μmol(photon) m^{-2} s^{-1}], but chlorosis was observed in 812HS. In the shading treatment [180 μmol(photon) m^{-2} s^{-1}], the leaves of 812HS were protected from chlorosis (Fig. 1). In other

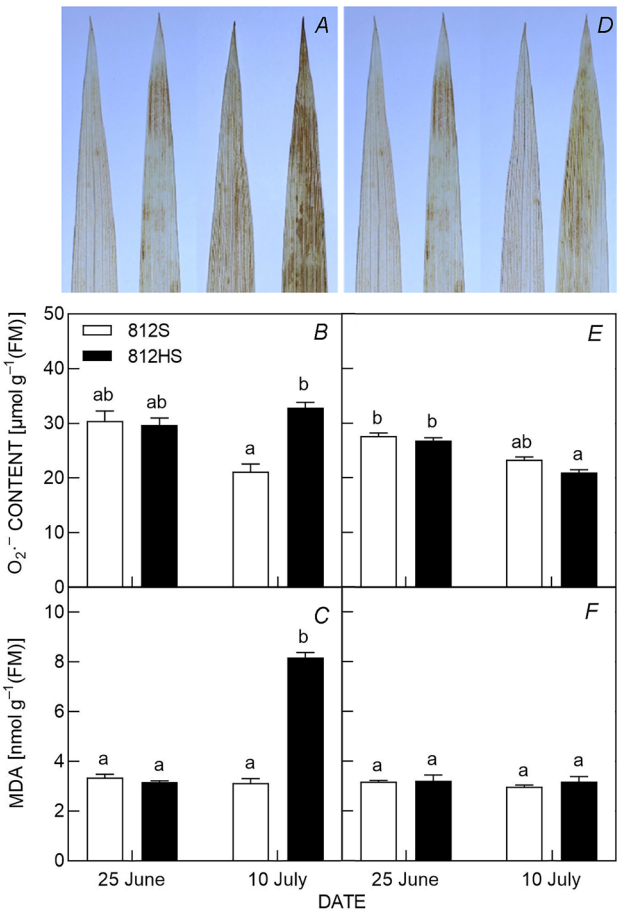

Fig. 4. Photooxidative damage of 812S and 812HS. Diaminobenzidine (DAB) staining (*A*), $O_2^{\bullet-}$ (*B*), and malondialdehyde (MDA) content (*C*) in the natural light treatment on 25 June and 10 July. DAB staining (*D*), $O_2^{\bullet-}$ (*E*), and MDA content (*F*) in the shading treatment. Data are mean ± SD, $n = 3$. Means with *different letters* are significantly different ($P<0.05$).

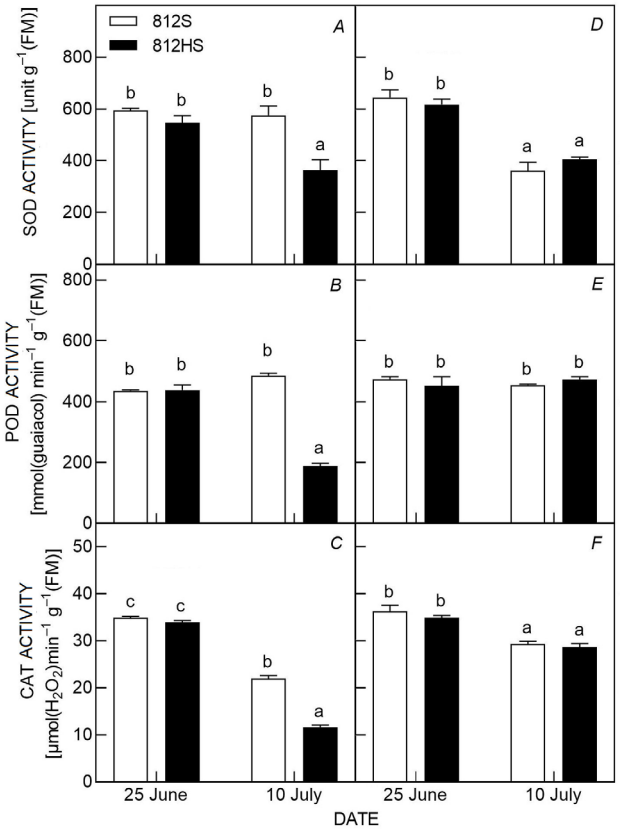

Fig. 5. Antioxidant enzyme activities in 812S and 812HS. (*A–C*) Antioxidant enzyme activities of the natural light treatment on 25 June and 10 July: superoxide dismutase (SOD) (*A*), peroxidase (POD) (*B*), catalase (CAT) (*C*). (*D–F*) Antioxidant enzyme activities of the shading treatment: SOD (*D*), POD (*E*), CAT (*F*). Data are mean ± SD, $n = 3$. Means with *different letters* are significantly different ($P<0.05$).

words, the decomposition of 812HS Chl was greater than the synthesis under high light. And there was a higher Chl *a*/*b* in 812HS under the natural light, which also means that both the transition of Chl *b* to Chl *a* and the breakdown of Chl *a* were accelerated. Generally, plants have more Chl *a* than Chl *b*, which is essential for survival, and the ratio of Chl *a*/*b* is always dominated by Chl *b* (Pattanayak *et al*. 2005, Luo *et al*. 2013). At the same time, there was a larger amount of ROS in 812HS when grown in natural light, convincingly demonstrating that Chl undergoes breakdown at high light. In contrast, Chl *a* and Chl *b* of 812HS were synthesized normally in the shading treatment, and the ratio of Chl *a*/*b* was close to that of 812S.

Carotenoids are light-harvesting pigments that serve to enhance the overall efficiency of photosynthetic light reactions and contribute to protecting photosynthetic organisms from the harmful effects of exposure to intense light, depending on where they exist in thylakoid membranes (Ashraf and Harris 2013). When presented in the fractionally free thylakoid lipid phase, carotenoids function as the scavengers of ROS; when in close contact with Chls, carotenoids bound to LHCs (Bassi and Dall'Osto 2021). With the growth of the rice, the carotenoids in 812S increased gradually but decreased in 812HS. In the shading treatment, the carotenoids in 812S and 812HS leaves showed almost no difference at different growth stages. Plants contain at least two types of carotenoids: β-carotene is found in both photosystems, and oxygenated carotenoids are found primarily in the LHCs; they are also called xanthophylls, including xanthophylls lutein (Lut), violaxanthin (Vio), neoxanthin (Neo), and zeaxanthin (Zea) once accumulated under excess light (García-Cerdán *et al*. 2020, Bassi and Dall'Osto 2021). The reaction rate of β-carotene is equal to xanthophyll *in vivo*. β-carotene was oxidized into β-carotene-5,8-endoperoxide, which was found to accumulate rapidly in leaves, and this accumulation was correlated with the extent of PSII photoinhibition and the loss of β-carotene under high light stress. However, xanthophyll endoperoxide contents were relatively low but activated and accumulated in excess light to dissipate excess energy (Müller *et al*. 2001,

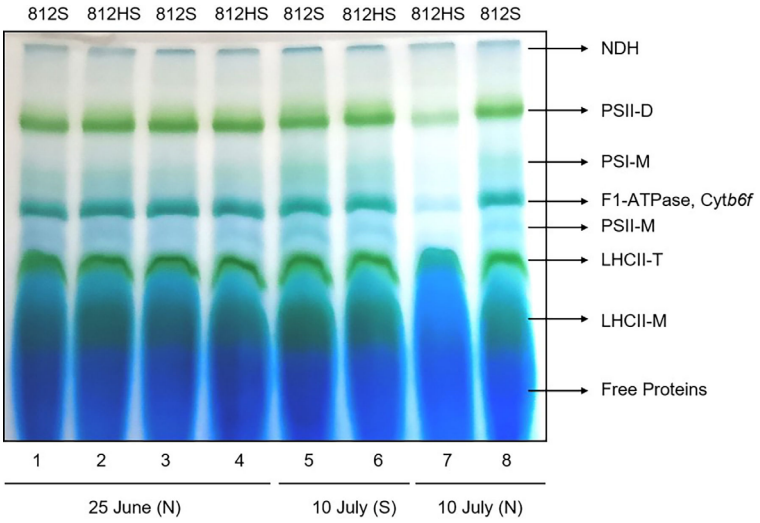

Fig. 6. BN-PAGE of thylakoid membrane protein complexes (10 μg chlorophylls) isolated from 812S and 812HS. Lanes 1, 2 (25 June) and 7, 8 (10 July) are the thylakoid membrane protein complexes content of the natural light treatment. Lanes 3, 4 (25 June) and 5, 6 (10 July) are the thylakoid membrane protein complexes content of the shading treatment. N – natural light, S – shaded light. NDH – NADH dehydrogenase-like; PSI-M – PSI monomers; PSII-D – PSII dimers; F1-ATPase – ATP synthase; Cyt b_6f – cytochrome b_6f complexes; PSII-M – PSII monomers; LHCII-T – LHC trimers; LHCII-M – LHC monomers.

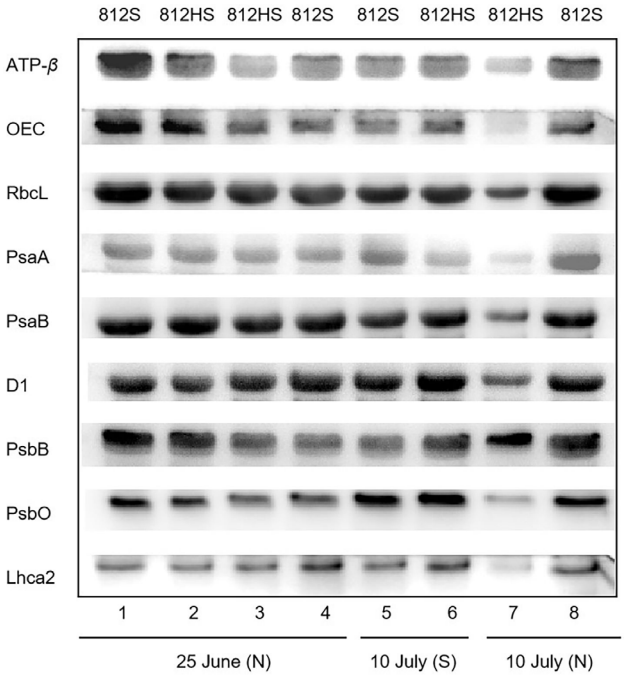

Fig. 7. Comparison of photosynthesis-associated protein subunits in the thylakoid membrane of 812S and 812HS. Lane 1, 2 (25 June) and 7, 8 (10 July) are the photosynthesis-associated protein subunits content in the natural light treatment. Lanes 3, 4 (25 June) and 5, 6 (10 July) are the photosynthesis-associated protein subunits content in the shading treatment. N – natural light, S – shaded light.

Ramel *et al*. 2012, 2013). In our study, the phenomenon of yellowing occurred in 812HS leaves due to the decrease of Chl. On the one hand, free Cars safely undergo rapid photooxidation due to the effects of ROS produced by PSII and PSI, converting ROS into $^3O_2$ and releasing excitation energy into heat by yielding the Car triplet excited states $(^3Car^*)^{\bullet}$ which accumulated with light intensity. On the other hand, Neo, Lut, and Vio have a specific function as a quencher of $O_2^{\bullet-}$, $^3Chl^*$, and $^1O_2$, respectively, and Vio is also de-epoxidized to Zea to enhance antioxidant and $^3Chl^*$ quenching activity. Meanwhile, β-carotene associated with photosystems undergoes oxidative degradation by producing β-cyclocitral, mitigating oxidative damage under excessive light conditions (Hashimoto *et al.* 2016, Bassi and Dall'Osto 2021). Though our study lacked quantification for different carotenoids, the total carotenoids in 812HS decreased under high light, making the leaves of 812HS sensitive to high light. In short, the specific protection mechanism of 812HS still needs to be further explored.

In our study, a decline in photosynthetic performance was positively correlated with the breakdown of photosynthetic pigments, except for the intercellular CO_2 concentration (C_i). Net photosynthetic rate (P_N), transpiration rate (E), and stomatal conductance (g_s) of 812HS decreased under strong light (Fig. 3), while the intercellular CO_2 concentration (C_i) increased, demonstrating that the decrease in P_N was limited to nonstomatal restriction theoretically. Nevertheless, the effect of the cuticle on C_i cannot be neglected, and the reason is that when the stomata are closed, CO_2 must be forced through the cuticle and into the leaf, resulting in C_i being over-valued compared to what it should be (Tominaga and Kawamitsu 2015). Jurczyk *et al.* (2016) reported that the decrease of P_N in cucumbers was associated with lower Rubisco activity and temperature, which caused C_i to increase (Bi *et al*. 2017). Therefore, the decrease in P_N and g_s may imply that the decline of P_N in 812HS was due to the lower Rubisco activity, which also corresponds with our results. On the other hand, the large accumulation of intercellular CO_2 in leaves also obstructed the transport of CO_2 required for photosynthesis to mesophyll cells, resulting in a decrease in photosynthetic carbon assimilation efficiency and the closure of epidermal stomata. Compared with the high-light treatment, P_N and E decreased a little in the shaded treatment, and C_i and g_s stayed at approximately the same level. These

results showed that high-light intensity caused obvious damage to the photosynthetic performance of 812HS, while the shaded treatment could effectively avoid photooxidation damage.

Plants always face the potential problem of excess energy in natural conditions. If the excess light energy cannot be consumed in time, it will lead to the accumulation of ROS, which plays a dual role in plants. A small amount of ROS is an important signaling molecule, but the accumulation of ROS caused by various biological stresses in plants results in serious oxidative damage to lipids, proteins, DNA, and other structures (Apel and Hirt 2004). Our study showed that the $O_2^{\bullet-}$ content in 812HS was significantly higher than that in 812S, indicating that excessive $O_2^{\bullet-}$ in 812HS could not be removed in time due to the strong light conditions, resulting in photooxidation. Once $O_2^{\bullet-}$ is produced, it rapidly disambiguates under enzymatic or nonenzymatic actions, producing H_2O_2 and O_2. Under natural light at 720 μmol(photon) m^{-2} s^{-1}, the leaves of 812HS became dark reddish brown, demonstrating that 812HS produced large amounts of H_2O_2. Meanwhile, H_2O_2 is a powerful inhibitor of the Calvin cycle, affecting the progress of photosynthesis.

Faced with excessive ROS injury, plants adopt an enzymatic scavenging system to remove ROS, such as $O_2^{\bullet-}$, H_2O_2, and OH^-, to protect cell membranes from membrane lipid peroxidation and other damage (Karuppanapandian *et al*. 2011). The main scavenger of $O_2^{\bullet-}$, SOD, removes $O_2^{\bullet-}$ by producing H_2O_2 and O_2 (Sudhakar *et al*. 2001). However, the SOD activity of 812HS was low, so the increased $O_2^{\bullet-}$ accumulated in leaves and transformed into excess H_2O_2 as the light intensity increased. Meanwhile, CAT and POD activity were also depressed, which contributed to the failure of the newly generated H_2O_2 to dissipate and to the accumulation of ROS.

Damage caused by ROS accumulation includes membrane lipid peroxidation. The changes in MDA content of membrane lipid peroxidation are often used as a measure of plant peroxidation damage (Ghanem *et al*. 2021). The MDA content in 812HS leaves was more than three times higher than that in 812S in the strong light treatment on 10 July, indicating that 812HS suffered membrane lipid peroxidation damage. Taken together, these results showed that 812HS had a lower tolerance to high-light intensity, and the photoprotection system was easily destroyed under high-light conditions, causing irreversible photooxidation damage.

Two major membrane protein complexes, PSI and PSII, embedded in chloroplast thylakoid membranes, are responsible for the primary photochemical reactions of photosynthesis. The main function of PSI is collecting 'light energy and transferring electrons through a series of redox centers (Chitnis 2001). The main functions of PSII include (*1*) driving photodecomposition of water, which provides electrons to the entire photosynthesis process; (*2*) reducing membrane-bound plastoquinone (PQ) by light energy (Iwata and Barber 2004, Fromme *et al*. 2006). They operate synergistically and are functionally linked by the PQ, Cyt b_6f complex, and plastocyanin (PC). The integrity and cooperation of chloroplast protein complexes are essential for complex photosynthesis. The photosynthetic electron transfer function of the chloroplast thylakoid membrane and the content of thylakoid membrane proteins decrease under stress (Tikkanen *et al*. 2014). In our study, the irreversible photooxidation of 812HS was triggered by strong light, and the membrane complexes of PSI and PSII core complex, monomer, trimer, Cyt b_6f, and LHCII were substantially degraded. Western blot also showed serious degradation of OEC protein, ATP-β subunit, and RbLs. All in all, photooxidation caused by excessive light damaged photosynthetic pigments and disrupted electron transport systems, manifesting themselves in reduced thylakoid membrane protein content. These conclusions were consistent with the degradation of pigments in 812HS leaves, decreased photosynthetic performance, and poor ability to repair the damage caused by photooxidation, which reasonably explained the phenomenon of yellowing in 812HS. Meanwhile, no significant degradation occurred in the thylakoid protein complexes of 812HS in the shaded treatment. These results indicate that shading may effectively reduce photooxidation caused by strong light and delay leaf senescence.

PsaA and PsaB are involved in the central heterodimer and electron transport chain (ETC), A_0 (A Chl *a*), A_1 (phylloquinone), and Fx (clusters of A 4Fe-4S) of P_{700}, which constitute the reaction center of PSI, where the initial steps of charge separation and ETC occur (Chitnis 2001). The PSII reaction center, composed of D1 (PsbA) and D2 (PsbD) subunits, is the site of the light-driven electron transfer reaction (Iida *et al*. 2008). When the light intensity is higher than the plant requires for electron flow in photosynthesis, it causes irreversible damage to PSII activity due to D1 protein degradation, which can only be repaired by *de novo* synthesis of D1 proteins (Edelman and Mattoo 2008). Immunoblotting analysis showed that the 812HS PSI core subunits (PsaA, PsaB) degraded under high light intensity, indicating that the reaction centers of both photosystems were injured by excessive light. Previous reports indicated that PSII damage precedes PSI damage and PSI is only injured when the electron flow of PSII exceeds the capacity of the PSI electron acceptor to process electrons irreversibly (Tikkanen *et al*. 2014). Our study confirmed that 812HS leaf chlorosis and irreversible photooxidation damage to PSI and PSII occurred under strong light conditions, proving that the rate of damage overtook the rate of repair. Compared to strong light conditions [720 μmol(photon) m^{-2} s^{-1}], the quantities of ATP-β subunit, PsaA, OEC, RbcL, D1, PsbB, PsbO, and Lhca2 in 812HS did not significantly increase under the lower light conditions [180 μmol(photon) m^{-2} s^{-1}]. These results indicate that the shaded treatment reduced the photooxidation damage of PSI and PSII and effectively avoided the degradation of photosynthesis-related proteins in leaves.

Conclusions: In summary, our study showed that the poor tolerance of 812HS to excessive light could initially induce a fast senescence process on the leaf tips, causing a range of physiological effects. Specifically, the H_2O_2,

$O_2^{\bullet-}$, and malondialdehyde content increased sharply in 812HS, which broke cellular redox homeostasis under excessive light conditions, damaging the antioxidant enzyme systems. Meanwhile, complexes embedded in thylakoid membranes, such as PSI and PSII core complex (PsaA, PsaB, D1), monomer and trimer of PSI and PSII, Cyt b_6f, and LHCII, were substantially degraded. Therefore, it is responsible for poor photosynthetic performance and the broken electron transport system. We hypothesize that carotenoids have a different effect in mitigating excess energy; however, when the rate of damage exceeds the rate of repair, this has little effect on photooxidation. Generally, the exact mechanism of photooxidation in 812HS requires further elucidation.

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