Defence response produced during photodynamic damage in transgenic rice overexpressing 5-aminolevulinic acid synthase

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Abstract

Photodynamic and photoprotective responses at different irradiances were investigated in transgenic rice (*Oryza sativa*) expressing *Bradyrhizobium japonicum* 5-aminolevulinic acid synthase (ALA-S). With high irradiance (HI) of 350 μmol m^{-2} s^{-1}, transgenic lines P5 and P14 showed a decrease in contents of chlorophyll (Chl) and the chloroplast-encoded gene *psbA* mRNA, whereas a decrease in light-harvesting Chl-binding proteins was observed only in P14. These effects were not observed in the wild-type (WT) line treated with HI or all of the lines treated with low irradiance (LI) of 150 μmol m^{-2} s^{-1}. HI resulted in a greater decrease in the quantum yield of photosystem 2 and a greater increase in non-photochemical quenching (NPQ) in the transgenic lines, particularly in P14, compared to WT. Photoprotective zeaxanthin contents increased at HI, even though carotenoid contents were lower in the transgenic lines compared to WT. When exposed to HI, superoxide dismutase greatly increased in transgenic lines P5 and P14, but peroxidase and glutathione reductase increased only in P14, in which more photodynamic damage occurred. Thus the greater expression of ALA-S in the transgenic plants developed the stronger protective functions, *i.e.* the increased values of NPQ and zeaxanthin, as well as more photodynamic reactions, *i.e.* decreased photosynthetic component and efficiency, in the photosynthetic complexes. However, the photodynamic reactions indicate that the antioxidant capacity was insufficient to cope with the severe stress triggered by photoactive porphyrins in the transgenic rice expressing ALA-S.

Additional keywords: 5-aminolevulinic acid synthase; antheraxanthin; antioxidant; chlorophyll; non-photochemical quenching; *Oryza*; photodynamic stress; violaxanthin; zeaxanthin.

Introduction

In plants, 5-aminolevulinic acid (ALA) is formed from the five-carbon skeleton of glutamate by three sequential enzymatic reactions of glutamyl-tRNA synthetase, glutamyl-tRNA hydrogenase, and glutamate-1-semialdehyde aminotransferase (Beale 1978). Eight ALA molecules are fused into porphyrins, which are then modified to protoporphyrin IX (Proto IX). The chelation of Proto IX with Mg^{2+} or Fe^{2+} and subsequent modifications lead to chlorophyll (Chl) or heme, respectively. ALA is formed in *Bradyrhizobium japonicum*, a bacterial species, by ALA synthase (ALA-S), which catalyzes the first step in heme synthesis, *i.e.* the condensation of glycine and succinyl-CoA, releasing carbon dioxide and CoA.

Treatment with low concentrations of ALA increases the growth and yield of radishes, kidney beans, barley, potatoes, and garlic by 10–60 % (Hotta *et al.* 1997). The ALA-forming activity correlates well with the steady-state contents of mRNA that encode light-harvesting chlorophyll-binding proteins, Lhc (Kruse *et al.* 1997). When ALA-treated plants are exposed to sunlight, excess tetrapyrroles absorb energy that is used in photochemical reactions (Tripathy and Chakraborty 1991) and photo-

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Abbreviations: ALA – 5-aminolevulinic acid; ALA-S – 5-aminolevulinic acid synthase; AOS – active oxygen species; APX – ascorbate peroxidase; CAT – catalase; Chl – chlorophyll; GR – glutathione reductase; HI – high irradiance; Lhc – light-harvesting chlorophyll-binding proteins; LI – low irradiance; NPQ – non-photochemical quenching; POD – peroxidase; PPFD – photosynthetic photon flux density; PS – photosystem; Proto IX – protoporphyrin IX; SOD – superoxide dismutase.
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sensitize the production of singlet oxygen, $^1O_2$ (Hopf and Whitten 1978). Active oxygen species (AOS) destroy vital proteins such as the photosystem 2 (PS2) D1 reaction centre protein as well as membrane lipids and pigments (Barber and Andersson 1992, Nishiyama *et al.* 2004). Rebeiz *et al.* (1984) reported that ALA can be used as a selective and biodegradable herbicide and insecticide.

Photoprotective processes prevent or minimize the generation of oxidizing molecules, scavenge AOS efficiently, and repair damage that inevitably occurs (Niyogi 1999, Suzuki and Mittler 2006). To protect the photosynthetic apparatus from photooxidative destruction, plants must dissipate excess photon energy by non-photochemical quenching (NPQ), the thermal dissipation of excess excitation energy in the PS2 antennae, and photochemical quenching, *i.e.* the transfer of electrons from PS2 to various acceptors within the chloroplast (Ort and Baker 2002). Xanthophylls are involved in the quenching of ^{3}Chl and $^1O_2$ and the inhibition of lipid peroxidation (Niyogi 1999, Frąckowiak and Smyk 2007). Non-radiative energy dissipation at PS2 is mediated by zeaxanthin and antheraxanthin (Jung 2004, Holt *et al.* 2005). Enzymatic antioxidant systems also function in the control of the toxic effects of AOS in plant tissues. Superoxide dismutase (SOD) acts as the first line of defence against AOS, dismutating superoxide (O_2^-) to H_2O_2. Peroxidase (POD) and catalase (CAT) subsequently detoxify H_2O_2 (Apel and Hirt 2004, Suzuki and Mittler 2006). SOD, ascorbate peroxidase (APX), monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase (GR) constitute the major defence system against AOS in chloroplasts (Dalton *et al.* 1986).

Transgenic rice plants expressing *B. japonicum* ALA-S had increased contents of ALA and the photoactive porphyrins Proto IX and protochlorophyllide, and showed symptoms of photobleaching (Jung *et al.* 2004). Greater drops in the F_v/F_m ratio, Chl contents, and shoot growth, and a greater increase in ALA were observed in line P14 than in line P5. The leaves of P5 and P14 were light green and light/pale green, respectively (Jung *et al.* 2004). We examined alterations in the chloroplast-related gene and protein to evaluate photodynamic damage in the photosynthetic machinery of wild-type (WT) and transgenic rice exposed to low (LI) or high (HI) irradiance. In addition, we compared AOS-scavenging enzymes, as well as the photoprotective apparatus involving NPQ and the xanthophyll cycle pigments, between WT and transgenic rice under ALA-induced photodynamic stress.

Materials and methods

Plant growth and irradiation: The T_2 generation of homozygous transgenic rice lines (*Oryza sativa* cv. Dongjin) expressing the *Bradyrhizobium japonicum* 5-aminlevulinic acid synthase gene was used for experiments. Scutellum-derived calli of rice were co-cultured with *Agrobacterium tumefaciens* LBA4404 harbouring the binary vector pGA1611:ALA-S (Jung *et al.* 2004). DNA encoding the plastid transit sequence for a tobacco plastid protoporphyrinogen oxidase (Lee *et al.* 2000) was used to import the *B. japonicum* ALA-S gene into plastids of rice. Seeds of untransformed and transgenic lines were kept for 3 d at 25 ºC in darkness on MS medium and then exposed to a photosynthetic photon flux density (PPFD) of either 150 µmol m^{-2} s^{-1} (LI) or 350 µmol m^{-2} s^{-1} (HI), with a 16/8 h light/dark cycle for 7 d. Leaves were taken for all experiments.

Isolation and analysis of RNA: Total RNA (10 µg) was isolated from leaves of WT or transgenic rice plants using the TRI reagent (*Sigma Chemical Co.*, St. Louis, MO, USA) and was fractionated on 1 % agarose gel containing formaldehyde using 20 mM 3-(N-morpholino)propanesulfuric acid (pH 7.0) as a running buffer. RNA samples were stained with ethidium bromide prior to blotting onto nylon membranes, and the blots were hybridized with a ^{32}P-labeled gene-specific probe for psbA, which encodes PsbA, the D1 protein of the PS2 reaction centre, at 60 ºC in 250 mM sodium phosphate buffer (pH 7.5), 7 % SDS, 1 % bovine serum albumin, and 1 mM EDTA. After hybridization, the RNA blot was washed twice with 2×SSC/0.1 % SDS and twice with 0.2×SSC/0.1 % SDS at 55 ºC. The radioactive signals of hybridized membranes were detected using a phosphor imaging system (*Fuji*, Japan).

Protein gel blot analysis: Leaves were homogenized in a homogenizer for 10 s in homogenization buffer (330 mM sorbitol, 50 mM HEPES, 2 mM EDTA, 0.1 % BSA, 4 mM DTT, pH 8.0) and filtered through *Miracloth*. The plastids were collected by centrifugation at 6 000×*g* for 2 min. Crude chloroplasts were used for gel blot analysis of the two antenna proteins of PS1 (Lhca2 and Lhca3) and the major Lhc of PS2 (Lhcb2). The proteins were separated using 14 % SDS-PAGE for Lhc and electroblotted onto *PVDF* membranes. Immunodetection was performed using standard procedures (*Boehringer*, Mannheim, Germany). The polyclonal antibodies against Lhca2, Lhca3, and Lhcb2 were purchased from *AgriSera* (Stockholm, Sweden).

Pigment extraction and analysis: For carotenoid analysis, 0.1 g of leaves was ground in 1 cm^3 of 100 % acetone containing 10 mg of $CaCO_3$. The extracts were centrifuged at 16 000×*g* for 10 min, and the resulting supernatants were collected. The pigments were separated by high-performance liquid chromatography using a *Waters 2690* System (*Millipore*, Milford, MA, USA) equipped with a *Waters 2487* Absorbance Detector

(*Millipore*) and a *Spherisorb ODS-1* column (5-µm particle size, 250.0×4.6 mm i.d.; *Alltech*, Deerfield, IL, USA). Solvent A (acetonitrile : methanol : 0.1 M Tris-HCl, pH 8.0, 72 : 8 : 3) was run isocratically from 0 to 4 min, followed by a 2.5-min linear gradient to 100 % solvent B (methanol : hexane, 4 : 1) at a flow rate of 2 cm^3 min^{-1}. The detector was set at 440 nm for the integration of the peak areas. Chl content was measured spectrophotometrically using the method of Lichtenthaler (1987).

Chl *a* fluorescence was measured *in vivo* using a pulse amplitude modulation fluorometer (*PAM-2000*, *Walz*, Effeltrich, Germany) after dark adaptation for 10 min. The quantum yield of electron transport through PS2 ($\Phi_{PS2} = \Delta F/F_m'$: $\Delta F = F_m' - F_t$; F_m', maximal fluorescence in light-adapted state; F_t, fluorescence in steady state), which indicates the quantum efficiency of PS2, was calculated as defined by Genty *et al*. (1989). This provides a quick and useful estimate of light reaction activity and photosynthetic rate. NPQ was also quantified, as previously done by Bilger and Björkman (1990), according to the Stern-Volmer equation, $NPQ = F_m/F'_m - 1$, where F'_m is the lowered maximal yield during photosynthetically active irradiation.

Antioxidant enzymes: Soluble proteins were extracted from 0.25 g of frozen leaves by homogenizing the leaf powder in 2 cm^3 of 100 mM potassium phosphate (pH 7.5) containing 2 mM EDTA, 1 % PVP-40, and 1 mM PMSF. Insoluble material was removed by centrifugation at 15 000×*g* for 20 min at 4 ºC. Equal amounts of protein were electrophoresed on 10 % non-denaturing polyacrylamide gels at 4 ºC for 90 min at a constant current of 30 mA. Gels were stained for SOD isoforms by soaking in 50 mM potassium phosphate (pH 7.8) containing 2.5 mM nitroblue tetrazolium (NBT) in darkness for 25 min, followed by soaking in 50 mM potassium phosphate (pH 7.8) containing 28 mM NBT and 28 µM riboflavin in darkness for 30 min (Rao *et al.* 1996). The gels were then irradiated for approximately 30 min. Staining of POD isozymes was achieved by incubating the gels in sodium citrate (pH 5.0) containing 9.25 mM *p*-phenylenediamine and 3.92 mM H_2O_2 for 15 min (Olson and Varner 1993). Catalase (CAT) activity was detected by incubating the gels in 3.27 mM H_2O_2 for 25 min, rinsing with water, and staining in a solution of 1 % potassium ferricyanide and 1 % ferric chloride for 4 min (Woodbury *et al.* 1971). Gels were stained for GR activity in 250 mM Tris-HCl (pH 7.5) containing 0.2 mg cm^{-3} 3-(4,5-dimethylthiazol-2-4)-2,5-diphenyl tetrazolium bromide, 0.2 mg cm^{-3} 2,6-dichlorophenolindophenol, 3.4 mM GSSG, and 0.5 mM NADPH in darkness for 1 h (Rao *et al.* 1996).

Results

Photodynamic damage in photosystems of transgenic rice overexpressing ALA-S: To assess the involvement of photosynthetic complexes in photodynamic stress, the gene expression of PsbA, which is the D1 protein of the PS2 reaction centre, was investigated in the ALA-S-expressing transgenic rice lines P5 and P14. The transgenic lines exhibited symptoms of photobleaching, which was caused by the increased contents of ALA, Proto IX, and protochlorophyllide (Jung *et al.* 2004). Under LI of 150 µmol m^{-2} s^{-1}, the mRNA contents of the chloroplast gene *psbA* were similar in the WT and transgenic lines P5 and P14, but the Chl content was slightly greater in P5 and P14 than in WT (Fig. 1 and Table 1). The transcript level of *psbA* was unchanged in the WT line under HI of 350 µmol m^{-2} s^{-1}, whereas it was significantly reduced in transgenic lines P5 and P14, with the greatest decrease in P14 (Fig. 1). The P14 line had higher levels of ALA-S activity and ALA than did the P5 line (Jung et al. 2004). In the transgenic lines exposed to HI, the decrease in *psbA* mRNA correlated well with a significant decrease in the Chl content (Fig. 1 and Table 1). In LI, contents of Chl *a*/*b*-binding proteins, including Lhca2 (PS1), Lhca3 (PS1), and Lhcb2 (PS2), did not differ between the WT and transgenic lines (Fig. 2). When exposed to HI, all the Lhc proteins decreased noticeably in transgenic line P14, but not in the P5 or WT lines (Fig. 2).

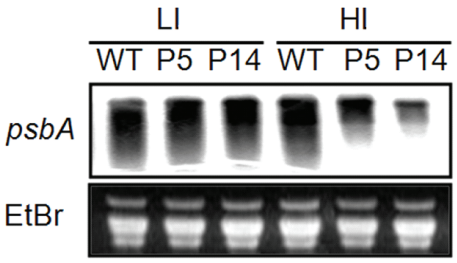

Fig. 1. RNA gel blot analysis of *psbA*, which encodes the PsbA protein (the D1 protein of the photosystem 2 reaction centre). Total RNA (10 µg) was blotted onto a nylon membrane as described in Materials and methods. Seeds of the wild-type (WT) line and transgenic lines P5 and P14 were grown on MS medium in darkness at 25 ºC for 3 d and then exposed to either a PPFD of 150 µmol m^{-2} s^{-1} (LI) or 350 µmol m^{-2} s^{-1} (HI) for 7 d. P5 and P14, T_2 generation of homozygous transgenic rice lines expressing the *B. japonicum* ALA-S gene; EtBr, ethidium bromide staining of RNA samples.

Non-enzymatic and enzymatic defence responses induced by photodynamic stress: We examined the photoprotective responses of ALA-S-expressing transgenic rice upon photodynamic damage under different irradiances. In LI, the quantum yield of electron transport through PS2 was similar in all lines (Table 2). HI decreased the quantum yield in transgenic lines P5 and P14, with a greater decrease in P14, as compared to LI.

Table 1. Carotenoid [mmol mol^{-1}(Chl *a*)] and chlorophyll [mg kg^{-1}(FM)] contents. Plants were subjected to the treatments described in Fig. 1, and portions of the leaves were taken for analysis. WT, wild type; P5 and P14, T_2 generation of homozygous transgenic rice lines expressing the *B. japonicum* ALA-S gene; N.D., not detected. Means ± SE of three replicates.

Pigment	LI			HI		
	WT	P5	P14	WT	P5	P14
Neoxanthin	29.4 ± 1.6	26.8 ± 2.4	28.9 ± 1.1	27.0 ± 0.9	19.6 ± 1.5	7.2 ± 1.0
Violaxanthin	53.1 ± 4.2	55.6 ± 2.9	60.3 ± 1.9	120.0 ± 5.5	69.5 ± 6.2	28.9 ± 3.4
Antheraxanthin	3.5 ± 0.8	2.5 ± 0.4	2.3 ± 0.2	7.1 ± 1.0	14.2 ± 2.1	6.9 ± 0.9
Lutein	79.0 ± 6.5	75.7 ± 9.6	85.3 ± 5.9	81.4 ± 7.6	45.6 ± 3.9	16.9 ± 3.5
Zeaxanthin	N.D.	N.D.	N.D.	3.5 ± 0.8	19.5 ± 2.3	16.9 ± 3.0
β-carotene	60.5 ± 7.8	64.5 ± 1.9	66.9 ± 7.4	69.1 ± 5.1	40.8 ± 2.0	7.5 ± 0.8
Total chlorophyll	1713 ± 11	1796 ± 26	1915 ± 45	1642 ± 95	830 ± 75	422 ± 8

HI did not cause any change in the quantum yield of the WT. In LI, the transgenic lines had a slightly higher NPQ than did the WT line (Table 2). In HI, the NPQ increased slightly in the WT, but increased by three times in the transgenic lines, as compared to responses in LI. No zeaxanthin was detected in any line under LI (Table 1). In response to HI, zeaxanthin was newly detected in all lines and the content was about five times higher in transgenic lines P5 and P14 than in the WT. The antheraxanthin content increased by two times in the WT line and by three to six times in the transgenic lines in HI, as compared to those exposed to LI (Table 1). The violaxanthin content in HI increased by two times in the WT, but decreased greatly in transgenic line P14. Other carotenoids, including neoxanthin, lutein, and β-carotene, were unchanged in the WT exposed to HI, but their contents decreased in transgenic lines P5 and P14, with a greater decrease in P14 (Table 1).

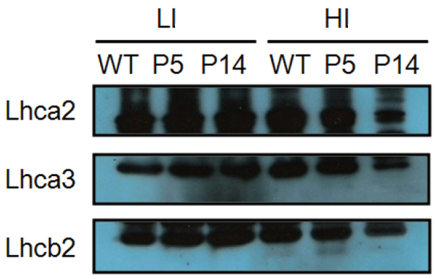

Fig. 2. Protein gel blot analysis of the light-harvesting chlorophyll-binding proteins Lhca and Lhcb. The expression of two antenna proteins of photosystem 1 (Lhca2 and Lhca3) and the major light-harvesting protein of photosystem 2 (Lhcb2) in leaves was assayed by immunoblotting. The plants were subjected to the treatments described in Fig. 1. LI, low irradiance; HI, high irradiance; WT, wild type; P5 and P14, T_2 generation of homozygous transgenic rice lines expressing the *B. japonicum* ALA-S gene.

Table 2. Quantum yield and non-photochemical quenching. Plants were subjected to the treatments described in Fig. 1. WT, wild type; P5 and P14, T_2 generation of homozygous transgenic rice lines expressing the *B. japonicum* ALA-S gene; quantum yield, quantum yield of electron transport through photosystem 2; NPQ, Stern-Volmer quenching, referred to as non-photochemical quenching. Means ± SE of three replicates.

Parameter	LI			HI		
	WT	P5	P14	WT	P5	P14
Quantum yield	0.648 ± 0.010	0.634 ± 0.030	0.637 ± 0.010	0.629 ± 0.010	0.421 ± 0.090	0.210 ± 0.000
NPQ	0.147 ± 0.060	0.169 ± 0.020	0.182 ± 0.060	0.208 ± 0.030	0.557 ± 0.110	0.621 ± 0.150

In LI, the activity of various antioxidant enzymes was similar in the WT line and transgenic lines P5 and P14 (Fig. 3). Chloroplast Cu/Zn-SOD (band 1) and mitochondrial Mn-SOD (band 2) were detected only in P5 and P14 plants exposed to HI (Fig. 3*A*). Cytosolic Cu/Zn-SODs (bands 3 and 4) increased in response to HI in transgenic lines P5 and P14, but not in the WT (Fig. 3*A*). POD activities did not differ in any of the lines exposed to LI, but in HI they were greater in P14 than in the WT and P5, mainly because of increases in POD isozymes 2 and 3 (Fig. 3*B*). However, in all lines examined, CAT activities were unchanged in response to HI and were similar among the different lines (Fig. 3*C*). An increase in GR activity was observed in transgenic line P14 exposed to HI, possibly as a response to increased photodynamic stress (Fig. 3*D*).

Discussion

When ALA-treated plants are irradiated, excess Proto IX accumulation produces $^1O_2$, which oxidizes unsaturated fatty acids on the cell surface (Rebeiz *et al*. 1984). Under HI, the pigment bleaching of transgenic rice plants overexpressing *B. japonicum* ALA-S appears to result from a higher content of Proto IX caused by the ectopic accumulation of ALA (Jung *et al*. 2004). In the present study, the ALA-S-expressing transgenic rice developed higher contents of not only photodynamic damage in the photosynthetic apparatus, but also photoprotective response and scavenging enzymes, in comparison to WT.

With an irradiance of 350 µmol m^{-2} s^{-1}, the transcript level of the chloroplast gene *psbA* was unchanged in the WT, whereas the transcript level was significantly reduced in transgenic lines P5 and P14, with a greater decrease in P14 (Fig. 1), in which more ALA-S activity occurred (Jung *et al*. 2004). Although the mechanism whereby oxidative stress causes a reduction in *psbA* mRNA is not known, the photodynamic stress indicated by the increase in malonyldialdehyde content and the decrease in F_v/F_m in the transgenic lines (Jung *et al*. 2004) might damage the D1 transcription system, thereby decreasing the turnover of D1. In the transgenic lines exposed to HI, the decrease in *psbA* mRNA correlated well with a significant decrease in Chl content (Fig. 1 and Table 1), which is consistent with previous observations of the structure and organization of the photosynthetic apparatus in Chl-deficient plants (Falbel *et al*. 1996, Härtel *et al*. 1997). The generation of $^1O_2$ in the PS2 reaction centre can result in damage to lipids, critical pigment cofactors, and protein subunits associated with PS2, especially the D1 protein, resulting in photo-oxidative inactivation of entire reaction centres (Barber and Andersson 1992, Aro *et al*. 1993). Chls assemble with the light-harvesting and reaction centre proteins of PS1 and PS2 (Green and Durnford 1996). The expression levels of Lhca2, Lhca3, and Lhcb2 were similar among all the lines exposed to LI, but decreased greatly in transgenic line P14 exposed to HI, suggesting that the Chl deficiency in P14 probably led to this degradation of the pigment-binding proteins in PS1 and PS2 (Table 1 and Fig. 2).

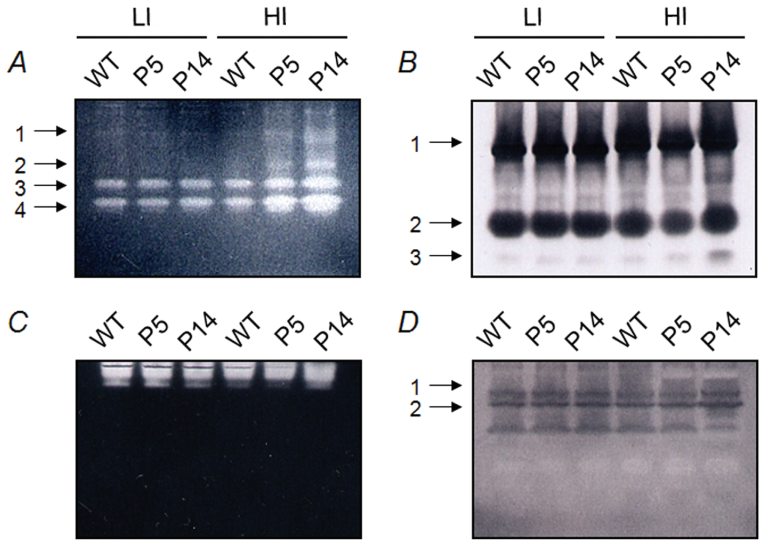

Fig. 3. Isozyme patterns of the scavenging enzymes superoxide dismutase (*A*), peroxidase (*B*), catalase (*C*), and glutathione reductase (*D*). Non-denaturing activity gels were prepared as described in Materials and methods. Incubation of the gels with KCN or H_2O_2 prior to staining for SOD activity suggested that SOD-2 is a Mn-SOD, whereas SOD-1, SOD-3, and SOD-4 are Cu/Zn-SOD enzymes (data not shown). Three forms of POD isozymes (POD-1, POD-2, and POD-3) were detected. Gels stained for GR activity revealed two GSSH-specific bands, GR-1 and GR-2. The plants were subjected to the treatments described in Fig. 1, and portions of the leaves were taken for analysis. LI, low irradiance; HI, high irradiance; WT, wild type; P5 and P14, T_2 generation of homozygous transgenic rice lines expressing the *B. japonicum* ALA-S gene.

Under some circumstances, damaged PS2 reaction centres may also be sites of thermal dissipation and scavengers of AOS (Krause 1988, Sharma *et al*. 1997, Trebst 2003). The increase in irradiance decreased the quantum yield of PS2, but greatly increased NPQ in the transgenic lines P5 and P14, which suggests efficient dissipation of excess photon energy (Table 2). However, the increase in energy dissipation through NPQ could not overcome photodynamic stress in the ALA-S-expressing transgenic plants (Figs. 1 and 2; Table 2). With the small Lhc protein, the dissipation of excess radiation *via* NPQ was more difficult, aggravating photodynamic damage in the transgenic plants. The protection of cells against $^1O_2$ is generally thought to be mediated by carotenoids, which are membrane-bound antioxidants that can quench ^{3}Chl and $^1O_2$, inhibit lipid peroxidation, and stabilize membranes (Demmig-Adams *et al*. 1996, Mittler *et al*. 2004). In response to HI, the new formation of zeaxanthin was five times greater in the P5 and P14 lines than in the WT, and the P5 line exhibited the greatest increase in antheraxanthin content compared to the other lines (Table 1). The binding of zeaxanthin and protons to Lhc may cause a conformational change that is necessary for thermal dissipation (Bilger and Björkman 1990, Ruban *et al*. 1993). The quenching of ^{1}Chl, $^1O_2$, and possibly also the inhibition of lipid peroxidation, are impaired in the absence of both zeaxanthin and lutein (Niyogi *et al*. 1997). Xanthophylls bound to the Lhc proteins are

located in close proximity to Chl for efficient quenching of ^{3}Chl and $^1O_2$ (Kühlbrandt *et al.* 1994). The overall lower contents of carotenoids, including neoxanthin, lutein, and β-carotene, were consistent with a loss of Lhc proteins in the transgenic lines exposed to HI (Fig. 2 and Table 1). This effect might be caused by impairment in the assembly of Chls and pigment-binding proteins and a deficiency in the increased need for redox equivalents in the photobleached transgenic plants expressing ALA-S.

The photodynamic stress in the transgenic plants necessitates the use of other components of the dissipative process, enzymatic antioxidants. Unlike the similar contents of activity of several antioxidant enzymes in all the lines exposed to LI, in HI newly produced chloroplast Cu/Zn-SOD and mitochondrial Mn-SOD activity and an increase in the content of cytosolic Cu/Zn-SODs occurred in the P5 and P14 lines (Fig. 3*A*), showing that these isozymes specifically responded to photodynamic stress induced by elevated ALA-S. The O_2^- radicals generated by the one-electron reduction of O_2 by PS1 are rapidly converted within the chloroplast to H_2O_2 by Cu/Zn-SOD (Asada 1994). Scavenging of H_2O_2 in plants can also be mediated by classical plant POD (Mittler *et al.* 2004). In HI, POD activity was greater in P14 than in the WT and P5 (Fig. 3*B*); content of CAT, which is indispensable for oxidative stress tolerance (Willekens *et al.* 1997), remained unchanged in response to HI (Fig. 3*C*). SOD and CAT significantly protect the PS2 complex against photoinhibition, whereas CAT has no protective effect with respect to PS1 (Tjus *et al.* 2001). The increase in chloroplast Cu/Zn-SOD and GR in transgenic line P14 (Fig. 3) might be evidence for porphyrin-mediated photodynamic stress through the chloroplast expression of ALA-S (Jung *et al.* 2004). Significant increases in SOD and small increases in POD and GR were observed in the transgenic lines, suggesting that the plants are trying to detoxify AOS. The balance among SOD, APX, and CAT activities is critical for suppressing toxic levels of O_2^- and H_2O_2 in a cell (Apel and Hirt 2004, Mittler *et al.* 2004). The localization of the photosensitizer to chloroplast membranes, coupled with the short half-life of $^1O_2$, suggests that the molecules most immediately modified by the photosensitization of porphyrin would likely be localized in or close to the plastid membrane (Ledford and Niyogi 2005). However, enzymatic antioxidant responses in ALA-S-expressing transgenic plants were not confined to chloroplasts, the location for the porphyrin-induced generation of active forms of oxygen and Chls, indicating photosensitization of the entire cell. Surplus protoporphyrinogen IX diffuses out of the plastid and is rapidly oxidized to Proto IX at the plasma membrane by non-specific protoporphyrinogen oxidase-like activity (Lee *et al.* 2000).

Non-enzymatic antioxidant mechanisms and scavenging enzymes are present to deal with the inevitable generation of photodynamic damage in transgenic plants expressing ALA-S. Additional ALA-synthesizing activity in the transgenic plants led to the development of more photodynamic reactions, as indicated by the reduced stability of proteins in the photosynthetic complexes, and of the stronger photoprotective functions. To prevent photooxidative stress at the chloroplast level, the transgenic plants exposed to HI have evolved complex systems, including Cu/Zn-SOD, GR, zeaxanthin, and NPQ. However, the equilibrium between production and scavenging of photodynamic stress appears to be perturbed by a severe stress situation triggered by the accumulation of porphyrins.

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