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Evaluation of the Defense System in Chloroplasts to Photooxidative Stress Caused by Paraquat Using Transgenic Tobacco Plants Expressing Catalase from *Escherichia coli*

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We evaluated the defense system in chloroplasts to photooxidative stress imposed by paraquat treatment under illumination in transgenic tobacco plants with increased tolerance to drought stress at a high light intensity produced by catalase from *Escherichia coli* targeted to chloroplasts [Shikanai et al. (1998) *FEBS Lett.* 428: 47]. At 24 h after the paraquat application, Chl was destroyed in the wild-type plants, but not in transgenic plants. Photosynthetic activities monitored by CO_2 fixation and Chl fluorescence were much less affected by the paraquat treatment in transgenic lines. The activities of chloroplastic ascorbate peroxidase (APX) isoenzymes decreased in parallel with the depletion of ascorbate (AsA) in leaves in both lines. Paraquat treatment had no effect on the transcript level of chloroplastic APX isoenzymes, while it significantly lowered the level of their proteins. These data suggest that the depletion of AsA in chloroplasts under severe stress conditions inactivates and degrades chloroplastic APX isoenzymes.

Key words: Ascorbate peroxidase — Catalase — Chloroplast — Paraquat treatment — Photooxidative stress — Transgenic tobacco.

Photosynthetic cells are prone to oxidative stress. The highly energetic reactions of photosynthesis and an abundant O_2 supply make the chloroplast a particularly rich source of active oxygen species (AOS) (Foyer et al. 1994, Allen 1995, Asada et al. 1998). Environmental stress is the major limiting factor in plant productivity. Much of the injury to plants imposed by stress exposure is associated with oxidative damage at the cellular level. The role of AOS in damage caused by stress in plants is indicated by the increased production of AOS and the increased oxidative damage in tissues during stress. Light, as the energy source for photosynthesis, is essential for plant life. However, excess light intensity can cause increased electron flow to O_2, resulting in greater production of O_2^- and H_2O_2 (Sen Gupta et al. 1993, Asada et al. 1998). In the photosynthetic carbon reduction (PCR) cycle of chloroplasts in higher plants, thiol-modulated enzymes, e.g., fructose-1,6-bisphosphatase (FBPase), sedoheptulose-1,7-bisphosphatase, $NADP^+$-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and phosphoribulokinase (PRK) are regulated by the system which consists of ferredoxin, thioredoxin *f* or *m*, and ferredoxin/thioredoxin reductase (Buchanan 1980, 1991). A low level (10 μM) of H_2O_2 inhibited the CO_2 fixation by 50% due to oxidation of the thiol-modulated enzymes in the PCR cycle (Kaiser 1976, Takeda et al. 1995). Accordingly, chloroplasts of higher plant cells have developed antioxidant defense systems including the water-water cycle consisting of ascorbate peroxidase (APX) isoenzymes and the regeneration system of reduced ascorbate (AsA).

Recently, manipulation of the expression of enzymes involved in the AOS-scavenging systems by gene transfer technology has provided new insight into the role of these enzymes in cellular compartments by allowing direct investigation of their function and interactions (Foyer et al. 1994). Much of this work has focused on superoxide dismutase (SOD), APX and glutathione reductase (GR) isoenzymes that were targeted in the cytosol or plastid (Sen Gupta et al. 1993, Pitcher et al. 1994, Aono et al. 1995a, b, Foyer et al. 1995, Slooten et al. 1995, Webb and Allen 1996). These experiments indicated that the modification of AOS-scavenging systems can lead to considerable changes in oxidative stress tolerance (Allen et al. 1997). To analyze the potential of the AOS-scavenging system of chloroplasts in higher plants, we introduced *E. coli* catalase encoded by *katE* into tobacco chloroplasts (Shikanai et al. 1998). The transgenic plants had increased tolerance to photooxidative stress imposed by drought at a high light intensity.

To explore in more detail the mechanism of tolerance to photooxidative damage conferred by the chimeric cata-

Abbreviations: AOS, active oxygen species; APX, ascorbate peroxidase [chlAPX, chloroplastic (stromal and thylakoid-bound) APX; cAPX, cytosolic APX; mAPX, microbody-bound APX; sAPX, stromal APX; tAPX, thylakoid-bound APX]; AsA, reduced ascorbate; DAsA, dehydroascorbate; DTT, dithiothreitol; FBPase, fructose-1,6-bisphosphatase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; $NADP^+$-GAPDH, $NADP^+$-glyceraldehyde-3-phosphate dehydrogenase; PCR cycle, photosynthetic carbon reduction cycle; PRK, phosphoribulokinase; PS, photosystem; SOD, superoxide dismutase.

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lase and the inhibition of chloroplastic APX (chlAPX) isoenzymes under the stress conditions, we have studied the effects of paraquat treatment on the physiological status, antioxidant enzymes and the antioxidant metabolites in tobacco leaves. The bipyridylium herbicide paraquat (methyl viologen) acts in chloroplasts in the light through the generation of O_2^- in a chain reaction, producing AOS. We examined the expression of catalase from *E. coli* in the chloroplast in relation to the capacity to protect the photosynthetic apparatus including thylakoid membranes as well as the stromal enzymes from photooxidative stress caused by paraquat application. The inhibition of chlAPX isoenzymes may be the primary mechanism of photooxidative damage in tobacco plants.

Materials and Methods

Plant material—Transgenic tobacco plants (T_2: T43-1) overexpressing *E. coli* catalase (*katE* gene) and wild-type plants (*Nicotiana tabacum* cv. Xanthi) were cultured for 7 weeks in a growth chamber under a 12-h light/12-h dark regime, with a moderate light intensity (300 μmol m^{-2} s^{-1}), 60% relative humidity, and day/night temperature of 25/20°C. No difference could be seen in growth or morphology between wild-type and transgenic plants as previously described (Shikanai et al. 1998).

Paraquat treatment—Plants were sprayed with 50 μM paraquat in 0.1% Tween 20 and exposed to light (300 μmol m^{-2} s^{-1}) for the indicated times. For the enzyme assays and the measurement of metabolites, discs (1.1 cm^2) from the third leaves of four plants in each line were harvested at 0 h, 6 h and 24 h after paraquat treatment, frozen in liquid N_2 and stored at −80°C. Differences were considered significant at the $p < 0.05$ level.

Enzyme extraction and assay—One unit of each enzyme activity except for SOD is defined as the amount required to change 1 μmol of substrate per min. Protein was determined by the method of Bradford (1976) with bovine serum albumin as a standard. Chl was measured by the method of Arnon (1949). Both cytosolic APX (cAPX) and microbody-bound APX (mAPX) isoenzymes are less sensitive to depletion of AsA than stromal APX (sAPX) and thylakoid-bound APX (tAPX); cAPX and mAPX have a half-inactivation time of approximately 60 min and over 24 h, respectively (Chen and Asada 1989, Miyake and Asada 1992, Ishikawa et al. 1998). On the basis of this characterization, the activities of the APX isoenzymes were separately assayed by the modified method reported by Amako et al. (1994). APX was measured by the decrease in absorbance at 290 nm (2.8 mM^{-1} cm^{-1}) due to AsA oxidation according to Shigeoka et al. (1980). Leaf tissues (1.1 $cm^2 \times 10$ discs) were ground to a fine powder in liquid N_2 and then homogenized in 1 ml of 50 mM potassium phosphate buffer (pH 7.6), 0.3 M sorbitol, 10 mM KCl, 5 mM $MgCl_2$, 5 mM AsA and 2% (W/V) polyvinylpyrrolidone using a pestle and mortar. The homogenate was centrifuged for 15 min at 100,000 × *g*. The soluble fraction contained activities of sAPX and cAPX isozymes, while the membrane fraction had activities of tAPX and mAPX isozymes. The supernatant (5 μl) obtained was added to 980 μl of 50 mM potassium phosphate buffer (pH 7.0) containing 10 μM H_2O_2. At 1, 2, 3 and 5 min after the start of the incubation, 10μl of 40 mM AsA was added to terminate the inactivation. The residual oxidizing activity of APX was then assayed by adding 5 μl of 20 mM H_2O_2. The ratio of cAPX and sAPX activities was calculated from the inactivation curve of each isoenzyme. Then the 100,000 × *g*-membrane fraction was washed and suspended in the same buffer. The suspended-membrane fraction contained activities of tAPX and mAPX isoenzymes; they were separately assayed by the same method using each half-inactivation time for measurement of activities of sAPX and cAPX isoenzymes as described above. GR activity was determined by measuring the rate of NADPH oxidation as the decrease in absorbance at 340 nm (6.22 mM^{-1} cm^{-1}) according to the method of Halliwell and Foyer (1978). Leaf tissues (1.1 $cm^2 \times 20$ discs) were ground to a fine powder in liquid N_2 and then homogenized in 1 ml of 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM AsA, 2% polyvinylpyrrolidone and 0.05% Triton X-100 using a pestle and mortar. The homogenate was centrifuged at 12,000 × *g* for 10 min to obtain the supernatant. The reaction mixture (1 ml) consisted of 100 mM Tris-HCl (pH 7.8), 2 mM EDTA, 0.05 mM NADPH, 0.5 mM oxidized glutathione (GSSG) and the enzyme. NADPH was added to start the reaction. Catalase activity was determined spectrophotometrically by following the rate of H_2O_2 disappearance at 240 nm, taking $\Delta\varepsilon$ at 240 nm as 43.6 M^{-1} cm^{-1} (Patterson et al. 1984). Leaf tissues (1.1 $cm^2 \times 10$ discs) were ground to a fine powder in liquid N_2 and then homogenized in 2 ml of 50 mM potassium phosphate (pH 7.0) and 2% polyvinylpolypyrrolidone using a pestle and mortar. The resulting lysate was centrifuged at 12,000 × *g* for 10 min, and the supernatant was used for the catalase assay. The reaction mixture contained 50 mM potassium phosphate (pH 7.0), 10.5 mM H_2O_2 and the enzyme solution. The reaction was run at 27°C for 2 min, and the initial linear rate was used to calculate the activity. PRK was assayed at 27°C in a reaction that was coupled with pyruvate kinase and lactate dehydrogenase (Takeda et al. 1995). Leaf tissues (1.1 $cm^2 \times 10$ discs) were ground to a fine powder in liquid N_2 and then homogenized with 1 ml of 100 mM Tris-HCl buffer (pH 8.0), 10 mM $MgCl_2$, 1 mM EDTA, 20 mM dithiothreitol (DTT) and 2% polyvinylpyrrolidone using a pestle and mortar. The homogenate was centrifuged for 10 min at 12,000 × *g*. The supernatant was used to assay PRK, FBPase, and $NADP^+$-GAPDH. The reaction mixture contained 100 mM Tris-HCl buffer (pH 8.0), 10 mM $MgCl_2$, 5 mM reduced glutathione (GSH), 1 mM ATP, 0.2 mM NADH, 50 mM KCl, 0.5 mM phospho*enol*-pyruvate, 2 units of pyruvate kinase, 5 units of lactate dehydrogenase, 2 units of ribose 5-phosphate isomerase, 2 mM ribose 5-phosphate and the enzyme solution. The activity was determined by monitoring the oxidation of NADH at 340 nm. FBPase was assayed at 27°C in a reaction that was coupled with glucose 6-phosphate dehydrogenase and phosphoglucose isomerase (Takeda et al. 1995). The reaction mixture contained 100 mM Tris-HCl buffer (pH 8.0), 10 mM $MgCl_2$, 0.4 mM $NADP^+$, 0.5 mM EDTA, 0.2 mM fructose 1,6-bisphosphate, 0.5 units of glucose-6-phosphate dehydrogenase, 1.5 units of phosphoglucose isomerase and the enzyme. Activity was determined by monitoring the reduction of $NADP^+$ at 340 nm. $NADP^+$-GAPDH was assayed at 27°C in a reaction mixture containing 100 mM Tris-HCl buffer (pH38.0), 10 mM $MgCl_2$, 0.2 mM NADPH, 5 mM ATP, 3 mM phosphoglycerate, 10 mM GSH, 2 units of phosphoglycerophosphate kinase and the enzyme. Activity was determined by monitoring the oxidation of NADPH at 340 nm (Takeda et al. 1995). Total SOD was assayed by its ability to inhibit the reduction of ferri cytochrome *c* by the O_2^- generated with a xanthine-xanthine oxidase system (McCord and Fridovich 1969). Leaf tissues (1.1 $cm^2 \times 20$ discs) were ground to a fine powder in liquid N_2 and then homogenized in 1 ml of 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 2% polyvinylpyrrolidone and 0.5% Triton.

The homogenate was centrifuged at 12,000 × *g* for 10 min. The supernatant obtained was filtered through a PD-10 column (Amersham Pharmacia Biotech, Uppsala, Sweden), and the elute was used for the SOD assay. One unit of activity was defined as the amount of enzyme required to inhibit cytochrome *c* reduction by 50%.

Gas-exchange and Chl fluorescence measurements—CO_2 fixation was measured with a portable photosynthesis system LI-6400 (Li-Cor, Lincoln, NE). Net CO_2 assimilation rates were measured using fully expanded leaves under the following conditions: 400 μmol m^{-2} s^{-1}, 1,000 μmol CO_2 mol^{-1}, 25°C, 60% relative humidity. Change of Chl fluorescence was measured at 25°C with a Mini PAM Chl Fluorometer (Waltz, Efeltrich, Germany). The minimum fluorescence yield (F_0) was determined after a 30-min dark adaptation, followed by illuminating the sample with a low-irradiance measuring light (approx. 0.12 μmol m^{-2} s^{-1}). A saturating pulse of white light (0.8 s, 10,000 μmol m^{-2} s^{-1}) was applied to determine the maximal fluorescence yield (F_m). The maximal quantum yield of photosystem (PS)II (F_v/F_m) was determined from the following equation: $F_v/F_m = F_m - F_0/F_m$.

Determinations of AsA and DAsA—AsA and DAsA were measured as described by Wise and Naylor (1987). Leaf tissues (1.1 cm^2 × 10 discs) were ground in liquid N_2 using a pestle and mortar with 2 ml of 5% $HClO_4$ and centrifuged at 10,000 × *g* for 5 min. A 100-μl aliquot of the leaf extract obtained was added directly to 900 μl of a 200 mM succinate buffer (pH 12.7, adjusted with NaOH) in a spectrophotometer. The final pH was approximately 6.0. The absorbance at 265 nm was recorded immediately and again 5 min after the addition of 5 units of AsA oxidase (Wako, from *Cucurbita* sp.). For determination of total AsA, the leaf extract was adjusted to pH 6.0 with 1.25 M K_2CO_3 and centrifuged at 10,000 × *g* for 5 min. The supernatant was incubated with 10 mM DTT in HEPES-KOH buffer (pH 7.5) for 10 min at 25°C. A 100-μl aliquot of the solution was added directly to 900 μl of a 200 mM succinate buffer (pH 6.0) in a spectrophotometer. The resultant solution was assayed as previously described. The difference between the total AsA and AsA contents was taken to be the content of DAsA.

Determination of GSH and GSSG—GSH and GSSG levels were determined according to the method of Griffith (1980). Leaf tissues (1.1 cm^2 × 10 discs) were ground using a pestle and mortar in liquid N_2 with 1 ml of 5% $HClO_4$ and centrifuged at 10,000 × *g* for 5 min. The supernatant was adjusted to pH 7.0 with 1.25 M K_2CO_3 and centrifuged at 10,000 × *g* for 5 min. The solution obtained was used for the assay of GSH and GSSG. The total GSH was measured by an enzymatic recycling assay based on GR in a reaction mixture (1 ml) containing 100 mM sodium phosphate buffer (pH 7.5), 5 mM EDTA, 0.2 mM NADPH, 0.6 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), and the supernatant. The reaction was started by the addition of 0.05 units of GR, and the reduction rate of DTNB was monitored at 412 nm for 3 min. GSSG was selectively measured by assaying the sample in which GSH was masked by treatment with 2-vinylpyrimidine. To 500 μl of the sample was added 10 μl of 1 M 2-vinylpyrimidine; this was vigorously mixed for 1 min then left at 25°C for 1 h. The resultant solution was assayed as previously described. The difference between the total GSH and GSSG contents was taken to be the content of GSH.

RNA extraction and northern blot analysis—We have previously described that the tAPX and sAPX isoenzymes arise from a common pre-mRNA, generated from an identical gene (*ApxII*), by alternative splicing of the 3′-terminal exons (Ishikawa et al. 1997). As a result, four mRNA variants, one form of tAPX (tAPX-I) and three forms of sAPX (sAPX-I, sAPX-II, and sAPX-III) are generated (Yoshimura et al. 1999). sAPX-II and sAPX-III mRNAs contained a sequence derived from exon 13 including the coding sequence of the membrane anchoring segment of tAPX as an untranslated region. Recently, we found that four mRNA variants also arise from common pre-RNA by alternative splicing of the 3′-terminal exons in the tobacco plants (unpublished data). Accordingly, the respective transcript levels of sAPX and tAPX isoenzymes are difficult to determine by Northern blot analysis. Therefore, in this experiment we detected the transcript levels of chlAPX isoenzymes using a cDNA of tAPX as a probe. Total RNA was isolated from tobacco leaves (1.0 g FW) according to the procedure of Logemann et al. (1987). Total RNA (30 μg each) was subjected to electrophoresis on 1.2% agarose gel containing 2.2 M formaldehyde and transferred to a Hybond N membrane (Amersham Pharmacia Biotech, Uppsala, Sweden). The membrane was Prehybridized at 55°C for 3 h in a buffer containing 5 × SSC, 5 × Denhard's solution, 1% SDS, and 100 μg ml^{-1} denatured salmon sperm DNA. The membrane was hybridized at 55°C for 12 h in the presence of the individual ^{32}P-random primed spinach cDNA of tAPX and washed twice at room temperature in 2 × SSC, 0.1% SDS for 10 min each, and in 0.1 × SSC, 0.1% SDS at 60°C for 1 h. The membrane was then exposed to an imaging plate, and the relative expression ratio of plastidic APX transcript was calculated with a Mac BAS 2000 (Fuji Photofilm, Tokyo, Japan) and shown as the mean values from three individual experiments.

Western blot analysis—To measure the protein levels of APX isoenzymes, 1.1 cm^2 of leaf tissues (30 discs) were ground to a fine powder in liquid N_2 and then homogenized with 1 ml of SDS-loading buffer [150 mM Tris-HCl (pH 6.8), 4% SDS, and 10% 2-Mercaptoethanol]. The homogenates were boiled for 5 min and centrifuged at 10,000 × *g* for 10 min. The supernatant (30 μl) was separated on a 12.5% slab gel, according to the method of Leammli (1970). The gels were stained with Coomassie Brilliant Blue R-250. For immunoblotting, the proteins were transferred to PVDF membranes, using an electroblot apparatus (Model 200/2.0, Bio-Rad, CA) at 13 V for 1 h. The membranes were treated with the monoclonal antibody-I raised against spinach chlAPX isoenzymes. Monoclonal antibody-I specifically reacted with chlAPX isoenzymes (unpublished data). Antibody binding was revealed using alkaline phosphatase as described earlier (Sambrook et al. 1989).

Results

Visible symptoms—To evaluate the efficiency of *E. coli* catalase introduced into tobacco chloroplasts under the photooxidative stress, 50 μM paraquat was sprayed on the wild-type and T43-1 plants under illumination at 300 μmol m^{-2} s^{-1} (Fig. 1A). At 6 h after spraying, no detectable damage was observed in either plant. However, at 24 h after the paraquat treatment destruction of Chl became apparent in wild-type plant, but not in the transgenic plants (Fig. 1A). When sprayed with 50 μM paraquat and exposed to a high light intensity (1,600 μmol m^{-2} s^{-1}), wild-type plants developed severe visible leaf injury after 24 h, while the transgenic plants did not exhibit the chlorosis seen in the wild-type plants (Fig. 1B). Neither the wild-type nor transgenic plants sprayed with paraquat in the dark showed

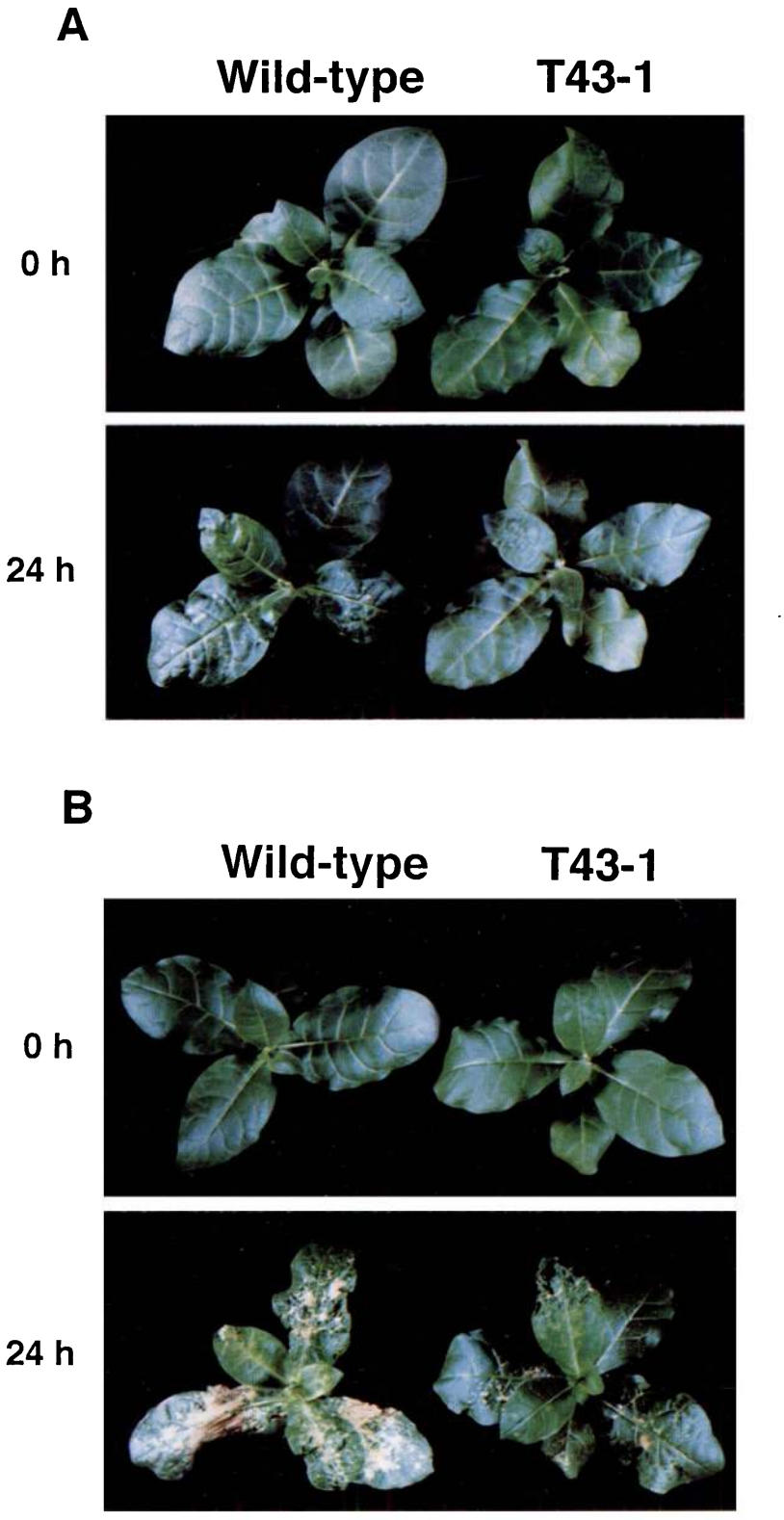

Fig. 1 Effects of paraquat treatment on wild-type and transgenic plants (T43-1). Both lines were sprayed with paraquat (50 μM) in 0.1% Tween 20 (25 ml) and exposed to light (300 μmol m^{-2} s^{-1}) (A) and high-intensity light (1,600 μmol m^{-2} s^{-1}) (B) for indicated times.

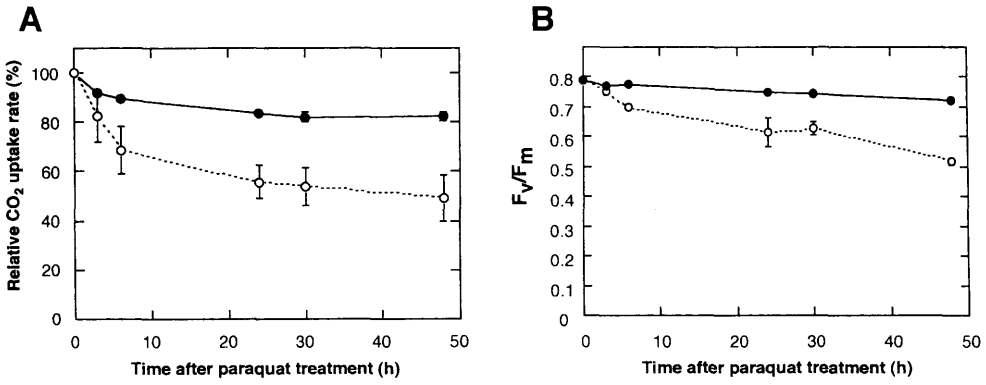

Fig. 2 Effects of paraquat treatment on the photosynthetic activity and the PSII activity (F_v/F_m) in wild-type and transgenic plants (T43-1). The CO_2 fixation of the third leaf in tobacco was measured at 1,000 μmol CO^2 mol^{-1}, 400 μmol m^{-2} s^{-1}, 25°C, 60% relative humidity. The PSII activity was performed at 25°C after dark adaptation for 30 min. Values presented are means ± SD expressed as % of initial values for each experiment in photosynthetic activity. A, photosynthetic activity; B, Chl fluorescence. Photosynthetic activities of wild-type and transgenic plants were 14.8 ± 1.3 and 14.7 ± 1.4 μmol CO^2 m^{-2} s^{-1}, respectively, before stress treatment. PSII activities of wild-type and transgenic plants were 0.792 ± 0.003 and 0.793 ± 0.006, respectively, before stress treatment. ○, wild-type; ●, T43-1.

degradation of Chl (data not shown). These results demonstrated that photooxidative damage is caused by the excess generation of AOS via O_2^- in chloroplasts exacerbated by paraquat under illumination and that transgenic tobacco plants had increased tolerance to photooxidative damage imposed by AOS.

Photosynthetic activity—To assess the resistance to oxidative stress in whole plants, photoinhibition during the first 24 h after the paraquat treatment was monitored as CO_2 fixation and PSII activity [variable fluorescence (F_v)/maximal fluorescence (F_m)] in leaves of transgenic and wild-type plants (Fig. 2A, B). The CO_2 fixation of wild-type plants decreased to 70% and 55% at 6 and 24 h after stress treatment, respectively, while that of transgenic plants decreased to 90% after 6 h and 83% after 24 h. The PSII activity (F_v/F_m) of wild-type plants decreased to 88% after 6 h, while the activity in the transgenic lines remained at a high level.

Effect of paraquat treatment on the activities involved in PCR cycle and AOS-scavenging system—We compared thiol-modulated enzymes in the PCR cycle and AOS-scavenging enzymes involved in the AsA-GSH cycle between wild-type and transgenic plants grown for 7 weeks under moderate conditions (Table 1). No significant difference in the activities of enzymes except for the activities of catalase and SOD was observed. The total activity of catalase in transgenic plants was approx. 3.2-fold higher than that in wild-type plants. Interestingly, the total SOD activity in transgenic plants has been found to be 1.7-fold higher than that in wild-type plants, when grown under normal conditions. Tanaka (1998) reported that transgenic rice overexpressing Cu/Zn-SOD in chloroplasts has enhanced total APX activity as much as SOD is enhanced. It has been reported that a small increase in APX occurs in cotton plants expressing chloroplast-targeted Mn-SOD (Payton et al. 1997). These data indicated that the balanced interaction of antioxidant enzymes may be necessary to obtain a substantial increase in stress tolerance. These problems may involve factors associated with the genes for AOS-scavenging enzymes rather than a problem with the host plant per se.

Figures 3 and 4 show the activities of thiol-modulated enzymes and AOS-scavenging enzymes and levels of antioxidants in the wild-type and transgenic plants at 6 and 24 h after the paraquat spraying. The activity derived from endogenous and chimeric catalases in transgenic plants was little affected for 24 h after the paraquat application, while the endogenous catalase in the wild-type plants was inactivated by 40%. The activities of PRK, $NADP^+$-GAPDH, and FBPase in the PCR cycle remained high in the transgenic lines, while those of the wild-type plants decreased to 70%, 50%, and 78% at 24 h after the paraquat treatment. The activities of sAPX and tAPX in wild-type plants decreased to 39% and 16%, respectively, at 24 h after stress treatment, while those in the transgenic plants decreased to 62% and 19%, respectively. The levels of mRNAs of chlAPX isoenzymes were scarcely changed under paraquat application in both lines. By contrast, the level of the proteins of chlAPX isoenzymes decreased during the stress conditions (Fig. 5). Interestingly, the activities of cAPX and GR increased at 24 h after the paraquat treatment, especially, in wild-type plants. At 6 h after the paraquat application, the total activity of SOD decreased to 83% in wild-type plants, while the activity in transgenic plants remained at the initial level (Fig. 4).

Changes in the level and ratio of AsA/DAsA and GSH/GSSG—The total AsA content (AsA + DAsA) of the leaves of the wild-type plants was approx. 3.4 μmol (mg

Table 1 Photosynthetic activities and enzyme activities and levels of antioxidants before stress treatment in wild-type and transgenic plants (T43-1)

	Wile-type	T43-1
CO_2 fixation (μmol CO_2 m^{-2} s^{-1})	14.8 ±1.3	14.7 ±1.4
PSII activity (F_v/F_m)	0.792±0.003	0.793±0.006
Thiol-modulated enzymes [U (mg protein)$^{-1}$]		
PRK	2.83 ±0.72	2.89 ±0.39
FBPase	0.11 ±0.14	0.11 ±0.01
$NADP^+$-GAPDH	1.14 ±0.07	1.09 ±0.19
AOS scavenging enzymes [(U (mg protein)$^{-1}$]		
Cytosolic APX	1.45 ±0.14	1.37 ±0.06
Stromal APX	0.34 ±0.03	0.39 ±0.02
Thylakoid-bound APX	0.15 ±0.03	0.15 ±0.02
GR	34.6 ±3.0 ($\times 10^{-3}$)	33.6 ±3.0 ($\times 10^{-3}$)
SOD	4.43 ±0.05*	7.75 ±1.62*
Catalase	28.9 ±3.2*	92.1 ±1.2*
Antioxidants [μmol (mg chlorophyll)$^{-1}$]		
AsA	2.98 ±0.04	3.04 ±0.17
DAsA	0.37 ±0.01	0.37 ±0.03
GSH	0.25 ±0.02	0.24 ±0.04
GSSG	0.067±0.008	0.072±0.029

Data are means±SD (n=3).
Symbol (*) in paired columns (wild-type, T43-1) refers to statically significant differences at $p<0.05$.

Chl)$^{-1}$, with 89% of the AsA present in the reduced form. No significant differences in the total AsA level and the ratio of AsA/DAsA between wild-type and transgenic plants before stress conditions were observed. A similar decrease in AsA occurred for both plants under the paraquat treatment (Fig. 4). In constant, the content of DAsA was hardly changed in both plants. At 6 h after the paraquat application, AsA levels of the wild-type and transgenic lines decreased to 76% and 83%, respectively. At 24 h after the spraying, the AsA level decreased to about 75% in both lines. It was found that chlAPX activities decreased in parallel with a decrease in the level of AsA in both wild-type and transgenic plants. The total GSH content (GSH+GSSG) and the ratio of GSH/GSSG were similar in both lines before the stress conditions. The total GSH pool size increased in both plants after the paraquat treat-

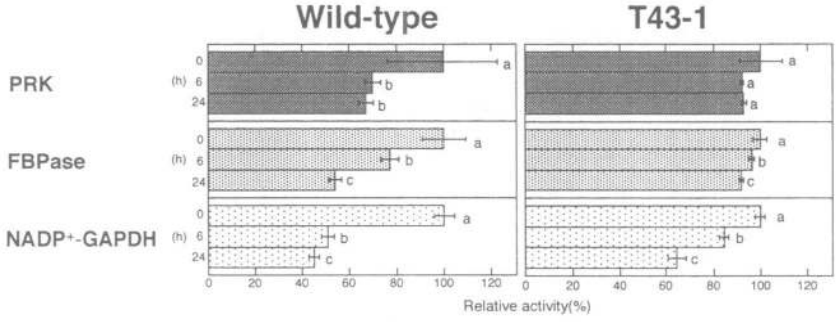

Fig. 3 Effects of paraquat treatment on the activities of thiol-modulated enzymes in wild-type and transgenic plants (T43-1). Third leaves of wild-type and transgenic plants were harvested at indicated times after paraquat treatment. Means±SD from three experiments are shown, expressed as percentages of the initial activities in each case for both wild-type and transgenic plant. Values without a common letter are significantly different according to t-test ($p<0.05$).

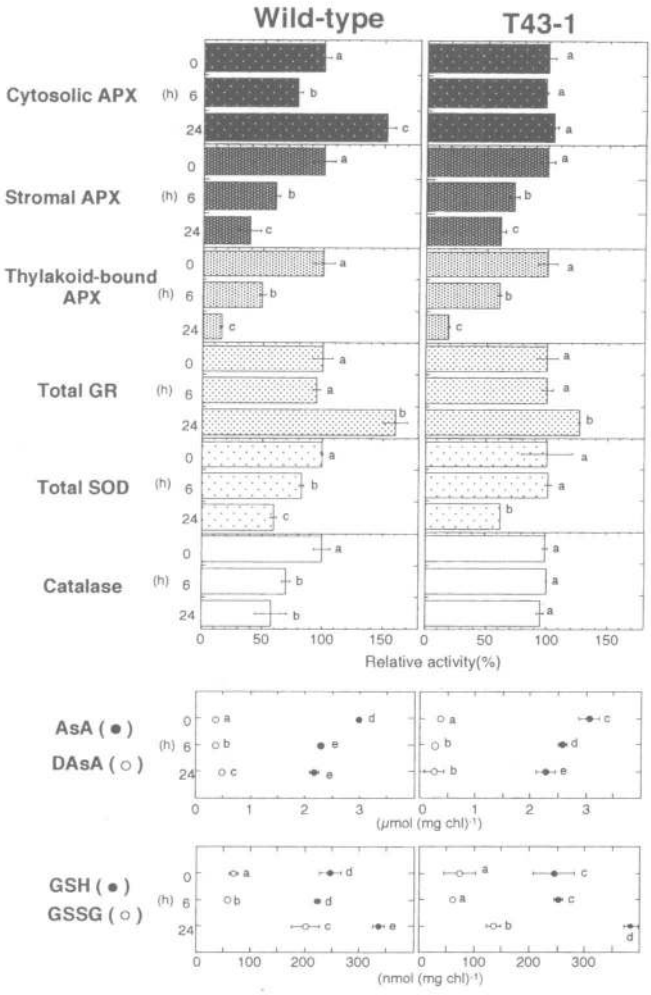

Fig. 4 Effects of paraquat treatment on AOS-scavenging enzymes and levels of antioxidants in wild-type and transgenic plants (T43-1). Third leaves of wild-type and transgenic plants were harvested at indicated times after paraquat treatment. Means ± SD from three experiments are shown, expressed as percentages of the initial activities in each case for both wild-type and transgenic plants. Values without a common letter are significantly different according to t-test ($p < 0.05$).

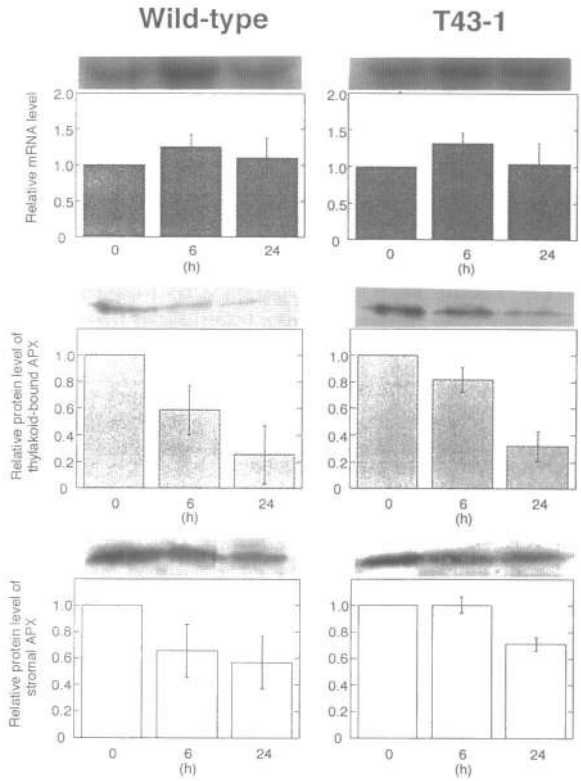

Fig. 5 Effects of paraquat treatment on transcript levels and proteins of chlAPX isoenzymes. Total RNAs (30 μg each) were isolated from spinach leaves, separated by electrophoresis, blotted onto a membrane, and hybridized with a cDNA encoding tAPX as described in the Experimental procedures section. mRNA levels in each sample were quantified by Mac BAS 2000, normalized to the respective 18S ribosomal RNA. Each extract aliquot was subjected to SDS-PAGE and immunoblotting as described in the Experimental procedures section and probed with a monoclonal antibody-I raised against spinach stromal APX isoenzyme. The quantitative intensity was determined by applying densitometry to video images of the blots (ATTO). mRNA levels and protein levels represented as the mean value and ±SD of three individual experiments.

ment. At 24 h after the paraquat spraying, the GSH levels of wild-type and transgenic lines increased approx. 1.4-fold and 1.6-fold, respectively, while the GSSG levels of wild-type and transgenic lines increased approx. 3.0-fold and 1.8-fold, respectively.

Discussion

To examine the tolerance to photooxidative stress by foreign catalase, we sprayed wild-type and T43-1 plants with 50 μM paraquat under illumination at 300 μmol $m^{-2} s^{-1}$ or 1,600 μmol $m^{-2} s^{-1}$. The applied paraquat is photoreduced by PSI and subsequently reoxidized by transfer of its electrons to O_2, forming O_2^- and then H_2O_2 (Bowler et al. 1992). The visible leaf injury of wild-type plants by the paraquat treatment demonstrated that AOS generated by paraquat under illumination caused the oxidative damage of plant cells and that the expression of *E. coli* catalase in transgenic lines resulted in increased antioxidant capacity and improved tolerance to photooxidative stress (Fig. 1). Changes in CO_2 fixation and PSII activity (F_v/F_m) in leaves between transgenic and wild-type plants indicated that during the early stages of the stress treatment, photosynthetic activities in transgenic lines were much less influenced by the stress treatment (Fig. 2). To analyze the effect of the chimeric catalase on protection from photooxidative damage, we studied changes in enzyme activities involved in the H_2O_2-scavenging system and

in the PCR cycle at 6 and 24 h after the paraquat treatment (Fig. 3, 4). In transgenic plants, the activities of photosynthesis, thiol-modulated enzymes, and catalase remained high compared with those in the wild-type plants after the paraquat application. In the wild-type and transgenic plants, the activities of chlAPX isoenzymes dramatically decreased at 24 h after the paraquat application. The activities of thiol-modulated enzymes were significantly different between the wild-type and transgenic lines under the stress conditions. The data suggest that chlAPX isoenzymes are much more strongly inactivated by oxidative stress than thiol-modulated enzymes, which are believed to be the enzymes most sensitive to H_2O_2 and that resistance to the oxidative stress of plants may be diminished by the instability of APX isozymes themselves under oxidative conditions.

On the basis of the data reported here, we propose a mechanism for resistance of transgenic plants to oxidative stress. During the early stages of photooxidative stress, O_2^- generated by paraquat is disproportionated to H_2O_2 by SOD, which is then scavenged by the resident enzyme systems, resulting in a low level of H_2O_2. However, when the AOS production far exceeds the endogenous AOS-scavenging capacity, the regulated balance between the generation system and scavenging system of AOS may be broken. Interestingly, the endogenous catalase in the wild-type plants was significantly inactivated during 24 h after exposure to the paraquat (Fig. 4), supporting the fact that endogenous catalase located in microbodies of higher plants is light sensitive and undergoes photoinactivation with subsequent degradation (Tel-Or et al. 1986, Feierabend et al. 1992, Hertwig et al. 1992). In contrast, the activities derived from endogenous and chimeric catalases in transgenic plants were hardly affected, suggesting that *E. coli* catalase may be relatively resistant to light-inactivation. Although *E. coli* catalase in the transgenic plants is present in chloroplasts as a soluble enzyme (Shikanai et al. 1998), it may be localized in the vicinity of the thylakoid membranes and in the producing site of AOS in chloroplasts. Consequently, under severe stress conditions, the foreign catalase can function to remove H_2O_2 instead of chlAPX isoforms and to protect the stromal enzymes, that is, thiol-modulated enzymes, and photosynthetic apparatus in thylakoid membranes from oxidative stress. It has been reported that a high level of H_2O_2 which can be generated under severe stress conditions could directly inactivate chlCu/Zn-SOD (Casano et al. 1997). At 6 h after the paraquat treatment, the total SOD activity decreased to 83% of the initial level in control plants, while in the transgenic plants the initial activity of SOD was retained (Fig. 4). It seems likely that the increased catalase also protects the degradation of SOD in chloroplasts of transgenic plants imposed by oxidative stress.

Why are chlAPX isoenzymes sensitive to photooxidative stress in both plant lines? Miyake and Asada (1996) reported that only 2 μM H_2O_2 inactivates chlAPX isoenzymes within several seconds, when the level of AsA is too low for the operation of the catalytic cycle of the APX isoenzymes. This finding leads to the suggestion that inactivation with the subsequent degradation product of the heme moiety of chlAPX isoenzymes under stress conditions may depend on the presence of AsA in the vicinity of the H_2O_2-scavenging site. A significant decrease in the total AsA occurred for both genotypes with the paraquat treatment; it was due mainly to that of AsA (reduced form) in both lines (Fig. 4). About 30% to 40% of the cellular AsA content is localized in the chloroplasts of higher plants, although chloroplasts occupy only 3 to 4% of the total cellular volume in a mature leaf mesophyll cell (Foyer et al. 1983, Gillham and Dodge 1986). Accordingly, it may be proposed that the AsA level in the chloroplasts of tobacco plants is significantly depleted under stress conditions. In fact, a decrease in AsA level was in harmony with the decreases in the activities of chlAPX isoenzymes in both lines with the paraquat treatment (Fig. 4). Interestingly, transcript levels of chlAPX isoenzymes were hardly altered, but their protein levels significantly decreased under stress conditions (Fig. 5). It is possible to assume that under stress conditions the generation rate of H_2O_2 in chloroplasts is several orders of magnitude higher than that estimated for non-stressed chloroplasts. When tobacco leaves suffer from photooxidative stress caused by paraquat treatment, the level of AsA in the vicinity of the chlAPX isoenzymes may decrease by the excess of AOS generated by paraquat treatment, which causes the irreversible damage of plastidic APX isoenzymes. Iturbe-Ormaetxe et al. (1998) reported that total APX activity and AsA content showed an identical reduction in pea plant under stress conditions induced by severe water deficit or paraquat. Conklin et al. (1997) reported that the *Arabidopsis* mutant vtc1 is deficient in AsA, accumulating approx. 30% of wild-type levels and is more sensitive to stress conditions than the corresponding wild-type such as O_3, UV-B light, and SO_2 that generate AOS. No differences that could account for the deficiency were found in the activities of enzymes that catalyze the oxidation or reduction of AsA. Among them, however, the overall activity of APX in the AsA-deficient *Arabidopsis* mutant vtc1 was significantly lower than that in the wild-type plants. The increase in the overall APX activity in the wild-type plants required the inclusion of AsA in the extraction buffer, while the enzyme activity in vtc1 did not depend upon. Taken together, these results supported the hypothesis that the decline in total APX activity in vtc1 may reflect a specific loss of chlAPX activities due to the lower endogenous AsA levels in this mutant. In addition, Arrigoni et al. (1997) observed a reduction in APX activity in *Lupines arbustus* seedlings in which AsA deficiency was induced by

lycorine treatment. Based on the data accumulated thus far, it is clear that a high level of endogenous AsA is essential to effectively maintain the antioxidant system that protects plants from oxidative damage due to biotic and abiotic stresses. Accordingly, manipulation of specific enzymes for AsA biosynthesis, including L-galactono-γ-lactone dehydrogenase, may allow us to possibly engineer plants containing stably increased levels of AsA and to improve their stress tolerance.

If the plant's defense against oxidative stress could be reinforced with a new gene and coordinated to maintain the appropriate physiological balance of all components, photooxidative stress tolerance would be improved. The expression of a single gene, *E. coli katE*, provided significant protective effects against photooxidative stress caused by drought under high-light intensity or paraquat application. However, it should be remembered that the stress regimes used are designed to produce detectable stress damage in a short period of time in the laboratory. It is not clear whether these increases in tolerance could have substantial effects in nature, such as plants exposed to frequent periods of severe stress throughout a growing season. Field tests of transgenic plants will surely provide the answer to these and other questions about the utility of enhancing the AOS-scavenging systems of crop plants.

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References

Allen, R.D. (1995) Dissection of oxidative stress tolerance using transgenic plants. *Plant Physiol.* 107: 1049–1054.

Allen, R.D. (1997) Use of transgenic plants to study antioxidant defenses. *Free Rad. Biol. Med.* 23: 473–479.

Amako, K., Chen, G.-X. and Asada, K. (1994) Separate assays specific for ascorbate peroxidase and guaiacol peroxidase and for the chloroplastic and cytosolic isozymes of ascorbate peroxidase in plants. *Plant Cell Physiol.* 35: 497–504.

Aono, M., Saji, H., Fujiyama, K. and Sugita, M. (1995a) Decrease in activity of glutathione reductase enhances paraquat sensitivity in transgenic *Nicotiana Tabacum*. *Plant Physiol.* 107: 645–648.

Aono, M., Saji, H., Sakamoto, A., Tanaka, K., Kondo, N. and Tanaka, K. (1995b) Paraquat tolerance of transgenic *Nicotiana Tabacum* with enhanced activities of glutathione reductase and superoxide dismutase. *Plant Cell Physiol.* 36: 1687–1691.

Arnon, D.I. (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Bata vulgaris*. *Plant Physiol.* 24: 1–15.

Arrigoni, O., De Gara, L., Paciolia, C., Evidente, A. and de Pinto, M.C. (1997) Lycorine: a powerful inhibitor of L-galactono-γ-lactone dehydrogenase activity. *J. Plant physiol.* 159: 362–364.

Asada, K., Endo, T., Mano, J. and Miyake, C. (1998) Molecular mechanism for relaxation of and protection from light stress. *In* Stress Responses of Photosynthetic Organisms. Edited by Satoh, K. and Murata, N. pp. 37–52. Elsevier, Amsterdam.

Bowler, C., Van Montagu, M. and Inzé, D. (1992) Superoxide dismutase and stress tolerance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 43: 83–116.

Bradford, M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248–254.

Buchanan, B.B. (1980) Role of light in the regulation of chloroplast enzymes. *Annu. Rev. Plant Physiol.* 31: 341–374.

Buchanan, B.B. (1991) Regulation of CO_2 assimilation in oxygenic photosynthesis: the ferredoxin/thioredoxin system. *Arch. Biochem. Biophys.* 288: 1–9.

Casano, L.M., Gómez, L.D., Lascano, H.R., González, C.A. and Trippi, V.S. (1997) Inactivation and degradation of Cu/Zn-SOD by active oxygen species in wheat chloroplasts exposed to photooxidative stress. *Plant Cell Physiol.* 38: 433–440.

Chen, G.-X. and Asada, K. (1989) Ascorbate peroxidase in tea leaves: occurrence of two isozymes and the difference in their enzymatic and molecular properties. *Plant Cell Physiol.* 30: 987–998.

Conklin, P.L., Pallanca, J.E., Last, R.L. and Smirnoff, N. (1997) L-Ascorbic acid metabolism in the ascorbate-deficient *Arabidopsis* mutant *vtc1*. *Plant Physiol.* 115: 1277–1285.

Feierabend, J., Schaan, C. and Hertwig. B. (1992) Photoinactivation of catalase occurs under both high- and low-temperature stress conditions and accompanies photoinhibition of photosystem II. *Plant Physiol.* 100: 1554–1561.

Foyer, C., Rowell, J. and Walker, D. (1983) Measurement of the ascorbate content of spinach leaf protoplasts and chloroplasts during illumination. *Planta* 157: 239–244.

Foyer, C.H., Lelandais, M. and Kunert, K.J. (1994) Photooxidative stress in plants. *Physiol. Plant.* 92: 696–717.

Foyer, C.H., Souriau, N., Perret, S., Lelandais, M., Kunert, K.J., Prurost, C. and Jouanin, L. (1995) Overexpression of glutathione reductase but not glutathione synthetase leads to increases in autooxidant capacity and resistance to photoinhibition in poplar trees. *Plant Physiol.* 109: 1047–1057.

Gillham, D.J. and Dodge, A.D. (1986) Hydrogen peroxide scavenging systems within pea chloroplasts. *Planta* 167: 246–251.

Griffith, O.W. (1980) Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal. Biochem.* 106: 207–212.

Halliwell, B. and Foyer, C.H. (1978) Properties and physiological function of a glutathione reductase purified from spinach leaves by affinity chromatography. *Planta* 139: 9–17.

Hertwig, B., Streb, P. and Feierabend, J. (1992) Light dependency of catalase synthesis and degradation in leaves and the influence of interfering stress condition. *Plant Physiol.* 100: 1547–1553.

Iturbe-Ormaetxe, I., Escuredo, P.R., Arrese-Igor, C. and Becana, M. (1998) Oxidative damage in pea plants exposed to water deficient or paraquat. *Plant Physiol.* 116: 173–181.

Ishikawa, T., Yoshimura, K., Sakai, K., Tamoi, M., Takeda, T. and Shigeoka, S. (1998) Molecular characterization and physiological role of a glyoxysome-bound ascorbate peroxidase from spinach. *Plant Cell Physiol.* 39: 23–34.

Ishikawa, T., Yoshimura, K., Tamoi, M., Takeda, T. and Shigeoka, S. (1997) Alternative mRNA splicing of 3′-terminal exons generates ascorbate peroxidase isoenzymes in spinach (*Spinacia oleracea*) chloroplasts. *Biochem. J.* 328: 795–800.

Kaiser, W.M. (1976) The effect of hydrogen peroxide on CO_2 fixation of isolated intact chloroplasts. *Biochim. Biophys. Acta* 440: 476–482.

Leammli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* 227: 680–685.

Logemann, J., Schell, J. and Willmitzer, L. (1987) Improved method for the isolation of RNA from plant tissues. *Anal. Biochem.* 163: 16–20.

McCord, J.M. and Fridovich, I. (1969) Superoxide dismutase, an enzymatic function for erythrocuprein (hemocuprein). *J. Biol. Chem.* 244: 6049–6055.

Miyake, C. and Asada, K. (1992) Thylakoid-bound ascorbate peroxidase in spinach chloroplasts and photoreduction of its primary oxidation product monodehydroascorbate radicals in thylakoids. *Plant Cell Physiol.* 33: 541–553.

Miyake, C. and Asada, K. (1996) Inactivation mechanism of ascorbate peroxide at low concentration of ascorbate; hydrogen peroxide decomposes compound I of ascorbate peroxide. *Plant Cell Physiol.* 37: 423–430.

Patterson, B.D., Payne, L.A., Chen, Y. and Graham, D. (1984) An inhibitor of catalase induced by cold in chilling-sensitive plants. *Plant Physiol.* 76: 1014–1018.

Payton, P., Allen, R.D., Trolinder, N. and Holaday, S. (1997) Over-expression of chloroplast-targeted Mn superoxide dismutase in cotton (*Gossypium hirsutum* L., cv. Coker 312) does not alter the reduction of photosynthesis after short exposures to low temperature and high light intensity. *Photosynth. Res.* 52: 233–244.

Pitcher, L.H., Repetti, P. and Zilinskas, B.A. (1994) Overproduction of ascorbate peroxidase protects transgenic tobacco plants against oxidative stress. *Plant Physiol.* Suppl 105: 116 abstr.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbour Laboratory Press, New York.

Sen Gupta, A., Heinen, J.L., Holaday, A.S., Burke, J.J. and Allen, R.D. (1993) Increased resistance to oxidative stress in transgenic plants that over-express chloroplastic Cu/Zn superoxide dismutase. *Proc. Natl. Acad. Sci. USA* 90: 1629–1633.

Shigeoka, S., Nakano, Y. and Kitaoka, S. (1980) Metabolism of hydrogen peroxide in *Euglena gracilis* Z. by L-ascorbic acid peroxidase. *Biochem. J.* 186: 377–380.

Shikanai, T., Takeda, T., Yamauchi, H., Sano, S., Tomizawa, K., Yokota, A. and Shigeoka, S. (1998) Inhibition of ascorbate peroxidase under oxidative stress in tobacco having bacterial catalase in chloroplast. *FEBS Lett.* 428: 47–51.

Slooten, L., Capiau, K., Van Camp, W., Van Montagu, M., Sybesma, C. and Inzé, D. (1995) Factors affecting the enhancement of oxidative stress tolerance in transgenic tobacco overexpressing manganese superoxide disumutase in the chloroplasts. *Plant Physiol.* 107: 737–750.

Takeda, T., Yokota, A. and Shigeoka, S. (1995) Resistance of photosynthesis to hydrogen peroxide in algae. *Plant Cell Physiol.* 36: 1089–1095.

Tanaka, K. (1998) Gene structures and expression control of active oxygen scavenging enzymes in rice. *In* Stress responses of photosynthetic organisms. Edited by Satoh, K. and Murata, N. pp. 53–68. Elsevier, Amsterdam.

Tel-Or, E., Huflejt, M.E. and Packer, L. (1986) Hydroperoxide metabolism in cyanobacteria. *Arch. Biochem. Biophys.* 246: 396–402.

Webb, R.P. and Allen, R.D. (1996) Overexpression of pea cytosolic ascorbate peroxidase confers protection against oxidative stress in transgenic *Nicotiana tabacum*. *Plant Physiol.* Suppl. 111: 48 abstr.

Wise, R.R. and Naylor, A.W. (1987) Chilling-enhanced photooxidation. The peroxidative destruction of lipids during chilling injury to photosynthesis and ultrastructure. *Plant Physiol.* 83: 272–277.

Yoshimura, K., Yabuta, Y., Tamoi, M., Ishikawa, T. and Shigeoka, S. (1999) Alternatively spliced mRNA variants of chloroplast ascorbate peroxidase isoenzymes in spinach leaves. *Biochem. J.* 338: 41–48.

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