Inhibition of ascorbate peroxidase under oxidative stress in tobacco having bacterial catalase in chloroplasts

Toshiharu Shikanai[a,c], Toru Takeda[b], Haruko Yamauchi[b], Satoshi Sano[a], Ken-Ichi Tomizawa[a], Akiho Yokota[a,c,*], Shigeru Shigeoka[b]

[a] *Research Institute of Innovative Technology for the Earth, Kizu, Kyoto 619-0292, Japan*
[b] *Department of Food and Nutrition, Faculty of Agriculture, Kinki University, Nakamachi, Nara 631-8505, Japan*
[c] *Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Nara 630-0101, Japan*

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Abstract To analyze the potential of the active oxygen-scavenging system of chloroplasts, we introduced *Escherichia coli* catalase into tobacco chloroplasts. Photosynthesis of transgenic plants was tolerant to high irradiance under drought conditions, while the wild plants suffered severe damage in photosynthesis under the same conditions. Irrespective of responses to the stress, ascorbate peroxidase was completely inactivated both in the transgenic and wild-type plants. These findings are contrary to the established idea that the ascorbate peroxidase-mediated antioxidative system protects chloroplasts from oxidative stress.

Key words: Ascorbate peroxidase; Catalase; Drought stress; Oxidative stress

1. Introduction

Inflow of CO_2 through stomata into chloroplasts and the subsequent fixation and reduction must be balanced with the formation of chemical energy in photosynthesis. A decrease in CO_2 influx obliges chloroplasts to synthesize superoxide (O_2^-) by direct reduction of O_2 by photosystem (PS) I. In chloroplasts, O_2^- is converted to hydrogen peroxide (H_2O_2) and O_2 by superoxide dismutase (SOD). Subsequently, H_2O_2 is decomposed by ascorbate peroxidase (APX) using ascorbate as an electron donor. Oxidized ascorbate is recycled to ascorbate by monodehydroascorbate reductase and the ascorbate-glutathione cycle [1,2]. Under environmental stress conditions, however, the antioxidative system cannot deal with excess generation of active oxygen species, which leads to irreversible inactivation of the photosynthetic apparatus.

To improve plant tolerance to oxidative stress, over-expression of antioxidative enzymes, SOD [3–7] and glutathione reductase (GSHR) [8] has been attempted. However, APX itself is instable in the absence of ascorbate in vitro [9]. Resistance to oxidative stress of plants may be diminished by the instability of APX itself under oxidative conditions.

In leaves of higher plants, H_2O_2 is also formed in bulk in peroxisomes during photorespiration [10]. H_2O_2 is decomposed by catalase that does not consume reducing power in its catalysis. To analyze the potential of the active oxygen-scavenging system of chloroplasts, we introduced *E. coli* catalase, HPII, encoded by *katE* [11], into tobacco chloroplasts. The enzyme has a higher affinity for H_2O_2 than plant catalase and does not require ascorbate for its stability.

2. Materials and methods

2.1. Vector construction and transformation of tobacco

A *Hin*dIII-*Sph*I fragment encoding the promoter and 57 amino acids transit peptide of the tomato *rbcS3C* gene [12] was translationally fused with the first methionine of the *E. coli katE* gene and cloned between *Hin*dIII and *Sst*I sites of pBI101 (Fig. 1). Transgenic tobacco plants (*Nicotiana tabacum* cv. Xanthi) were regenerated from leaf disks infected with *Agrobacterium tumefaciens* LBA4404 carrying this construct. Transgenic plants were cultured in a growth chamber at a moderate light intensity (300 μmol m^{-2} s^{-1}) with 60% relative humidity at 25°C, as well as wild-type plants.

2.2. Western analysis

Total leaf protein (25 μg) was subjected to SDS-PAGE and followed by immunoblotting with a mouse antiserum against *E. coli* HPII.

2.3. Subcellular fractionation

Total leaf extracts from the wild type and T43-1 were fractionated on a 1.3–2.5 M sucrose gradient. Activities of marker enzymes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [13] and hydroxypyruvate reductase (HPR) [14] were monitored along with catalase activity [15].

2.4. Assays of enzyme activities and photosynthesis

Activities of ribulose 5-phosphate kinase (PRK) [13], cytosolic and chloroplast isoforms of APX [16] and glutathione reductase (GSHR) [17] were measured using leaf extracts by the methods cited. CO_2 fixation was measured with a portable photosynthesis system (Li-Cor, Lincoln, NE). Chlorophyll fluorescence was measured in mature leaves using a PAM-2000 fluorometer (Heinz Walz, Effeltrich, Germany) [18].

3. Results and discussion

3.1. Introduction of E. coli catalase into tobacco chloroplasts

To introduce *Escherichia coli* catalase into tobacco chloroplasts, the tomato sequence encoding promoter and transit peptide of *rbcS3C* [12] was translationally fused with *E. coli katE*. The tomato *rbcS3C* promoter regulates light-dependent transcription mainly in leaves [19], and its transit peptide functions for targeting the chimeric catalase into chloroplasts [20]. Eight tobacco clones carrying the *E. coli katE* were selected by Southern hybridization. Total leaf catalase activity at the T_0 generation ranged from once to twice that of wild-type tobacco. The level of the total catalase activity in transgenic lines was strictly controlled by light (data not shown), suggesting that the increase is due to the *katE* expression under the control of the *rbcS3C* promoter. T_1 plants containing approximately three-fold higher catalase activity than the

*Corresponding author: at affiliation c. Fax: (81) (743) 72-5569.
E-mail: yokota@bs.aist-nara.ac.jp

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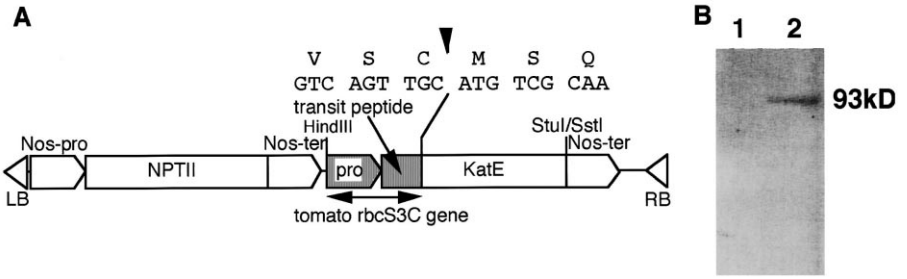

Fig. 1. Transformation of tobacco plants with *E. coli katE*. A: Construction of a transformation vector. Partial nucleotide and amino acid sequences near the junctioning site are shown. The arrowhead indicates the proteolytic processing site. B: Western blot analysis. Total protein (25 μg) from wild-type (lane 1) and T43-1 (lane 2) leaves was subjected to SDS-PAGE and followed by immunoblotting with a mouse antiserum against *E. coli* HPII.

wild-type plants were originated from lines T6-1, T24-2 and T43-1 and were used for further analyses.

Western blot analysis using a mouse antiserum against *E. coli* HPII detected a single 93-kDa signal corresponding to a subunit of HPII [11] in T43-1 (Fig. 1B, lane 2), suggesting that the signal peptide was processed from the chimeric catalase. Since no signal corresponding to intrinsic catalase (57 kDa) was detected with the leaf extracts from the wild-type and T43-1 plants (Fig. 1B, lanes 1 and 2), the antiserum was specific to *E. coli* catalase.

To determine whether the *E. coli* catalase was imported into chloroplasts, we analyzed its subcellular localization in the leaves of T43-1 (Fig. 2). Considering the distribution of the HPR activity, fractions 4–7 and 14–16 contained peroxisomes. Chloroplasts were marked with GAPDH, the activity of which was split into two fractions. These may correspond to intact (fractions 26–29) and partly injured (fractions 35–42) chloroplasts. Peroxisomes were slightly contaminated by chloroplasts, as deduced from the distribution of the GAPDH activity. In wild-type tobacco, catalase activity predominantly migrated in peroxisomal fractions. In addition to the peroxisomal fractions, the chloroplast fractions from T43-1 contained a higher catalase activity (165 ± 27 μmol/mg protein/min), which was not detected in the wild-type tobacco (Fig. 2, fractions 28–44). Western blot analysis using anti-HPII antibody showed that the introduced *E. coli* catalase was exclusively localized in chloroplast fractions of T43-1 (data not shown). Thus, we concluded that the chimeric catalase was imported into chloroplasts, where the transit peptide was removed.

3.2. Evaluation of tolerance to oxidative stress

To evaluate the efficacy of *E. coli* catalase introduced into tobacco chloroplasts in mediating resistance to oxidative stress, leaf disks from the wild-type and T43-1 plants were incubated with various concentrations of paraquat under illumination at 300 μmol m^{-2} s^{-1} (Fig. 3). Electrons are transferred from PS I to oxygen via paraquat, and the resulting O_2^- is converted to H_2O_2 by SOD in chloroplasts. While the wild-type leaf disks bleached in the presence of 0.25 μM paraquat, the T43-1 leaf disks remained green even at 1 μM paraquat (Fig. 3).

Resistance to oxidative stress was then evaluated with whole plants. T_1 progeny plants of T6-1, T24-2 and T43-1, as well as wild-type plants, were cultured in a growth chamber at a moderate light intensity (300 μmol m^{-2} s^{-1}) with periodic watering for six weeks and then transferred to stress conditions that plants often encounter in nature (illumination at 1600 μmol m^{-2} s^{-1} and 60% relative humidity without watering). Although the wild-type and transgenic plants grew normally under moderate light conditions with periodic watering, resistance to the stress conditions of these plants was different. In the leaves of wild-type plants, the degradation of chlorophyll was visible after 24 h of the stress treatment and severe chlorosis occurred after 72 h (Fig. 4A, B and C). However, transgenic plants did not show any chlorosis at least for 96 h under the stress conditions (Fig. 4D, E and F). We concluded that introduction of *E. coli* catalase into tobacco chloroplasts efficiently improved plant resistance to photooxidation caused by drought stress at the high-light intensity.

3.3. Resistant mechanism to drought stress

To analyze the mechanism of the observed resistance, enzyme activities of PRK, catalase, cytosolic and chloroplast isoforms of APX and GSHR, as well as the rate of CO_2 fixation, were compared between the wild-type and T43-1 plants (Fig. 5). The plants were analyzed after 48 h stress treatment, when visible injury was evident in the wild-type plants (Fig. 4). Surprisingly, chloroplast isoforms of APX

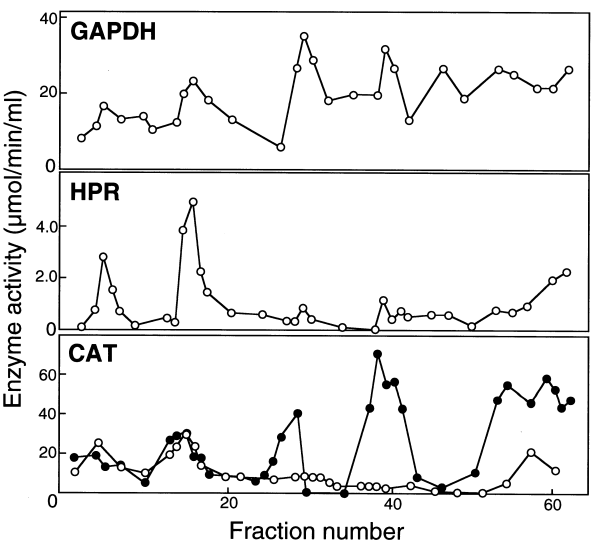

Fig. 2. Subcellular localization of *E. coli* catalase. Total leaf extracts from the wild type (open circles) and T43-1 (closed circles) were fractionated on an 1.3–2.5 M sucrose gradient. Activities of marker enzymes, GAPDH and HPR, were monitored to determine fractions containing chloroplasts and peroxisomes, respectively, along with catalase activity (CAT).

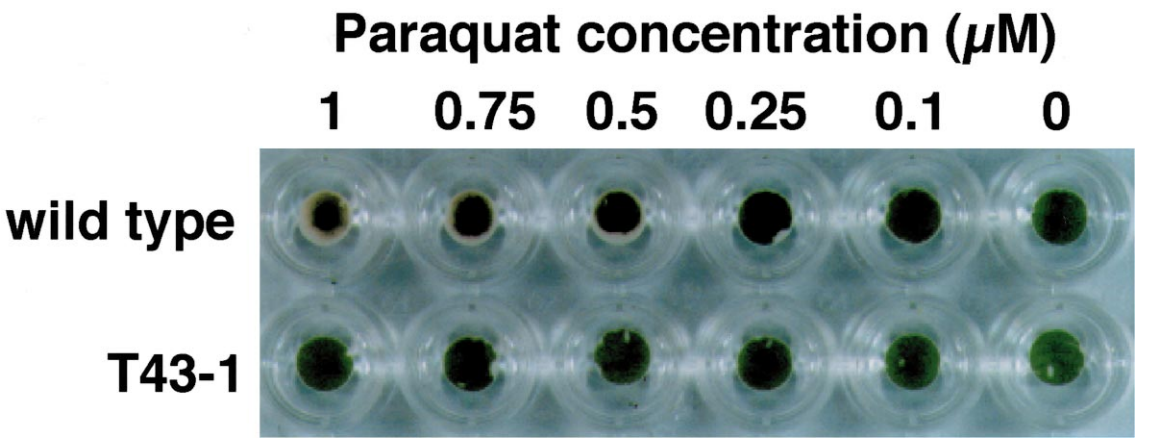

Fig. 3. Effects of paraquat on leaf disks from the wild-type and T43-1 plants. Disks were placed in the dark for 1 h and then illuminated at 300 μmol m^{-2} s^{-1} for 18 h at 25°C.

were completely inactivated both in the wild-type and T43-1 plants under the stress conditions used. It is worth noticing that chloroplast isoforms of APX were completely inactivated with PRK still active, indicating that APX is much more strongly inactivated by oxidative stress than PRK, which has been believed to be one of the enzymes most sensitive to active oxygen species [21]. We therefore must reconsider the idea that the endogenous scavenging system in chloroplasts functions to protect the photosynthetic apparatus, including PRK, from active oxygen species under stress conditions. Under the oxidative stress conditions, excess generation of H_2O_2 may cause the depletion of reduced ascorbate, which inactivates APX [9,22,23]. The ascorbate-glutathione cycle may function only under mild environmental conditions.

Whereas APX activity was completely inactivated, catalase activity was not inhibited after exposure to the stress conditions for 48 h in T43-1 (Fig. 5). Irreversible processes of photoinhibition, which were accelerated by the inactivation of the ascorbate-glutathione cycle, should be decelerated by catalase in chloroplasts of T43-1. Although endogenous peroxisomal catalase was inactivated in the wild-type plants (Fig. 5), the resistance to oxidative stress cannot be compared directly between *E. coli* HPII and the endogenous tobacco catalase, due to their different subcellular localizations. However, we showed that HPII was much more resistant to oxidative stress than chloroplast APX (Fig. 5). We believe that the different tolerance of the enzymes to oxidative stress should explain the improved resistance to the stress conditions in the transgenic tobacco.

In chloroplasts the endogenous scavenging system is located

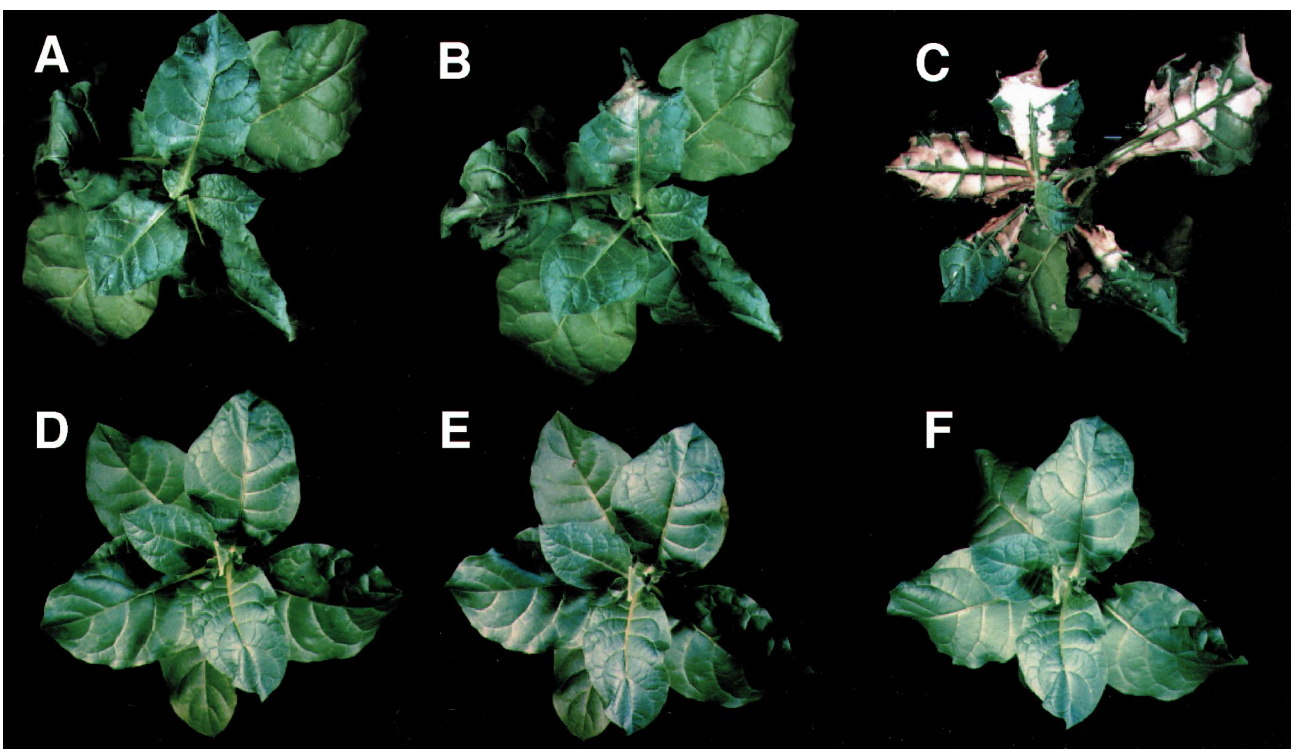

Fig. 4. Typical leaves of the wild-type (A, B and C) and T43-1 (D, E and F) tobacco plants exposed to drought stress (60% relative humidity without watering) at high light intensity (1600 μmol m^{-2} s^{-1}) for 24 h (A and D), 48 h (B and E) and 72 h (C and F).

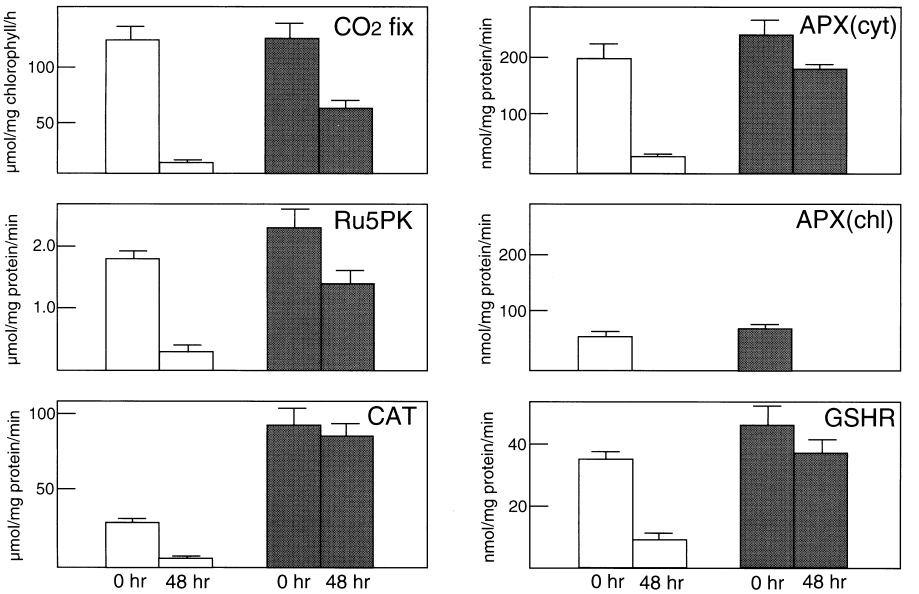

Fig. 5. Changes in activities of CO_2 fixation and enzymes after a shift from moderate light conditions (300 μmol m^{-2} s^{-1}) at 60% relative humidity with periodic watering to drought conditions (60% relative humidity without watering for 48 h) at high light intensity (1600 μmol m^{-2} s^{-1}). Total leaf activities of PRK, catalase (CAT) and GSHR were determined, as well as activities of cytosolic (cyt) and chloroplast (chl) isoforms of APX. The wild-type (open bars) and T43-1 (shaded bars) plants were analyzed before (0 h) and after (48 h) the stress treatment. Error bars show S.D.

both on the stromal thylakoid membranes and in the stroma. The membrane-bound system prevents active oxygen species from leaking from the stromal thylakoid membranes, where the superoxide radical, O_2^-, is generated by the direct reduction of O_2 by PS I [24]. On the other hand, the stromal system functions to protect several enzymes from oxidative damage caused by active oxygen species leaking from their generation sites. Since catalase activity was localized in the water-soluble fraction of T43-1 chloroplasts (data not shown), the *E. coli* catalase should function in the stroma. To evaluate the effect of *E. coli* catalase on protection of photosynthetic apparatus in thylakoid membranes, we compared variable chlorophyll fluorescence (F_v)/maximal chlorophyll fluorescence (F_m) between wild-type and T43-1 leaves (Table 1). The decrease in F_v/F_m, which was relevant to the inactivation of D1-protein in PS II, was significantly higher in the wild-type plants than in T43-1. The *E. coli* catalase was enough to protect photosynthetic apparatus in thylakoid membranes such as D1-protein of PS II as well as the stromal enzymes from oxidative stress (Table 1 and Fig. 5).

A significant difference between two H_2O_2-scavengers, APX and catalase, is the consumption of NADPH in the former. Since oxidation of NADPH by the ascorbate-glutathione cycle provides PS I with additional electron acceptors, APX may be superior to catalase in the ability of discarding excess electrons. This would be true under the mild environmental conditions. Considering the enzyme stability under oxidative stress conditions (Fig. 5), however, catalase is much superior to APX as a stress-resistant enzyme.

Table 1
Changes in chlorophyll fluorescence (F_v/F_m) under stress conditions

	0 h	24 h
Wild type	0.73 ± 0.04	0.45 ± 0.03
T43-1	0.73 ± 0.04	0.67 ± 0.04

Chlorophyll fluorescence was measured in mature leaves before (0 h) and after (24 h) the stress treatment using a PAM-2000 fluorometer (Heinz Walz, Effeltrich, Germany). Data represent means ± S.D. of the results from six repeated experiments with two independent plants. The decrease in F_v/F_m represents the photoinhibition under drought stress conditions.

Although transgenic approaches to increase plant tolerance to oxidative stress have focused on the over-expression of SOD, inconsistencies in the phenotype of the transgenic plants have been reported [3–7]. One possible explanation for the discrepancy between these results is that the over-expression of SOD could confer the resistance to plants in the limited range of oxidative stress due to the sensitivity of the endogenous scavenging system to active oxygen species [25]. In fact, our results indicated that the ascorbate-glutathione cycle could function only in the early steps of photoinhibition due to its lability under oxidative stress. To improve plant resistance to oxidative stress efficiently, qualitative modification of the antioxidative machinery should be more important than quantitative modifications.

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