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Response of the antioxidative system to photooxidative stress in transgenic tobacco plants overexpressing thylakoid membrane-bound ascorbate peroxidase

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Introduction

Under optimal conditions, the reduction of O_2 in the electron transfer system in chloroplasts, microsomes, and mitochondria is unavoidable and leads to the production of active oxygen species (AOS) such as the superoxide radical (O_2^-) and H_2O_2. Chloroplasts, especially, are a potentially powerful source of oxidants. The generation rate of O_2^- by the thylakoid-bound electron carrier on the reducing side of PS I as well as that by the peripheral ferredoxin was about 10% of the electron transfer rate, as observed by the fixation rate of CO_2 at saturating light intensity (Asada, 1999). The AOS oxidize the target molecules, such as the D1 protein in the PS II reaction center and the reaction center complex of PSI in chloroplasts. Furthermore, in the Calvin cycle, thiol-modulated enzymes, e.g., fructose-1,6-bisphosphatase and phosphoribulokinase, which are regulated by the ferredoxin/thioredoxin system, are inactivated to 50% by 10 μM H_2O_2 due to the oxidation of thiol residues in these enzymes (Takeda et al. 1995). Antioxidants and antioxidant enzymes, such as ascorbate (AsA), glutathione (GSH), superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase, function to interrupt the cascades of uncontrolled oxidation in some organelles. APX isoenzymes are distributed in the stroma and thylakoid membrane (tAPX) in chloroplasts, microbodies, mitochondria, and cytosol (Shigeoka et al., 2001). In the chloroplasts of higher plants, the water-water cycle consisting of the thylakoid membrane-attached SOD, tAPX, and the AsA-regeneration system participates in the detoxification of AOS and the dissipation of excess photon energy. Recently, we found that the decrease in activities of chloroplastic APX isoenzymes is caused by the depletion and/or the redox change of AsA in tobacco and spinach under photooxidative stress, indicating that the antioxidative systems of chloroplasts are very sensitive to photooxidative stress (Yoshimura et al. 2000; Miyagawa et al. 20000). Here, we created transgenic tobacco plants (TpTAP-12) overexpressing tAPX in chloroplasts and evaluated the defense system in chloroplasts in response to photooxidative stress conditions.

Materials and methods

For the construction of transgenic tobacco plants (*Nicotiana toabacum* cv. Xanthi), the spinach tAPX cDNA, encoding the chloroplast transit sequence, the catalytic active domain, and the thylakoid membrane-binding sequence, was ligated into a plant binary vector, the pBI121 linker derived from the Ti-plasmid, in which the tAPX was placed under the control of the cauliflower mosaic virus (CaMV) 35S promoter in a sense orientation. The recombinant plasmid was introduced into *Agrobacterium tumefaciens* strain LBA 4404, which was transformed into the tobacco plants as described previously (Shikanai et al. 1998). Transgenic tobacco plants and wild-type plants were cultured for 7 weeks in a growth chamber under a 12-h photoperiod with a moderate light intensity (300 µmol m^{-2} s^{-1}), 60% relative humidity, and day/night temperature of 25/20°C. Paraquat (PQ) stress were caused by spraying with 50 µM paraquat in 0.1% Tween 20 and exposing to high light (1600 µmol m^{-2} s^{-1}) or moderate light (300 µmol m^{-2} s^{-1}) for the indicated times. For the assays, discs (1.1 cm^2) were prepared from the third leaves from the top in each line. The antioxidant amounts, enzyme assays, and measurements of photosynthetic parameters were determined as described previously (Miyagawa et al. 2000).

Results and discussion

Creation of transgenic tobacco overexpressing tAPX-- Wild-type tobacco plants were infected *Agrobacterium* transformed the transgene containing the spinach tAPX cDNA under the control of CaMV 35S promoter. A total of 50 independent transformed lines were produced. The original transgenic lines were self-pollinated to produce the T_2 progeny. No differences were observed in growth or morphology between wild-type and transgenic plants.

Expression level of endogenaous tAPX-- To analyze the expression of tAPX derived from the transgene, Northern blotting and Western blotting were carried out. High amounts of the mRNA and protein derived from the transgene were detected in the leaves of transgenic lines such as TpTAP-12, 17, and 42 but not in wild-type plants (data not shown). The expression level of tAPX protein in all transgenic lines was about 20-fold higher than that of exogenous tAPX in wild-type plants. The intact chloroplasts were prepared from wild-type and TpTAP-12 lines and then separated into thylakoid membrane and soluble stroma fractions as described previously (Miyagawa et al., 2000; Yoshimura et al., 2000). The tAPX protein from the transgene was detected in the intact chloroplasts and the thylakoid membrane fractions from transgenic plants (data not shown). The APX activity in the thylakoid membrane fraction from the TpTAP-12 plant was 20-fold higher than that of the wild-type plants (data not shown). Thus, we concluded that the tAPX protein derived from the transgene is imported into chloroplasts and sorted on the thylakoid membrane in transgenic plants.

Measurements of physiological parameter--- To evaluate the physiological parameter in wild-type and TpTAP-12 plants, the CO_2 fixation, PSII activity, antioxidant levels (AsA and GSH), and activities of antioxidative enzymes (APX, SOD, MDAR, DAR, and GR) were measured. No significant differences were observed in any of the parameters except for the tAPX activities between wild-type and TpTAP-12 plants, indicating that the overexpression of tAPX in chloroplasts has no effect on plant metabolism under normal conditions.

Response of transgenic and wild-type plants to PQ treatment--- To evaluate the effect of the overexpression of tAPX on the protection to the photooxidative stress, 50 µM PQ was sprayed on wild-type and TpTAP-12 plants under illumination at 1600 µmol m^{-2} s^{-1}. The wild-type plants developed more severe visible leaf injury, as seen in the degradation of chlorophyll, than TpTAP-12 plants at 24 h after spraying (data not shown), indicating that photooxidative damage is caused by the excess generation of AOS via O_2^- in chloroplasts exacerbated by PQ under illumination and that transgenic plants had increased tolerance to

photooxidative damage. To analyse in detail the response of the antioxidative systems in chloroplasts to mild-photooxidative stress, wild-type and TpTAP-12 plants were sprayed with 50 µM PQ under illumination at 300 µmol m^{-2} s^{-1}. The CO_2 fixation and PSII activity of wild-type plants decreased at 6 h after PQ treatment, while those of transgenic plants remained at a high level (Fig. 1). The activities of FBPase, GAPDH, and PRK remained at high levels in the TpTAP-12 plants, while those of the wild-type plants decreased to 43%, 20%, and 71%, respectively, 24 h after PQ treatment (data not shown). The activity of the AsA-regeneration enzymes, MDAR and DAR, did not change after PQ treatment, while the GR activity increased significantly in both wild-type and TpTAP-12 plants. Total GSH content in leaves of both plants increased to about 250% after PQ treatment,

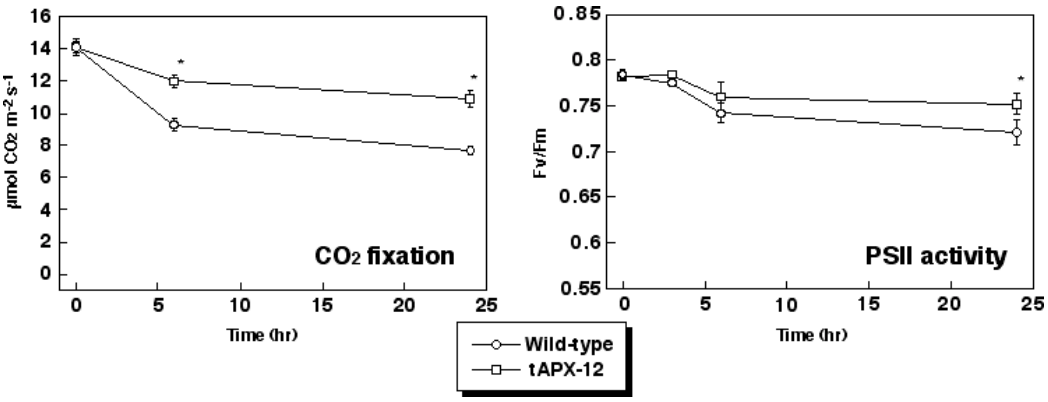

Fig. 1 Effects of PQ treatment on photosynthetic activity and PS II activity (Fv/Fm). The CO_2 fixation of the third leaf in tobacco was measured at 1,000 µmol CO_2 mol^{-1}, 400 µmol m^{-2} s^{-1}, 25˚C, 60% relative humidity. The PS II activity was performed at 25˚C after dark adaptation for 30 min.

whereas no significant difference in the redox status [GSH/(GSH+GSSG)] was observed.

Recently, we found that the inactivation of chlAPX isoenzymes is caused by the depletion of AsA under photooxidative stress conditions (Miyagawa et al. 2000; Yoshimura et al. 2000). The AsA content in wild-type plants decreased 24 h after PQ treatment (Fig. 2); its change was due to the decrease in the reduced form, leading to a significant change in the redox status of AsA. By contrast, in TpTAP-12 plants, the AsA content and its redox status hardly changed after the PQ treatment. As shown in Figure 3, the tAPX activity remarkably decreased 24 h after PQ treatment in wild-type plants, while no significant difference in activity of tAPX was observed during the oxidative stress conditions in TpTAP-12 plants. These observations suggest that the enhanced level of tAPX in the chloroplasts of transgenic plants increases the H_2O_2-scavenging capacity, leading to the maintenance of the level and redox status of AsA in chloroplasts and the protection of photosynthesis from photooxidative stress conditions.

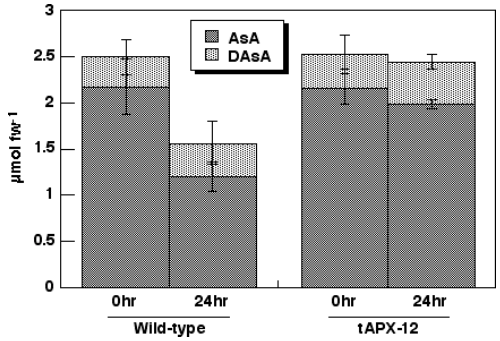

Fig. 2 Effects of PQ treatment on AsA and DAsA contents. Values presented are means ± SD from three experiments.

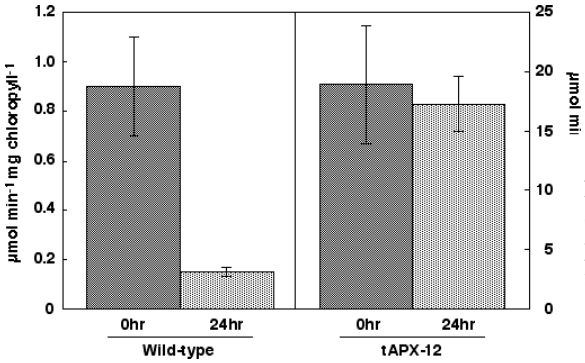

Fig. 3 Effect of PQ treatment on tAPX activity. Values presented are means ± SD from three experiments.

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