Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Reduction of methylviologen-mediated oxidative stress tolerance in antisense transgenic tobacco seedlings through restricted expression of *StAPX**

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Received July 10, 2012; Revision accepted Dec.18, 2012; Crosschecked June 17, 2013

Abstract: Ascorbate peroxidases are directly involved in reactive oxygen species (ROS) scavenging by reducing hydrogen peroxide to water. The tomato thylakoid-bound ascorbate peroxidase gene (*StAPX*) was introduced into tobacco. RNA gel blot analysis confirmed that *StAPX* in tomato leaves was induced by methylviologen-mediated oxidative stress. The sense transgenic seedlings exhibited higher tAPX activity than that of the wild type (WT) plants under oxidative stress conditions, while the antisense seedlings exhibited lower tAPX activity. Lower APX activities of antisense transgenic seedlings caused higher malondialdehyde contents and relative electrical conductivity. The sense transgenic seedlings with higher tAPX activity maintained higher chlorophyll content and showed the importance of tAPX in maintaining the optimal chloroplast development under methylviologen stress conditions, whereas the antisense lines maintained lower chlorophyll content than WT seedlings. Results indicated that the over-expression of *StAPX* enhanced tolerance to methylviologen-mediated oxidative stress in sense transgenic tobacco early seedlings, whereas the suppression of *StAPX* in antisense transgenic seedlings showed high sensitivity to oxidative stress.

Key words: Methylviologen, Oxidative stress, *StAPX,* Stress tolerance, Transgenic tobacco seedling
doi:10.1631/jzus.B1200190 **Document code:** A **CLC number:** Q945

1 Introduction

Reactive oxygen species (ROS) are generated as by-products of plant cellular metabolism including superoxide anion radical ($O_2^- \cdot$), hydrogen peroxide (H_2O_2), hydroxyl radical, and so on. Under environmental stress, ROS overproduction in plant cells can damage cellular components including DNA, proteins, and membrane lipids (Mittler, 2002). Meanwhile, plants have developed a number of homeostatic antioxidant mechanisms to protect themselves against ROS. These mechanisms include ROS-scavenging enzymes and low molecular weight antioxidants, such as superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11), and catalase (CAT, EC 1.11.1.6), as well as ascorbic acid (AsA), glutathione, and phenolic compounds (Asada, 1999). These antioxidants mechanisms can protect plants from ROS damage by scavenging the toxic ROS or reducing the damages of uncontrolled oxidation in certain organelles.

APX plays an important role in eliminating H_2O_2 by utilizing ascorbate as its specific electron donor to reduce H_2O_2 to H_2O (Noctor and Foyer, 1998). APX isozymes are localized in such

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* Project supported by the Natural Science Foundation of Jiangsu Province (No. BK2010344), the Opening Foundation of State Key Laboratory of Crop Biology (No. 2011KF11), the Postdoctoral Science Foundation of China (No. 2011M500867), and the National Natural Science Foundation of China (No. 31071338)

organelles as chloroplasts, mitochondria, peroxisomes, and cytosols. Chloroplastic APX can be found anchored to the thylakoid membrane (tAPX) and stroma (soluble sAPX) (Shigeoka *et al*., 2002). Chloroplasts are the major sources of $O_2^-\cdot$ and H_2O_2 which result from highly energetic reactions that take place there. Since CAT is predominately located in the peroxisomes, H_2O_2 is mainly scavenged by APX in chloroplasts. Two enzymes are involved in ROS detoxification in chloroplasts. One is SOD which is responsible for converting $O_2^-\cdot$ into H_2O_2. The other one is APX which is responsible for removing H_2O_2. The enhancement of chloroplast antioxidant defenses has proved to be one of the most effective ways of protecting plant cells from abiotic stress (Ishikawa and Shigeoka, 2008; Sato *et al*., 2011; Saxena *et al*., 2011). Antisense tobacco lines suggested that suppression of *tAPX* in tobacco may be lethal and *tAPX* antisense wheat lines resulted in lower photosynthetic carbon assimilation (Yabuta *et al*., 2002; Danna *et al*., 2003).

To clarify the contribution of tAPX in protecting plants against oxidative stress, we evaluated transgenic tobacco seedlings with overexpression of *tAPX* or with suppression of *tAPX* against oxidative stress induced by methyl viologen (MV), an ROS-generating herbicide. The results indicated that introducing *StAPX* into tobacco early seedlings changed the tolerance to oxidative stress.

2 Materials and methods

2.1 Plant materials and growth conditions

Wild type tobacco (*Nicotiana tabacum* NC89) seedlings (WT), sense transgenic lines (T_3-2 and T_3-6), and antisense transgenic lines (TA_3-2) were used as plant materials. The seeds were germinated under sterile conditions in Petri dishes containing MS basal medium (Murashige and Skoog, 1962) supplemented with 50 μg/ml kanamycin (WT seeds in MS medium without kanamycin). The sprouts were then planted in 10-cm diameter plastic pots (one plant per pot) filled with sterilized soil and grown in a greenhouse with temperature of 25–30 °C/15–20 °C (day/night regime) and a relative humidity of (75±10)%.

2.2 Methylviologen treatments

Four-week-old tobacco seedlings were subjected to oxidative stress by spraying with MV (0, 50, 100, and 200 μmol/L) dissolved in 1 g/L Tween-20 solution using a spray booth for 5 d. Four-week-old tomato plants were sprayed with 0, 50, 100 and 200 μmol/L MV solutions for 5 d. The stressed leaves were collected from about 3–4 seedlings per treatment, immediately frozen in liquid nitrogen and stored at −80 °C. Control samples were sprayed with water, grown under the same conditions as the stressed plants.

2.3 RNA gel blot analysis

Total RNA was extracted using the Trizol reagent extraction procedure from all treated tomato leaves (1 g fresh weight). Total RNA was extracted from the untreated leaves of transgenic tobacco lines and WT plants. Total RNA of 20 μg was subjected to electrophoresis on 1.2% agarose gel containing 2.2 mol/L formaldehyde and then transferred onto Hybond N^+ membranes. Pre-hybridization took place at 65 °C for 24 h. The membranes were hybridized at 42 °C for 36 h. A 0.5-kb fragment from the 3′ partial cDNA of *StAPX* was used as gene-specific probe and labeled with [a-^{32}P] dCTP by the random prime labeling method. The membranes were washed twice after hybridization and then exposed to an imaging plate. The relative expression ratio of *tAPX* transcript was calculated using a phosphor screen imaging system.

2.4 Activity assays of antioxidant enzyme and AsA

Soluble protein was quantified according to Bradford (1976)'s method. The tAPX activity was assayed according to the method described by Amako *et al*. (1994).

SOD activity was assayed according to the method of Giannopolitis and Ries (1977) by measuring the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT).

CAT activity was measured according to the method of Aebi (1974) by decomposition of H_2O_2 at 240 nm. The reaction was initiated by H_2O_2 (10 mmol/L). One unit of CAT activity was defined as mmol/L H_2O_2 degraded per minute.

AsA was determined according to the method of Kampfenkel *et al.* (1995). Each leaf tissue sample (0.5 g) from transgenic and WT plants was

homogenized in 2-ml ice-cold 60 g/L trichloroacetic acid (TCA), and then centrifuged for 5 min at 13000×*g* (4 °C). The supernatant was immediately assayed for AsA and dehydroascorbate (DHA) contents. The following solutions are used: 0.2 ml sample (6% TCA for blank), 0.2 mol/L phosphate buffer (pH 7.4), 0.2 ml distilled water (ddH_2O), 1 ml 6% TCA, 0.8 ml 42% H_3PO_4, 0.8 ml 4% 2,2′-bipyridyl, and 0.4 ml 3% $FeCl_3$. The assay tube was incubated at 42 °C for 1 h and the light absorbance was determined at 525 nm.

2.5 Malondialdehyde (MDA) content and relative electrical conductivity (REC) measurements

The 0.5 g fresh leaves were ground in a grinding medium containing 10% TCA. The homogenate was centrifuged at 4000×*g* for 10 min. Tris-buffered acetate (TBA; 0.6%) of 2 ml was added to 2 ml supernatant and mixed. The liquid was boiled for 15 min, cooled quickly, and then centrifuged. The water phase was used to determine light absorbance at 532, 600, and 450 nm.

REC was determined according to the method of Sui *et al*. (2008).

2.6 Chlorophyll fluorescence measurement

The photosynthetic activity was measured by chlorophyll fluorescence determination of photochemical efficiency (Fv/Fm), which represented the maximum quantum yield of photosystem II (PSII). Fv/Fm was measured using a portable chlorophyll fluorescence meter (FMS2, Hansatech, UK). Four-week-old tobacco seedlings were sprayed with 200 μmol/L MV dissolved in 1 g/L Tween-20 solution for 5 d (25 °C, 24 h photoperiod, 600 μmol/(m^2·s) photosynthetic photon flux density (PPFD)). After 30 min of dark adaptation, measurements were conducted on the third-fourth leaves of plants at 25 °C, using saturating light flashes (3000 μmol/(m^2·s)).

2.7 Percentage of leaf sensitivity against MV, leaf disk assay for sensitivity against MV, and chlorophyll contents

The percentage of leaf damage that appeared on the leaves sprayed by MV was evaluated 5 d after treatment: 0% meant no damage and 100% meant fully damaged on leaves.

A leaf disks experiment (Lee *et al*., 2007) was used to analyze MV damage. The leaves of tobacco seedlings were transferred to 9.0-cm Petri dishes, each containing 20 ml of MV solution at various concentrations (0, 50, 100, and 200 μmol/L). Each Petri dish incubated at 25 °C for 24 h under continuous white light (100 μmol/(m^2·s)). The effect of MV on leaf disks was analyzed by monitoring the phenotypic changes.

The chlorophyll contents in the seedling leaves were determined according to Hemavathi *et al*. (2010) after 200 μmol/L MV spraying.

3 Results

3.1 Expression of *StAPX* in tomato leaves under oxidative stress conditions

Expression of *StAPX* in tomato leaves analyzed by RNA gel blot showed that the expression levels of *StAPX* increased under MV stress condition (Fig. 1), which indicated that the expression of *StAPX* in tomato was induced by MV-oxidative stress. Fig. 1a shows that the expression level was relatively high after 200 μmol/L MV treatment for 5 d, and Fig. 1b shows that the highest expression level was at 4 d under 200 μmol/L MV stress conditions.

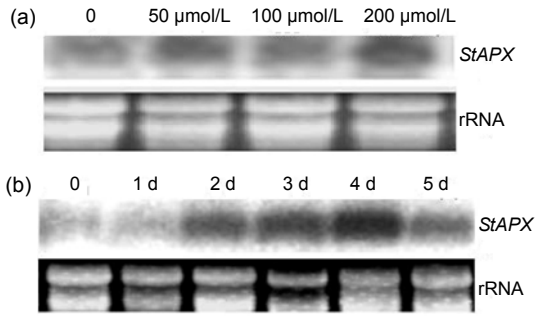

Fig. 1 Expression analysis of *StAPX* by RNA gel blot in tomato leaves under MV stress

(a) The expression of *StAPX* under 0, 50, 100, and 200 μmol/L MV stress conditions for 5 d; (b) The expression of *StAPX* under 200 μmol/L MV stress conditions for 0, 1, 2, 3, 4, and 5 d

3.2 Expression of *StAPX* in transgenic lines

The analysis of the predicted amino acid sequence of *StAPX* from other higher plants identified that *StAPX* cDNA encodes a tAPX protein anchored to the thylakoid membrane via a C-terminal transmembrane domain. *StAPX* was introduced into

tobacco under the control of the cauliflower mosaic virus 35S promoter (Sun *et al*., 2010). Twevel individual kanamycin-resistant transgenic lines (T_1) of tobacco were checked by polymerase chain reaction (PCR). The transgenic lines possessed single copy number. T_2 seeds were tested for segregation of the kanamycin-resistant trait. In 12 different T_2 lines, the segregation ratio of kanamycin-resistant:kanamycin-sensitive was about 3:1. T_3 lines were isolated and prepared for further analysis. The sense transgenic lines did not show obvious differences in vegetative or reproductive growth, while the antisense transgenic lines were slightly stunted in growth. From the tested lines, we selected T_3-2 (sense transgenic line), T_3-6 (sense transgenic line), and TA_3-2 (antisense transgenic line) for further analysis. The results of RNA gel blot showed that two sense transgenic lines had strong positive signals and WT plant had a weak signal, while almost no signal was found in the antisense transgenic line (Fig. 2).

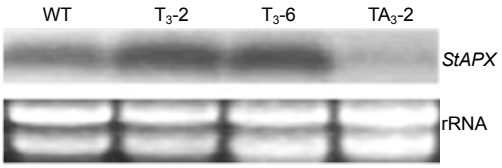

Fig. 2 Expression of *StAPX* in transgenic tobacco lines and WT plants under normal conditions
Total RNA of 20 μg was analyzed by RNA gel blot using 3′ partial cDNA of *StAPX* as a gene-specific probe. The ethidium bromide staining of the RNA gel is shown as a loading control (rRNA)

3.3 Changes of antioxidative enzyme activity and AsA content under stressed conditions

A more than two-fold increase in tAPX activity was observed in both sense transgenic lines compared to those in WT plants, and tAPX activity in these antisense transgenic lines was about 50% of the control (WT plants) level under normal conditions. When exposed to 200 μmol/L MV treatment for 5 d, the tAPX activities of WT and TA_3-2 decreased about 70.4% and 81.5%, respectively; however, the tAPX activities in T_3-2 and T_3-6 remained their original values (Fig. 3a).

There were almost no differences in the activities of SOD among the transgenic lines and WT under normal conditions. SOD activities in TA_3-2 seedlings declined rapidly under MV stress conditions, while those in T_3-2 and T_3-6 lines were declined much slower (Fig. 3b).

The activities of CAT in T_3-2 and T_3-6 lines declined slightly slower than those in TA_3-2 and WT seedlings under 200 μmol/L MV stress conditions, while there were almost no differences among them under normal conditions. Under the MV stress conditions for 5 d, there were very low CAT activities in antisense transgenic seedlings (Fig. 3c).

The content of AsA was reduced in parallel by the time of MV treatment. The contents of AsA in WT and TA_3-2 plants were reduced to 27.1% and 24.5%, respectively, while those in T_3-2 and T_3-6 lines reduced to 38.4% and 41.8% after 200 μmol/L MV treatment for 5 d, respectively (Fig. 3d). At the same time, the content of dehydroascorbate (DHAsA) increased in all the tested lines. DHAsA contents markedly increased in the sense transgenic seedlings compared with those in antisense transgenic seedlings (data not shown), which indicated that there was less AsA reacting with H_2O_2, inducing less DHAsA and H_2O in antisense transgenic lines.

3.4 Changes of MDA and REC contents under stressed conditions

There were slight increases in MDA and REC in sense transgenic lines as compared to those in the WT and antisense transgenic plants. MDA contents in T_3-2, T_3-6, WT, and TA_3-2 seedlings increased about 122.58%, 119.20%, 214.28%, and 345.59%, respectively, after 200 μmol/L MV treatments for 5 d (Fig. 4a). After 200 μmol/L MV treatment for 5 d, the REC of T_3-2 and T_3-6 increased by 104.70% and 101.20%, whereas the contents of REC in the WT and TA_3-2 lines increased by 164.91% and 216.80%, respectively (Fig. 4b). The sense transgenic lines showed a reduction of membrane damage compared to WT, whereas antisense transgenic plants showed an aggravation of membrane damage under stressed conditions.

3.5 Changes of chlorophyll fluorescence and chlorophyll content

The Fv/Fm of tested leaves was evaluated to determine the degree of damage induced by MV stress on the photosynthetic apparatus. Compared with the normal condition, the Fv/Fm of WT and TA_3-2 seedlings decreased, respectively, by 61.97%

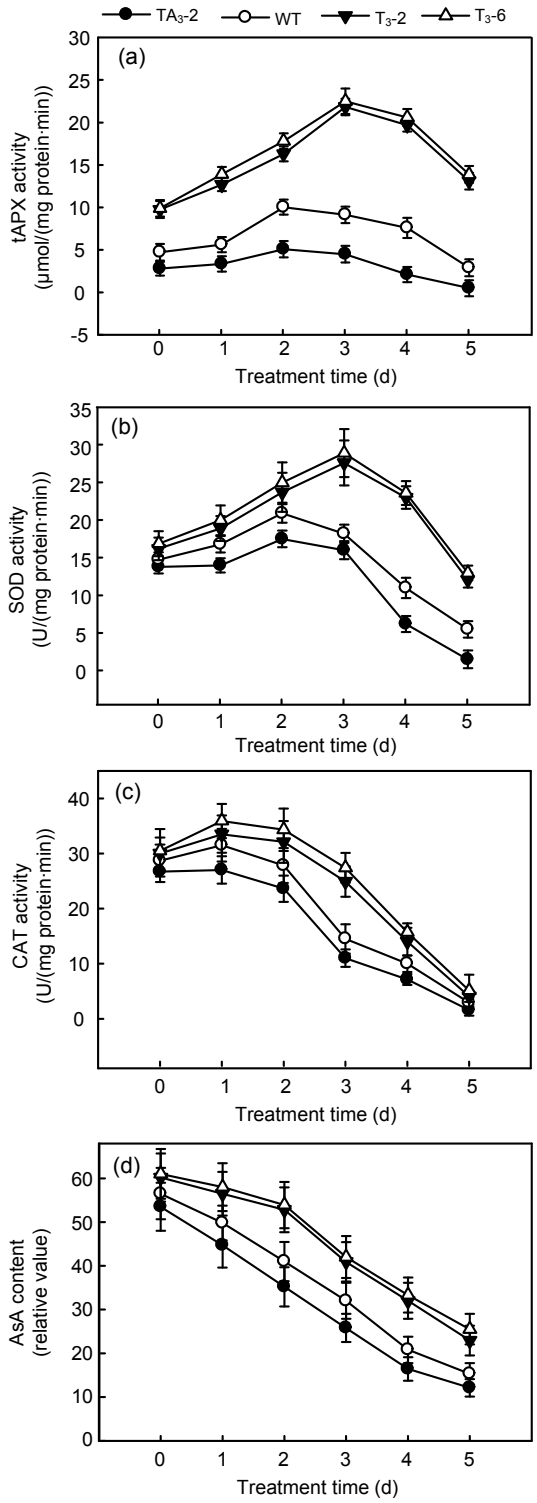

Fig. 3 Changes of antioxidant protective substance activities under 200 μmol/L MV stress conditions for 5 d in WT and transgenic tobacco seedlings

(a) tAPX activities; (b) SOD activities; (c) CAT activities; (d) AsA content. Data are presented as mean±SD (n=3)

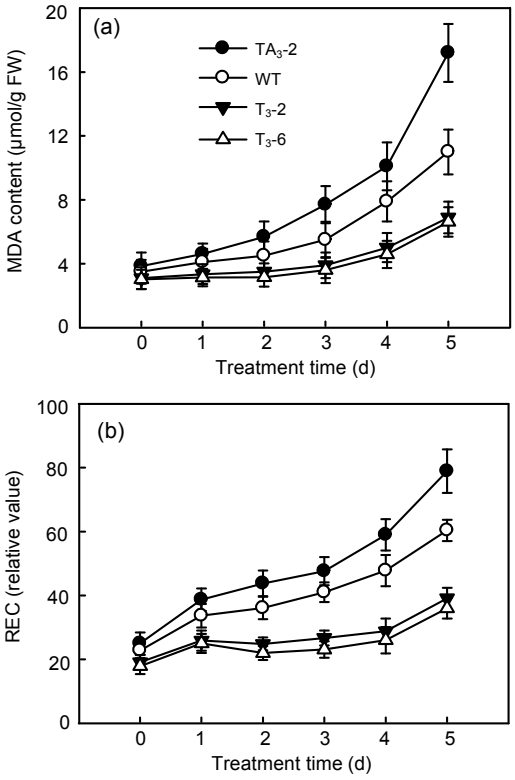

Fig. 4 Changes of membrane damage in WT and transgenic tobacco seedlings under MV stress conditions

Changes of MDA content (a) and REC (b) after 200 μmol/L MV treatment for 5 d. Data are presented as mean±SD (n=3). FW: fresh weight

and 74.64% after 200 μmol/L MV treatments for 5 d, while the Fv/Fm of T_3-2 and T_3-6 seedlings decreased by 42.69% and 39.98%, respectively (Fig. 5a).

When leaves were subjected to MV, the chlorophyll content decreased. Moreover, the chlorophyll content in the antisense transgenic lines was significantly lower than those of WT and sense transgenic lines. The chlorophyll contents of WT and TA_3-2 seedlings were significantly reduced to 43.06% and 19.33%, respectively. However, T_3-2 and T_3-6 seedlings exhibited 63.26% and 67.91% chlorophyll content, respectively, after 200 μmol/L MV treatments for 5 d (Fig. 5b).

3.6 Phenotypic differences and leaf disk assay under stressed conditions

To further assess oxidative stress tolerance, the tested seedlings were evaluated for visible damage after spraying with solutions containing 0, 50, 100,

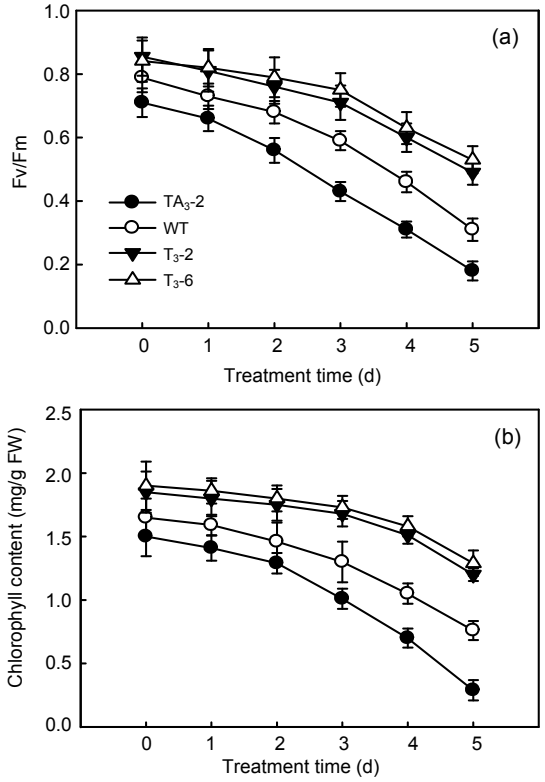

Fig. 5 Changes of Fv/Fm in WT and transgenic tobacco seedlings (a) and chlorophyll contents in tested tobacco seedlings (b) after 200 μmol/L MV treatment for 5 d

Data are presented as mean±SD (n=3). FW: fresh weight

and 200 μmol/L MV for 5 d. Severe necrosis was observed in the leaves of WT and antisense transgenic seedlings when exposed to MV stress conditions, whereas only partial necrosis was observed in the leaves of the sense transgenic lines. Visible leaf damage on tested plants became more severe with increased MV concentration. At the same time, leaves treated with water remained green in both WT and transgenic lines. After 200 μmol/L MV treatments for 5 d, WT and TA_3-2 plants showed 45% and 67% leaf damage, respectively, whereas T_3-2 and T_3-6 showed only 25% and 21% leaf damage, respectively.

The leaf disk assay was observed clearly from the leaf disks derived from WT, sense and antisense transgenic lines after 200 μmol/L MV treatments for 5 d. Leaf disks treated with distilled water remained green in both WT and transgenic lines. When leaf disks were subjected to MV, the chlorophyll content decreased. There was more obvious degreening in antisense lines leaf disks than in WT and sense transgenic plants (data not shown).

4 Discussion

MV is thought to be a very effective electron acceptor and MV catalyzes the photo-reduction of O_2 thereby accelerating the production of $O_2^-\cdot$ and H_2O_2 (Cornic *et al*., 2000). The electron transfer chain of the chloroplasts is the best-documented source of H_2O_2 (Asada, 1999). It has been reported that chloroplast APX is the primary target of MV-induced oxidative stress (Mano *et al*., 2001). The expression of *StAPX* was enhanced by MV-mediated oxidative stress (Fig. 1), indicating that *StAPX* was possibly regulated at the transcriptional level under MV stress conditions. The level of membrane per-oxidation of sense transgenic seedlings was obviously lower than those of WT and antisense transgenic seedlings (Fig. 4). This suggested that over-expression of *StAPX* in sense transgenic tobacco played an important role in protecting the structure of cell membrane whereas suppression of *StAPX* in antisense transgenic tobacco had an adverse effect on the protection of cell membrane structure.

Photoreductions of molecular oxygen lead to the formation of $O_2^-\cdot$ on the stromal side of the thylakoid memebrane via photosystem I. $O_2^-\cdot$ is highly reactive and is dismutated to H_2O_2 rapidly by thylakoid-assosiate Cu/Zn SOD. H_2O_2 in turn is reduced to water by APXs and peroxiredoxins, which is the water-water cycle (Shigeoka *et al*., 2002). The SOD activity was positively correlated with the change of tAPX activity and SOD activities in sense transgenic seedlings were higher than those in WT and antisense transgenic seedlings under MV stress conditions (Figs. 3a and 3b). To our understanding, this is due to efficient removal of H_2O_2 as a result of high tAPX activity. Accordingly, lower H_2O_2 content in chloroplasts accelerates higher SOD activity. These results indicate that APXs are of significance in proper scavenging of H_2O_2 in chloroplasts. A similar conclusion has been reported by Giacomelli *et al*. (2007). At the same time, a higher activity of tAPX did not increase CAT activity, and a decline in CAT activity was observed under MV stress condition for 2 d (Fig. 3c). It could be caused by the fact that some of the enzymes (such as CAT) were sensitive to ROS or the scavenging of H_2O_2 by APX possibly compensated in part for the decreased activities of CAT in

tobacco seedlings under oxidative conditions. Further study is necessary to fully develop a detailed explanation of this process.

Under stressed conditions, the abrupt increase in excitation energy led to the accumulation of H_2O_2. The accumulation of H_2O_2 was linked to the translation activity in chloroplasts and led to enhanced photoinhibition due to impairment of the photosystem II repair cycle under stressed conditions (Nishiyama *et al*., 2006). Fv/Fm in the tested lines was reduced after MV treatment, while the reduction was higher in WT and antisense transgenic seedlings than in sense transgenic seedlings (Fig. 5a). The results indicated that tAPX activity is crucial for photo-protection under stressed conditions.

Leaf injury by MV treatment is often used to assay the resistance of plants to oxidative stress (Yoshimura *et al*., 2004). The presence of MV somewhat hinders the greening of plants. The results showed that severe necrosis was observed in the leaf of antisense transgenic seedlings while partial necrosis was observed in the sense transgenic seedlings. At the same time, the chlorophyll content was contrarily correlative to the degree of necrosis, which showed that T_3-2 and T_3-6 lines with the higher tAPX activity maintained the higher chlorophyll content than TA_3-2 lines under MV stress conditions. The results indicated the crucial importance for tAPX in maintaining optimal chloroplast development. The antisense transgenic lines were slightly stunted in growth, which demonstrated that flowering time and longevity are also tightly correlated with the resistance to oxidative stress (Kurepa *et al*., 1998), implying the physiological importance of tAPX in tobacco plants even under normal growth conditions.

The results demonstrated changed APX activity in transgenic tobacco seedlings and the tolerance to MV-mediated oxidative stress. The results clearly indicated that tAPX, as one of the antioxidant enzymes, plays an important role in the effective protection of plants against environmental stress.

Compliance with ethics guidelines

Wei-hong SUN, Yong WANG, Hua-gang HE, Xue LI, Wan SONG, Bin DU, and Qing-wei MENG declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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