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Overexpression of tomato *tAPX* gene in tobacco improves tolerance to high or low temperature stress

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Abstract

In order to investigate the function of chloroplast ascorbate peroxidase under temperature stress, the thylakoid-bound ascorbate peroxidase gene from tomato leaf (*TtAPX*) was introduced into tobacco. Transformants were selected for their ability to grow on medium containing kanamycin. RNA gel blot analysis confirmed that *TtAPX* in tomato was induced by chilling or heat stress. Over-expression of *TtAPX* in tobacco improved seed germination under temperature stress. Two transgenic tobacco lines showed higher ascorbate peroxidase activity, accumulated less hydrogen peroxide and malondialdehyde than wild type plants under stress condition. The photochemical efficiency of photosystem 2 in the transgenic lines was distinctly higher than that of wild type plants under chilling and heat stresses. Results indicated that the over-expression of *TtAPX* enhanced tolerance to temperature stress in transgenic tobacco plants.

Additional key words: *Lycopersicum esculentum*, malondialdehyde, *Nicotiana tabacum*, photosystem 2, thylakoid-bound ascorbate peroxidase, transgenic tobacco.

Introduction

Temperature stress affects severely the growth, yield and quality of plants. Reactive oxygen species (ROS) tends to increase if plants were exposed to stress conditions such as low or high temperature. Therefore, plants have developed antioxidant mechanisms to protect themselves against ROS. These mechanisms employ antioxidant enzymes, 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 non-enzymatic antioxidants, such as ascorbic acid (AsA), reduced glutathione and phenolic compounds (Asada 1999).

The chloroplasts are the primary source of ROS, as electrons escaping from the photosynthetic electron transfer system interact with molecular oxygen, thereby resulting in the generation of superoxide anion radical ($O_2^{\bullet -}$). SOD catalyzes conversion of $O_2^{\bullet -}$ into oxygen and H_2O_2, and then APX reduces H_2O_2 to H_2O. This water-water cycle operates rapidly removing ROS at their site of generation (Asada 1999). Plants mainly use APX as the enzyme scavenging H_2O_2 in chloroplasts (Asada 1992). APX isoforms are distributed in at least four distinct cell compartments, the thylakoid membrane (tAPX) and stroma (sAPX) in chloroplasts, the cytosol (cAPX), the mitochondria (mitAPX) and the peroxisomes (mAPX).

It has been suggested that the membranes are primarily damaged under temperature stress (Kratsch and Wise 2000). Since more than 50 % of APX in the chloroplast is tAPX, the transition of thylakoid membrane phase under stress conditions can affect the activity of tAPX and consequently the scavenging of H_2O_2. Improvements in stress tolerance were observed in transgenic plants with over-expression of *tAPX* (Murgia *et al*. 2004, Lee *et al*. 2007). However, when challenged with photoinhibitory treatments at high irradiance or low temperature, the

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Abbreviations: APX - ascorbate peroxidase; AsA - ascorbic acid; CAT - catalase; CK - control; F_0 - initial fluorescence; F_v/F_m - variable to maximum fluorescence ratio (maximum photochemical efficiency of PS 2); MDA - malondialdehyde; $O_2^{\bullet -}$ - superoxide radical; PBS - phosphate-buffered saline; P_N - net photosynthetic rate; PS 2 - photosystem 2; REL - relative electrolyte leakage; ROS - reactive oxygen species; SDS - sodium dodecyl sulfate; SOD - superoxide dismutase; SSC - standard saline citrate; tAPX - thylakoid-bound ascorbate peroxidase; TBA - Tris-buffered acetate; TCA - trichloroacetic acid; *tAPX* - tomato thylakoid-bound ascorbate peroxidase gene; WT - wild type plant.

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tAPX over-expressing *Arabidopsis* lines showed no increased resistance with respect to controls (Murgia *et al*. 2004). These facts rose following questions. Can the over-expression of *TtAPX* gene change the resistance of the tobacco seeds and seedlings to temperature stress? Is the over-expression of *TtAPX* gene related to PS 2 photoprotection under temperature stress? To answer these questions, we generated transgenic tobacco plants with over-expression of *TtAPX* and studied their tolerance to high and low temperature stresses.

Materials and methods

Plants and treatments: Two transgenic tobacco lines (T_2-3 and T_2-6), wild tobacco (*Nicotiana tabacum* L. cv. NC89) plants and wild tomato (*Lycopersicum esculentum* Mill. cv. Zhongshu 4) plants were grown in plastic pots (one plant per pot) filled with sterilized soil in a greenhouse at day/night temperature of 25 - 30/15 - 20 °C, 14-h photoperiod and photon flux density (PFD) of 300 - 600 μmol m^{-2} s^{-1}. When the sixth leaf was fully expanded, the plants were exposed to temperature stresses (4 °C for 12 h or 42 °C for 24 h, respectively). The stressed leaves were immediately frozen in liquid nitrogen and stored at -80 °C until used. The other parts of tobacco seedlings were utilized for phenotypic analysis after stress treatments.

RNA gel blot analysis: Total RNA was extracted from tomato leaves using the Trizol reagent (*Invitrogen*, Carlsbad, USA) according to the manufacturer's instructions. 20 μg of total RNA was separated in 1.2 % denaturation formaldehyde agarose gel and then transferred onto *Hybond* N^+ membrane (*GE Healthcare*, Beijing, China). RNA was fixed on membrane by crossing-linking under UV radiation. Pre-hybridization was performed at 42 °C for 12 h. The 3' partial cDNA 0.5 kb of *TtAPX* was used as gene-specific probe and labeled with [a-^{32}P]dCTP by the random prime labeling method (*Prime-a-Gene-Labeling System*, *Promega,* Madison, USA). After 24 h hybridization, filters were washed subsequently in 2× SSC (1× SSC was 0.15 M NaCl, 0.015 M sodium citrate, pH 7) with 0.2 % SDS and 0.2× SSC with 0.2 % SDS at 65 °C. Autoradiography was performed at -80 °C. The membrane was exposed to an imaging plate and the relative expression ratio of *TtAPX* transcript was analyzed using the *Molecular Imager Fx-Plus* (*Bio-Rad*, Ivry sur Seine, France).

Construction of expression plasmid and plant transformation: The full length of *TtAPX* was amplified by RT-PCR. The cDNA amplification products were fused into pMD-18T vector and sequenced. Both fragments were cut with *Sal*I and *Xba*I and then ligated into the *Sal*I - *Xba*I site of the binary vector pBI121 (with replacement of the GUS fragment) under the control of the cauliflower mosaic virus 35S (CaMV35S) promoter. The sense expression plasmids (pBI121-*TtAPX*) were transformed into tobacco plants (3 to 4-week-old) by *Agrobacterium*-mediated cotyledon infiltration. Discs infected with *A. tumefaciens* were incubated on medium for inducing shoots. After a few weeks, the regenerated shoots were transferred to a root-inducing medium. Each media contained 50 μg cm^{-3} kanamycin and 250 μg cm^{-3} sodium cefotaxime.

The transgenic tobacco plants (T_0 generation) were self-pollinated using glassine envelopes, resulting in the production of T_1 seeds. T_2 plants were selected with kanamycin (50 μg cm^{-3}) and grown on MS agar medium at 25 °C, a 16-h photoperiod and PFD 600 μmol m^{-2} s^{-1}. Three-week-old seedlings were transplanted into pots with soil and grown in the greenhouse.

Stress treatments and seed germination: WT and transgenic surface-sterilized tobacco seeds were sown in Petri dishes containing 30 cm^3 of MS medium. Approximately 40 seeds were placed on each 9-cm Petri dish. Five of these dishes were transferred to a growth room with temperature 42 °C for 48 h, and five were transferred to a growth room with temperature 4 °C for 48 h. Then all of them were transferred back to normal conditions (25 °C). The control plants (plants which were not subject to a temperature stress) were sown at 25 °C. Germination was scored after 15 d.

Enzyme activity assays and H_2O_2 content measurements: WT and transgenic tobacco seedlings (6-week-old) were allowed to grow under 42 °C for 24 h or 4 °C for 24 h. Control plants were grown at 25 °C.

Chloroplasts were isolated from 50 g of fresh leaves according to the method of Robinson *et al*. (1983). The leaves were homogenized in a blender in 200 cm^3 ice-cold medium containing 330 mM sorbitol, 2 mM AsA, 30 mM 2-N-morpholino-ethanesulfonic acid (pH 6.5) and 0.1 % bovine serum albumin. The homogenate was filtered through six layers of cheese cloth and centrifuged at 2000 *g* for 3 min. The pellet was suspended with 4 cm^3 PBS for measurement of chloroplast APX activity.

APX activity was assayed according to the method of Chen and Asada (1989). Enzyme activity was measured in a reaction mixture consisting of 50 mM phosphate buffer (pH 7.0), 0.5 mM AsA and 0.2 mM H_2O_2 at 290 nm (coefficient of absorbance, $\varepsilon = 2.8$ mM^{-1} cm^{-1}). The result was calculated in terms of 1 mmol AsA oxidized per min. SOD activity was assayed according to the method of Giannopolitis and Ries (1977). The soluble protein content was measured following the method of Bradford (1976).

H_2O_2 content was determined by homogenizing 500-mg leaf samples in 100 mM phosphate buffer (pH 7.0) according to the method of Sairam and Srivastava (2002). The concentration of H_2O_2 was estimated by measuring the absorbance of the titanium-hydroperoxide complex and using a standard curve plotted with known concentration

of H_2O_2. The absorbance was determined at 415 nm.

Malondialdehyde (MDA) content and relative electrolyte leakage (REL) measurements: Fresh leaves (0.5 g) without midrib were thoroughly ground in a grinding medium containing 10 % TCA. The homogenate was centrifuged at 4 000 *g* for 10 min. 2 cm^3 Tris-buffered acetate (TBA; 0.6 %) were added to 2 cm^3 supernatant (2 cm^3 distilled water were added to the control supernatant) and mixed. The liquid was boiled for 15 min and cooled quickly and then centrifuged. The water phase was used to determine the absorbance at 532, 600 and 450 nm.

Six leaf discs (0.8 cm) were put into 10 cm^3 distilled water and vacuum infiltrated for 30 min, and then the initial electric conductance (S_1) was measured after surged for 3 h. Then a cuvette was filled with leaf discs and distilled water, and the mixture was cooked 30 min to measure the final electric conductance (S_2). The REL was calculated as: REL (%) = $S_1/S_2 \times 100$.

Chlorophyll a fluorescence and net photosynthetic rate measurements: The maximal photochemical efficiency of PS 2 (F_v/F_m) was measured by using a portable Chlorophyll fluorescence meter (*FMS2*, *Hansatech*, Norfolk, UK) according to Kooten and Snel (1990). Measurements were conducted at 25 °C on the fourth to fifth dark-adapted leaves. The minimum fluorescence (F_0) was determined under low modulated irradiance and the maximal fluorescence (F_m) was determined after a 0.8 s of saturating irradiance (7000 μmol m^{-2} s^{-1}). The F_v/F_m of PS 2 was expressed as $(F_m - F_0)/F_m$.

Net photosynthetic rate (P_N) was measured with a portable photosynthetic system (*CIRAS-2*, *PP Systems*, Boston, USA) at 25 °C under ambient CO_2 concentration of 360 μmol mol^{-1} and PFD of 800 μmol m^{-2} s^{-1}. Before P_N measurement, tobacco plants were induced for about 30 min at 25 °C (under 100 μmol m^{-2} s^{-1} PFD) to make the stomata open and then adapted for about 15 min at PFD of 600 μmol m^{-2} s^{-1}.

Statistical analysis: The significance of differences between wild type and transgenic plants in measured parameters was tested by one-way analysis of variance (*ANOVA*) and by Student's t-test.

Results

A cDNA designated as *TtAPX* was isolated from tomato leaves. The full-length sequence of the cDNA consisted of 1 369 bp nucleotides and a 1 130 bp open reading frame at position 88 - 1218 bp, encoding a 377-residue polypeptide. The deduced amino acid sequence of the cDNA showed that it encoded a polypeptide of approximately 42.1 kDa. This cDNA of *TtAPX* is submitted to the *GenBank* databases under accession number AF413573 (Wang *et al.* 2002) (http://www.ncbi.nlm.nih.gov). The analysis of the predicted amino acid sequence of *TtAPX* from other higher plants clearly identified *TtAPX* cDNA as encoding a *tAPX* protein targeted to the chloroplast (data not shown).

The results showed that the expression levels of *TtAPX* increased under low or high temperature stress and the expression level varied with the time of heat stress treatment, whereas varied slightly with the time of chilling treatment (Fig. 1). This indicated that the expression of *TtAPX* in tomato was induced by temperature stress, especially by heat stress. *TtAPX* was found to be expressed constitutively and the expression level was higher in leaves than in other organs (Fig. 2), so leaves were used as materials in the following RNA gel blot.

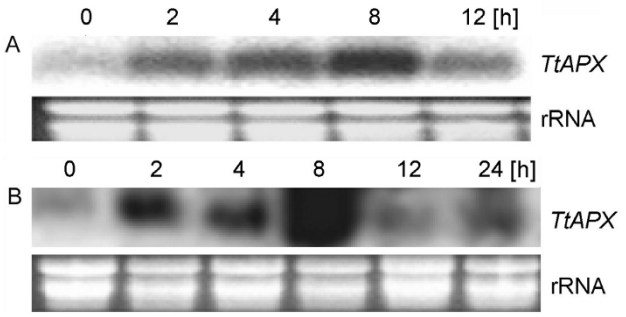

Fig. 1. Expression of *TtAPX* by RNA gel blot in tomato under temperature stress. The ethidium bromide stained rRNA band is shown as a loading control. *A*, *B* - the expression of *TtAPX* at 4 and 42 °C, respectively.

Twelve individual kanamycin-resistant transgenic lines (T_0) of tobacco were used. Each transgenic line seemed to represent an independent integration event since a specific DNA fragment in each line was observed by genomic DNA gel blot analysis. However, there were no PCR products observed in WT tobacco (data not shown). The different homozygous T_2 lines were used as experimental materials. The transgenic lines did not show any obvious differences in vegetative or reproductive growth.

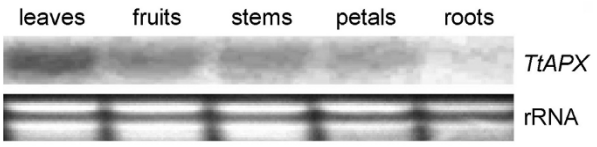

Fig. 2. Expression of *TtAPX* in different organs of tomato at 25 °C. Total RNA was extracted from leaves, fruits, stems, petals and roots of wild tomato plants.

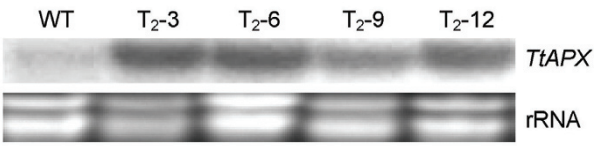

Fig. 3. Expression of *TtAPX* in transgenic tobacco lines and WT plants. T_2-3, T_2-6, T_2-9 and T_2-12: different transgenic lines; WT: wild type plants.

To investigate the expression of the *TtAPX*, four lines named T_2-3, T_2-6, T_2-9 and T_2-12 were selected for RNA gel blot analysis. From the four lines we selected T_2-3 and T_2-6 for physiological measurement. RNA gel blot analysis showed that all kanamycin-resistant plants had strong positive signals, while a weak signal was found in the WT plants (Fig. 3). The result indicated that *TtAPX* had been introduced into the tobacco genome and over-expressed at the RNA level.

On the MS medium at 25 °C (CK), the germination rate of both lines and WT was about 96.0 %. For 4 °C treatment, the germination rate of T_2-3 and T_2-6 was 85.0 and 82.5 %, respectively. For 42 °C treatment, the germination rate of T_2-3 and T_2-6 was 80.0 and 76.8 %, respectively. However, the germination rates of WT were 53.3 % (4 °C) and 53.0 % (42 °C), respectively. The result suggested that over-expression of *TtAPX* increased germination rate in the transgenic tobacco seeds under temperature stress. Early seedlings of WT were wilted under stress conditions, whereas transgenic seedlings appeared to remain growing slowly.

The APX activity of T_2-3 and T_2-6 was higher than that in WT plants under normal conditions. At 4 °C for 12 h, the APX activity of WT increased by about 90.0 % of the original values, whereas the activity of T_2-3 and T_2-6 was 205.3 and 203.6 %, respectively (Fig. 4*A*). At 42 °C for 9 h, the APX activity of WT increased by about 133.5 % of the original values, whereas the activity of T_2-3 and T_2-6 was 266.0 and 254.7 %, respectively (Fig. 4*B*).

SOD activity in transgenic plants was always higher than that of WT plants. SOD activity in all the tested plants firstly increased during stress treatment, reached maximum and then decreased, and increased again during recovery (Fig. 4*C*,*D*).

H_2O_2 contents were negatively correlated with the changes of APX activities (Fig. 4). When compared to control conditions, the increments of H_2O_2 content in T_2-3 and T_2-6 were 55.0 and 59.0 % under chilling stress for 24 h, respectively. However, the increment of H_2O_2 content in WT was 140.0 %. In the same way, H_2O_2 content in transgenic plants increased more slowly in transgenic than in WT plants under stress (Fig. 4*E*,*F*).

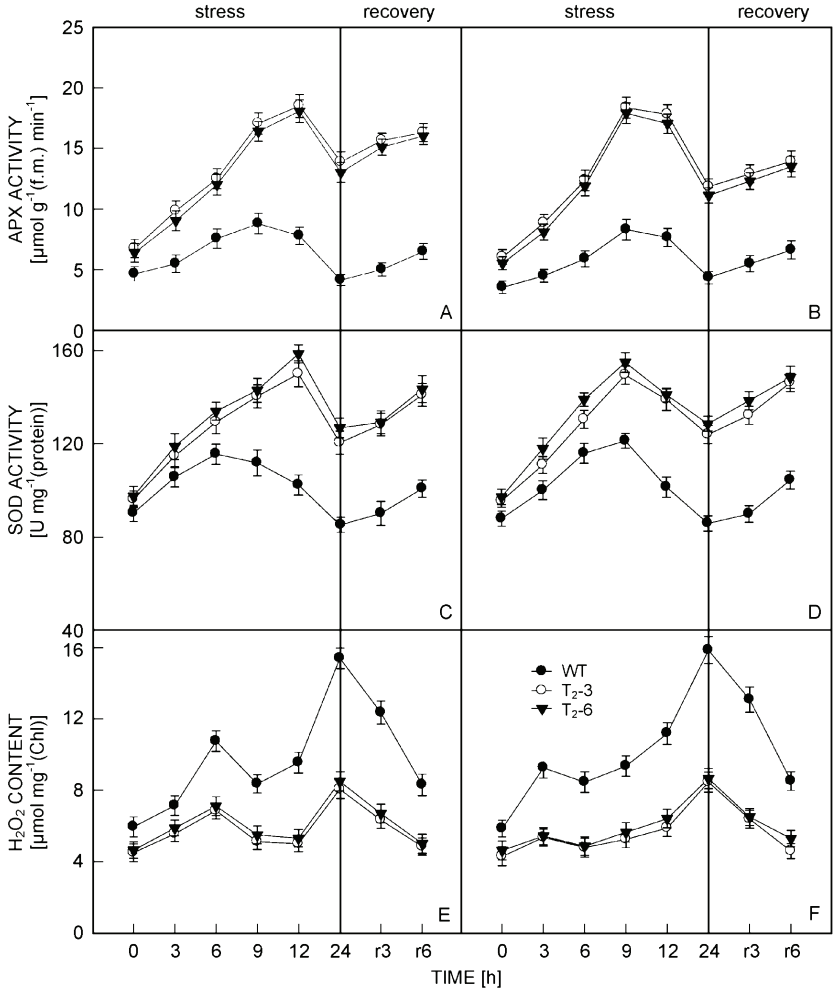

Fig. 4. Changes of APX activities (*A*, *B*), SOD activities (*C*, *D*) and H_2O_2 contents (*E*, *F*) in tobacco leaves under temperature stress. and during recovery. *A*, *C*, *E* - 4 °C; *B*, *D*, *F* - 42 °C. Data presented here are means ± SD of four independent experiments.

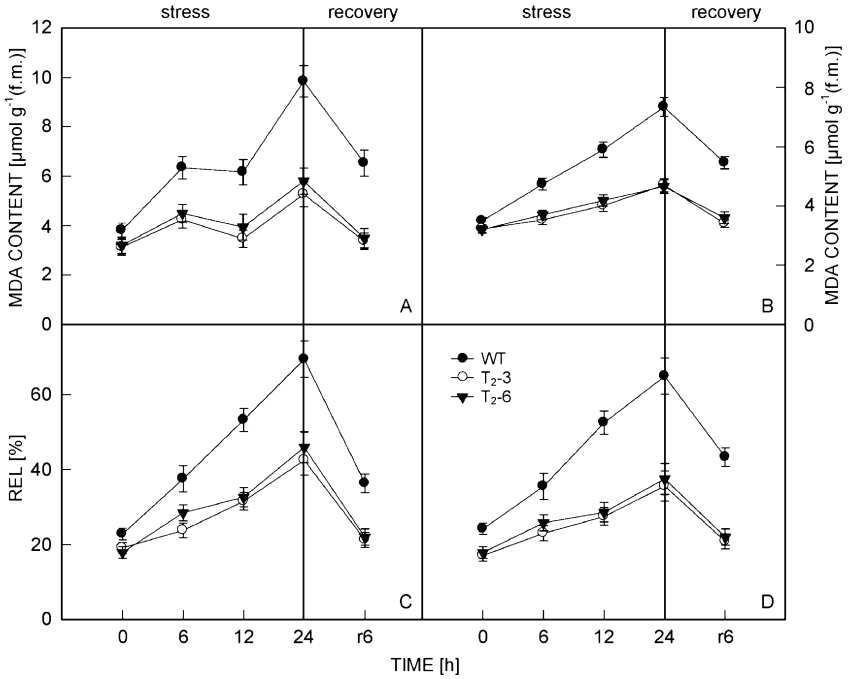

Fig. 5. Changes of MDA content (*A*, *B*) and relative electrolyte leakage (REL; *C*, *D*) in tobacco leaves under temperature stress and during recovery. *A*, C - 4 °C; *B*, *D* - 42 °C stress. Data presented here are means ± SD of four independent experiments.

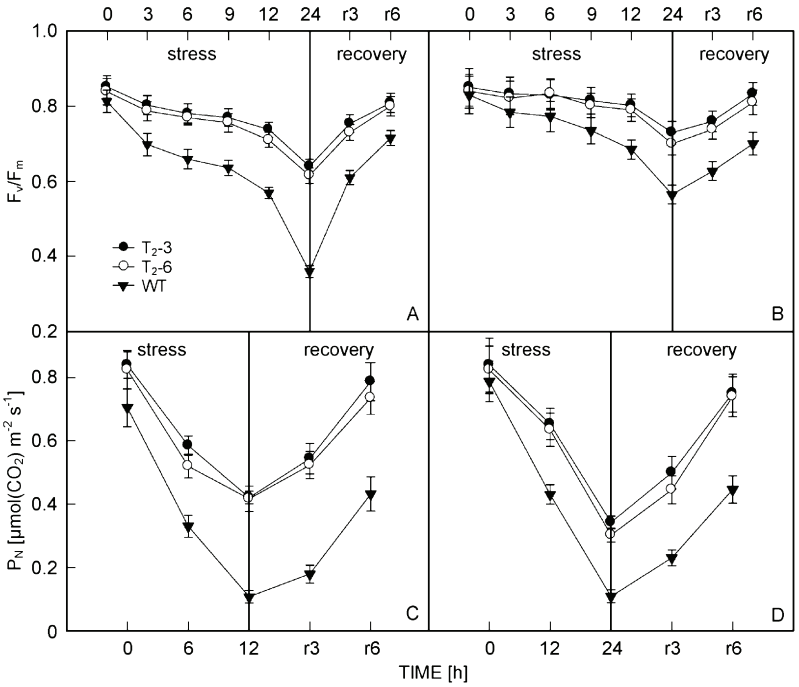

Fig. 6. Changes of F_v/F_m (*A*, *B*) and P_N (*C*, *D*) in tobacco plants under temperature stress and during recovery. *A*, *C* - 4 °C; *B*, *D* - 42 °C.; Data presented here are means ± SD of four independent experiments.

MDA is one of important markers of membrane system subjected to damage. MDA contents of WT plants were higher than those of transgenic lines under temperature stress. MDA contents in WT, T_2-3 and T_2-6 lines increased about 142.8, 74.7 and 81.5 %, respectively, under chilling stress for 24 h (Fig. 5*A*). MDA contents in WT, T_2-3 and T_2-6 lines increased about 110.3, 45.9 and 45.6 %, respectively, under thermal stress for 24 h (Fig. 5*B*).

After treatment for 24 h at 4 °C , the REL of T_2-3 and T_2-6 increased by 143.4 and 149.0 % of initial values,

whereas it increased to 204.2 % of initial values in the WT (Fig. 5*C*). Fig. 5*D* showed that heat treatment resulted in less ion leakage from the transgenic plants than from the wild type. These results indicated that membrane damage was more serious in WT plants than that in transgenic plants under temperature stress.

F_v/F_m decreased under temperature stress, but the F_v/F_m of transgenic plants was higher than that of WT plants. The F_v/F_m of WT decreased by 55.7 % under chilling stress for 24 h and by 31.9 % under heat stress for 24 h, respectively (Fig. 6*A*,*B*). However, the F_v/F_m of T_2-3 and T_2-6 lines decreased only by 23.7 and 26.8 % at 4 °C for 12 h (Fig. 6*A*), and by 14.2 and 16.7 % at 42 °C for 24 h (Fig. 6*B*), respectively.

P_N of WT and transgenic plants decreased under high or low temperature stress, and the decrease of P_N was more obvious in WT compared to transgenic plants (Fig. 6*C*,*D*). These results showed the important role of *TtAPX* over-expression in protection of the photosynthetic apparatus under temperature stress.

Discussion

The expression levels of APX gene varied with the time of stress treatments (Shi *et al*. 2001, Kavitha *et al.* 2008). Therefore it was necessary to examine the expression pattern of *TtAPX* in tomato. The result showed that expression of *TtAPX* was enhanced by chilling and heat stresses (Fig. 1). In contrast, constitutive expression observed in tAPX activity and no significant changes in tAPX activity were found in other experiments (Yoshimura *et al*. 2000, Shigeoka *et al*. 2002, Song *et al*. 2005). This inconsistency may be due to variations of the experimental set-up used, such as different plants, different plant organs or tissues, differential time points of plant sampling, different method of stress treatments.

Some studies found that the *tAPX*-over-expressing plants showed increased resistance with respect to controls (Yabuta 2002, Murgia *et al*. 2004, Song *et al*. 2005). However, results in this study indicated that the increase of APX activity in transgenic tobacco plants was due to over-expression of *TtAPX*, as confirmed by RNA gel blot analysis. APX activity increased more substantially in the transgenic plants than WT plants when exposed to temperature stresses (Fig. 4*A*,*B*). Consistent closely with APX activity, the expression of T*tAPX* followed a similar pattern, indicating that *TtAPX* was regulated at the transcriptional level.

Heat stress reduced germination rate and early growth in *Amaranthus lividus,* increased accumulation of H_2O_2 while decreased APX activity (Bhattacharjee 2008). However, the low content of MDA and H_2O_2 and high activity of APX improved the chilling tolerance of Manila grass (Wang *et al*. 2009). There are similar results in this study. Higher APX and SOD activities of T_2-3 and T_2-6 contributed to the decrease in REL and H_2O_2 and MDA contents (Figs. 4, 5). The ability of H_2O_2 scavenging in transgenic plants was higher obviously than that of WT plants. This suggested that over-expression of *TtAPX* in transgenic tobacco played an important role in preventing excessive H_2O_2, thereby enhancing the tolerance in temperature stress.

F_v/F_m in all plants was reduced under temperature stresses, while the reduction was higher in WT than that in transgenic plants (Fig. 6). Also P_N decreased more significantly and recovered more slowly in WT plants than in transgenic plants (Fig. 6). Zhou *et al*. (2004) and Song *et al*. (2005) found that abiotic stress-induced decrease in the photosynthesis and increase in ROS production was partly compensated by the water-water cycle, followed by significant increase in the activity of antioxidant enzymes. It suggested that over-expression of *TtAPX* played an important role in removing H_2O_2 and minimizing photo-oxidative damage during the early stage of temperature stress. Further work is required to explore the effect of *TtAPX* on PS 2 and PS 1 photoinhibition under temperature stress.

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