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Effect of water stress on yield and nutrition quality of tomato plant overexpressing *StAPX*

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Abstract

We investigated the effect of water stress on yield and quality of tomato plants overexpressing *Solanum lycopersicum* thylakoid-bound ascorbate peroxidase gene (*StAPX*). APX activity, hydrogen peroxide content, net photosynthetic rate of tomato leaves, and yield and nutrition quality of tomato fruits were measured under soil moisture 70, 60, and 50 % of full field capacity. Results show that the capability of APX for scavenging hydrogen peroxide induced by water stress was higher in the transgenic than the wild type (WT) plants. The yield of fruits of the transgenic tomato plants was higher than that of WT plants under water stress and the fruit nutrition quality was not different. These results indicate that overexpression of *StAPX* might improve water stress tolerance in the transgenic tomato plants.

Additional key words: chlorophyll, H_2O_2 scavenging, net photosynthetic rate, proline, *Solanum lycopersicum*, sugars, thylakoid-bound ascorbate peroxidase

Introduction

Drought stress is one of the serious problems in agriculture (Jacob 2008, Vats *et al*. 2011). Among others, drought stress can cause ion imbalance and production of reactive oxygen species (ROS; *e.g*., Mittler 2002). Ascorbate peroxidases (APX, EC 1.11.1.11) are directly involved in ROS scavenging by reducing H_2O_2 to water. Several studies found that plants overexpressing *APX* show increase of stress resistance (Murgia *et al*. 2004, Lee *et al*. 2007, Sato *et al*. 2011, Saxena *et al*. 2012).

In order to study the influence of the thylakoid membrane APX (TAPX) in chloroplasts on water stress resistance, *Solanum lycopersicum* thylakoid-bound ascorbate peroxidase gene (*StAPX*) was introduced into tomato under the control of the cauliflower mosaic virus 35S promoter. The transgenic lines were selected for their ability to grow on medium containing kanamycin and identified by polymerase chain reaction (PCR). Further, transgenic tomato plants overexpressing *StAPX* (T_2 generation) were explored to investigate physiological and biochemical parameters in their leaves and fruits under different irrigation conditions.

Materials and methods

Agrobacterium-mediated transformation of tomato plants: The *StAPX* cDNA was isolated from tomato leaves by reverse trascriptase - polymerase chain reaction (RT-PCR). The PCR amplification conditions and gene sequence data were reported previously (Sun *et al*. 2010). The full-length *StAPX* cDNA was ligated into the

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Abbreviations: APX - ascorbate peroxidase; EDTA - ethylenediaminetetraacetic acid; PBS - phosphate buffered saline; PPFD - photosynthetic photon flux density; P_N - net photosynthetic rate; PS II - photosystem II; ROS - reactive oxygen species; SM - soil moisture, *StAPX* - *Solanum lycopersicum* thylakoid-bound ascorbate peroxidase gene; T - transgenic tomato line; TAPX - thylakoid-bound ascorbate peroxidase; WT - wild type plant.
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expression vector pBI121 (with replacement of the GUS fragment) under the control of the cauliflower mosaic virus 35S (35S-CaMV) promoter. The pBI121-*StAPX* constructs were introduced into *Agrobacterium tumefaciens* LBA4404 by the freezing transformation method. The tomato cotyledons were infected with *A. tumefaciens* by the infiltration method (Horsch 1985) and grown in a growth room at temperature of 25 °C, relative humidity of 75 ± 5 %, a 16-h photoperiod, and photosynthetic photon flux density (PPFD) of 600 - 1 000 μmol m^{-2} s^{-1}. Discs of the cotyledons infected with *A. tumefaciens* were incubated on medium to induce shoots. The regenerated shoots were transferred to a root-inducing medium after a few weeks. Both media contained 50 μg cm^{-3} kanamycin and 250 μg cm^{-3} sodium cefotaxime. The transgenic plants were screened using kanamycin selection and PCR identification.

The transgenic T_0 generations were self-pollinated with glassine envelopes resulting in the production of T_1 seeds. The T_1 seeds were screened in Murashige and Skoog (MS) culture medium supplemented with 50 μg cm^{-3} kanamycin. About two-week-old seedlings were transplanted into sterilized soil pots and grown in a greenhouse at day/night temperatures of 25 - 30/15 - 20 °C and the natural photoperiod. Different homozygous lines (T_2) were used for experimental analysis. The transgenic lines were identified by PCR with the 35S-CaMV promoter primer (5′-GTAATCTCCACTGACGTAAG-3′) and the gene downstream primer (5′-TTTAGCATATAT GACTACGGC-3′).

Plants and stress treatments: The seeds of transgenic lines (T_2-2 and T_2-4) and wild type (WT) tomato (*Solanum lycopersicum* cv. Zhongshu 6) were germinated between pieces of moistened filter paper at 25 °C for 5 d. The germinated seeds were then planted into bed filled with sterilized soil and grown in a greenhouse. After two weeks, three water treatments were applied: *1*) normal irrigation - soil moisture (SM) of 70 ± 5 % of full field capacity, *2*) mild drought treatment - SM 60 ± 5 %, and *3*) moderate drought treatment - SM 50 ± 5 %. These treatments ended when the last flower head bore fruit. The tested plants were irrigated by drip irrigation and soil moisture was determined at 10:00 and 15:00 with *HH2* portable apparatus with *ML2* detector (*Delta-T Devices*, Cambridge, UK).

Measurements of TAPX activity and H_2O_2 content in tomato leaves: Chloroplasts were isolated according to the method of Robinson *et al*. (1983) from 20 g of fresh leaves of plants treated for 0, 7, 14, 21, 28, and 35 d. The chloroplast pellet was suspended in 0.05 M phosphate buffered saline (PBS) with 5 mM EDTA (pH 7.8) for measurement of APX activity and the other part of the pellet was suspended in acetone for measurement of H_2O_2. APX activity was assayed spectrometrically according to the method of Chen and Asada (1989). The absorbance was determined at 290 nm and the result was calculated in terms of 1 μmol ascorbic acid (AsA) oxidized per min. H_2O_2 content was determined spectrometrically as well according to the method of Ferguson *et al*. (1983). The absorbance was determined at 415 nm. At least three replicate measurements were used for each treatment.

Measurements of net photosynthetic rate (P_N) and the chlorophyll content: Tomato plants were acclimated at temperature of 25 °C and PPFD of 100 μmol m^{-2} s^{-1} for about 30 min, to make the stomata open, and then under PPFD of 800 μmol m^{-2} s^{-1} for about 15 min. Then, P_N was measured with a portable photosynthetic system (*CIRAS-2, PP Systems*, Norfolk, UK) at 25 °C, PPFD of 800 μmol m^{-2} s^{-1}, ambient CO_2 concentration of 360 μmol mol^{-1} and relative humidity of 75 ± 5 %. The chlorophyll contents in the fresh leaves of tested plants were measured according to the method of Hemavathi *et al*. (2009).

Measurements of content of proteins, free proline, and soluble sugars: Total protein content of fresh tomato leaves or fruits was determined according to the method of Bradford (1976). Coomassie brilliant blue *G250* was used for staining and absorbance was determined at 595 nm.

Free proline content was quantified according to the method of Meloni *et al*. (2004). Sulfosalicylic acid was used to dissolve the proline, acidic ninhydrin for staining, and finally, absorbance was determined at 520 nm.

Soluble sugar content was determined according to the method of Zhao *et al*. (2002). Absorbance was determined at 630 nm after the reaction of soluble sugars with anthrone.

Measurement of yield and quality of fruits: The number of tomato fruits in each plant were counted after fruit maturity (about 90 d after planting) and fresh mass was determined. Organic acid content was determined by the titration method. A 10 g of fruits was ground in an ice-bath and distilled water was added to give a constant volume of 100 cm^3. The mixture was filtrated and then 2 dribblets of 1 % (m/v) phenolphthalein were added to 20 cm^3 of the filtrate. NaOH was used for titration and the end point was determined when the pink colour appeared and was maintained for 0.5 min.

Ascorbic acid (AsA; vitamin C) content was determined spectrophotometrically in UV region. A 10 g of ground tomato fruits was mixed with distilled water to give a constant volume of 1 000 cm^3. The liquid mixture was filtrated, centrifuged for 10 min, and then 5 cm^3 of Cu(II)-EDTA was added. AsA content was determined at 267 nm. Lycopene content was quantified according to the method of Zhang and Ding (2001). Petroleum ether was used to dissolve out lycopene and absorbance was determined at 502 nm.

Statistical analysis: At least three replicate measurements were used for each treatment. Statistical significances of differences were analyzed by Student's *t*-test.

Results

The analysis of the predicted amino acid sequence of *StAPX* from other higher plants was described by Sun *et al*. (2010). The kanamycin-resistant lines obtained did not show obvious difference from wild plants in both vegetative and reproductive growth. Each of the transgenic line seemed to represent an independent integration event observed by genomic DNA gel blot analysis. Several T_2 transgenic lines were selected for identification by PCR with the 35S-CaMV promoter primer and the gene downstream primer. There was an intense 1 400 bp band corresponding in size to the *StAPX* product in each transgenic plant but not in the WT plants (Fig. 1). Among them, T_2-2 and T_2-4 were selected for physiological measurement. The measured values of T_2-2 and T_2-4 showed only slight differences and the values of T_2-2 were used to analyses mentioned below.

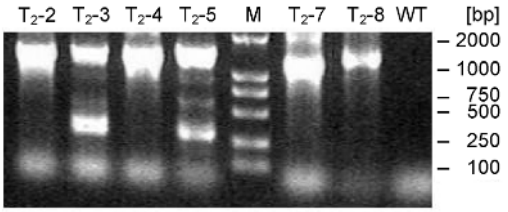

Fig. 1. Identification PCR of transgenic tomato plants by using the genome template. *Lane* M - DNA marker; *lanes* T_2-2 to T_2-5, T_2-7, and T_2-8 - different transgenic lines; *lane* WT - wild type plants.

There were about 3-fold increase in TAPX activity in the transgenic lines comparing with the WT plants on day 0. APX activities in the both types of tested plants firstly increased and then decreased under moderate water stress. However, TAPX activities in the transgenic lines were evidently higher than those in the WT plants. For example, when exposed to 50 % SM for 28 d, the TAPX activities of T_2-2 and WT were about 0.76 and 0.57 of those at day 0, respectively. The TAPX activity in T_2-2 was 3.53 times higher than that of WT on day 28 (Fig. 2*A*).

H_2O_2 content in all the tested plants constantly increased with the decline of SM and H_2O_2 content in the transgenic lines was lower than that in the WT plants (Fig. 2*B*).

Chlorophyll content in the transgenic lines was significantly higher than in the WT plants when they were subjected to water stress (Fig. 2*C*, Table 1). The decrease of P_N was more serious in the WT than in transgenic plants under water stress conditions (Fig. 2*D*, Table 1). Compared with the control, P_N in the T_2-2 and WT plants decreased by 32.8 and 54.03 %, respectively, under 50 % SM for 28 d (Fig. 2*D*).

Total protein content of T_2-2 and WT at 50 % SM for 28 d was about 1.19 and 0.93 of the control, respectively (Table 2). Free proline content as well as content of soluble sugars in both types of the tested plants changed little due to the water stress (Table 2).

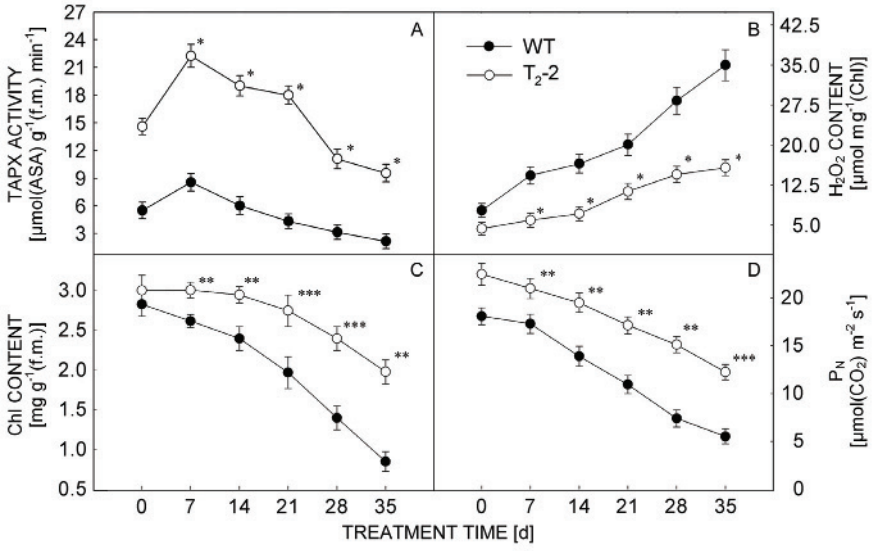

Fig. 2. TAPX activity (*A*), H_2O_2 content (*B*), chlorophyll (Chl) content (*C*), and net photosynthetic rate (P_N) (*D*) in leaves of WT and transgenic (T_2-2) tomato plants grown at soil moisture of 50 % for 35 d. Means ± SE, $n \geq 3$, *, **, *** - significant differences between T_2-2 and WT at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$.

Table 1. The TAPX activity, H_2O_2 content, chlorophyll content, and net photosynthetic rate (P_N) of tomato leaves under different SM for 28 d. Means ± SE, $n \geq 3$, *, ** - significant differences between T_2-2 and WT at $P \leq 0.05$ and $P \leq 0.01$.

SM [%]	Plants	TAPX activity [μmol(AsA) g^{-1}(f.m.) min^{-1}]	H_2O_2 content [μmol mg^{-1}(Chl)]	Chl content [mg g^{-1}(f.m.)]	P_N [μmol m^{-2} s^{-1}]
70	T_2-2	13.95 ± 0.30*	4.95 ± 0.05**	3.35 ± 0.14**	22.01 ± 0.65*
	WT	4.90 ± 0.06	7.50 ± 0.06	2.98 ± 0.10	17.88 ± 0.44
60	T_2-2	13.24 ± 0.55*	5.93 ± 0.06*	3.21 ± 0.12**	18.88 ± 0.50*
	WT	4.05 ± 0.03	13.51 ± 0.12	2.37 ± 0.11	12.75 ± 0.41
50	T_2-2	11.08 ± 0.78*	14.51 ± 0.83*	2.40 ± 0.10**	15.13 ± 0.55*
	WT	3.14 ± 0.08	28.30 ± 1.85	1.40 ± 0.13	7.41 ± 0.43

Table 2. The content of proteins, free proline, and soluble sugars of tomato leaves at 0 d and 28 d under different SM. Means ± SE, $n \geq 3$, **, *** - significant differences between T_2-2 and WT at $P \leq 0.01$ and $P \leq 0.001$, respectively..

SM [%]	Plants	Soluble proteins [mg g^{-1}(f.m.)]		Free proline [μg g^{-1}(f.m.)]		Soluble sugars [mg g^{-1}(f.m.)]	
		0 d	28 d	0 d	28 d	0 d	28 d
70	T_2-2	0.41 ± 0.05***	0.45 ± 0.05**	0.52 ± 0.06**	0.59 ± 0.06**	0.50 ± 0.04**	0.58 ± 0.06**
	WT	0.31 ± 0.05	0.34 ± 0.04	0.49 ± 0.04	0.56 ± 0.04	0.48 ± 0.05	0.50 ± 0.04
60	T_2-2	0.53 ± 0.06**	0.82 ± 0.06**	0.57 ± 0.04**	0.71 ± 0.05**	0.66 ± 0.10***	0.71 ± 0.10**
	WT	0.41 ± 0.04	0.49 ± 0.05	0.56 ± 0.06	0.65 ± 0.06	0.53 ± 0.10	0.60 ± 0.09
50	T_2-2	0.45 ± 0.03**	0.54 ± 0.04**	0.60 ± 0.05***	0.56 ± 0.05**	0.55 ± 0.04**	0.59 ± 0.05**
	WT	0.34 ± 0.04	0.32 ± 0.03	0.59 ± 0.05	0.52 ± 0.04	0.50 ± 0.05	0.53 ± 0.05

The yield of a single plant decreased with the decline of SM. However, single fruit mass and yield of a single plant in the transgenic lines were evidently higher than those in the WT plants under water stress conditions (Table 3). The sugar-acid ratios in the transgenic tomato fruits were 1.02, 0.97, and 1.00 of those in WT under 70, 60, and 50 % SM, respectively, suggesting that quality of the transgenic tomato fruits is almost the same as that of WT (Table 4).

The nutrition quality was affected by SM. Ascorbic acid (vitamin C) content in the tested tomato fruits increased gradually along with the decline of SM whereas soluble protein content changed slightly and lycopene content changed fluctuantly. The vitamin C content in the transgenic tomato fruits was 1.09, 1.18, and 1.23 of that in WT under 70, 60, and 50 % SM, respectively (Table 4). The results indicate that nutrition quality of the transgenic tomato fruits might be somewhat better to that of WT under water stress conditions.

Table 3. The yield of tomato fruits from plants grown under different SM for 28 d. The fruits were harvested 90 d after planting. Means ± SE, $n \geq 3$, *, ** - significant differences between T_2-2 and WT at $P \leq 0.05$ and $P \leq 0.01$, respectively.

SM [%]	Plants	Fruit number [$plant^{-1}$]	Fruit mass [kg]	Fruit yield [kg $plant^{-1}$]
70	T_2-2	10	0.219 ± 0.01*	2.195 ± 0.30*
	WT	10	0.202 ± 0.02	2.018 ± 0.25
60	T_2-2	12	0.167 ± 0.01**	2.004 ± 0.33**
	WT	11	0.156 ± 0.01	1.650 ± 0.30
50	T_2-2	11	0.150 ± 0.01**	1.655 ± 0.35*
	WT	10	0.123 ± 0.005	1.230 ± 0.28

Discussion

Drought stress induces closure of stomata, leaf wilting, structural and functional damages of cell membrane, and ROS accumulation (Reddy *et al*. 2004). Previous studies show that decrease in non-cyclic electron transport caused by abiotic stresses could be partially compensated by water-water cycle (Shigeoka *et al*. 2002). At the same moment, activities of antioxidant enzymes, including APX, and content of non-enzymatic antioxidants such as ascorbic acid (vitamin C), increased (Mýtinová *et al*. 2010, Li *et al*. 2012). The increase of APX activity in transgenic plants could decrease the accumulation of H_2O_2 and lighten the damage induced by ROS under drought stress (Fig. 2).

The results show that reduction of P_N was higher in

Table 4. The content of soluble sugars and organic acids, sugar/organic acid ratio, and content of proteins, ascorbic acid, and lycopene of tomato fruits. Plants were grown under different SM for 28 d. The fruits were harvested 90 d after planting. Means ± SE, n = 5, *, **, *** - significant differences between T_2-2 and WT at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively.

SM [%]	Plants	Soluble sugars [%]	Organic acids [%]	Sugar/acid ratio	Proteins [mg g^{-1}(f.m.)]	Ascorbic acid [mg g^{-1}(f.m.)]	Lycopene [μg g^{-1}(f.m.)]
70	T_2-2	2.15 ± 0.20*	0.33 ± 0.03***	6.57	3.55 ± 0.25*	0.42 ± 0.01***	18.00 ± 0.51**
	WT	2.18 ± 0.31	0.34 ± 0.03	6.46	3.20 ± 0.35	0.39 ± 0.01	18.05 ± 0.55
60	T_2-2	2.50 ± 0.33**	0.39 ± 0.03***	6.34	3.31 ± 0.30***	0.49 ± 0.05**	18.45 ± 1.03*
	WT	2.46 ± 0.30	0.38 ± 0.03	6.54	2.85 ± 0.30	0.42 ± 0.04	18.70 ± 0.50
50	T_2-2	2.80 ± 0.29*	0.43 ± 0.05***	6.51	2.65 ± 0.25*	0.52 ± 0.05**	17.51 ± 1.05*
	WT	2.70 ± 0.35	0.41 ± 0.05	6.52	2.06 ± 0.12	0.42 ± 0.02	17.56 ± 1.01

the WT than in the transgenic seedlings (Fig. 2*D,* Table 1) indicating the crucial importance of TAPX in protection from photo-damage under water stress conditions. At the same time, the transgenic lines maintained the higher chlorophyll content (Fig. 2*C*, Table 1). The chloroplasts are thought to be the primary source of ROS and TAPX played a key role in protecting photosynthetic apparatus in the transgenic plants from oxidative damage under drought stress. In the same sense as above, other studies reported that suppression of *tAPX* in antisense tobacco lines may be lethal (Yabuta *et al*. 2002) and that *tAPX* antisense wheat lines show lower photosynthetic carbon assimilation (Danna *et al*. 2003).

Osmotic adjustment is thought to be one of adaptive reactions to resist drought stress in plants. However, other crops, such as tomato, accumulate little amount of osmotically active substances under water stress (Reddy *et al*. 2004, Li *et al*. 2010). In the present study, the accumulations of soluble protein, soluble sugars and free proline in tomato leaves were not outstanding (Table 2), which suggests that osmotic adjustment might not be important for tomato plant resistance to water stress.

Different SMs affect dry matter accumulation and nutrition quality of the tomato fruits. The yield of tomato fruits was the maximum and the quality was the best when SM was 70 - 75 % (Chen *et al*. 2006, as well as this study). The net photosynthetic rate in the transgenic plants was evidently higher than that in the WT plants (Fig. 2*D*, Table 1), and the yield and the quality of the transgenic tomato fruits tended to be better than those of WT under the stress conditions (Tables 3,4).

In conclusion, the transgenic tomato plants had higher yield than WT and the fruits had similar nutrition quality as WT under the water stress. The transgenic tomato plants with overexpression of *StAPX* had better stress-resistance and could be recommended to drought stress conditions.

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