

Response of xanthophyll cycle and chloroplastic antioxidant enzymes to chilling stress in tomato over-expressing glycerol-3-phosphate acyltransferase gene

N. SUI*, M. LI**, X.-Y. LIU*, N. WANG*, W. FANG*, and Q.-W. MENG*,***

College of Life Sciences, Key Lab of Crop Biology of Shandong Province, Shandong Agricultural University, Tai'an, 271018, P.R. China*

Shandong Academy of Agricultural Sciences, Ji'nan, 250100, P.R. China**

Abstract

Over-expression of chloroplastic glycerol-3-phosphate acyltransferase gene (*LeGPAT*) increased unsaturated fatty acid contents in phosphatidylglycerol (PG) of thylakoid membrane in tomato. The effect of this increase on the xanthophyll cycle and chloroplast antioxidant enzymes was examined by comparing wild type (WT) tomato with the transgenic (TG) lines at chilling temperature (4 °C) under low irradiance (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Net photosynthetic rate and the maximal photochemical efficiency of photosystem (PS) 2 (F_v/F_m) in TG plants decreased more slowly during chilling stress and F_v/F_m recovered faster than that in WT plants under optimal conditions. The oxidizable P700 in both WT and TG plants decreased during chilling stress under low irradiance, but recovered faster in TG plants than in the WT ones. During chilling stress, non-photochemical quenching (NPQ) and the de-epoxidized ratio of xanthophyll cycle in WT plants were lower than those of TG tomatoes. The higher activities of superoxide dismutase (SOD) and ascorbate peroxidase (APX) in TG plants resulted in the reduction of O_2^- and H_2O_2 contents during chilling stress. Hence the increase in content of unsaturated fatty acids in PG by the over-expression of *LeGPAT* could alleviate photoinhibition of PS2 and PS1 by improving the de-epoxidized ratio of xanthophyll cycle and activities of SOD and APX in chloroplast.

Additional key words: ascorbate peroxidase; glycerol-3-phosphate acyltransferase; hydrogen peroxide; *Lycopersicon*; net photosynthetic rate; photosystems 1 and 2; quenching; superoxide dismutase.

Introduction

Chilling stress inhibits photosynthesis by the process of photoinhibition (Aro *et al.* 1993). Chloroplast membrane is the primary position to be damaged under chilling stress (Kratsch and Wise 2000). Since Lyons and Raison (1970) considered that chilling could impair membrane permeability by the transition of membrane lipids from a liquid-crystalline phase to a gel phase, many experiments have suggested that chilling tolerance is related to the composition and structure of plant membrane lipids (Somerville 1995, Nishida and Murata 1996, Murata and Los 1997). Tolerance to chilling stress is closely

connected with the fatty acid unsaturation of plant membrane lipids (Somerville and Browse 1991). The chilling resistance of higher plants is closely correlated with the amounts of *cis*-unsaturated fatty acids in phosphatidylglycerol (PG) of chloroplast membranes (Nishida and Murata 1996). The main factor that determines the contents of *cis*-unsaturated fatty acids in PG is the substrate selectivity of glycerol-3-phosphate acyltransferase (GPAT: EC 2.3.1.15) in chloroplasts (Roughan and Slack 1982). In addition, PG molecules are important for both the formation and function of photosynthetic

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***Corresponding author; fax: +86 538 8226399, e-mail: qwmeng@sdaa.edu.cn

Abbreviations: A – antheraxanthin; APX – ascorbate peroxidase (EC 1.11.1.11); AsA – ascorbate acid; F_v/F_m – maximal photochemical efficiency of PS2; F_0 – initial fluorescence; F_v – variable fluorescence; F_m – maximum yield of fluorescence; GPAT – glycerol-3-phosphate acyltransferase; *LeGPAT* – *Lycopersicon esculentum* glycerol-3-phosphate acyltransferase gene; H_2O_2 – hydrogen peroxide; NPQ – non-photochemical quenching; O_2^- – superoxide radical; PBS – phosphate buffer solution; PFD – photon flux density; PG – phosphatidylglycerol; P_N – net photosynthetic rate; PS – photosystem; P700 – PS1 reaction centre; RC – reaction centre; ROS – reactive oxygen species; SOD – superoxide dismutase (EC 1.15.1.1); TG – transgenic; V – violaxanthin; WT – wild type; Z – zeaxanthin.

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apparatus (Hagio *et al.* 2002, Xu *et al.* 2002, Domonkos *et al.* 2004). The majority of PG molecules are localized in the thylakoid membranes that are the site of oxygenic electron transport (Wada and Murata 1998). Thus, the changes of fatty acid species in PG influence the photosynthetic function of photosystem (PS) 1 and PS2 and the activities of chloroplast antioxidant enzymes.

During chilling stress, reactive oxygen species (ROS) are produced, which are associated with the photo-inhibition of PS1 (Sonoike 1996, Sonoike *et al.* 1997, Li *et al.* 2003, 2004b). Using thylakoids or intact chloroplasts of spinach, Jakob and Heber (1996) found that PS1 photoinhibition occurred concomitant with the accumulation of active oxygen species. Superoxide dismutase (SOD) can convert O_2^- to H_2O_2 and O_2 , and plays a major role in the defence against superoxide-derived oxidative stress in plant cells. Other enzymatic and non-enzymatic reactions can contribute to H_2O_2 generation (Pang *et al.* 2005). Ascorbate peroxidase (APX) reduces H_2O_2 to water with ascorbic acid (AsA) as specific electron donor and is thus the most important plant peroxidase in H_2O_2 detoxification in chloroplasts (Foyer and Haliwell 1976, Noctor and Foyer 1998). Since more than fifty percent of APX in the chloroplasts is thylakoid-bound, the transition of membrane lipids from a liquid-crystalline phase to a gel phase can affect the activity of thylakoid-bound APX and consequently affect the scavenging of ROS.

In the long-term evolution of plants, many protective mechanisms were formed to adapt to all kinds of stress. One of the protective mechanisms is xanthophyll cycle, in which violaxanthin (V) transforms to zeaxanthin (Z) *via* antheraxanthin (A) during irradiance stress (and reverse at dark). An involvement of xanthophyll cycle in the process of thermal energy dissipation has been confirmed *e.g.* by the analysis of *Chlamydomonas* and *Arabidopsis*

mutants (Niyogi 1999). The xanthophyll cycle may transform harmlessly the excitation energy into heat and thereby prevent the formation of damaging active oxygen species. In this way it protects the photosynthetic apparatus against photodamage (Demmig-Adams and Adams 1996, Huner *et al.* 1998, Müller *et al.* 2001, Li *et al.* 2003, 2004b). The xanthophyll cycle-dependent NPQ may protect the photosynthetic apparatus from inactivation and damage caused by excess photon energy (Horton *et al.* 1994). The roles of the xanthophyll cycle, the water-water cycle, and their cooperation have been discussed by Li *et al.* (2003, 2005). Lipids have several roles in the hypothetical violaxanthin-cycle signal transduction cascade (Yamamoto 2006). High membrane fluidity and low pigment to lipid ratio are required for optimal incorporation of xanthophylls into model lipid membranes (Yamamoto and Bangham 1978, Hieber *et al.* 2004). The cycle's activity and migration of Z to target pigment-binding proteins take place within the lipid phase of the thylakoid. Moreover, V, the substrate for V de-epoxidase (VDE), must be "activated" with lipids. Yamamoto *et al.* (1975) reported all chloroplast lipids examined supported VDE activity. MGDG was the most effective, followed by digalactosyldiacylglycerol (DGDG), phosphatidylcholine (PC), sulfoquinovosyldiacylglycerol (SQDG), phosphatidylinositol (PI), and phosphatidylglycerol (PG). However, the relationship between unsaturation of fatty acids in PG and xanthophyll cycle remains unclear.

In the present study, we used two transgenic (TG) tomato lines over-expressing *LeGPAT*, which increase unsaturation of fatty acids in PG of thylakoid membrane, together with the wild type (WT) plants, to investigate the role of xanthophyll cycle and chloroplast antioxidant enzymes in protecting PS2 and PS1 under chilling stress.

Materials and methods

Plants and chilling treatments: The full-length *LeGPAT* cDNA was sub-cloned into the expression vector pBI121 downstream of the 35S-CaMV promoter to form sense constructs (pBI-*LeGPAT*). The 35S-CaMV *LeGPAT* constructs were first introduced into *Agrobacterium tumefaciens* LBA4404 by the freezing transformation method and verified by PCR and sequencing. Leaf disk transformation using WT tomato plants was performed as described by Horsch *et al.* (1985). Discs infected with *A. tumefaciens* were incubated on medium for inducing shoots. After a few weeks, the regenerated shoots were transferred to medium for inducing roots. Both media contained cefotaxime sodium (250 g m^{-3}) and kanamycin (50 g m^{-3}). TG plants were screened using kanamycin selection generated by the incubation of transformed tomato leaf disks (Holsters *et al.* 1978). TG plants infected with *A. tumefaciens* carrying *LeGPAT* gene were detected by PCR after the first screening with kanamycin

(50 g m^{-3}) (data not shown). Twenty-five individual kanamycin-resistant lines were obtained from tissue culture. These initial kanamycin-resistant plants were named T₀. Seeds of two lines named T₀-5 and T₀-19 were selected for physiological measurement. There were no obvious morphological differences between the TG and WT plants.

The seeds of T₀-5 and T₀-19 of tomato (*Lycopersicon esculentum* cv. Zhongshu 4) over-expressing *LeGPAT*, as well as the WT plants of the same cv. were used and germinated between moistened filter paper at 25 °C for 3 d. Sprouted burgeons were then planted in 13.5 cm-diameter plastic pots (one plant per pot) filled with sterilized soil and grown at 25–30/15–20 °C (day/night) under a 14 h photoperiod ($300\text{--}400\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$ PFD) in a greenhouse. The progeny obtained from T₀-5 and T₀-19 was named T₁-5 and T₁-19. When tomato plants were 3- to 4-months old, they were treated for 0, 3, 6, 9, 12 h

at 4 °C. The treated leaves were then frozen in liquid nitrogen and stored at -80 °C until further use for the determinations of (A+Z)/(V+A+Z), antioxidant enzyme activities, and ROS content.

Fatty acid composition: Tomato leaf tissue was harvested from 3- to 4-month-old tomato plants and frozen immediately in liquid nitrogen. Lipids were extracted as described by Siegenthaler and Eichenberger (1984) and separated by two-dimensional thin layer chromatography (Xu and Siegenthaler 1997). For quantitative analysis, individual lipids were scraped from the plates and used to prepare fatty acid methyl esters. Fatty acid composition of individual lipids was determined by gas chromatography as described by Chen *et al.* (1994).

Net photosynthetic rate (P_N) and chlorophyll (Chl) a fluorescence: Plants were treated at 4 °C for 0, 1, 3, 6, 9, or 12 h and P_N was measured with a portable photosynthetic system (CIRAS-2, PP Systems, UK) at 25 °C under a concentration of ambient CO₂ (360 $\mu\text{mol mol}^{-1}$) and a PFD of 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Before P_N measurement, tomato plants were induced for about 30 min at 25 °C and a PFD of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ to make the stomata open and then adapted for about 15 min at a PFD of 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Chl fluorescence was measured with a portable fluorometer (FMS2, Hansatech, England) according to the protocol of van Kooten and Snel (1990). The minimal fluorescence (F_0) with all PS2 reaction centres (RCs) open was determined by a modulated radiation which was low enough not to induce any significant variable fluorescence (F_v). The maximal fluorescence (F_m) with all RCs closed was determined by a 0.8 s pulse of saturating radiation of 7 000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ on dark-adapted leaves. The maximal photochemical efficiency (F_v/F_m) of PS2 was expressed as the ratio $F_v/F_m = (F_m - F_0)/F_m$. Non-photochemical quenching (NPQ) was estimated as $\text{NPQ} = F_m/F_m' - 1$, where F_m' is the maximum yield of fluorescence in light-acclimated leaves (Schreiber *et al.* 1994).

After the treatments of plants, F_v/F_m was measured at 4 °C and then recovered at 25 °C. Before chilling stress, plants were adapted in darkness for more than 2 h to measure F_v/F_m . During chilling, plants were adapted in darkness for 15 min before the F_v/F_m measurement. Before this measurement, plants were treated for 0, 1, 3, 6, 9, or 12 h at 4 °C and recovered for 1, 2, 5, 8, 12, or 24 h at 25 °C. Before measurement of NPQ, plants were treated for 0, 1, 3, 6, 9, or 12 h at 4 °C.

Absorbance at 820 nm: Oxidation and reduction of P700

was measured at 820 nm with a PEA (Plant Efficiency Analyzer) senior (Hansatech, UK), as described by Schansker *et al.* (2003). The oxidizable P700 was measured at chilling stress (4 °C) and recovered at 25 °C under low irradiance of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Before measurement of the oxidizable P700, plants were treated for 0, 1, 3, 6, 9, or 12 h at 4 °C and recovered for 1, 2, 5, 8, 12, or 24 h at 25 °C.

Photosynthetic pigments were extracted with 0.5 g of ice-cold acetone and quantified by reversed phase high performance liquid chromatography based on a method described by Li *et al.* (2003).

Activities of antioxidant enzymes in chloroplasts: Chloroplasts were isolated from 50 g of fresh leaves according to Robinson *et al.* (1983). The leaves were homogenized in a blender in 200 cm³ ice-cold medium containing 330 mM sorbitol, 30 mM 2-N-morpholinoethanesulfonic acid (pH 6.5), 2 mM ascorbic acid, and 0.1 % bovine serum albumin. The homogenate was filtered through six layers of cheese cloth and centrifuged at 2 000×*g* for 3 min. The pellet was suspended with 4 cm³ PBS for measurement of chloroplastic APX and SOD activities.

APX activity was determined according to Jimenez *et al.* (1997). SOD assay was performed as described by Giannopolitis and Ries (1977). The soluble protein content was measured following the method of Bradford (1976).

Analysis of O₂⁻ and H₂O₂ contents: The assay for O₂⁻ was performed as described by Wang and Luo (1990). Fresh leaves without midrib were thoroughly ground in an ice bath in a grinding medium containing 0.05 M phosphate buffer (pH 7.8). The homogenate was centrifuged at 5 000×*g* for 10 min at 4 °C. The supernatant with phosphate buffer (pH 7.8) and 10 mM hydroxylammonium chloride was incubated at 25 °C for 20 min, then 17 mM *p*-aminobenzene sulfonic acid and 7 mM α -naphthylamine were added, and the mixture was incubated at 25 °C for 20 min. Finally, ethyl ether was added into the mixture that was centrifuged at 1 500×*g* for 5 min. The water phase was used to determine the absorbance at 530 nm. The O₂⁻ generation was calculated per g fresh mass of leaves.

H₂O₂ content was determined according to the Sairam and Srivastava (2002) method. The concentration of H₂O₂ was estimated by measuring the absorbance of the titanium-hydroperoxide complex and using a standard curve plotted with known concentration of H₂O₂.

Results

Characterization of the tomato cDNA clone: A cDNA designated as *LeGPAT* was isolated from tomato leaves. The full-length sequence of the cDNA consists of 1 770 bp nucleotides and a 1 314 bp open reading frame at position 71–1 384 bp, encoding a 437-residue polypeptide. This cDNA of *LeGPAT* is submitted to the *GenBank* databases under accession number DQ459433 (address is as follows: <http://www.ncbi.nlm.nih.gov>).

Changes of fatty acid composition in WT and TG plants: The fatty acid composition of PG was affected

Table 1. Fatty acid composition of PG in leaves of wild type (WT) and transgenic (T₁-5, T₁-19) tomato plants. – present in trace amount (<0.1 % of total fatty acids). **p*<0.05, significant level (*t*-test). Means±SD (*n* = 3) of three measurements on each of three plants are presented as mole percentage. Standard deviations between triplicates were <3 % of the indicated values.

	Fatty acid [%]					
	16:0	16:1 (3t)	18:0	18:1	18:2	18:3
WT	25.46±1.02	16.26±0.09	7.20±0.25	8.90±0.04	24.69±1.01	17.38±2.23
T ₁ -5	14.81±0.80	18.93±0.12	–	4.93±0.99	35.61±1.65*	25.72±1.09*
T ₁ -19	15.36±1.76	19.69±0.11	1.03±0.23	3.10±0.75	38.18±0.21*	22.64±3.26

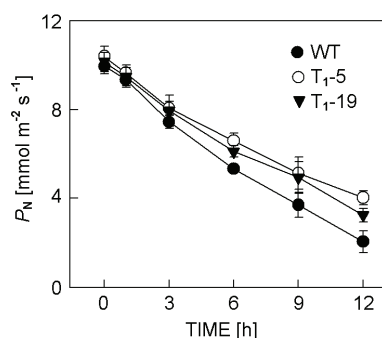


Fig. 1. Changes of net photosynthetic rate (P_N) under chilling stress in wild type (WT) and transgenic (T₁-5, T₁-19) tomato plants. P_N was measured under ambient CO₂ (360 $\mu\text{mol mol}^{-1}$) and chilling temperature of 4 °C. Before measurement of P_N , tomato plants were placed for about 30 min at 25 °C and a PFD of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ to make the stomata open and then adapted for about 15 min at a PFD of 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Means±SD of five measurements on each of five plants.

P_N of WT and TG plants under chilling stress: The increase of *cis*-unsaturated fatty acids in TG tomatoes did not significantly influence P_N relative to the WT under normal condition. P_N of WT and TG plants markedly decreased during chilling stress (4 °C), and the decrease of P_N was more obvious in WT compared to TG plants (Fig. 1).

Photoinhibition of photosystems and xanthophyll cycle under chilling stress: Photoinhibition of PS2 was estimated by measuring the maximal photochemical efficiency of PS2 (F_v/F_m). F_v/F_m obviously decreased in

significantly by over-expression of *LeGPAT* (Table 1). A higher content of 18:2 and 18:3 was detected in TG plants. But the contents of saturated fatty acids of 16:0 and 18:0 decreased in TG plants compared with those in WT plants. The relative contents of total *cis*-unsaturated fatty acids in PG increased from 51.0 % in the WT to 66.3 % in T₁-5 and 63.9 % in T₁-19. This indicated that the over-expression of *LeGPAT* in tomato leaves increased the content of *cis*-unsaturated fatty acids in PG of thylakoid membranes.

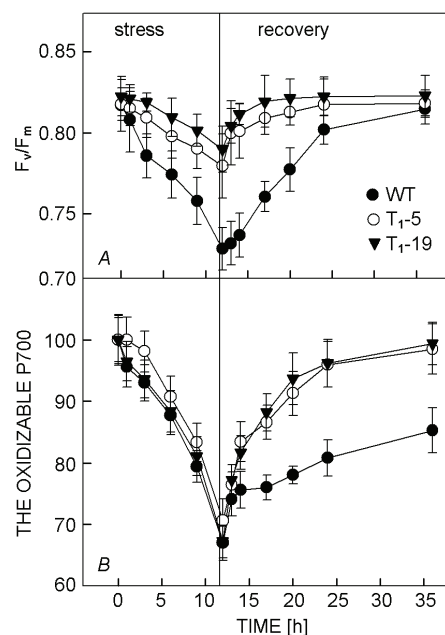


Fig. 2. Effect of chilling stress on (A) F_v/F_m and (B) the oxidizable P700 in wild type (WT) and transgenic (T₁-5, T₁-19) tomato plants and the subsequent recovery. The measurement of F_v/F_m and the oxidizable P700 were made during chilling treatment (4 °C) for 0, 1, 3, 6, 9, or 12 h and subsequent recovery (25 °C) for 1, 2, 5, 8, 12, or 24 h. Means±SD of five measurements on each of five plants.

WT plants during chilling stress (4 °C) relative to the TG plants (Fig. 2A). At the end of 12 h chilling stress at 4 °C, F_v/F_m in WT, T₁-5, and T₁-19 decreased by about 10.8,

4.7, and 4.0 %, respectively. In addition, the recovery of F_v/F_m in TG plants was also quicker than that in WT plants. F_v/F_m of T₁-5 and T₁-19 recovered completely in 8 h, while F_v/F_m of WT only recovered after 24 h in optimum temperature, and reached only about 95.2 % of the original value (Fig. 2A).

The oxidizable P700 markedly decreased both in WT and TG plants during chilling under low irradiance (Fig. 2B). There were no evident differences between WT and TG plants during chilling stress. When tomato plants were transferred to 25 °C and a PFD of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$, the oxidizable P700 of transgenic plants recovered more quickly than that of WT plants. After a 24-h recovery, the

oxidizable P700 recovered to about 98.5, 99.4, and 85.3 % of the original values in T₁-5, T₁-19, and WT plants, respectively.

Both NPQ and the de-epoxidized ratio of the xanthophyll cycle, (A+Z)/(V+A+Z), increased in both WT and TG plants (Fig. 3A,B) at chilling temperature. NPQ and (A+Z)/(V+A+Z) of TG plants markedly increased relative to that of WT during chilling stress. At the end of stress, NPQ increased by about 39.9 and 37.8 % of the original values in T₁-5 and T₁-19, and by 28.6 % in WT, respectively. At the same time, (A+Z)/(V+A+Z) increased by about 36.0 % in WT, 52.9 % in T₁-5, and 47.2 % in T₁-19, respectively.

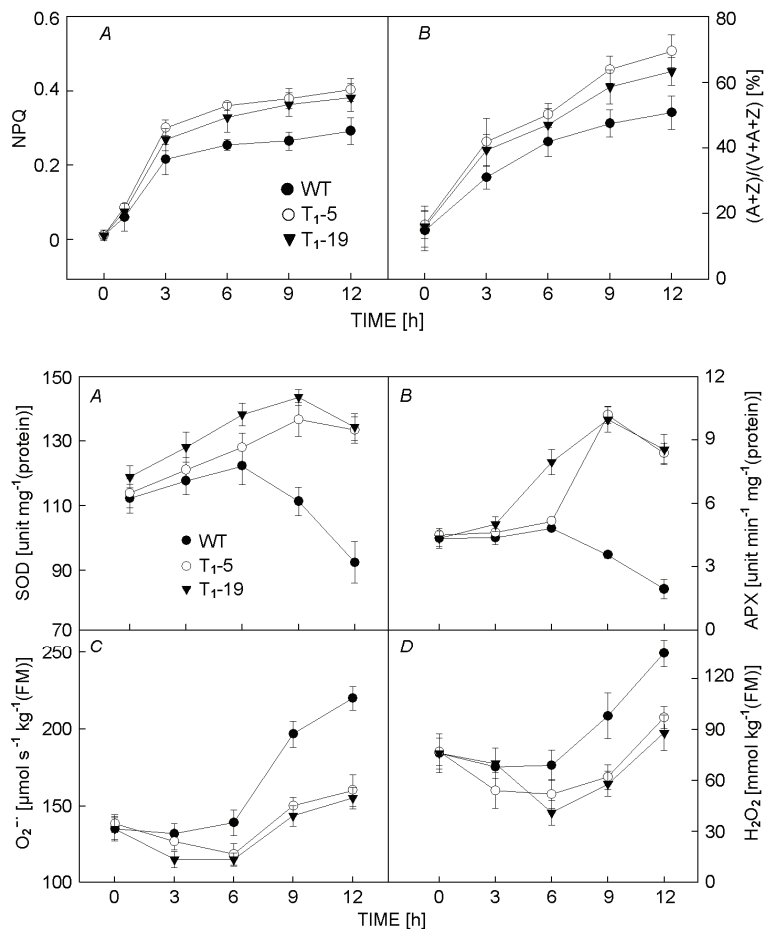


Fig. 3. Responses of (A) non-photochemical quenching (NPQ) and (B) the conversion state of xanthophyll cycle (A+Z)/(V+A+Z) in wild type (WT) and transgenic (T₁-5, T₁-19) tomato plants at chilling temperature (4 °C) under low irradiance of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Means \pm SD of five measurements on each of five plants.

Fig. 4. The activities of (A) superoxide dismutase (SOD) and (B) ascorbate peroxidase (APX), and the contents of (C) $\text{O}_2^{\cdot -}$ and (D) H_2O_2 in leaves of wild type (WT) and transgenic (T₁-5, T₁-19) tomato plants at chilling temperature (4 °C) under low irradiance (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PFD). Means \pm SD of 3 measurements on each of three plants.

Chloroplast SOD and APX activities of WT plants increased during first 6 h of chilling stress and then decreased, whereas the SOD and APX activities of TG plants increased during first 9 h of chilling stress and then slightly decreased (Fig. 4A,B). After 6-h chilling stress, SOD and APX activities of TG plants were higher than those of WT plants. At the end of chilling stress, the SOD and APX activities of WT decreased by about 18.1 % and 55.2 % of the original values, respectively, whereas the activities of both enzymes in leaves of TG plants were higher than at the beginning of chilling treatment.

The contents of $\text{O}_2^{\cdot -}$ and H_2O_2 in WT increased after 6 h chilling stress, while the contents of $\text{O}_2^{\cdot -}$ and H_2O_2 of TG plants increased only after 9 h chilling stress (Fig. 4C,D). Both $\text{O}_2^{\cdot -}$ and H_2O_2 contents increased more markedly in WT plants than in the TG plants. At the end of chilling stress, the $\text{O}_2^{\cdot -}$ content in leaves of WT, T₁-5, and T₁-19 plants increased by about 63.0, 15.7, and 14.8 % of initial values, respectively, and H_2O_2 contents increased by about 77.6, 26.0, and 15.8 % of initial values, respectively. This indicated that the higher SOD and APX activities of T₁-5 and T₁-19 contributed to the decrease of $\text{O}_2^{\cdot -}$ and H_2O_2 contents.

Discussion

Glycerol-3-phosphate acyltransferase (GPAT) catalyzes the initial and committed step of glycerolipid synthesis. *LeGPAT* is a member of *GPAT* expressed in chloroplast, which has been proved by the expression of p35S-*LeGPAT*-GFP conducts in cowpea mesophyll protoplasts observed with confocal microscopy (data not shown). The substrate selectivity of *LeGPAT* is similar to GPAT in chilling-resistant plants, showing a preference for oleic acid (18:1) (data not shown). Over-expression of *LeGPAT* in T₁-5 and T₁-19 resulted in the increase of unsaturated fatty acid content of PG (Table 1). The content of saturated fatty acids of PG in the thylakoid membrane was related to plant sensitivity to chilling stress, which was closely correlated with membrane fluidity. The importance of membrane fluidity in temperature tolerance had been widely discussed in various mutation analyses, TG, and physiological studies (*e.g.* Orvar *et al.* 2000, Sung *et al.* 2003). Increasing *cis*-unsaturated fatty acid contents of PG could increase tolerance of plants to chilling (Nishida and Murata 1996).

The xanthophyll cycle-dependent NPQ is a very important mechanism for the protection of PS2 against excess irradiance under chilling temperature (Demmig-Adams and Adams 1996, Xu *et al.* 1999, Li *et al.* 2003, 2004a). This mechanism alleviates the excitation pressure on PS2 RCs by diverting photon energy into heat (Gray *et al.* 1996). When WT and TG tomatoes were treated with chilling temperature, the formation of Z and A as well as energy dissipation by NPQ increased much more in TG lines than in WT plants (Fig. 3). The photoinhibition of PS2 in WT was more severe than in TG tomatoes (Fig. 2A). Hence the increased xanthophyll cycle-dependent energy dissipation protected PS2 RCs in the TG lines T₁-5 and T₁-19. However, there was no significant difference in the oxidizable P700 content between WT and TG lines during chilling stress (Fig. 2B), which suggests that the increase of xanthophyll cycle-dependent NPQ in T₁-5 and T₁-19 alleviated PS2 photoinhibition, but had lesser effect on PS1. The higher amount of excess energy in WT might thus aggravate photoinhibition of PS2, limiting the electron transport to PS1, and protecting PS1 from further photoinhibition (Tjus *et al.* 1998, Li *et al.* 2003).

Chilling temperature inhibits carbon assimilation (Savitch *et al.* 2000, Allen and Ort 2001, Yu *et al.* 2002), resulting in the scarcity of effective electron acceptors and the accumulation of reducing power on the acceptor side of PS1 (Havaux and Davaud 1994, Terashima *et al.* 1994, Sonoike 1996) and the damage of some PS1 component (Tjus *et al.* 1998). As the CO₂ assimilation was inhibited in our case (Fig. 1), the limited amount of electron acceptors could cause the increase of stromal over-reduction, which would contribute to the accumulation of reactive oxygen species and electron recombination of P700 (Li *et al.* 2003). However, the slower

recovery of the oxidizable P700 in WT could be attributed not only to the limitation of electron acceptors and stromal over-reduction but probably also to the damage of PS1 components (related to the damage of FeS centres) as has been suggested previously (Inoue *et al.* 1989, Sonoike *et al.* 1995, Sonoike 1996, Tjus *et al.* 1998). The increase of *cis*-unsaturated fatty acid contents of PG in TG tomato plants might maintain higher SOD and APX activities and thus reduce the degradation of PS1 components.

ROS such as the superoxide anions (O₂^{•-}), hydrogen peroxide (H₂O₂), and hydroxyl radicals (OH[•]) are formed in all aerobic cells as by-products of normal metabolic processes (Asada 1992), especially under environmental stress, resulting in oxidative damage at the cellular level. Excitation energy that is not used for photochemistry and not dissipated as fluorescence or heat can be transferred to molecular oxygen, creating highly damaging ROS (Foyer *et al.* 1994, Niyogi 1999, Apel and Hirt 2004). On one hand, plants can use O₂ as terminal electron acceptor in both photorespiration and Mehler reaction to protect the chloroplasts from photodamage (Osmond and Grace 1995, Li *et al.* 2004b). On the other hand, the production of ROS might be related to more electrons transported to Mehler reaction caused by the inhibition of the Calvin cycle and photorespiration during chilling stress (Li *et al.* 2003, 2004b). Meanwhile, plants contain a series of non-enzymatic antioxidants, such as ascorbate, glutathione, flavonoids, and carotenoids, and enzymatic antioxidants, such as SOD, APX, and catalase (CAT) (Song *et al.* 2005). These antioxidants function properly to scavenge the toxic ROS and protect the plants from ROS damage (Noctor and Foyer 1998). The efficient removal of ROS from the chloroplast is a key factor for plant stress tolerance. However, CAT activity is not found in the chloroplasts. SOD and APX are the key enzymes to scavenge O₂^{•-} and H₂O₂ in chloroplasts. In our case, the activity of SOD decreased more and the accumulation of O₂^{•-} was higher in WT relative to TG lines of tomato upon their exposure to chilling under low irradiance (Fig. 4A,C). APX, which eliminates peroxides by converting AsA to dehydroascorbate, is one of the most important enzymes playing a crucial role in eliminating toxic H₂O₂ from plant cells (Foyer *et al.* 1994). The activity of APX was higher when the content of unsaturated fatty acids was increased (Fig. 4B). The co-operative up-regulation of APX activity resulted in H₂O₂ reduction in T₁-5 and T₁-19 (Fig. 4D). The reduction of O₂^{•-} and H₂O₂ contents in TG lines then probably decreased the damage of PS1.

In conclusion, over-expression of *LeGPAT* increased the content of *cis*-unsaturated fatty acids in PG of TG tomato plants, which improved the de-epoxidized ratio of xanthophyll cycle and activities of chloroplast SOD and APX, resulting in the reduced contents of O₂^{•-} and H₂O₂.

Powerful removal of O_2^- and H_2O_2 was possibly vital in protecting photosynthetic apparatus against chilling stress under low irradiance by alleviating photoinhibition of PS2 and PS1. To our knowledge, the results presented

here are the first suggestion of a relationship between the *cis*-unsaturated fatty acids in PG and xanthophyll cycle and chloroplast antioxidant enzymes in tomato.

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