The increase in unsaturation of fatty acids of phosphatidylglycerol in thylakoid membrane enhanced salt tolerance in tomato

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Abstract

Overexpression of chloroplastic glycerol-3-phosphate acyltransferase gene (*LeGPAT*) in tomato increased *cis*-unsaturated fatty acid content in phosphatidylglycerol (PG) of thylakoid membrane. By contrast, suppressing the expression of *LeGPAT* decreased the content of *cis*-unsaturated fatty acid in PG. Under salt stress, sense transgenic plants exhibited higher activities of chloroplastic antioxidant enzymes, lower content of reactive oxygen species (ROS) and less ion leakage compared with the wild type (WT) plants. The net photosynthetic rate (P_N) and the maximal photochemical efficiency (F_v/F_m) of photosystem II (PSII) decreased more slightly in sense lines but more markedly in the antisense ones, compared to WT. D1 protein, located in the reactive center of the PSII, is the primary target of photodamage and has the highest turnover rate in the chloroplast. Under salt stress, compared with WT, the content of D1 protein decreased slightly in sense lines and significantly in the antisense ones. In the presence of streptomycin (SM), the net degradation of the damaged D1 protein was faster in sense lines than in other plants. These results suggested that, under salt-stress conditions, increasing *cis*-unsaturated fatty acids in PG by overexpression of *LeGPAT* can alleviate PSII photoinhibition by accelerating the repair of D1 protein and improving the activity of antioxidant enzymes in chloroplasts.

Additional key words: ascorbate peroxidase; D1 protein; glycerol-3-phosphate acyltransferase; phosphatidylglycerol; salt stress.

Introduction

Plants are frequently exposed to various kinds of environmental stresses which can adversely affect both growth and reproductive success in their natural habitats. They have developed mechanisms that allow them to withstand these stresses. One such mechanism involves the regulation of fatty acid unsaturation in membrane lipids (Moon *et al*. 1995). The ratio of saturated to unsaturated fatty acids and the phospholipid composition in thylakoid membranes play a significant role in protecting the photosynthetic apparatus from environmental stresses, especially cold- and salt stress (Nishida and Murata 1996, Sakurai *et al*. 2003, 2007). PG is the only phospholipid in thylakoid membranes, which are the site of oxygenic electron transport in PSII (Wada and Murata 1998). Therefore, the changes of fatty acid species in PG affect the photosynthetic function of PSII and the activities of chloroplastic antioxidant enzymes. The main factor that determines the content of *cis*-unsaturated fatty acids in PG is the substrate selectivity of glycerol-3-phosphate acyltransferase (GPAT) in chloroplasts (Roughan and Slack 1982).

In our previous studies, the level of *LeGPAT* protein

Received 21 September 2009, *accepted* 13 May 2010.
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Abbreviations: APX – ascorbate peroxidase; AsA – ascorbic acid; DGDG – digalactosyldiacylglycerol; F_v/F_m – the maximal photochemical efficiency of PSII; *LeGPAT* – *Lycopersicon esculentum* glycerol-3-phosphate acyltransferase gene; MGDG – monogalactosyldiacylglycerol; P_N – net photosynthetic rate; PG – phosphatidylglycerol; PPFD – photosynthetic photon flux density; PSI(II) – photosystem I (II); PVDF – polyvinylidene fluoride; ROS – reactive oxygen species; SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis; SM – streptomycin; SOD – superoxide dismutase; SQDG – sulfoquinovosyldiacylglycerol; 16:0 – palmitic acid; 16:1(3t) – 3-trans-hexadecenoic acid; 18:0 – stearic acid; 18:1 – oleic acid; 18:2 – linoleic acid; 18:3 – linolenic acids.
Acknowledgments: We thank professor Lixin Zhang, Institute of Botany, Chinese Academy of Sciences, for the D1-specific antibodies. This research was supported by the State Key Basic Research and Development Plan of China (2009CB118500), the Natural Science Foundation of China (30871458), Program for Changjiang Scholars and Innovative Research Team in University (Grant IRT0635) and the Natural Science Foundation of Shandong province (Y2007D50).

and the mRNA were examined. Western hybridization revealed the presence of strong positive protein signals corresponding to *LeGPAT* in sense plants. The content and activity of LeGPAT were higher in sense plants than in WT, and the increase of *cis*-unsaturated fatty acids in PG enhanced the tolerance to low temperature in tomato (Sui *et al.* 2007b). When photosynthetic organisms are exposed to salt stress, fatty acids of membrane lipids are desaturated (Huflejt *et al.* 1990). By comparing *desA*$^-$/*desD*$^-$ mutant cells with WT cells, Allakhverdiev *et al.* (1999) found that the function of unsaturation of fatty acids in membrane lipids enhanced the tolerance of *Synechocystis* cells to salt stress. The loss of the oxygen-evolving activity of PSII was much more rapid in *desA*$^-$/*desD*$^-$ cells than in WT cells under 0.5 M NaCl stress and the recovery of its activity was much faster in WT cells than in *desA*$^-$/*desD*$^-$ cells. Using the method of gene engineering, Allakhverdiev *et al.* (2001) increased the unsaturation of fatty acids in the membrane lipids of *Synechococcus*, and found that the PSII activity of isolated thylakoid membranes from *desA*$^+$ cells was more resistant to salt stress than that of isolated membranes from WT cells. Therefore, these findings indicated that unsaturation of fatty acids in thylakoid membranes enhanced the tolerance of the photosynthetic apparatus to salt stress.

Superoxide dismutase (SOD) can convert $O_2^{\bullet -}$ to H_2O_2 and O_2, and plays a significant role in defending plant cells against superoxide-derived oxidative stress (Sui *et al.* 2007a). Ascorbate peroxidase (APX) reduces H_2O_2 to water using ascorbic acid (AsA) as the specific electron donor and is thus the most important peroxidase in H_2O_2 detoxification in plant chloroplasts (Foyer and Halliwell 1976, Noctor and Foyer 1998). Chloroplastic SOD and APX played an important part in improving salt tolerance in transgenic rice plants (Tanaka *et al*. 1999).

D1 protein is the primary site of photodamage and has the highest turnover rate in the chloroplast (Mattoo *et al.* 1999). It is considered that the extent of unsaturation of fatty acids in PG affects the turnover of D1 protein by modifying the molecular environments of PSII reaction center (Moon *et al*. 1995). Salt stress slightly inhibited the transcription and translation of the *psbA* genes encoding the D1 protein (Allakhverdiev *et al*. 2002), whereas pulse-chase experiments revealed that salt stress inhibited the synthesis of D1 de novo in *Synechococcus sp. PCC*7942 (Ohnishi and Murata 2006). In addition, salt stress represses the repair of photodamaged PSII by inhibiting the degradation and synthesis of D1 protein. The repair of PSII includes the step of insertion of the precursor to the D1 protein into the membrane (Allakhverdiev and Murata 2008). The unsaturation of fatty acids in PG might accelerate the insertion, resulting in the enhanced rate of repair. Therefore, the specific step accelerated by the enhanced unsaturation of fatty acids in PG needs to be clarified.

In the present study, we selected *LeGPAT* transgenic plants and WT tomato to investigate the relationship between unsaturated fatty acids in PG and salt tolerance. The results showed that the unsaturated fatty acids in PG played an important role in accelerating the degradation of the damaged D1 protein from the reaction center of PSII during salt stress.

Materials and methods

Plant material and treatments: The WT tomato (*Lycopersicon esculentum* cv. Zhonshu 4), T_2 sense lines [T_2-19(+), T_2-5(+)] and T_2 antisense lines [T_2-16(-), T_2-2(-)] were planted in plastic pots (15 cm in diameter, 10 cm high, one plant per pot) filled with vermiculite. The plants were cultivated with Hoagland nutrient solution (the amount of irrigation was enough to avoid water stress and maintain the natural growth of plants) and grown at 25/20°C (day/night) with a 14-h photoperiod (300–400 μmol m^{-2} s^{-1} PPFD) in a greenhouse. When the sixth leaf was fully expanded, each plant was watered with 250 ml of 200 mM NaCl solution once a day.

Fatty acid composition analysis: Tomato leaf tissue was harvested from 6- to 7-week-old 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 (TLC) on silica gel plates (Xu and Siegenthaler 1997). Lipid spots were detected by brief exposure to I_2 vapour. For quantitative analysis, individual lipids were separated by TLC, scraped from the plates, and used to prepare fatty acid methyl esters. The fatty acid composition of individual lipids was determined by gas chromatography as previously described (Chen *et al.* 1994). Fatty acid methyl esters were analysed by FID-GC on a capillary column (length: 30 m; thickness: 0.33 μm; internal diameter: 0.32 mm) with N_2 as the carrier gas at a flow rate of 36.6 ml min^{-1}. The column was maintained isothermally at 150°C.

Relative electric conductivity: Six leaf discs (0.8 cm in diameter) were put into 10 ml of distilled water and vacuumized for 30 min, and then surged for 3 h to measure the initial electric conductance (S1) (25°C). A cuvette was filled with leaf discs and distilled water, the mixture was cooked (100°C) for 30 min and then reduced to room temperature (25°C) to determine the final electric conductance (S2). The relative electric conductivity (REC) was evaluated as: REC = S1×100/S2.

Net photosynthetic rate (P_N) was measured with a portable photosynthetic system (*CIRAS-2, PP Systems*,

Hitchin, Hertfordshire, UK) under the condition of a concentration of ambient CO_2 (360 μmol mol^{-1}), a PPFD of 800 μmol m^{-2} s^{-1}, leaf temperature 25 ± 1°C and relative air humidity 85–90%.

Chlorophyll (Chl) fluorescence was measured with a portable fluorometer (*FMS2, Hansatech*, King's Lynn, UK) according to the protocol described by Van Kooten and Snel (1990). The minimal fluorescence (F_0) with all PSII reaction centers open was determined by modulated light (about 10 μmol m^{-2} s^{-1}) which was low enough not to induce any significant variable fluorescence (F_v). The maximal fluorescence (F_m) with all reaction centers closed was determined by 0.8-s saturating light of 8,000 μmol m^{-2} s^{-1} on a dark-adapted (15 min) leaf. The maximal photochemical efficiency (F_v/F_m) of PSII was expressed as: $F_v/F_m = (F_m - F_0)/F_m$.

Growth performance of WT and transgenic plants under salt stress: Surface-sterilized seeds were germinated on 0.72% (w/v) agar-solidified MS medium (Murashige and Skoog 1962) supplemented with 3% (w/v) sucrose. 5-day-old seedlings were transferred to 0.72% (w/v) agar-solidified MS medium and MS medium supplemented with 150 mM NaCl, respectively. After one month, the plants were photographed, and the fresh mass, root length, and height of plants were determined.

Activities of antioxidant enzymes and the content of ROS 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 ml 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. Half of the pellets were suspended with 4 ml PBS for measurement of chloroplastic APX, SOD activities, and $O_2^{\bullet -}$ content. APX activity was determined according to Jimenez *et al.* (1997). SOD assay was performed as described by Giannopolitis and Ries (1977). The other half were suspended with acetone for measurement of Chl content and H_2O_2. The Chl content was measured following the method of Arnon (1949). The H_2O_2 content was determined according to Sairam and Srivastava's method (2002). The assay for $O_2^{\bullet -}$ content was performed as described by Wang and Luo (1990).

Thylakoid membrane preparation: Thylakoid membranes were prepared according to the method of Zhang *et al.* 1999. The tomato leaves were homogenized in an ice-cold isolation buffer containing 400 mM sucrose, 50 mM HEPES, pH 7.8, 10 mM NaCl, 2 mM EDTA and 2 mM $MgCl_2$ and filtered through three layers of pledget. The filtrate was centrifuged at 5,000 × *g* for 10 min. The thylakoid pellets were washed with isolation buffer, recentrifuged, and finally suspended in the isolation buffer. The resulting thylakoid membranes were frozen in liquid N_2 and stored at –70°C before use.

SDS-PAGE and western blot analysis: Thylakoid membrane proteins were denatured and separated using 15% polyacrylamide gradient gel that contained 6 M urea. Separated proteins were electroblotted to PVDF membrane and then probed with polyclonal antibodies raised in rabbits against the full-length D1 protein. The secondary antibody was peroxidase-conjugated goat anti-rabbit IgG. The D1 protein antibody was used at a dilution of 1:500 and the secondary antibody was used at 1:5,000 dilution.

Incubation of leaves with streptomycin: In order to block the synthesis of D1, leaves were incubated with streptomycin (SM) after plants were watered with salt solution for 3 days. Detached leaves were placed with their petiole in a 3 mM aqueous solution of SM and illuminated for 2 h in ventilated air at room temperature. Thylakoid membranes of these leaves were prepared.

Results

Changes of fatty acid composition: Compared with WT plants, there was no significant change in monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) or sulfoquinovosyldiacylglycerol (SQDG) in transgenic plants (Table 1). However, the fatty acid composition of PG was markedly different between WT and transgenic plants. The content of *cis*-unsaturated fatty acids and 16:01(3t) in sense plants was higher than in WT, with a marked increase in the content of *cis*-unsaturated fatty acids. By contrast, the content of *cis*-unsaturated fatty acids and 16:01(3t) in antisense plants was lower than in WT. The levels of *cis*-unsaturated fatty acids in T_2-19(+), T_2-5(+), WT, T_2-16(-), and T_2-2(-) were 57.8, 55.5, 53.6, 51.7, and 49.3%, respectively. These results indicated that overexpression of *LeGPAT* in tomato plants increased the content of *cis*-unsaturated fatty acids in PG. After salt stress for 6 days, the level of *cis*-unsaturated fatty acids of PG in T_2-19(+), T_2-5(+), WT, T_2-16(-), and T_2-2(-) were 63.8, 61.4, 52.3, 48.3, and 47.8%, respectively (Table 2). Compared with WT, the content of *cis*-unsaturated fatty acids of PG increased in the sense lines, but their content decreased in the antisense ones.

Influence of salt stress on P_N: In normal growth conditions, there was little difference in P_N between WT and transgenic lines. During salt stress, the P_N of WT and transgenic plants decreased markedly (Fig. 1*A*).

Table 1. Fatty acid composition of thylakoid membrane lipids in WT and transgenic tomato leaves. Present at trace levels (<0.1% of total fatty acid). Means ± SD (n = 3) of three measurements on each of three plants are expressed as mole percentage. Standard deviations between triplicates was <3% of the indicated values.

Lipid	Strain	Fatty acid composition [mol%] 16:00	16:01(3t)	18:00	18:01	18:02	18:03
MGDG	T_2-19(+)	4.11 ± 0.8	0.56 ± 0.5	16.47 ± 0.4	0.71 ± 0.4	4.54 ± 0.6	73.6 ± 0.9
	T_2-5(+)	3.86 ± 0.7	0.41 ± 0.9	16.02 ± 0.5	1.7 ± 0.6	5.3 ± 0.8	72.71 ± 0.4
	WT	3.12 ± 0.6	0.82 ± 0.8	17.1 ± 0.6	1.24 ± 0.8	4.38 ± 0.9	73.32 ± 0.3
	T_2-16(-)	4.11 ± 0.8	0.95 ± 0.4	15.15 ± 0.4	1.72 ± 0.9	4.3 ± 0.7	73.77 ± 0.6
	T_2-2(-)	3.55 ± 0.3	0.84 ± 0.3	17.04 ± 0.2	1.38 ± 0.2	4.56 ± 0.3	72.56 ± 0.6
DGDG	T_2-19(+)	20.98 ± 0.5	-----	0.98 ± 0.8	7.57 ± 0.5	5.91 ± 0.5	64.55 ± 0.5
	T_2-5(+)	21.79 ± 0.4	-----	1.27 ± 0.6	6.69 ± 0.6	5.98 ± 0.8	64.27 ± 0.8
	WT	22.57 ± 0.6	0.17 ± 0.6	1.18 ± 0.3	6.41 ± 0.4	6.91 ± 0.6	64.65 ± 0.8
	T_2-16(-)	23.87 ± 0.9	-----	1.39 ± 0.5	5.56 ± 0.7	5.24 ± 0.3	63.92 ± 0.5
	T_2-2(-)	22.15 ± 0.2	-----	1.33 ± 0.9	6.05 ± 0.5	6.17 ± 0.6	64.3 ± 0.7
SQDG	T_2-19(+)	60.09 ± 0.5	-----	0.16 ± 0.8	11.68 ± 0.4	8.36 ± 0.3	19.72 ± 0.5
	T_2-5(+)	59.24 ± 0.7	-----	-----	10.04 ± 0.7	10.52 ± 0.4	20.22 ± 0.7
	WT	59.7 ± 0.5	-----	0.19 ± 0.6	10.35 ± 0.8	9.32 ± 0.5	20.45 ± 0.2
	T_2-16(-)	59.96 ± 0.8	-----	-----	10.03 ± 0.9	10.69 ± 0.6	19.32 ± 0.9
	T_2-2(-)	60.3 ± 0.9	-----	0.18 ± 0.5	10.13 ± 0.6	10.12 ± 0.6	19.27 ± 0.8
PG	T_2-19(+)	1.23 ± 0.9	40.96 ± 0.6	-----	10.31 ± 0.2	32.08 ± 0.6	15.42 ± 0.6
	T_2-5(+)	1.89 ± 0.8	42.63 ± 0.5	-----	12.05 ± 0.4	29.38 ± 0.7	14.05 ± 0.8
	WT	17.86 ± 0.7	34.81 ± 0.9	0.07 ± 0.5	11.47 ± 0.3	28.85 ± 0.9	13.35 ± 0.5
	T_2-16(-)	27.12 ± 0.6	25.43 ± 0.8	0.15 ± 0.3	9.23 ± 0.4	26.89 ± 0.7	11.18 ± 0.8
	T_2-2(-)	26.02 ± 0.2	24.14 ± 1.0	0.11 ± 0.6	10.18 ± 0.2	28.79 ± 0.8	10.75 ± 0.6

Table 2. Fatty acid composition of PG in WT and transgenic tomato leaves after salt stress for 6 days. Present at trace levels (<0.1% of total fatty acid). Means ± SD (n = 3) of three measurements on each of three plants are expressed as mole percentage. Standard deviations between triplicates was <3% of the indicated values.

Lipid	Strain	Fatty acid composition [mol%] 16:00	16:01(3t)	18:00	18:01	18:02	18:03
PG	T_2-19(+)	0.52 ± 0.2	35.47 ± 0.3	0.11 ± 0.4	7.18 ± 0.2	32.62 ± 0.5	24.08 ± 0.7
	T_2-5(+)	0.88 ± 0.5	38.04 ± 0.5	-----	7.43 ± 0.6	33.99 ± 0.3	20.02 ± 0.9
	WT	12.54 ± 0.4	34.69 ± 0.7	0.29 ± 0.6	6.9 ± 0.1	26.02 ± 0.2	19.42 ± 0.5
	T_2-16(-)	28.14 ± 0.2	22.63 ± 0.7	0.88 ± 0.3	5.62 ± 0.8	24.29 ± 0.1	18.42 ± 0.4
	T_2-2(-)	20.15 ± 0.7	28.86 ± 0.1	0.4 ± 0.8	3.61 ± 0.5	25.24 ± 0.4	19.01 ± 0.7

Compared with WT, the decrease of P_N in antisense lines was more obvious than that in sense plants. After salt stress for 7 days, the P_N of T_2-19(+), T_2-5(+), WT, T_2-16(-), and T_2-2(-) decreased by 48.2, 52.4, 62.3, 72.3, and 72.5%, respectively. These results demonstrated that the increased content of *cis*-unsaturated fatty acids of PG in transgenic tomato plants played an important role in protecting the photosynthetic apparatus from salt stress.

The increase of *cis*-unsaturated fatty acids of PG alleviates photoinhibition of PSII: In order to investigate the function of *cis*-unsaturated fatty acids of PG in alleviating photoinhibition of PSII, F_v/F_m was determined. Under salt stress, the F_v/F_m in all plants decreased. F_v/F_m in the antisense plants decreased more obviously relative to WT, whereas F_v/F_m of the sense lines was maintained at a higher level (Fig. 1*B*). At the end of the salt stress period, the F_v/F_m of T_2-19(+), T_2-5(+), WT , T_2-16(-), and T_2-2(-) decreased by 5.9, 6.2, 10.4, 14.3, and 16.4% compared with initial value, respectively. These results indicated that the PSII reaction center was damaged more seriously in WT than that in the sense plants. The antisense lines suffered the most severe damage.

Effect of salt stress on seedling growth: To observe the growth performance of the WT and transgenic plants under salt stress for a long time, 5-day-old WT and transgenic seedlings were transferred to glass bottles containing MS agar media and MS agar media supplemented with 150 mM NaCl, respectively. The growth pattern was observed after 30 days (Fig. 2, Table 2). Under normal growth conditions, all plants grew well but plant growth was inhibited under salt stress. Compared with WT,

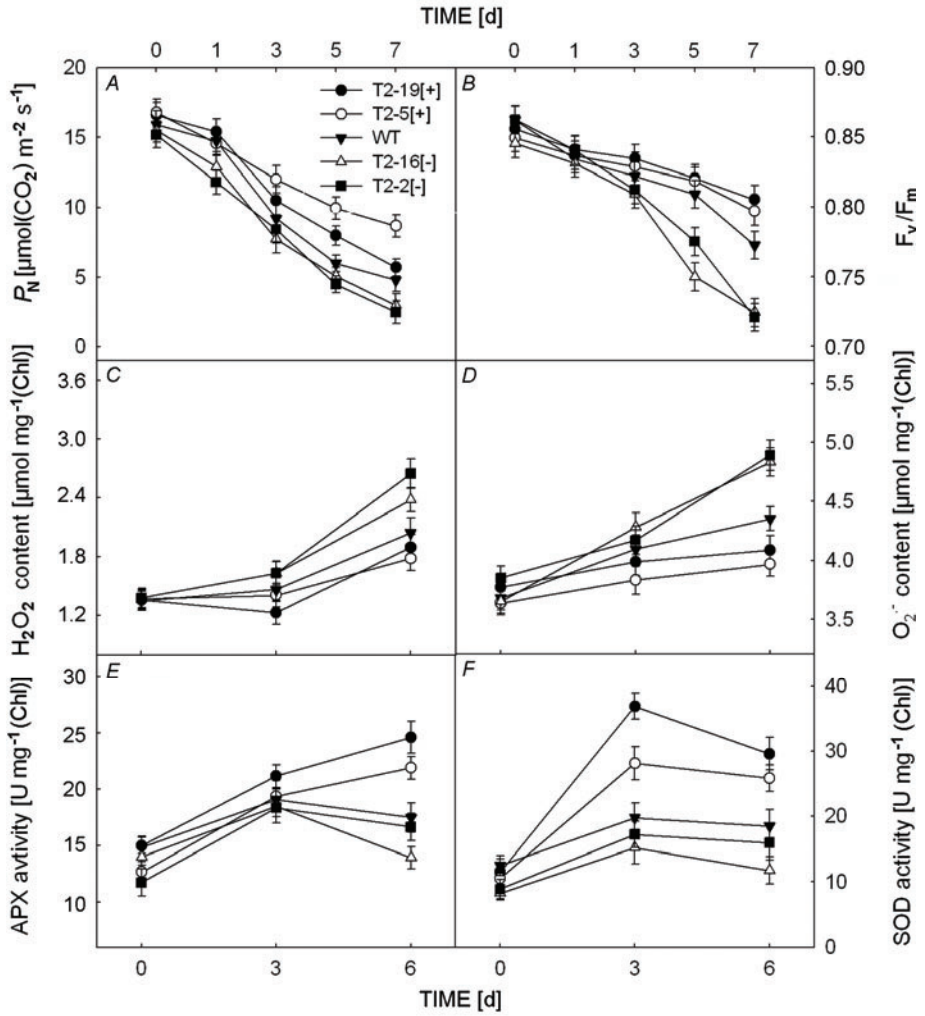

Fig. 1. Changes in P_N and F_v/F_m in WT and transgenic plants under salt stress. Plants were irrigated with 200 mM NaCl once a day. P_N (*A*) and F_v/F_m (*B*) were measured at 0, 1, 3, 5, and 7 days at the PPFD of 800 μmol m^{-2} s^{-1}. Means ± SD (n = 5) of five measurements on each five plants. The content of H_2O_2 (*C*) and $O_2^{\bullet -}$ (*D*), the activities of ascorbate peroxidase (APX) (*E*) and superoxide dismutase (SOD) (*F*) in chloroplasts of WT and transgenic plants under salt stress. Means ± SD, (n = 3) of three measurements on each of three plants.

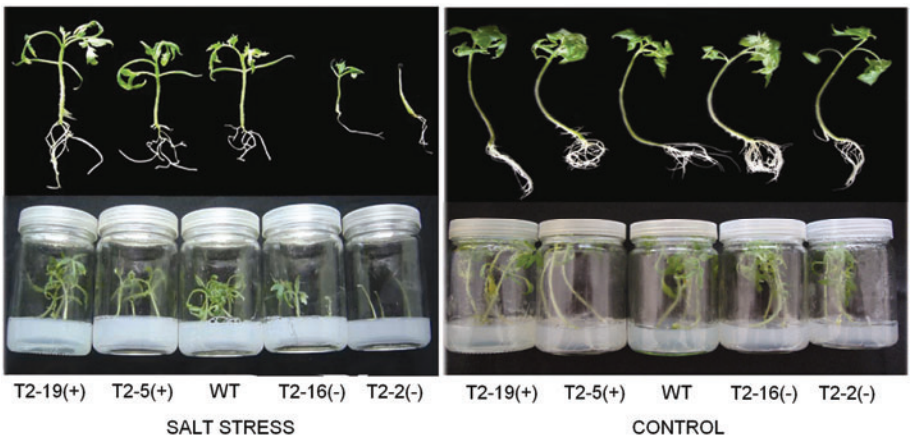

Fig. 2. Effect of salt stress on seedling growth of WT and transgenic plants. 5-day-old WT and transgenic seedlings were transferred to glass bottles containing MS agar media (B) and MS agar media supplemented with 150 mM NaCl (A), respectively. Fresh mass, root length and plant height were measured after 30 days. Means ± SD (n = 15) of fifteen measurements on each of fifteen plants.

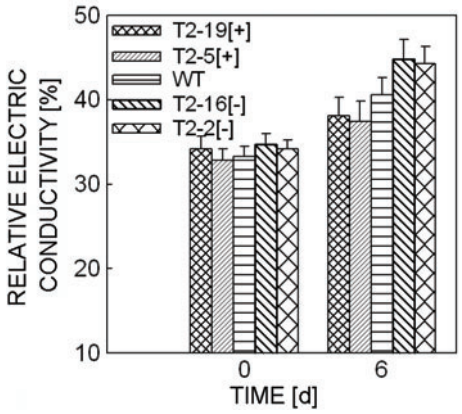

Fig. 3. Relative electric conductivity in WT and transgenic plants were measured under 200 mM NaCl for 6 days. Means ± SD (n = 5) of five measurements on each of five plants.

sense plants grew better under salt stress. They accumulated significantly more organic substance and root development was observed to be better. Under salt stress, the growth of the antisense lines was more severely inhibited.

Activities of antioxidant enzymes and the content of ROS in chloroplasts: APX and SOD activities of chloroplasts in WT increased during the first 3 days of salt stress and then decreased, but the activity of APX in sense lines continued to increase during salt stress (Fig. 1*E,F*). SOD activity in sense lines was maintained at a higher level than in WT under salt stress. The changes of APX and SOD activities in the sense lines were similar to those in WT, whereas the acitivities of APX and SOD in antisense plants were always lower than those in WT. The content of $O_2^{\bullet -}$ and H_2O_2 in the chloroplasts of all plants tested increased during salt stress. Relative to WT, the content of $O_2^{\bullet -}$ and H_2O_2 in sense plants increased more slightly, whereas both $O_2^{\bullet -}$ and H_2O_2 increased more markedly in antisense plants (Fig. 1*C,D*). At the end of the 6-day salt-stress period, the content of $O_2^{\bullet -}$ in the chloroplasts of T_2-19(+), T_2-5(+), WT , T_2-16(-), and T_2-2(-) increased by 9.9, 9.1, 12.9, 32.3, and 27.1% of initial values, respectively, and H_2O_2 content increased by about 39.4, 30.1, 51.4, 72.2, and 91.4% compared with the initial value, respectively. These results demonstrated that the higher activities of SOD and APX in sense plants markedly inhibited the increase of of $O_2^{\bullet -}$ and H_2O_2 content compared with WT, whereas the results observed in antisense lines were contrary to those in sense plants.

Effect of salt stress on relative electric conductivity: After salt stress for 6 days, the relative electric conductivity increased in both WT and transgenic plants. Compared with WT, there was less ion leakage from sense lines. Contrary to sense plants, there was more ion leakage from antisense lines than from WT (Fig. 3). At the end of the 6-day salt-stress period, the relative electric

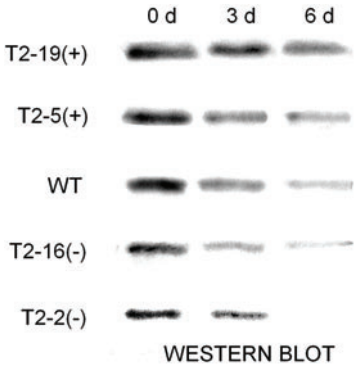

Fig. 4. Western blot analysis for WT and transgenic plants. Thylakoid membranes were isolated from WT and transgenic plants as in indicated times. Proteins were separated by SDS-PAGE and then probed with D1 antibody. Means ± SD (n = 3) of three measurements on each of three plants.

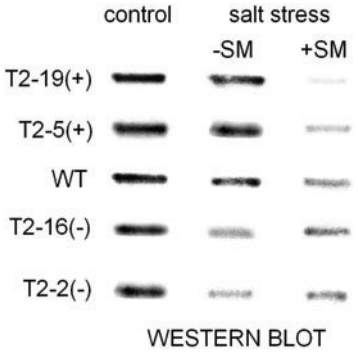

Fig. 5. The unsaturation fatty acids of PG accelerated degradation of D1 protein under salt stress. The leaves were incubated with SM for 3 h after salt stress for 3d and then the synthesis of D1 was inhibited. Thylakoid membranes were isolated from WT and transgenic plants as in indicated times. Proteins were separated by SDS-PAGE and then probed with D1 antibody. Means ± SD (n = 3) of three measurements on each of three plants.

conductivity of T_2-19(+), T_2-5(+), WT, T_2-16(-), and T_2-2(-) increased by 11.4, 14.0, 21.9, 29.1, and 29.5% of the initial value, respectively. These results indicated that compared with WT, membrane damage was less in the sense plants, and more serious in the antisense lines under salt stress.

Effects of salt stress on the content of D1 protein during photoinhibition of PSII: Western blot analysis was performed to investigate the effect of salt stress on the levels of D1 protein. The WT and 4 lines were exposed to 200 mM NaCl for 0, 3, and 6 d and the changes in D1 protein content were investigated. Western blot analysis indicated that the content of D1 protein in all lines tested decreased during salt stress, with a prominent decrease observed in the antisense lines compared with WT (Fig. 4). In the presence of SM, the net degradation of D1 was faster in the sense lines than in WT, in contrast to the results in the antisense lines (Fig. 5).

Discussion

The PSII complex contains a large number of lipid molecules, which was found by X-ray crystallography (Jordan *et al.* 2001). Biochemical analysis of the lipid content of PSII indicates a number of integral lipids, their composition being similar to the average lipid composition of the thylakoid membrane (Loll *et al*. 2007). The abundant lipid molecules might facilitate the turnover of D1 protein under environmental stresses and PG molecules might play a role in maintaining the functional structure of PSII (Wada and Murata 2007).

The relationship between PG and the chilling tolerance of plants has been intensively investigated (Murata *et al*. 1992, Sui *et al*. 2007b). However, the function of PG in higher plants in response to salt stress is still unclear. In our previous studies, *LeGPAT* preferred oleic acid (18:1) to palmitic acid (16:0) as the substrate in tomato (Sui *et al*. 2007b). There was no evident difference in the substrate specificity between the sense trangenic plants and WT, but the transgenic plants with overexpression of *LeGPAT* increased the total activity of GPAT. The content of unsaturated fatty acids of PG in T_2-19(+) and T_2-5(+) increased relative to WT, but suppressed-expression of *LeGPAT* contributed to the decreased content of the unsaturated fatty acids in T_2-16(-) and T_2-2(-) (Table 1). Under salt stress, compared with normal growth conditions, the unsaturated fatty acids content of PG in the sense lines increased, but the content in the antisense ones decreased (Table 2). It is possible that PG can enhance the tolerance of plants to salt stress by the increase of *cis*-unsaturated fatty acids.

Salt stress can reduce the growth and development of plants, and inhibit cell division and expansion (Mahajan and Tuteja 2005). Hagio *et al.* (2002) have indicated that PG contributed to the development of chloroplasts. Chloroplasts are the main location for plant photosynthesis which provides energy and substance for growth and development. After salt stress, compared with normal growth conditions, the fresh mass, length of root, the process of lateral root formation, and plant height of the sense lines were slightly inhibited. By contrast, the growth of antisense plants was markedly inhibited (Table 3, Fig. 2). Relative to the antisense ones, the sense lines showed more tolerance to salt stress than WT. Therefore, it is deduced that the increase in unsaturated fatty acids of PG molecules might play a significant role in maintaining the functional structure of PSII. The higher activity of PSII in the sense lines can improve the rate of photosynthesis, thus providing more substance and energy for plant growth.

High Na^+ levels can lead to reduction in photosynthesis and production of ROS (Yeo 1998). During salt stress, ROS are mainly formed in chloroplasts, resulting in oxidative damage at the cellular level. ROS can be scavenged by enzymatic antioxidants in chloroplasts, such as SOD and APX. Because there is no catalase (CAT) activity found in chloroplasts, SOD and APX are the key enzymes for scavenging ROS in chloroplasts and thus protect plants from stresses. In contrast to T_2-16(-) and T_2-2(-), P_N and F_v/F_m of the T_2-19(+) and T_2-5(+) decreased less than in WT (Fig. 1*A,B*). This indicated that photoinhibition of PSII in WT was more severe than in the sense plants and less severe than in the antisense lines.

The increase in fatty acids unsaturation of PG serves to maintain the structural integrity of PSII and PSI, support the development of chloroplasts and enhance the binding of protein subunits to the PSII and PSI complex. It seems likely that unsaturation of fatty acids of PG provides some structural flexibility within the reaction center. Under salt stress, the flexible surroundings in chloroplasts are favourable to thylakoid membrane-binding antioxidant enzymes which scavenge ROS. In our experiment, we discovered that T_2-19(+) and T_2-5(+) could maintain higher APX and SOD activities and lower H_2O_2 and $O_2^{\bullet -}$ levels than WT (Fig. 1*C,D*). The reduction of $O_2^{\bullet -}$ and H_2O_2 levels in sense tomato plants

Table 3. Effect of salt stress on seedling growth of WT and transgenic plants. WT and transgenic seedlings were transferred to glass bottles containing MS agar media (control) and MS agar media supplemented with 150 mM NaCl for 30 days. Fresh mass, root length and plant height were measured after 30 days. Means ± SD (n = 15) of fifteen measurements on each of fifteen plants.

	Strains	Plant height [cm]	Root length [cm]	Fresh mass [g]
Control	T_2-19(+)	8.14 ± 0.07	5.81 ± 0.08	0.396 ± 0.07
	T_2-5(+)	7.98 ± 0.05	5.56 ± 0.05	0.389 ± 0.06
	WT	7.58 ± 0.06	5.34 ± 0.05	0.385 ± 0.06
	T_2-16(-)	7.52 ± 0.04	5.33 ± 0.07	0.382 ± 0.03
	T_2-2(-)	7.49 ± 0.06	5.30 ± 0.1	0.381 ± 0.09
Salt stress	T_2-19(+)	4.35 ± 0.05	5.08 ± 0.06	0.240 ± 0.04
	T_2-5(+)	4.02 ± 0.06	4.82 ± 0.03	0.232 ± 0.03
	WT	3.59 ± 0.02	4.37 ± 0.06	0.172 ± 0.05
	T_2-16(-)	3.37 ± 0.08	3.95 ± 0.04	0.100 ± 0.06
	T_2-2(-)	2.68 ± 0.05	4.11 ± 0.12	0.090 ± 0.07

could alleviate the damage to PSII. Under salt stress, lots of ROS were produced in chloroplasts. When ROS could not be cleared by antioxidant enzymes in time, the superfluous ROS could transfer from chloroplasts to the cytoplasm and attack the plasma membrane. ROS could weaken the fluidity of membrane lipids and damage normal membrane function, resulting in lipid peroxidation. Consequently, membrane leakage occurred and the ultrastructure of cells changed. The increase in unsaturation of fatty acids of PG in the sense lines allowed the activity of antioxidant enzymes to be maintained, relative to WT. Therefore, there were fewer ROS transfering to the cytoplasm, and damage to the plasma membrane was less (Fig. 3).

D1 protein was the primary target for photodamage in the PSII complex when plants suffered photoinhibition (Aro *et al.* 1993, Andersson and Aro 2001). The degradation of damaged D1 from the PSII was the first step in the repair process of D1. The unsaturation of fatty acids in membrane lipids is a key factor in enhancing the tolerance of PSII to salt stress by accelerating the repair of D1 (Allakhverdiev and Murata 2008). The reaction center subunits D1 and D2 are enclosed by a belt of 11 lipids providing a flexible environment for the exchange of D1 (Loll *et al*. 2007). However, the repair of PSII involves many steps, and the specific steps regulated by the unsaturation of fatty acids in PG remained to be clarified. In the sense lines, D1 protein could be maintained at a higher level compared with other plants during salt stress (Fig. 4). Under salt stress, in the presence of SM, the net degradation of D1 in the sense lines was more rapid than that in the antisense ones (Fig. 5). Therefore, the increase in unsaturation of fatty acids of PG can improve the rate of degradation of D1 protein. ROS could attack the sensitive site of PSII, and inhibit the *de novo* synthesis of proteins in PSII by inhibiting the elongation of peptides and, in particular, the D1 protein (Takahashi and Murata 2006). In antisense plants, there were a large number of ROS (Fig. 1*C*) because of the decreasing activities of antioxidant enzymes (Fig. 1*E*). The total content of D1 protein was less than that in WT and sense lines during salt stress (Fig. 4) and the net degradation of D1 protein was more rapid than in other lines (Fig. 5). Therefore, we considered that, on the one hand, the increased *cis*-unsaturation of PG can be beneficial to the degradation of damaged D1 from the reaction center of PSII, leaving more sites for the insertion of newly synthesized D1. On the other hand, the increased *cis*-unsaturation of PG can enhance the activity of antioxidant enzymes in scavenging the excess ROS and improve the process of peptide elongation, accelerating *de novo* synthesis of D1 protein. As far as we know, the results shown here are the first evidence of a relationship among the *cis*-unsaturated fatty acids in PG and D1 protein and chloroplastic antioxidants in tomato under salt stress.

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