DOI: 10.1007/s11099-013-0014-5 PHOTOSYNTHETICA 51 (2): 238-244, 2013

Enhancement of low-temperature tolerance in transgenic tomato plants overexpressing *Lefad7* through regulation of trienoic fatty acids

X.Y. LIU[*], Y.B. TENG[*], B. LI[**], and Q.W. MENG[**,+]

College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou, 310036, P. R. China[*]
College of Life Science, State Key Laboratory of Crop Biology, Shandong Agricultural University, Tai'an, 271018, P. R. China[**]

Abstract

We studied how tomato (*Lycopersicon esculentum* Mill.) chloroplast omega-3 fatty acid desaturase gene (*Lefad7*) overexpression enhanced low-temperature (LT) tolerance in transgenic tomato plants. In these plants, the content of linolenic acid (18:3) markedly increased and, correspondingly, the content of linoleic acid (18:2) decreased. Similar changes were found after 6 h under LT (4°C) treatment. Under LT stress, wild type (WT) tomato plants showed a much greater increase in relative electrolyte leakage and malondialdehyde (MDA) contents compared with transgenic plants. Transgenic plants exhibited higher activities of antioxidative enzymes and a lower content of reactive oxygen species (ROS). Transgenic plants maintained a relatively higher level of the net photosynthetic rate (P_N) and chlorophyll (Chl) content than WT plants under LT stress. Taken together, we suggested that overexpression of *Lefad7* enhanced LT tolerance by changing the composition of membrane lipids in tomato plants, with the increased content of trienoic fatty acids and reduced content of dienoic fatty acids that led to series of physiological alterations.

Additional key words: chloroplast omega-3 fatty acid desaturase gene; *Lycopersicon esculentum*.

Introduction

Low temperature is an important environmental factor that inhibits plant growth and development in many tropical and subtropical crops (Kargiotidou *et al.* 2008). Plants can be severely impacted by frequent LT stress, associated with global environmental changes (Chapin *et al.* 2000, Kargiotidou *et al.* 2008). The quality and yield of important, low-temperature-sensitive, vegetable crops, such as cucumber, tomato, and sweet pepper, might be impaired severely, when subjected to LT stress. Much research has focused on the generic pathway of plant response to LT stress, including those studying how plants perceive extracellular stress, and how signals, *via* the membrane, activate large and complex intracellular signaling cascades, resulting in the expression of multiple cold-responsive genes (Huang *et al*. 2012). Among these LT responses, the injuries, caused by changes in temperature, are thought to take place primarily in the membrane (Kratsch and Wise 2000), and LT tolerance is closely connected to a composition of plant membrane lipids, especially the levels of unsaturated fatty acids (Somerville 1995, Nishida and Murata 1996, Murata and Los 1997, Iba 2002, Yu *et al.* 2009). In higher plants, the chloroplast membrane contains generally 70% of fatty acids and it shows the distinct characteristic of a higher content of polyunsaturated fatty acids (PUFAs), especially trienoic fatty acids (TAs), namely linolenic (18:3) and hexa-decatrienoic (16:3) acids (Browse and Somerville 1991). TAs are

Received 8 December 2011, *accepted* 28 November 2012.
[+]Corresponding author; phone: +86 538 88249606, fax: +86 538 88249606, e-mail: qwmeng@sdau.edu.cn
Abbreviations: APX – ascorbate peroxidase; *AtFAD7 – Arabidopsis thaliana* omega-3 fatty acid desaturase; Chl – chlorophyll; DAs – dienoic fatty acids; DGDG – digalactosyldiacylglycerol; FADs – fatty acid desaturases; IUFA – index of unsaturated fatty acid; *Lefad7 – Lycopersicon esculentum* omega-3 fatty acid desaturase gene; LT – low-temperature; MDA – malondialdehyde; MGDG – monogalactosyldiacylglycerol; PFD – photon flux density; PG – phosphatidylglycerol; P_N – net photosynthetic rate; PUFAs – polyunsaturated fatty acids; ROS – reactive oxygen species; SOD – superoxide dismutase; SQDG – sulfoquinovosyldiacylglycerol; TAs – trienoic fatty acids; WT – wild type; 16:1(3t) – trans-hexadecenoic acids; 16:3 – hexadecatrienoic acids; 18:1 – oleic acid; 18:2 – linoleic acid; 18:3 – linolenic acids.
Acknowledgements: This research was supported by a grant from the Major State Basic Research Development Program of China (973 program) (No. 2009CB118505) and the Natural Science Foundation of Zhejiang Province (Y3110327).

predominantly found in the membrane glycerolipids, and they make up about 95% of PUFAs, esterified to monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), the most abundant lipids in the chloroplast membranes of vegetative cells (Browse *et al.* 1989, Miquel and Browse 1992).

Enhanced unsaturation of fatty acids during cold-acclimation is thought to be due to the increase of desaturase activities (Cheesbrough 1989, Williams *et al.* 1992). Higher plants have two pathways for PUFAs biosynthesis - prokaryotic and eukaryotic. Oleic acid (18:1) is catalyzed to 18:3 *via* 18:2 by fatty acid desaturases (FADs) in the prokaryotic and eukaryotic pathways, and 16:1 was catalyzed to 16:3 *via* 16:2 by FADs only in the prokaryotic mode. Omega-3 fatty acid desaturases are key enzymes for the formation of TAs from the desaturation of lipid-linked dienoic fatty acids (DAs). In *Arabidopsis thaliana*, there are 3 genes, namely *fad3*, *fad7*, and *fad8*, encoding the omega-3 fatty acid desaturases. Proteins encoded by *fad7* and *fad8* are located in the plastid membrane, and the protein encoded by *fad3* is located in the microsomal membrane (Browse *et al.* 1986, Lemieux *et al.* 1990, McConn *et al.* 1994, Somerville and Browse 1996). Recently, it was reported that soybean FAD7 is localized in the chloroplast thylakoids (Andreu *et al*. 2007). Transgenic overexpression of the chloroplast omega-3 fatty acid desaturase gene can enhance the cold tolerance of tobacco (Kodama *et al.* 1994). Overexpressing the *Arabidopsis fad7* or *fad8* in tobacco increased TAs and decreased DAs in transgenic plant leaf tissue (Nishiuchi and Iba 1998, Murakami *et al.* 2000). Overexpression *fad3* and *fad7*, which led to the increase of 18:3 acids in leaves, resulted in a better maintenance of membrane fluidity and increased tolerance to cold and chilling in *Arabidopsis* (Routaboul *et al.* 2000). Increasing TAs in chloroplast membranes can enhance the LT tolerance in plants during the early growth stage (Iba 2002). The role of omega-3 desaturases in plant tolerance to low temperature has been established (Upchurch 2008). Because of the relationship among *fad7*, unsaturated fatty acids, and LT tolerance, the increase of the TAs, especially the overexpression of *fad7*, could be a potential method to improve the LT tolerance of diverse crops.

Tomato (*Lycopersicon esculentum* Mill.) is an important low-temperature-sensitive crop, grown throughout the world. In previous studies, we isolated the *fad7* from tomato and overexpressed *Lefad7* in tomato plants. Transgenic tomato plants overexpressing *Lefad7* had greater LT tolerance compared with WT (Liu *et al.* 2008). The goal of the present study was to determine, whether the overexpression of *Lefad7* increases the LT tolerance. The objective was also to define the modification of membrane lipid composition, especially changes of TAs and DAs contents, and other physiological parameters in two transgenic plant lines and WT exposed to a chilling temperature of 4°C for 6 h. Our data suggest that overexpression of *Lefad7* enhanced the LT tolerance by the increase of TAs content and reduction of DAs content (Kodama *et al.* 1994, Iba 2002). We found that the increase of TAs was associated with a series of physiological alterations and that this might further enhance the LT tolerance.

Materials and methods

Plant materials and treatments: We have generated f transgenic tomato lines, overexpressing *Lefad7* under control of 35S-CaMV promoter. T_1-7 and T_1-11, which expressed different *Lefad7* levels, were chosen as experimental, transgenic materials (Liu *et al.* 2006, 2008). The seeds of WT and transgenic tomato plants (of T_2 generation) were germinated on moistened filter paper at 25°C for 3 d. Sprouted seeds were then planted in soil in the greenhouse under a 14/10 h photoperiod at 25°C and a light intensity of 500–600 μmol m^{-2} s^{-1}. Sixth fully expanded leaves were harvested from one-month-old tomato plants. Detached leaves with petioles were placed in water and the leaves were exposed to a LT stress (4°C) under a low light (100 μmol m^{-2} s^{-1}) for 6 h in a chilled chamber *(i.e.,* cold treatment). Then the leaves of treated and untreated control (*i.e*. grown under 25°C, 100 μmol m^{-2} s^{-1}) were frozen immediately in liquid nitrogen and stored at –80°C.

Fatty acid detection: Leaves were harvested from one-month-old tomato plants and frozen immediately in liquid nitrogen. Lipids were extracted and separated by two-dimensional thin layer chromatography (TLC). 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 was determined by gas chromatography as previously described (Su *et al.* 1980, Xu and Siegenthaler 1997).

Membrane permeability determinations: Membrane permeability was determined by measuring the relative electrolyte conductivity following the method of Santos *et al*. (2001) with some modification. Briefly, leaf samples were washed in deionized water to remove surface-adhered ions. Fresh samples (10 leaf disks) were added to a glass flask containing 20 ml of deionized water. The flasks were shaken at 120 cycle min^{-1}. Samples were incubated 6 h at 25°C and the conductivity of solution (ECo) was measured with a microdigital conductivity meter (*DDS-11D*, China). After this measurement, samples were killed by autoclaving at 110°C for 10 min. When the temperature of the flasks declined to 25°C, 20 ml of deionized water were added, and the total conductivity (ECt) was measured. Relative electrolyte conductivity was calculated as (ECo/ECt) × 100.

MDA content determination: MDA contents were determined by the thiobarbituric acid (TBA) reaction described previously (Hara *et al.* 2003, Guo *et al.* 2007). The amount of TBA reactive substance (TBARS) was calculated from the difference in absorbance at 532 nm using extinction coefficient of 155 mM^{-1} cm^{-1}.

Activities of anti-oxidative enzymes and ROS analysis: Leaves were homogenized in a blender in 200 ml ice-cold medium containing 330 mM sorbitol, 30 mM 2-N-morpholino-ethanesulfonic 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 ml PBS for measurement of chloroplastic ascorbate peroxidase (APX, EC 1.11.1.11) and superoxide dismutase (SOD, EC 1.15.1.1) activities. APX activity was determined according to Jimenez *et al*. (1997). The SOD assay was performed as described by Giannopolitis and Ries (1977). A *UV-1601* spectrophotometer (*SHIMADZU*, Japan) was used to measure optical density. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of nitro-blue tetrazolium. The assay for $O_2^{\cdot -}$ was performed as described by Wang and Luo (1990). H_2O_2 content was determined according to the method of Sairam and Srivastava (2002).

Assay of P_N and determination of Chl content: Attached leaves were treated at 4°C for 6 h and P_N was measured with a portable photosynthetic system (*CIRAS-2*, *PP Systems*, UK) at 25°C and 4°C, under a concentration of ambient CO_2 (360 μmol mol^{-1}), and a PFD of 600 μmol m^{-2} s^{-1}. Before P_N measurement, tomato plants were adapted at 25 °C and a PFD of 100 μmol m^{-2} s^{-1} for about 30 min to make stomata open and then adapted for about 15 min at a PFD of 600 μmol m^{-2} s^{-1}.

A leaf sample (0.25 g) was macerated in 80% acetone/water (v/v) and a small amount of $CaCO_3$, the extract was then filtered through two layers of nylon and centrifuged in sealed tubes at 15,000 × *g* for 5 min. The supernatant was collected and the absorbance read at 652 nm using a *UV-1601* spectrophotometer (*SHIMADZU*, Japan) to calculate the Chl content by the method of Bruinsma (1961).

Results

Fatty acid composition: Under normal conditions, the 18:3 content of the *Lefad7* transgenic plant leaves was significantly higher than the 18:3 content of WT leaves. Under normal conditions, the increase in 18:3 content was associated with the decrease in 18:2 in both the types of plants (Fig. 1*A*). Under LT stress, 18:3 leaf contents of both types of plants increased over that found under the normal conditions (Fig. 1*B*), however, the transgenic plants had a higher 18:3 content than WT plants. Under LT stress, the 18:2 contents of both trangenic and WT plants correspondingly decreased (Fig. 1*B*). The index of unsaturated fatty acid (IUFA = 18:1 × 1 + 18:2 × 2 + 18:3 × 3) increased after cold treatment. Before the treatment, IUFA was 194.0, 192.0, and 184.2% in leaves of T_1-7, T_1-11, and WT plants, respectively. Under LT stress, the IUFA was 203.9, 201.6, and 196.9% in T_1-7, T_1-11, and WT plants, respectively.

We determined the fatty acid composition of the main polar lipids, of which MGDG and DGDG of all the lipids contained the greatest amounts of 18:3 in the leaves of transgenic lines and WT plants. Under LT stress, the 18:3 content in transgenic lines and WT plants increased with a slight decrease of 18:2 in MGDG, DGDG, SQDG, and PG compared with the values before stress. The 18:3 content in MGDG, SQDG, and PG of transgenic line T_1-7, where the expression level of *Lefad7* was the highest (Liu *et al.* 2008), was about 15% higher than in WT after the treatment, but it was similar in the DGDG of T_1-7, T_1-11, and WT plants (Table 1). We also found that the proportion of the individual glycerolipids in the leaves of

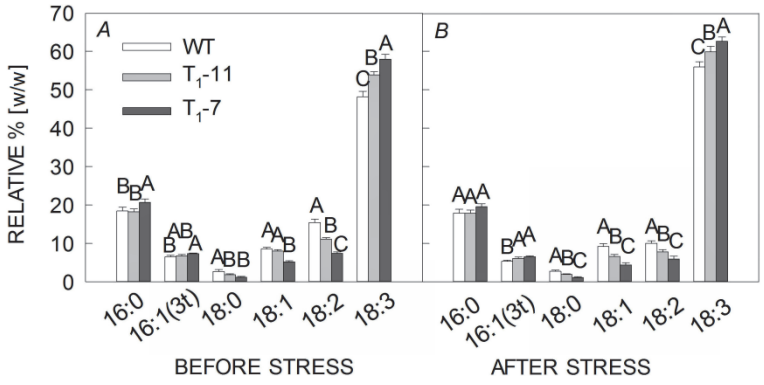

Fig. 1. Fatty acid composition of leaf tissue of WT and transgenic (T_1-11, T_1-7) tomato leaves at different conditions. *A*: The plants were grown at 25ºC; *B*: The detached plants were treated with 4ºC for 6 h. Means ± SD; $n = 5$. $P<0.05$. Significant level (*Duncan*'s test).

Table 1. Effect of low temperature (4°C, 6 h) on fatty acid composition of individual leaf polar lipids in WT and two transgenic tomato lines (T_1-7, T_1-11). Means ± SD (n = 9) of 3 measurements on each of 3 plants are presented as mole percentage [mol%]. P<0.05. Significant level (*Duncan*'s test). Standard deviations between triplicates were < 3% of the indicated values.

Lipid	Genotype	Fatty acid composition [mol%] 16:0	16:1(3t)	18:0	18:1	18:2	18:3
MGDG	WT	2.34 ± 0.48^c	0	0.23 ± 0.03^b	23.94 ± 0.80^a	1.71 ± 0.40^b	71.77 ± 1.35^c
	T_1-11	3.85 ± 0.45^b	0	0.58 ± 0.06^a	14.13 ± 0.40^b	2.97 ± 0.26^a	78.47 ± 0.53^b
	T_1-7	4.70 ± 0.06^a	0	0.31 ± 0.05^b	11.54 ± 0.55^c	0.58 ± 0.21^c	82.74 ± 1.25^a
DGDG	WT	14.42 ± 0.89^c	0	3.26 ± 0.95^a	1.68 ± 0.16^a	9.52 ± 1.13^a	71.72 ± 0.62^c
	T_1-11	17.01 ± 0.62^b	0	2.46 ± 0.26^b	1.69 ± 0.08^a	5.31 ± 0.43^b	73.53 ± 0.36^b
	T_1-7	20.46 ± 0.63^a	0	1.22 ± 0.32^c	1.12 ± 0.07^b	2.83 ± 0.37^c	74.37 ± 0.37^a
SQDG	WT	36.45 ± 1.38^a	0	2.81 ± 0.28^a	4.29 ± 0.32^a	17.87 ± 0.41^a	42.58 ± 1.26^c
	T_1-11	31.70 ± 1.1^b	0	1.61 ± 0.20^c	6.98 ± 0.21^a	12.83 ± 0.38^b	46.88 ± 1.05^b
	T_1-7	34.46 ± 0.65^a	0	1.89 ± 0.08^b	1.96 ± 0.09^a	13.49 ± 0.40^b	48.20 ± 0.68^a
PG	WT	18.26 ± 1.02^a	21.30 ± 1.25^c	4.53 ± 0.71^a	6.78 ± 0.53^a	10.95 ± 0.37^a	38.18 ± 1.25^c
	T_1-11	18.93 ± 0.89^a	24.27 ± 0.72^b	2.82 ± 0.11^b	3.62 ± 0.12^b	9.90 ± 0.45^b	40.46 ± 0.82^b
	T_1-7	18.66 ± 1.24^a	26.14 ± 0.83^a	0.77 ± 0.13^c	2.80 ± 0.12^c	6.61 ± 0.26^c	45.02 ± 1.07^a

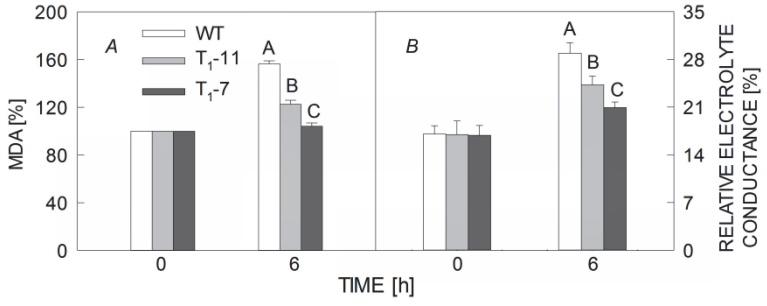

Fig. 2. Effects of low temperature (4°C, 6 h) on MDA (*A*) and relative electrolyte conductance (*B*) in WT and transgenic tomato leaves. Means ± SD; n = 5. P<0.05. Significant level (*Duncan*'s test).

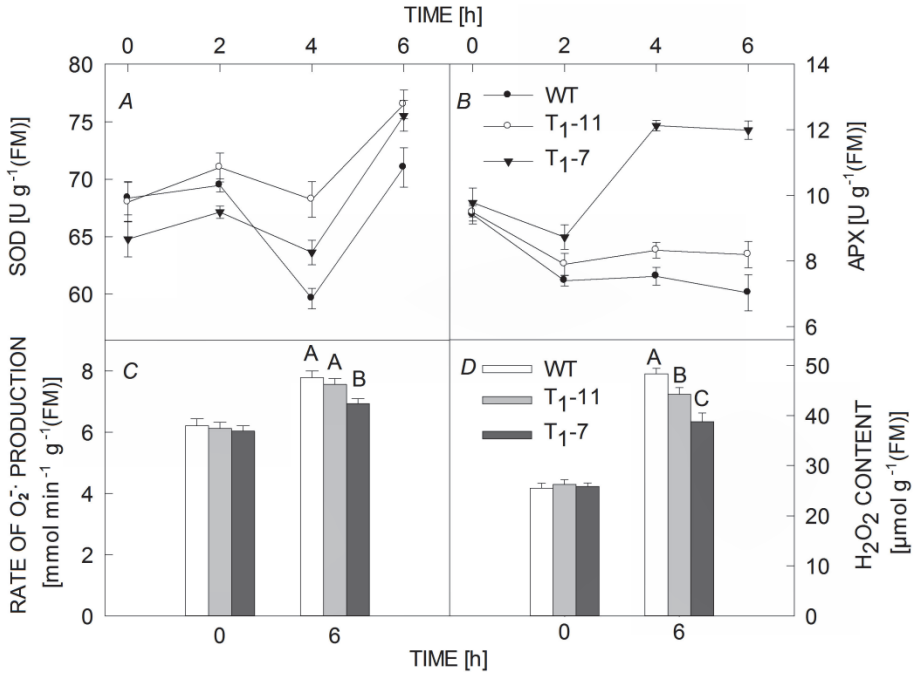

Fig. 3. Effects of low temperature (4°C) on (*A*) superoxide dismutase (SOD) activity (2, 4, and 6 h), (*B*) ascorbate peroxidase (APX) activity (2, 4, and 6 h), (*C*) $O_2^{\cdot-}$ (4 h), and (*D*) H_2O_2 (4 h) content in wild type (WT) and transgenic tomato leaves under the light of 100 µmol(photon) $m^{-2} \cdot s^{-1}$. Means ± SD; n = 5. P<0.05. Significant level (*Duncan*'s test).

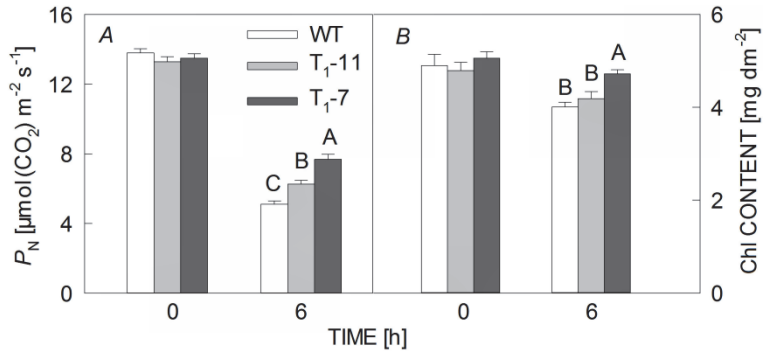

Fig. 4. Effects of low temperature (4ºC, 6 h) on net photosynthetic rate (P_N) (*A*) and chlorophyll (Chl) content (*B*) in wild type (WT) and transgenic tomato leaves. Means ± SD; $n = 5$. $P<0.05$. Significant level (*Duncan*'s test).

transgenic plants was similar to that in the WT plants both before (Liu *et al.* 2008) and after the treatment (data not shown).

MDA and index of injury: Under normal growth conditions, transgenic plants exhibited a similar MDA level and membrane permeability compared with WT. After cold treatment, the membrane lipid peroxidation level increased both in WT and transgenic plants, with the lesser increase in MDA in the transgenic plants than in WT (Fig. 2*A*). In addition, the electrolyte conductance increased both in WT and transgenic plants, while the increase was lesser in transgenic plant than in WT (Fig. 2*B*).

Activities of antioxidative enzymes and ROS content: After cold treatment, the activity pattern of SOD increased first within 1 h, then decreased, and afterwards increased again after 4 h both in the transgenic and WT plants (Fig. 3*A*). The activity of APX decreased first within 1 h, then increased, afterwards decreased again after 4 h during the LT stress in both transgenic lines and WT plants (Fig. 3*B*). Compared with untreated controls, in transgenic plants, the activities of SOD increased by 3.9, 12.5, and 16.6% in WT, T_1-11, and T_1-7, respectively (Fig. 3*A*). The activities of APX were 1.7 fold higher in T_1-7 compared with WT after the cold treatment (Fig. 3*B*). After the cold treatment, we detected a lower level of ROS, including $O_2^{\cdot -}$ and H_2O_2, in transgenic lines compared with the WT plants (Fig. 3*C*,*D*). Among these ROS changes, the increase of $O_2^{\cdot -}$ in WT, T_1-11, and T_1-7 was 25.3, 23.5 and 14.93%, respectively (Fig. 3*C*), and the increase of $O_2^{\cdot -}$ in WT, T_1-11, and T_1-7 was 89.7, 68.6, and 50.0% , respectively (Fig. 3*D*).

P_N and Chl content: When *Lefad7* was overexpressed in tomato, the increased 18:3 content did not significantly influence P_N and Chl content under normal growth conditions (Fig. 4*A*,*B*, *i.e.*, before stress). After the cold treatment, P_N and Chl content in WT markedly decreased, while they declined slowly in transgenic plants compared with WT plants, especially in T_1-7. Meanwhile, the Chl content was similar in T_1-7 plants both under LT stress and under normal conditions (Fig. 4).

Discussion

It was reported that omega-3 fatty acid desaturase activity is rate-limiting for the production of TAs (Iba *et al*. 1993). Therefore, we could modify the content of DAs and TAs by changing omega-3 fatty acid desaturase gene expression. Previously, we demonstrated that the overexpression of *Lefad7* in transgenic tomato resulted in enhanced tolerance to LT stress (Liu *et al*. 2008). Here, we attempted to determine how the overexpression of *Lefad7* might confer the LT tolerance in tomato.

In many plants, 18:3 content increased, accompanying cold acclimation, and a positive relationship is observed between a higher degree of fatty acid desaturation and cold tolerance (Zhang *et al.* 2005). We found that the overexpression of the *Lefad7* did not significantly affect the synthesis of each lipid class in tomato (data was not shown), but the total 18:3 contents increased both in transgenic lines and WT plants under LT stress (Fig. 1). We had previously observed that the upregulation of *Lefad7* transcript level could change the 18:3 content in tomato (Fig. 1, Table 1, Liu *et al*. 2008). The 18:3 contents of membrane lipids, MGDG, DGDG, and PG, catalyzed by plastid omega-3 fatty acid desaturase, LeFAD7, changed markedly in the WT chloroplast compared with trangenic lines during LT stress (Table 1) (Liu *et al.* 2008). It should be noted that 18:3 content in lipids of the transgenic lines was higher than that in WT plants both before and after the cold treatment (Table 1, Liu *et al.* 2008).

Our data suggests that the levels of TAs were important for the tomato growth under low temperature. The level of TAs was related to *Lefad7* transcripts level, and higher 18:3 contents in transgenic lines had alleviated the photoinhibition of photosystems PSI and PSII under chilling at low irradiance (Liu *et al.* 2008). The membrane is thought to be the primary site of injury during LT stress (Kratsch and Wise 2000), and chilling symptoms are associated with a decrease in membrane fluidity and an increase in membrane leakage permeability

(Upchurch 2008). The increase in the degree of unsaturation appears to be a major factor that determines membrane fluidity (Routaboul *et al.* 2000). Fatty acid desaturases may modulate the membrane fluidity by modifying the unsaturated level of membrane lipid to respond to low temperature (Upchurch 2008, Teixeira *et al.* 2010). Both the integrity of biological membranes and functions of integral membrane proteins are sensitive to low temperature, because cold stress alters the membrane environment and potentially the tertiary and quaternary structures of membrane proteins. We measured the electrolyte leakage to test the level of damage to lipid membrane after LT treatments (Barclay and McKersie 1994). Moreover, membrane lipid peroxidation has been observed in many species under cold stress (Fryer *et al.* 1998, Griffiths *et al.* 2000), and it was associated with the electrolyte leakage caused by the low temperature (Alonso *et al.* 1997). In our study, we found that the relative electrolyte leakage and MDA level of leaves in transgenic plants increased less than that in WT plants. By this criterion, the membrane injury was more severe in WT than in transgenic lines. This suggests that the higher TAs content in the chloroplast lipids of the transgenic plants might prevent the membrane damage during LT stress (Table 1, Fig. 2). It is known that elevated cellular lipid peroxidation is one of initial events associated with LT injury (Burdon *et al.* 1994, O'Kane *et al.* 1996), suggesting that membrane lipid peroxidation can alter the activities of enzymes located in the membrane. The increase in antioxidative enzyme activity in plants under stress has been considered to be essential for a plant adaptation during cold stress (Mittler 2002). Our experiments showed that the activities of antioxidative enzymes were higher in transgenic plants than in WT ones and it might mitigate the membrane damage due to oxidation and lipid peroxidation (Burdon *et al*. 1994) (Fig. 3). The ROS content in transgenic plants leaves was lower compared with WT ones (Fig. 3), suggesting that the relative level of antioxidative enzyme activity was sufficient to maintain a relatively lower ROS steady-state level in transgenic plants compared with WT ones. In addition, we measured higher P_N and Chl contents in transgenic plant leaves compared with WT leaves after cold stress (Fig. 4), suggesting that increased TAs in the photosynthetic membrane were required to maintain a chloroplast function during LT stress (Routaboul *et al.* 2000). It is relevant to point out here that in our experiments, plant leaves (detached leaves with petiole) were exposed to low temperature for 6 h. It is likely that the effect of chilling stress on the entire plant is more severe than on detached leaves. Therefore, future work should seek to verify current results by chilling treatment of the whole plants.

Global changes likely bring both altered seasonal low and high temperatures, resulting in a greater potential for damage to crop productivity. Many important commercial food crops, such as tomato, are sensitive to low temperature. Frequently, LT stress can result in a substantial damage to tomato fruit yield and quality. Therefore, it is important to improve the LT tolerance in tomato. We demonstrated that overexpression of *Lefad7* enhanced the LT tolerance of tomato plants through increasing TAs. Increasing TAs further enhanced the stability of the membrane and reduced the membrane damage. It has also been reported that overexpression of omega-3 fatty acid desaturase genes can improve the LT tolerance in other plants, such as *Arabidopsis*, rice, soybean (Iba *et al.* 1993, Shimada *et al.* 2000, Andreu *et al.* 2010). These results indicate that it might be possible to improve a resistance to LT stress efficiently in economical important crops by increasing *fad7* expression. Further, we might be able to improve the overall transgenic expression of omega-3 fatty acid desaturase gene, like *Lefad7,* by using gene constructs containing a cold-inducible promoter.

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