Overexpression of *PbDHAR2* from *Pyrus sinkiangensis* in Transgenic Tomato Confers Enhanced Tolerance to Salt and Chilling Stresses

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Abstract. Ascorbic acid (AsA) is a major antioxidant and redox buffer in plants. Dehydroascorbate reductase (DHAR; EC 1.8.5.1) catalyzes the conversion of dehydroascorbate (DHA) to AsA and is crucial for AsA regeneration. In this study, we developed transgenic tomato plants that overexpressed *PbDHAR2* to investigate whether *PbDHAR2* could limit the deleterious effects of salt and chilling stresses. These transgenic plants contained significantly higher AsA levels than the wild-type (WT) plants. Overexpression of *PbDHAR2* increased the expression of the AsA-glutathione (GSH) cycle genes in transgenic lines under salt and chilling stresses. In addition, the transgenic lines subjected to salt and chilling stresses showed higher levels of antioxidant enzyme activity, lower malondialdehyde (MDA) levels, and higher chlorophyll contents than the WT. Thus, our results demonstrate that the regulation of *PbDHAR2* during AsA regeneration contributes to enhanced salt and chilling tolerance in tomato.

When plants are subject to environmental stresses, including salt, chilling, heat, and drought, they are damaged because of oxidative stress caused by the accumulation of reactive oxygen species (ROS) in plant cells (Apel and Hirt, 2004). ROS affect plant growth, development, and crop productivity. Many cellular components, such as nucleic acids, carbohydrates, and proteins, are damaged oxidatively (Asada, 2006; Foyer and Noctor, 2005; Mittler et al., 2004). Therefore, plants have developed a set of complex adaptation mechanisms to reduce the deleterious effects of ROS. These mechanisms employ several enzymatic and nonenzymatic scavenging systems, such as antioxidant enzymes, including superoxide dismutase (SOD; EC 1.15.1.1), peroxidase (POD; EC 1.11.1.6), catalase (CAT; EC 1.11.1.6), and antioxidant compounds, such as AsA (Arrigoni, 1994; Conklin, 2001; Farooq et al., 2013).

AsA is a cofactor for many enzymes; it can control cell division and growth (Conklin, 2001; Gallie, 2013a; Gallie, 2013b; Potters et al., 2000); and it improves resistance to a number of environmental stresses, such as salt stress (Zhang et al., 2012), high temperature (Larkindale et al., 2005), oxidative stress (Bai et al., 2013), and inappropriate light levels (Pék et al., 2011).

Most AsA are localized in the cytoplasm, whereas ≈10% are localized in the apoplast, and 12% to 30% are found in the chloroplasts (Horemans et al., 2000; Pignocchi and Foyer, 2003). It has been reported that an AsA-GSH pathway exists in cytosol, chloroplasts, mitochondria, and peroxisomes, and represents an important antioxidant defense system against H₂O₂ (Davey et al., 2000; Potters et al., 2000). In the AsA-GSH cycle, antioxidant enzymes, including ascorbate peroxidase (APX; EC 1.11.1.11), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), DHAR, and glutathione reductase (GR; EC 1.6.4.2), play an important role in protecting plants against environmental stresses. AsA is oxidized to monodehydroascorbate (MDHA) that reduces H₂O₂ to water. MDHA is unstable and rapidly disassociates into AsA and DHA. DHA returns to the cytosol for reduction to AsA by DHAR. Thus, DHAR allows the plant to recycle DHA before it is lost, which suggests that DHAR is indirectly involved in ROS scavenging by the AsA-GSH pathway (Giovannoni, 2007; Harb et al., 2010; Melino et al., 2009; Potters et al., 2000; Wang et al., 2010).

DHAR is a ubiquitous protein and *DHAR* genes form a distinct gene family. Shimaoka et al. (2000) reported that two major *DHAR* genes existed in spinach leaves. One *DHAR* gene (*DHAR1*) is in cytosol, and another *DHAR* gene (*DHAR2*) is in chloroplasts. Two major DHARs existed in the form of a gene family and occupied at least 90% of total DHAR activity. *DHAR* genes involved in abiotic stress are known, including *DHAR1*

involved in salt and temperature stresses (Eltayeb et al., 2006; Ushimaru et al., 2006; Wang et al., 2010), and *DHAR2* involved in salt stress in tobacco (Kwon et al., 2003; Lee et al., 2007). Unraveling of these signalling factors offers a valuable approach for engineering salt and chilling tolerance.

Although *DHAR* genes have been isolated from several plants (Chen et al., 2003; Shimaoka et al., 2000; Urano et al., 2000; Wang et al., 2010; Zou et al., 2006), there is no report of the function of the *PbDHAR2* gene in pear. It is noticeable that knowledge of the DHAR cascade of fruit crops under abiotic stress is scarce compared with other plants, including Arabidopsis, tobacco, and tomato. Pear (*Pyrus* spp.) is one of the most important fruits in the world and the content of AsA in pears is quite low compared with other plants, such as kiwifruit (Koutinas et al., 2010) and pepper (Aloni et al., 2008). We wanted to know whether overexpression of *PbDHAR2* had contributed to increased AsA content. On the other hand, the pear is a perennial ligneous plant and only slow progress has been made in the improvement of salt and chilling stresses via traditional cross-hybridization procedures. As a first step toward creation of pear transgenic plants with enhanced stress tolerance, efforts have been made to isolate and characterize *PbDHAR2* gene in this study. Moreover, to gain a better understanding of the *PbDHAR2* gene, transgenic tomato lines and WT plants were used to investigate the effects of increased AsA accumulation and any consequent changes in physiology under different stress conditions. We examined some chemical changes that took place after the plants were subjected to salt or chilling stress to evaluate the mechanism controlling *PbDHAR2* functions and its potential application in *Pyrus* plant biotechnology.

Materials and Methods

Gene isolation and construction of the plant expression vector. Total RNA was extracted from the flesh of pear, *Pyrus sinkiangensis* Yu 'Kuerlexiangli', using the cetyltrimethylammonium bromide (CTAB) method as described by Gambino et al. (2008). The cDNA synthesis was carried out according to the manufacturer's protocol using a ReverTra Ace qPCR RT Kit (ToYoBo, Osaka, Japan). The primers for the *PbDHAR2* gene coding sequence (Pbr 016672.1) were constructed using the National Center for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) (Wu et al., 2013). The full length of the *PbDHAR2* gene was amplified using a D2-1 primer and polymerase chain reaction (PCR) (Table 1). To construct a vector for plant transformation, the *PbDHAR2* gene was amplified by PCR with the D2-2 primer including *NcoI* and *BstEII* restriction sites on their respective 5'-ends (Table 1). Then the *PbDHAR2* PCR fragment was digested with *NcoI* and *BstEII* and inserted into the pCambia1301 binary vector to replace the *GUS* gene. The process

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was controlled by the CaMV 35S promoter. The vector containing the *PbDHAR2* gene sequence was introduced into *Agrobacterium tumefaciens* strain EHA105 and used to transform Micro-Tom tomato plants.

Plant transformation and generation of transgenic plants. *Agrobacterium*-mediated transformation of leaf discs was carried out according to Huang et al. (2011) to generate transgenic tomato plants. The leaf discs were selected in Murashige and Skoog (MS) medium containing 2.0 mg·L⁻¹ zeatin (ZT), 10 mg·L⁻¹ hygromycin, and 400 mg·L⁻¹ cefotaxime (sodium salt) for adventitious bud regeneration. Regenerated shoots were transferred to 0.5 MS medium supplemented with 0.2 mg·L⁻¹ indole-3-butyric acid to promote rooting. Genomic DNA was isolated from the leaves of the WT and the transgenic plants. The presence of the *PbDHAR2* transgenic lines in hygromycin-resistant seedlings (T0) were identified by PCR using the D2-1 primer (Table 1). Transgenic tomatoes were allowed to self pollinate. The genomic DNA of the transgenic plants

(T1) was confirmed by PCR using the specific HYG primer (accession number AAF65341.1) to verify the presence of the *PbDHAR2* transgene. Overexpression of the *PbDHAR2* gene was examined by semiquantitative PCR [reverse transcription (RT)-PCR] using the D2-3 primer and three selected putative transgenic plants (Table 1). The *Actin* gene was used as the internal control (Table 1). RT-PCR amplification of the *PbDHAR2* was performed using 100 ng of cDNA as a template with the following program: 3 min denaturing at 94 °C, followed by 35 cycles of 30 s at 94 °C, 1 min at 60 °C, 30 s at 72 °C, and a 10 min extension at 72 °C. T2 seeds of the overexpressing lines were used in the stress tolerance analysis.

Abiotic stress treatments. The stress tolerance capacity of the WT and the transgenic plants were subjected to NaCl stress or 4 °C stress. At least 17 seedlings of each lines were transplanted into pots (7 × 7 cm square pots, 8 cm high) containing vermiculite and turf soil (1:1) at 14 h light cycle, 22 ± 4 °C and

60% relative humidity (RH). The WT and the transgenic plants were selected for the NaCl and the chilling (4 °C) treatments when the plants were 40 d old. Salt stress was applied by irrigating the plants with 0.5 Hoagland nutrient solution containing 50 mM NaCl every 5 d for 4 weeks. Plants (40 d old) grown in normal conditions were subjected to 4 °C (14 h light/10 h darkness) for 1 week as in the chilling stress. The samples were then harvested and stored at -80 °C until needed.

AsA and DHA content assay. AsA and DHA contents were measured essentially as described by Li et al. (2009) but with minor modifications. Two hundred milligrams of fresh leaves from the same position of plants was homogenized in 8 mL 6% (w/v) metaphosphoric acid (HPO₃) on ice to prevent AsA oxidation. The homogenate was centrifuged for 20 min at 12,000 g and the supernatant was analyzed for AsA and total ascorbate (AsA + DHA, T-AsA) content. Total AsA content was determined by initially incubating the samples for 15 min at

Table 1. Primer sequences used for cloning, vector construction, transgenic confirmation, and expression analysis.

Genes	Primers	GenBank ID	Sequences (5'–3')	
			Forward	Reverse
<i>PbDHAR2</i>	D2-1	Pbr016672.1	GCTCCAGCATCACTCTTCGC CTGCCATGGGCTCCAGCATCACTCTTCGC (<i>NcoI</i> site is underlined)	ACACCCGTCACAACCTTTT CGGGTAACCCACACCCGTCACAACCTTTT (<i>BstEII</i> site is underlined)
<i>PbDHAR2</i>	D2-2	—	GGCGCCACGGCACAA	CCCAAGTCTACCAACTTCAGGT
<i>PbDHAR2</i>	D2-3	—	ATGGCAGACGGAGAGATATCA	GCCTTTGCAATCCACATCTGCTG
<i>Actin</i>	—	—	AGGGCGAAGAATCTCGTGCT	TCGCCTCGCTCCAGTCAATG
<i>HYGROMYCIN</i>	HYG	AAF65341.1	CTCTTCGAGCGAGAGTCGTT	CGTTCATAAAGCTGCACTCGT
<i>DHAR1</i>	—	AY971873.1	CTCTGGTAAGCTGAAGGGGG	AGGATGCTCTTGATTGAAGTGG
<i>MDHAR</i>	—	NM_001247084.1	TGCTAGCTTTGCCAACCGAT	GGGTTCTCCCAAGCCTTTT
<i>APX</i>	—	NM_001247702.1

Fig. 1. Identification of transgenic lines containing *PbDHAR2*. (A) Polymerase chain reaction (PCR) analysis for the presence of the *PbDHAR2* gene. WT, wild-type; L1–L16, transgenic lines. (B) PCR analysis using the HYG primer in the WT and the transgenic lines. L1, L5, and L6 transgenic lines contained the *PbDHAR2*. (C) Gene expression analysis of *PbDHAR2* in the WT and the transgenic lines by reverse transcription-PCR analysis.

42 °C in 200 mM phosphate buffer solution (pH 7.4) and 1.5 mM dithiothreitol (DTT). After incubation, 0.2 mL of 0.5% (w/v) N-ethylmaleimide (NEM) was added for 2 min at room temperature to remove excess DTT. AsA content was determined in a similar manner except that 0.4 mL deionized H₂O was substituted for DTT and NEM.

Color was developed with the addition of 1 mL 10% trichloroacetic acid (TCA), 0.8 mL 42% o-phosphoric acid, 0.8 mL 2% 2,2-dipyridyl, and 0.4 mL 3% (w/v) FeCl₃. The reaction mixtures were then incubated at 42 °C for 1 h and quantified at 525 nm. A standard curve of AsA was established and used for quantification.

Expression analysis by quantitative real-time PCR. The genes involved in the tomato AsA-GSH cycle from the WT and transgenic line plants before and after salt and chilling treatment were analyzed by quantitative real-time PCR (qPCR) using the SYBR green dye method. Total RNA was extracted from the WT and the transgenic lines leaves using the CTAB method (Gambino et al., 2008) and then treated with DNase I (Invitrogen, Carlsbad, CA). RNA integrity was assessed by 1% agarose gel electrophoresis, and the concentration of extracted RNA was determined by NanoDrop (Thermo). The cDNA synthesis was carried out according to the manufacturer's protocol using a ReverTra Ace qPCR RT Kit (ToYoBo, Japan). The relative expressions of *DHAR1* (accession number AY971873.1), *MDHAR* (accession number NM_001247084.1) and *APX* (accession number NM_001247702.1) genes were determined by qPCR using the LightCycler® 480II Real-Time PCR System (Roche, Basel, Switzerland). A 20 µL total reaction volume, containing 1 µL prediluted cDNA, 10 µL LightCycler® 480 SYBR Green I Master (Roche, Mannheim, Germany), and 200 nm forward and reverse primers (Table 1) were used. *Actin* was used as an internal control to normalize the relative expression levels of the analyzed genes in tomato. Relative

Fig. 2. Overexpression of the *PbDHAR2* gene increased stress tolerance following salt and chilling treatment in the transgenic lines. (A) The phenotypes of the wild-type (WT) and the transgenic lines after salt stress. (B) The phenotypes of the WT and the transgenic lines after chilling stress. Asterisks show that values are significantly different between the WT and the transgenic lines under the same growth conditions.

Fig. 3. Analysis of ascorbic acid (AsA) content, dehydroascorbate (DHA) content, and AsA:DHA ratio of the wild-type (WT) and the transgenic lines before and after salt and chilling stresses. (A) AsA content in the WT and transgenic lines under normal, salt, and chilling conditions. (B) DHA content in the WT and transgenic lines under normal, salt, and chilling conditions. (C) AsA/DHA ratio in the WT and transgenic lines under normal, salt, and chilling conditions. Asterisks show that values are significantly different between the WT and the transgenic lines under the same growth conditions (**P* < 0.05; ***P* < 0.01).

expression of a gene was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Analysis of antioxidant enzyme activity, and measurement of the MDA and chlorophyll contents. The activities of three antioxidant enzymes, SOD, POD, and CAT, were measured as in previous reports (Huang et al., 2010; Mittova et al., 2000).

MDA and chlorophyll (chlorophyll a + chlorophyll b) content were evaluated in leaves taken from the WT and the transgenic lines. MDA content was measured as described in previous studies (Huang et al., 2010; Zhang et al., 2012). A sample (0.1 g) of leaf tissue was extracted with 2 mL of 5% (w/v) TCA and the homogenate was centrifuged at 3000 rpm for 10 min. Then, 2 mL of 10% TCA containing 0.67% barbituric acid (TBA) was added to the supernatant. The mixture was boiled at 100 °C for 30 min and centrifuged again after the mixture had been cooled on ice. Finally, the MDA content was determined by measuring the optical density at 532, 450, and 600 nm. Chlorophyll content was measured as described by Huang et al. (2010) and Liu et al. (2008) with minor modifications. Briefly, the chlorophyll content was extracted from ≈ 0.1 g of leaves directly into a 10 mL extract (ethanol:ace-

tone:H₂O = 4.5:4.5:1) at room temperature in the dark. The crude extraction was centrifuged for 2 min at 3000 rpm, which left the leaves completely white. The resultant supernatant was used for assessing absorbance at 663, 645, and 440 nm.

Statistical analysis. The SPSS 17.0 statistical software package was used for all statistical analyses. Each sample was replicated three times and the results expressed as means with standard deviations ($\pm$ SD), at a significance level of $P < 0.05$ and $P < 0.01$.

Results

Identification and regeneration of transgenic plants. The 1048 base pair (bp) long *PbDHAR2* gene was isolated and cloned from *P. sinkiangensis*. The gene was transformed into the pCAMBIA1301 binary vector. After sequence confirmation, the construct was introduced into *A. tumefaciens* strain EHA105. A specific 1kb amplification corresponding to *PbDHAR2* was detected with 16 putative transgenic lines (T0), and no expected band was observed in WT plants (Fig. 1A). In total, three transgenic lines (T1) were confirmed by PCR using specific HYG primer (Fig. 1B). Furthermore, RT-PCR analysis using the D2-3 primer showed that

PbDHAR2 was detected in transgenic lines, but it was not detected in WT plants (Fig. 1C). Two of the lines (L1 and L5), had higher *PbDHAR2* gene expression levels, so these lines (T2) were selected for further experiment analysis.

Assessment of stress tolerance in WT and transgenic lines. The WT and the transgenic lines were subjected to salt and chilling stresses to allow the role of *PbDHAR2* to be studied in detail. There were no apparent differences between the WT and transgenic lines before they were subjected to salt and chilling stresses. The morphological appearances of L1 and L5 were better than that of WT, as more leaves remained green and grew strongly in the transgenic lines (Fig. 2A). Similarly, improved phenotypes were observed after the transgenic plants were subjected to chilling treatment compared with the WT plants (Fig. 2B). This finding implied that the overexpression of the *PbDHAR2* gene may enhance tolerance to salt and chilling stresses.

The AsA content in the leaves of the WT plants and the transgenic lines were measured to determine the metabolic consequences of *PbDHAR2* overexpression. We found that the AsA content in the two transgenic lines ($6.03 \text{ mg}\cdot\text{g}^{-1}$ FW and $6.28 \text{ mg}\cdot\text{g}^{-1}$ FW for

Fig. 4. Quantitative real-time polymerase chain reaction analysis of ascorbic acid-glutathione cycle genes expression levels in the wild-type (WT) and the transgenic lines under normal, salt, and chilling conditions. (A) *DHAR1*, (B) *MDHAR*, and (C) *APX* expression levels. Asterisks show that values are significantly different between the WT and the transgenic lines under the same growth conditions (* $P < 0.05$; ** $P < 0.01$).

L1 and L5, respectively) were 1.44- and 1.50-fold higher than in the WT (4.13 mg·g⁻¹ FW) (Fig. 3A), and DHA content had slightly increased in the L1 and L5 (Fig. 3B). The AsA:DHA ratio in the L1 (16.46) and L5 (17.54) were higher than that of the WT (12.84) (Fig. 3C). Following salt and chilling stresses, both AsA content and AsA:DHA ratio of leaves increased in the WT and the transgenic lines. The AsA content had slightly increased by 1.46- and 1.54-fold after salt stress and by 1.51- and 1.49-fold after chilling stress in the L1 and L5 compared with the WT, respectively (Fig. 3A). The AsA:DHA ratio in the L1 and L5 were 2.36- and 1.61-fold higher than in the WT under salt treatment (Fig. 3C). This indicated that *PbDHAR2* was an important positive regulator of AsA content.

Expression analysis of AsA-GSH cycle genes in the WT and the transgenic lines when under stress. qPCR analysis was performed on the WT and the transgenic lines before and after they were subjected to the two stresses to investigate the effect of the stresses on the expression of AsA-GSH cycle genes (Fig. 4). These genes involved in AsA recycling (*DHAR1*, *MDHAR*, and *APX*). The increased tolerance of salt in plants overexpressing *PbDHAR2* was associated with increased levels of expression of genes in-

involved in AsA recycling compared with WT plants (Fig. 4). A similar effect on gene expression was observed under chilling stress. However, the transgenic lines response to salt stress were more pronounced than their response to chilling stress. These results suggested that overexpression of *PbDHAR2* enhanced the expression levels of AsA recycling genes to regulate the AsA content, which led to reduced stress damage in transgenic plants.

Analysis of antioxidant enzyme activity and metabolite levels. ROS can be scavenged by enzymatic and nonenzymatic pathways. Three antioxidative enzymes (SOD, POD, and CAT) play significant roles in the response to stress. Under normal growth conditions, the activities of the three enzymes showed no significant differences in the WT and the transgenic lines, except for POD activity in L1 (Fig. 5). Under salt and chilling stresses, the SOD, POD, and CAT activities were enhanced in the transgenic lines (Fig. 5A–C). This result indicated that the transgenic lines can reduce ROS accumulation and enhance stress tolerance.

Under normal conditions, the MDA contents in the WT and the transgenic lines were no significantly difference. However, after salt treatment, the MDA content in the WT

increased 2.65-fold, whereas it only increased 2.54-fold and 2.03-fold in L1 and L5, respectively (Fig. 6A). Changes to MDA under chilling treatment were correlated with changes in the salt treatment in the WT and the transgenic lines. Similarly, the chlorophyll content in the WT and the transgenic lines was not significantly different under normal conditions, but under salt and chilling stresses the chlorophyll content fell in both the WT plants and the transgenic lines (Fig. 6B). However, chlorophyll content in the WT was significantly lower than in the transgenic lines. These results supported the hypothesis that AsA recycling is an essential part of the enhanced salt and chilling tolerance conferred by *PbDHAR2*.

Discussion

The role of AsA in plant growth, development and the response to various stresses, including salt, chilling, and oxidative stresses, has been well documented (Arrigoni, 1994; Conklin, 2001; Giovannoni, 2007; Horemans et al., 2000; Linster and Clarke, 2008). The DHAR protein catalyzes DHA to AsA in AsA-GSH cycle (Chen and Gallie, 2005; Gallie, 2013b). Thus, the *PbDHAR2* gene may contribute to regulating

Fig. 5. Analysis of antioxidant activity in the wild-type (WT) and the transgenic lines under normal, salt, and chilling conditions. (A) Superoxide dismutase (SOD), (B) peroxidase (POD), and (C) catalase (CAT) activities. Asterisks show that values are significantly different between the WT and the transgenic lines under the same growth conditions (* $P < 0.05$; ** $P < 0.01$).

Fig. 6. Analysis of metabolite levels in the wild-type (WT) and the transgenic lines under normal, salt, and chilling conditions. (A) Malondialdehyde (MDA) and (B) chlorophyll contents. Asterisks show that values are significantly different between the WT and the transgenic lines under the same growth conditions (* $P < 0.05$; ** $P < 0.01$).

AsA levels when a plant is subjected to abiotic stresses. The overexpression of *DHAR1* significantly increased DHAR activities and AsA contents in potato (Qin et al., 2011). It has been reported that the DHAR protein regulated AsA synthesis by elevating the expression of the *DHAR1* gene in tobacco (Chen et al., 2003), but the regulatory mechanism for the *PbDHAR2* gene from *P. sinkiangensis* is still unclear.

In this study, the vector containing the *PbDHAR2* gene was transformed into tomato plants. The *PbDHAR2* gene was successfully introduced and was overexpressed in the L1 and L5 (Fig. 1C). The AsA content significantly rose in the transgenic lines, which indicated that *PbDHAR2* overexpression could regulate AsA content (Fig. 3A). The AsA:DHA ratio in the transgenic lines were higher than in the WT (Fig. 3B). It was suggested that *PbDHAR2* overexpression in the transgenic lines would increase the likelihood that DHA is converted to AsA before decaying, that would lead to the increase in AsA in the transgenic plants. Furthermore, AsA content in plants can be elevated by increasing the expression of *DHAR1*, which affects AsA recycling (Chen et al., 2003). Ushimaru et al. (2006) found that the overexpression of *DHAR1* did not significantly increase the AsA content, but it did enhance *Arabidopsis* plant tolerance to high tempera-

ture stress. In contrast, a study by Haroldsen et al. (2011) supported our conclusion that the overexpression of *DHAR1* produced a 1.6-fold increase in AsA content in tomato compared with the WT. Therefore, *PbDHAR2* overexpression was able to increase the content of AsA, which provided us a new direction to improve the AsA content of pear flesh in the future work.

According to the phenotype analysis, the *PbDHAR2* overexpression lines showed stronger plant growth when subjected to salt and chilling stresses than the WT plants (Fig. 2). These results further confirmed that *PbDHAR2* overexpression could enhance plant resistance to abiotic stresses.

ROS production is induced in response to various stresses (Noctor and Foyer, 1998). In plant cells, ROS can be transformed into H_2O_2 , and the AsA-GSH recycling pathway, including *DHAR1*, *MDHAR*, and *APX*, can scavenge H_2O_2 by converting it into H_2O using AsA (Mittova et al., 2000). We used biochemical and physiological analysis to determine whether ROS accumulation in the transgenic lines was less than in the WT. Our results showed the expression levels of the AsA-GSH cycle genes were upregulated significantly in the transgenic lines under salt and chilling stresses (Fig. 4), implying these regulatory genes participate in the AsA cycle and lead to greater accu-

mulation of AsA. Haroldsen et al. (2011) reported plants overexpressing *DHAR1* and *MDHAR* had higher levels of total AsA compared with plants grown in growth chambers. Overexpression of *APX* increased salt tolerance in tobacco (Wu et al., 2014). Therefore, the increased levels of *DHAR1*, *MDHAR*, and *APX* expression influenced the levels of AsA, thereby resulting in the reduced damage to the plant cells, which might explain the lower levels of ROS in the transgenic lines.

Plants have several enzymatic scavenging systems for ROS detoxification, such as SOD, POD, and CAT (Miller et al., 2010). In our study, the transgenic lines showed higher SOD, POD, and CAT activities under salt and chilling stresses compared with the WT plants (Fig. 5). Abiotic stresses can improve the MDA content (Del Rio et al., 2005). We found the L1 and L5 had a lower content of MDA and a greater content of chlorophyll compared with the WT plants when under salt and chilling stresses (Fig. 6). The transgenic lines lowered their ROS levels by increasing the activities of their enzymatic and nonenzymatic scavenging systems, which explains why the transgenic lines have better phenotypes compared with WT. Interestingly, an increasing number of reports have shown that AsA content rise when plants are under stress (Eltayeb et al.,

2007; Fotopoulos et al., 2006; Lee et al., 2007). Wang et al. (2010) found that the expression of *Arabidopsis DHAR1* protected against oxidative stress. These findings are in agreement with a previous report by Chen and Gallie (2006).

In conclusion, *PbDHAR2* is an important positive regulator of AsA content. The AsA-GSH cycle genes were upregulated by *PbDHAR2* overexpression under salt and chilling stresses, and the enhanced stress tolerance was also correlated with a rise in antioxidant enzymes and antioxidants, which led to more efficient ROS scavenging. These results indicated that overexpression of *PbDHAR2* improved stress tolerance in the L1 and L5. Therefore, the *PbDHAR2* gene has the potential to be a candidate gene for enhancing salt and chilling stresses tolerance in the future.

Literature Cited

- Aloni, B., L. Karni, G. Deventurero, E. Turhan, and H. Aktas. 2008. Changes in ascorbic acid concentration, ascorbate oxidase activity, and apoplastic pH in relation to fruit development in pepper (*Capsicum annuum* L.) and the occurrence of blossom-end rot. *J. Hort. Sci. Biotechnol.* 83:100–105.
- Apel, K. and H. Hirt. 2004. Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* 55:373–399.
- Arrigoni, O. 1994. Ascorbate system in plant development. *J. Biochem. Biom.* 26:407–419.
- Asada, K. 2006. Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant Physiol.* 141:391–396.
- Bai, T., P. Ma, C. Li, R. Yin, and F. Ma. 2013. Role of ascorbic acid in enhancing hypoxia tolerance in roots of sensitive and tolerant apple rootstocks. *Sci. Hort.* 164:372–379.
- Chen, Z. and D.R. Gallie. 2005. Increasing tolerance to ozone by elevating foliar ascorbic acid confers greater protection against ozone than increasing avoidance. *Plant Physiol.* 138:1673–1689.
- Chen, Z. and D.R. Gallie. 2006. Dehydroascorbate reductase affects leaf growth, development, and function. *Plant Physiol.* 142:775–787.
- Chen, Z., T.E. Young, J. Ling, S.C. Chang, and D.R. Gallie. 2003. Increasing vitamin C content of plants through enhanced ascorbate recycling. *Proc. Natl. Acad. Sci. USA* 100:3525–3530.
- Conklin, P. 2001. Recent advances in the role and biosynthesis of ascorbic acid in plants. *Plant Cell Environ.* 24:383–394.
- Davey, M.W., M.V. Montagu, D. Inzé, M. Sanmartin, A. Kanellis, N. Smimoff, I.J.J. Benzie, J.J. Strain, D. Favell, and J. Fletcher. 2000. Plant L-ascorbic acid: Chemistry, function, metabolism, bioavailability and effects of processing. *J. Sci. Food Agr.* 80:825–860.
- Del Rio, D., A.J. Stewart, and N. Pellegrini. 2005. A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutr. Metab. Cardiovas.* 15:316–328.
- Eltayeb, A.E., N. Kawano, G.H. Badawi, H. Kaminaka, T. Sanekata, T. Shibahara, S. Inanaga, and K. Tanaka. 2007. Overexpression of monodehydroascorbate reductase in transgenic tobacco confers enhanced tolerance to ozone, salt and polyethylene glycol stresses. *Planta* 225:1255–1264.
- Eltayeb, A.E., N. Kawano, G.H. Badawi, H. Kaminaka, T. Sanekata, I. Morishima, T. Shibahara, S. Inanaga, and K. Tanaka. 2006. Enhanced tolerance to ozone and drought stresses in transgenic tobacco overexpressing dehydroascorbate reductase in cytosol. *Physiol. Plant* 127:57–65.
- Farooq, M., M. Irfan, T. Aziz, I. Ahmad, and S.A. Cheema. 2013. Seed priming with ascorbic acid improves drought resistance of wheat. *J. Agron. Crop Sci.* 199:12–22.
- Fotopoulos, V., M. Sanmartin, and A.K. Kanellis. 2006. Effect of ascorbate oxidase overexpression on ascorbate recycling gene expression in response to agents imposing oxidative stress. *J. Expt. Bot.* 57:3933–3943.
- Foyer, C.H. and G. Noctor. 2005. Redox homeostasis and antioxidant signaling: A metabolic interface between stress perception and physiological responses. *Plant Cell* 17:1866–1875.
- Gallie, D.R. 2013a. L-ascorbic Acid: A multifunctional molecule supporting plant growth and development. *Scientifica* 2013:795964.
- Gallie, D.R. 2013b. The role of L-ascorbic acid recycling in responding to environmental stress and in promoting plant growth. *J. Expt. Bot.* 64:433–443.
- Gambino, G., I. Perrone, and I. Griboaldo. 2008. A rapid and effective method for RNA extraction from different tissues of grapevine and other woody plants. *Phytochem. Anal.* 19:520–525.
- Giovannoni, J.J. 2007. Completing a pathway to plant vitamin C synthesis. *Proc. Natl. Acad. Sci. USA* 104:9109–9110.
- Harb, J., B. Khraiweh, J. Streif, R. Reski, and W. Frank. 2010. Characterization of blueberry monodehydroascorbate reductase gene and changes in levels of ascorbic acid and the antioxidative capacity of water soluble antioxidants upon storage of fruits under various conditions. *Sci. Hort.* 125:390–395.
- Haroldsen, V.M., C.L. Chi-Ham, S. Kulkarni, A. Lorence, and A.B. Bennett. 2011. Constitutively expressed DHAR and MDHAR influence fruit, but not foliar ascorbate levels in tomato. *Plant Physiol. Biochem.* 49:1244–1249.
- Horemans, N., H. Foyer, C.G. Potters, and H. Asard. 2000. Ascorbate function and associated transport systems in plants. *Plant Physiol. Biochem.* 38:531–540.
- Huang, X.S., J.H. Liu, and X.J. Chen. 2010. Overexpression of PtrABF gene, a bZIP transcription factor isolated from *Poncirus trifoliata*, enhances dehydration and drought tolerance in tobacco via scavenging ROS and modulating expression of stress-responsive genes. *BMC Plant Biol.* 10:230.
- Huang, X.S., T. Luo, X.Z. Fu, Q.J. Fan, and J.H. Liu. 2011. Cloning and molecular characterization of a mitogen-activated protein kinase gene from *Poncirus trifoliata* whose ectopic expression confers dehydration/drought tolerance in transgenic tobacco. *J. Expt. Bot.* 62:5191–5206.
- Koutinas, N., T. Sotiropoulos, A. Petridis, D. Almaliotis, E. Deligeorgis, I. Therios, and N. Voulgarakis. 2010. Effects of preharvest calcium foliar sprays on several fruit quality attributes and nutritional status of the kiwifruit cultivar *Tsechelis*. *HortScience* 45:984–987.
- Kwon, S.Y., S.M. Choi, Y.O. Ahn, H.S. Lee, H.B. Lee, Y.M. Park, and S.S. Kwak. 2003. Enhanced stress-tolerance of transgenic tobacco plants expressing a human dehydroascorbate reductase gene. *J. Plant Physiol.* 160:347–353.
- Larkindale, J., J.D. Hall, M.R. Knight, and E. Vierling. 2005. Heat stress phenotypes of *Arabidopsis* mutants implicate multiple signaling pathways in the acquisition of thermotolerance. *Plant Physiol.* 138:882–897.
- Lee, Y.P., S.H. Kim, J.W. Bang, H.S. Lee, S.S. Kwak, and S.Y. Kwon. 2007. Enhanced tolerance to oxidative stress in transgenic tobacco plants expressing three antioxidant enzymes in chloroplasts. *Plant Cell Rpt.* 26:591–598.
- Li, M., D. Liang, F. Pu, F. Ma, C. Hou, and T. Lu. 2009. Ascorbate levels and the activity of key enzymes in ascorbate biosynthesis and recycling in the leaves of 22 Chinese persimmon cultivars. *Sci. Hort.* 120:250–256.
- Linster, C.L. and S.G. Clarke. 2008. L-Ascorbate biosynthesis in higher plants: The role of VTC2. *Trends Plant Sci.* 13:567–573.
- Liu, J.-H., H. Inoue, and T. Moriguchi. 2008. Salt stress-mediated changes in free polyamine titers and expression of genes responsible for polyamine biosynthesis of apple shoots. *Environ. Expt. Bot.* 62:28–35.
- Livak, K.J. and T.D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25:402–408.
- Melino, V.J., K.L. Soole, and C.M. Ford. 2009. Ascorbate metabolism and the developmental demand for tartaric and oxalic acids in ripening grape berries. *BMC Plant Biol.* 9:145.
- Miller, G., N. Suzuki, S. Ciftci-Yilmaz, and R. Mittler. 2010. Reactive oxygen species homeostasis and signalling during drought and salinity stresses. *Plant Cell Environ.* 33:453–467.
- Mittler, R., S. Vanderauwera, M. Gollery, and F. Van Breusegem. 2004. Reactive oxygen gene network of plants. *Trends Plant Sci.* 9:490–498.
- Mittova, V., M. Volokita, M. Guy, and M. Tal. 2000. Activities of SOD and the ascorbate-glutathione cycle enzymes in subcellular compartments in leaves and roots of the cultivated tomato and its wild salt-tolerant relative *Lycopersicon pennellii*. *Physiol. Plant.* 110:42–51.
- Noctor, G. and C.H. Foyer. 1998. Ascorbate and glutathione: Keeping active oxygen under control. *Annu. Rev. Plant Biol.* 49:249–279.
- Pék, Z., P. Szuvandzsiev, A. Nemenyi, L. Helyes, and A. Lugasi. 2011. The effect of natural light on changes in antioxidant content and color parameters of vine-ripened tomato (*Solanum lycopersicum* L.) fruits. *HortScience* 46:583–585.
- Pignocchi, C. and C.H. Foyer. 2003. Apoplastic ascorbate metabolism and its role in the regulation of cell signalling. *Curr. Opin. Plant Biol.* 6:379–389.
- Potters, G., N. Horemans, R.J. Caubergs, and H. Asard. 2000. Ascorbate and dehydroascorbate influence cell cycle progression in a tobacco cell suspension. *Plant Physiol.* 124:17–20.
- Qin, A., Q. Shi, and X. Yu. 2011. Ascorbic acid contents in transgenic potato plants overexpressing two dehydroascorbate reductase genes. *Mol. Biol. Rpt.* 38:1557–1566.
- Shimaoka, T., A. Yokota, and C. Miyake. 2000. Purification and characterization of chloroplast dehydroascorbate reductase from spinach leaves. *Plant Cell Physiol.* 41:1110–1118.
- Urano, J., T. Nakagawa, Y. Maki, T. Masumura, K. Tanaka, N. Murata, and T. Ushimaru. 2000. Molecular cloning and characterization of a rice dehydroascorbate reductase. *FEBS Lett.* 466:107–111.
- Ushimaru, T., T. Nakagawa, Y. Fujioka, K. Daicho, M. Naito, Y. Yamauchi, H. Nonaka, K. Amako, K. Yamawaki, and N. Murata. 2006. Transgenic *Arabidopsis* plants expressing the rice dehydroascorbate reductase gene are resistant to salt stress. *J. Plant Physiol.* 163:1179–1184.
- Wang, Z., Y. Xiao, W. Chen, K. Tang, and L. Zhang. 2010. Increased vitamin C content accompanied by an enhanced recycling pathway

- confers oxidative stress tolerance in *Arabidopsis*. *J. Integr. Plant Biol.* 52:400–409.
- Wu, G., G. Wang, J. Ji, H. Gao, W. Guan, J. Wu, C.F. Guan, and Y. Wang. 2014. Cloning of a cytosolic ascorbate peroxidase gene from *Lycium chinense* Mill. and enhanced salt tolerance by overexpressing in tobacco. *Gene* 543:85–92.
- Wu, J., Z. Wang, Z. Shi, S. Zhang, R. Ming, S. Zhu, M.A. Khan, S. Tao, S.S. Korban, H. Wang, N.J. Chen, T. Nishio, X. Xu, L. Cong, K. Qi, X. Huang, Y. Wang, X. Zhao, J. Wu, C. Deng, C. Gou, W. Zhou, H. Yin, G. Qin, Y. Sha, Y. Tao, H. Chen, Y. Yang, Y. Song, D. Zhan, J. Wang, L. Li, M. Dai, C. Gu, Y. Wang, D. Shi, X. Wang, H. Zhang, L. Zeng, D. Zheng, C. Wang, M. Chen, G. Wang, L. Xie, V. Sovero, S. Sha, W. Huang, S. Zhang, M. Zhang, J. Sun, L. Xu, Y. Li, X. Liu, Q. Li, J. Shen, J. Wang, R.E. Paull, J.L. Bennetzen, J. Wang, and S. Zhang. 2013. The genome of the pear (*Pyrus bretschneideri* Rehd.). *Genome Res.* 23:396–408.
- Zhang, Z., J. Wang, R. Zhang, and R. Huang. 2012. The ethylene response factor AtERF98 enhances tolerance to salt through the transcriptional activation of ascorbic acid synthesis in *Arabidopsis*. *Plant J.* 71:273–287.
- Zou, L., H. Li, B. Ouyang, J. Zhang, and Z. Ye. 2006. Cloning and mapping of genes involved in tomato ascorbic acid biosynthesis and metabolism. *Plant Sci.* 170:120–127.