Enhanced Ascorbic Acid Accumulation through Overexpression of Dehydroascorbate Reductase Confers Tolerance to Methyl Viologen and Salt Stresses in Tomato

Qingzhu LI, Yansu LI, Chaohan LI and Xianchang YU

Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing, China

Abstract: As an important antioxidant for plants and humans, L-ascorbic acid (AsA, vitamin C) can scavenge reactive oxygen species (ROS) and can be regenerated from its oxidized form in a reaction catalyzed by dehydroascorbate reductase (DHAR). To analyse the effect of overexpressing *DHAR* on tomato (*Solanum lycopersicum*), an expression vector containing potato cytosolic *DHAR* (*DHAR1*) or chloroplastic *DHAR* (*DHAR2*) cDNA driven by a cauliflower mosaic virus 35S promoter was transferred into tomato plants. Compared with the wild type (WT), *DHAR1* overexpression increased DHAR activity and AsA content in both leaves and fruits, while *DHAR2* overexpression increased DHAR activity and AsA content mainly in leaves. *DHAR1* and *DHAR2* overexpression increased the chlorophyll content and photosynthetic rate of transgenic lines, but had no effect on plant height and stem diameter. Furthermore, the germination rate, plant fresh weight, seedling length and chlorophyll content of transgenic *DHAR1* and *DHAR2* plants under salt stress were higher than those of WT plants. In addition, the transgenic plants also exhibited considerable tolerance to oxidative damage induced by methyl viologen (MV). Taken together, these results indicated that overexpressing potato *DHAR1* and *DHAR2* enhanced the level of AsA in tomato and, consequently, increased the tolerance of tomato to salt and MV stress.

Keywords: chloroplastic *DHAR*; cytosolic *DHAR*; oxidative damage; stress tolerance; vitamin C

L-Ascorbic acid (AsA, vitamin C) has important biological functions in both plants and animals. It participates in diverse biological processes, such as pathogen defence mechanisms and modulation of plant growth and morphology (Smirnoff & Wheeler 2000). Most importantly, AsA plays an essential role as an antioxidant in several pathways, and protects plants from oxidative damage by scavenging free radicals and reactive oxygen species (ROS) (Loewus 1999). In plants, AsA is highly abundant and accumulates to 2–25 mmol/l (Davey *et al*. 2000). However, humans lack the ability to synthesize AsA, so it must be obtained from food regularly. Recent studies indicated that the increased AsA intake (from 60 to 200 mg/day) may be beneficial for human health (Carr & Frei 1999; Levine *et al*. 1999). Therefore, an increased AsA content in plant foods would increase their nutritive value.

A variety of abiotic stresses lead to the production of reactive oxygen species (ROS) in plants (Smirnoff 1998). The presence of ROS leads to the induction of antioxidative enzymes, which are able to reduce the oxidizing environment (Blokhina *et al*. 2003). AsA is oxidized to monodehydroascorbate (MDHA) and then to dehydroascorbate (DHA) in the process of enzymatic and non-enzymatic ROS scavenging reactions. DHA undergoes reversible spontaneous hydrolysis to 2,3-diketogulonic acid or is recycled to AsA by dehydroascorbate

reductase (DHAR), which uses glutathione (GSH) as a reductant. Thus DHAR-catalyzed rapid regeneration of AsA is necessary to maintain the antioxidative capacity of AsA (Chen *et al*. 2003). In recent years, many studies have investigated the *DHAR* gene and its function. Researchers found out that the overexpression of *DHAR* in different plants, including *Arabidopsis,* tobacco, wheat, and potato, could improve ascorbate recycling and increase the level of AsA, which in turn could improve tolerance to numerous stresses, such as salt, high light, high temperature and methyl viologen (MV) stresses (Chen *et al*. 2003; Kwon *et al*. 2003; Chen & Gallie 2004; Dolatabadian *et al*. 2008; Qin *et al*. 2011). These results indicate that DHAR not only has important physiological functions, but also can be applied to improve the AsA content and stress tolerance of crop plants.

However, there has been no study to examine the effect of overexpressing potato *DHAR* genes in transgenic tomato plants. In the present study, the potato *DHAR1* and *DHAR2* genes were introduced into tomato to investigate the effect of *DHAR* overexpression on AsA content of tomato and the change in the intracellular redox state of transgenic tomato plants overexpressing *DHAR1* or *DHAR2* genes and the effect of *DHAR* overexpression on plant tolerance to methyl viologen and salt stresses.

MATERIAL AND METHODS

Plant materials and plant growth conditions

Solanum lycopersicum cv. Sy12f was used as the wild type. The fully-grown transgenic plants (T_0 generation) were self-pollinated using glassine envelopes to produce T_1 seeds. Seeds of transgenic lines that were selected with 100 mg/l kanamycin solution on moistened filter paper were grown in a growth room at 28°C for 3 days. Sprouted seedlings were transplanted into 10 cm diameter pots containing a commercial mineral-mixed soil for 3–4 weeks. The seedlings were transplanted into soil and grown in a greenhouse at 26 ± 4°C and 50–60% relative humidity with an 11 h light and 13 h dark cycle. The average photon flux density (PFD) in the morning (9:00) was 570 ± 10 μmol/m^2/s and in the afternoon (13:00) it was 1170 ± 50 μmol/m^2/s. Plants were watered every 4 days. For the analysis of gene expression and enzyme activity, functional leaves (fourth to sixth leaves) and mature fruits (45 days after self-pollination) were used as samples.

Construction of plant expression vector and tomato transformation

cDNAs encoding two isoforms of *DHARs* localized in the cytosol (*DHAR1*, GenBank ID: EF030707) and chloroplasts (*DHAR2*, GenBank ID: FJ477252) from potato were isolated and characterized, and plant expression vectors were constructed as described by Qin *et al*. (2011) (Figure 1a). Transgenic tomato plants expressing potato *DHAR* using the cauliflower mosaic virus (CaMV) 35S promoter in the pBI-121 binary vector were generated using *Agrobacterium tumefaciens* -mediated leaf-disk transformation (Horsch *et al*. 1985). Disks infected with *A. tumefaciens* were incubated on Murashige and Skoog (MS) medium (pH 5.8) supplemented with 2.0 mg/l zeatin (ZT), 0.5 mg/l IAA, 500 mg/l kanamycin and 500 mg/l carbenicillin (Cb) to induce shoots. Regenerated shoots showing kanamycin resistance were selected on MS medium supplemented with 250 mg/l cefotaxime sodium, 50 μg/l kanamycin, 2.0 mg/l ZT, and 0.25 mg/l indoleacetic acid (IAA). After several weeks, the regenerated shoots were transferred to a root-inducing medium comprising 1/2 MS medium supplemented with 0.15 mg/l IAA.

Identification of transgenic plants and analysis of transcript expression

Genomic DNA was isolated from 0.5 g young leaves by the CTAB method (Qin *et al*. 2011). The primer (5'-GTAATCTCCACTGACGTAAG-3'), which was derived from the CaMV 35S promoter sequence, was chosen as the forward primer. The presence of cytosolic *DHAR* and chloroplastic *DHAR* was confirmed by PCR analysis using the primer in combination with the cytosolic *DHAR* reverse primer (5'-CCATCGAACTGCTAACTAG-3') and the chloroplastic *DHAR* reverse primer (5'-TCAGGCTATCATTCAGTG-3'), respectively.

Real-time RT-PCR was used for the analysis of target gene expression in transgenic plants. Leaves which can photosynthesize with a net photosynthetic rate > 0 (termed as functional leaves in this article) and fruits were used as fresh material. Transgenic lines D1-2, D1-4, D1-5, D2-3, D2-5 and

D2-6 were used for the analysis. For real-time RT-PCR, gene-specific primers for cytosolic *DHAR* (forward: 5'-CCAGAATACCAAGGCTGAAGAAA-3'; reverse: 5'-TGCTAACTACCCGAGACACAACA-3') and chloroplastic *DHAR* (forward: 5'-ATCAATGGCGGCTTCACTC-3'; reverse: 5'-CTTTACCTTCGGGGCTTATCTTC-3') were designed, and the elongation factor 1-α gene (GenBank accession number: AB061263) (forward: 5'-ATTGGAAACGGATATGCTCCA-3'; reverse: 5'-TCCTTACCTGAACGCCTGTCA-3') was chosen as a control. All primers were synthesized by TaKaRa Company (Otsu Shinga, Japan). SYBR green RT-PCR amplification of each cDNA was performed using the SYBR PrimeScript^{TM} RT-PCR Kit (TaKaRa) and a LightCycler 2.0 real-time PCR machine (Roche, Mannheim, Germany). Real-time RT-PCR was carried out with the following program: 95°C for 10 s; 40 cycles of 95°C for 5 s and 60°C for 60 s. Relative quantification of the mRNA level was computed by the comparative Ct ($2^{-\Delta\Delta Ct}$) method (Bubner & Baldwin 2004).

Enzyme assays and AsA determination

Activity of DHAR was measured using the method of Chen and Gallie (2004) with slight modification. Fresh material of leaves (0.5 g) or fruits (0.1 g) was ground in cold extraction buffer (50 mmol/l Tris-HCl, pH 7.2, 0.3 mol/l mannitol, 1 mmol/l EDTA, 1% bovine serum albumen, 0.05% L-cysteine and 2% (w/v) polyvinylpyrrolidone), and then centrifuged at 4°C at 12 000 × g for 10 min. The 3 ml reaction mixture contained 50 mmol/l phosphate buffer (pH 7.0), 0.1 mmol/l EDTA, 0.5 mmol/l DHA, 3.5 mmol/l GSH and 100 µl enzyme extract. The increase in absorbance at 290 nm was monitored, and the activity was calculated using an absorbance coefficient of 14.3 mmol/l/cm.

Activity of monodehydroascorbate reductase (MDHAR) was determined using the method of Jimenez *et al.* (1997) with slight modification. The reaction mixture contained 100 mmol/l Tris-HCl (pH 6.3), 0.2 mmol/l NADH, 1 mmol/l AsA, and 1 u AsA oxidase. The reaction was initiated by adding AsA oxidase to the mixture, thus generating the substrate MDHA. Activity was measured as the AsA oxidase-induced oxidation of NADH. The reaction was monitored at 340 nm (extinction coefficient for NADH 6.2 mmol/l/cm).

Activity of L-galactono-1,4-lactone dehydrogenase (GalLDH) was determined according to the method of Tabata *et al.* (2001). Ascorbate peroxidase (APX) activity was determined using the method described by Pignocchi *et al.* (2006). AsA content was measured according to the method of Luwe *et al.* (1993).

Determination of net photosynthetic rate and chlorophyll content

Net photosynthetic rate (P_n) was measured with a portable photosynthetic system (CIRAS-2, PP Systems, Herts, UK) between 9:30 and 10:30. The PFD was controlled to 800 ± 10 µmol/m^2/s and the relative humidity was controlled to 70–80% by the system. Chlorophyll content was determined according to the method of Arnon (1949).

Leaf disk assay for MV sensitivity and determination of chlorophyll content

Damage induced by MV was analysed by a leaf disk method as described by Lee *et al.* (2007) with slight modifications. Leaf disks (8 mm diameter) from the third leaves of five different T_1 plants of the D1-4 and D2-5 transgenic lines and wild-type plants were floated on 5 ml MV solution at different concentrations (0, 2, 50, or 100 µmol/l) in 5 cm Petri dishes. Fifteen leaf disks were placed in each Petri dish, which were incubated at 25°C for 24 h under continuous light of 100 µmol/m^2/s. The effect of MV on leaf disks was analysed by monitoring phenotypic changes and measuring the chlorophyll content. The chlorophyll content in the leaf disks was quantified according to the procedure of Upadhyaya *et al.* (2010).

Salt stress treatment

Transgenic lines D1-4 and D2-5 and wild-type tomato seeds were surface-sterilized for 1–2 min in 70% ethanol, 15 min in 4% sodium hypochlorite, and then rinsed thoroughly with sterile distilled water. Thereafter, the seeds were placed on MS medium containing 0 or 100 mmol/l NaCl in closed Petri dishes or plant tissue culture containers and incubated under 100 µmol/m^2/s PFD at 25°C/22°C (day/night) with a photoperiod of 16 h/8 h (light/dark).

Germination rate was measured after 12 days of salt stress treatment. After 40-day treatment,

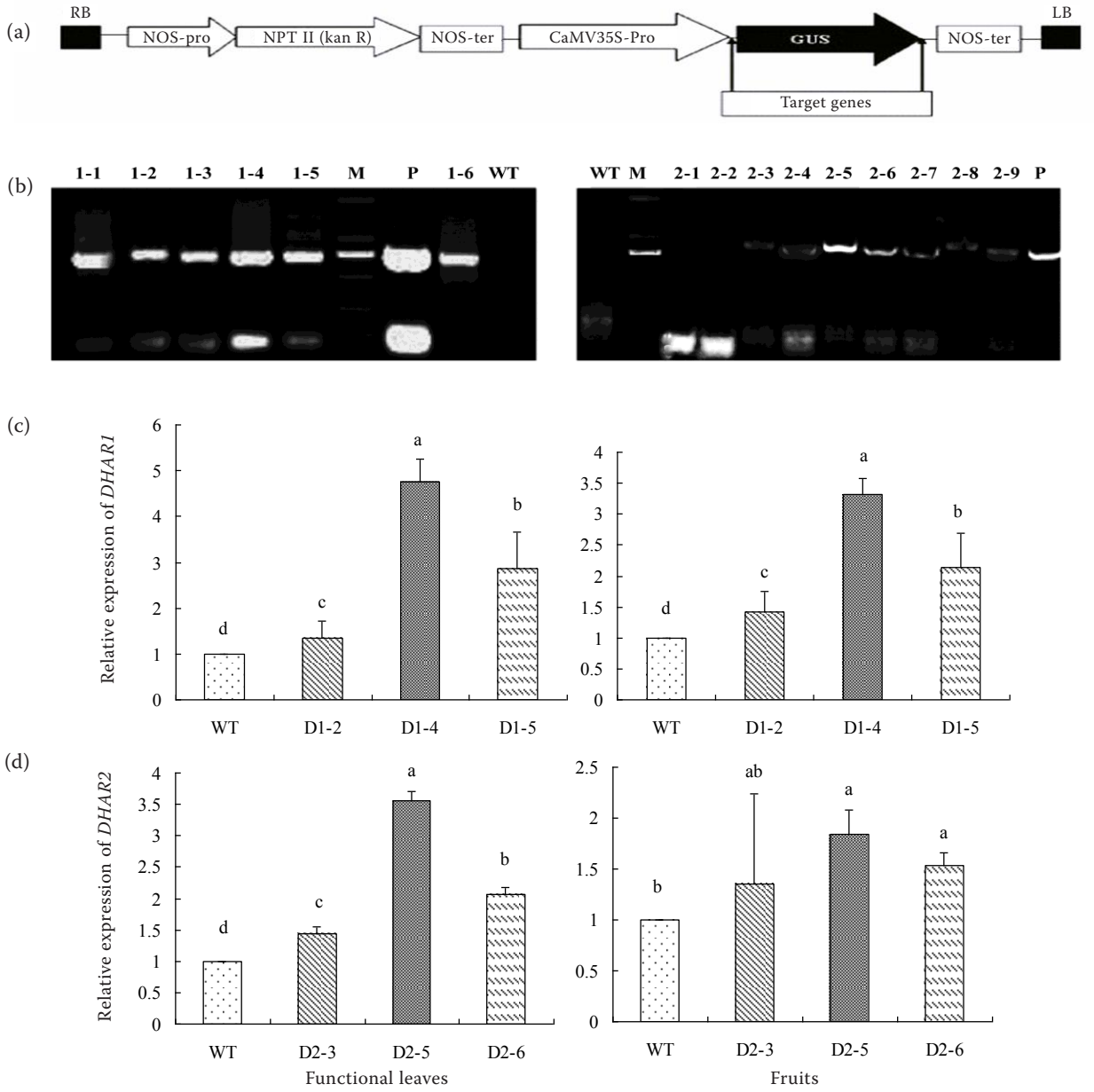

Figure 1. Development of tomato plants overexpressing *DHAR1* and *DHAR2* and expression of the genes in transgenic plants; (a) vector construct for expressing the *DHAR* genes in transgenic tomato plants;the plasmid pBI-121 contained the ORF of the target genes under control of the CaMV35S promoter and NOS terminator, in a binary vector for *Agrobacterium* transformation; kanamycin resistance genes (NPTII) were used as selection markers; LB – left T-DNA border; RB – right T-DNA border; NOS-pro – nopaline synthase promoter; GUS – protein-coding region of a gene for β-glucuronidase; the insertion sites of *DHAR1* and *DHAR2* are *Xba* I + *Sal* I and *Xba* I + *Sac* I; (b) confirmation of *DHAR1* (b-1) and *DHAR2* (b-2) transformants by PCR analysis; P – positive control (PCR with recombinant pBI vector carrying *DHAR1* or *DHAR2*); M – marker; WT – wild-type plant; *DHAR1* transgenic lines are represented by D1-1, D1-2, D1-3, D1-4, D1-5, and D1-6, and *DHAR2* transgenic lines are represented by D2-3, D2-4, D2-5, D2-6, D2-7, D2-8, and D2-9; (c) Relative expression of *DHAR1* in transgenic tomato plants; (d) relative expression of *DHAR2* in transgenic tomato plants; to analyse the expression of *DHAR1* and *DHAR2*, real-time RT-PCR was performed on mRNA extracted from functional leaves and mature fruits of *DHAR1* (D1-2, D1-4, D1-5) and *DHAR2* (D2-3, D2-5, D2-6) transformants as detailed in the text; the relative quantification of *DHAR1* and *DHAR2* expression was calculated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method; data are expressed as means ± SD of three replicates; the same letter above bars indicates a non-significant difference at the $P < 0.05$ probability level

growth parameters (plant height, plant fresh weight) and chlorophyll content were measured.

Statistical analysis

Values presented are means ± standard deviations (SD). Statistical analyses were carried out by analysis of variance (ANOVA) using DPS software. Duncan's multiple range test was applied to examine the significance of differences between treatments.

RESULTS

Expression of *DHAR1* and *DHAR2* in transgenic plants

The analysis of PCR products amplified from *DHAR1* transgenic lines (D1-1 to D1-6) and *DHAR2* transgenic lines (D2-3 to D2-9) confirmed that transformants carrying *DHAR1* and *DHAR2* were generated (Figure 1b). The lines D1-2, D1-4, D1-5, D2-3, D2-5, and D2-6 were chosen as representative *DHAR1* and *DHAR2* transgenic lines. The real-time RT-PCR analysis using cDNA from confirmed transformants and WT showed that, compared with WT, *DHAR1* expression in the D1-2, D1-4 and D1-5 lines was increased 1.3, 4.7 and 2.9 fold in functional leaves, and 1.4, 3.3 and 2.1 fold in mature fruits, respectively; *DHAR2* expression in the D2-3, D2-5 and D2-6 lines was increased 1.5, 3.6 and 2.0 fold in functional leaves, and 1.3, 1.8 and 1.5 fold in fruits, respectively (Figure 1c, d). These results indicated that the two potato *DHAR* genes were successfully introduced into tomato plants, and that the expression of both *DHAR1* and *DHAR2* was enhanced in transgenic plants.

Enzyme activities and AsA content in T_1 generation of *DHAR* transgenic plants

Compared with wild-type plants (WT), the DHAR activity of D1-2, D1-4 and D1-5 lines increased by 36.6%, 87.8% and 43.9% in functional leaves, and by 5.7%, 28% and 12% in fruits, respectively. By contrast, the D2-3, D2-5 and D2-6 lines, respectively, exhibited 18%, 117.1% and 39.1% higher DHAR activity than WT in functional leaves, but the DHAR activity in fruits was not significantly changed (Table 1).

The AsA and AsA+DHA contents were also increased in the six transgenic lines. The AsA contents of D1-2, D1-4 and D1-5 increased by 20.3%, 90.6% and 39.1% in functional leaves, and by 15.2%, 36.4% and 21.2% in fruits, respectively (Table 2). With regard to D2-3, D2-5 and D2-6, AsA contents were 14.1%, 84.4% and 14.0% higher, respectively, in functional leaves in these lines than in WT, but they did not show a significant change in fruits (Table 2). Compared with WT, the DHA content of D2-5 decreased by 6.9% in functional leaves, but that of the other five transgenic lines was not significantly changed either in functional leaves or in fruits (Table 2).

Thus, the total ascorbate (AsA + DHA) content of the six transgenic lines showed a similar but smaller change compared to that of AsA content. For example, compared to WT plants, total ascorbate increased 19.0%, 35.5% and 22.4% in D1-2, D1-4 and D1-5 in functional leaves and 9.1%, 23.6% and 14.5% in their fruits, respectively; in D2-3, D2-5 and D2-6, total ascorbate increased in functional leaves 5.6%, 47.7% and 6.5% , respectively, but it did not show a significant change in fruits (Table 2). The ratio of AsA/DHA, a valuable indicator of the available AsA level, was also significantly enhanced in leaves of the six transgenic lines (Table 2).

An increase in AsA could result from increased biosynthesis or regeneration. To investigate the former possibility, the level of GalLDH, which catalyzes the terminal step in ascorbate biosynthesis, was measured in leaves of tomato plants (Table 1). GalLDH activity in the six *DHAR*-overexpressing lines was not significantly different from that of wild-type plants, indicating that the observed increase in AsA content in *DHAR*-overexpressing plants did not result from increased biosynthesis. Moreover, no significant change in the activities of MDHAR and APX, which are also two key enzymes in the AsA-GSH cycle, was observed, implying that the increase in AsA content in *DHAR*-overexpressing plants was not closely related to MDHAR and APX activity (Table 1).

Growth parameters, net photosynthetic rate and chlorophyll content of T_1 generation *DHAR* transgenic plants

To examine the effect of increased DHAR activity and AsA content, which resulted from the overexpression of *DHAR*, on physiological characteristics

Table 1. Activities of DHAR, MDHAR, APX, and GalLDH in functional leaves and mature fruits (in U/g FW) of *DHAR1* and *DHAR2* transformants and wild-type plants (WT); *DHAR1* transgenic lines are represented by D1-2, D1-4 and D1-5, and *DHAR2* transgenic lines are represented by D2-3, D2-5 and D2-6; data are expressed as means ± SD of three replicates; values within a row followed by the same letter are not statistically different at the $P < 0.05$ probability level

		WT	D1-2	D1-4	D1-5	D2-3	D2-5	D2-6
Functional leaves	DHAR	4.11 ± 0.15^{d}	5.59 ± 0.89^{c}	7.72 ± 0.64^{b}	5.92 ± 0.16^{c}	4.96 ± 0.20^{cd}	8.92 ± 0.61^{a}	5.70 ± 0.49^{c}
	MDHAR	5.36 ± 0.46^{a}	5.42 ± 0.74^{a}	5.69 ± 0.34^{a}	5.72 ± 0.18^{a}	5.23 ± 0.48^{a}	5.41 ± 0.38^{a}	5.40 ± 0.37^{a}
	APX	34.15 ± 1.07^{a}	35.20 ± 1.62^{a}	33.17 ± 1.70^{a}	34.19 ± 1.73^{a}	33.85 ± 1.54^{a}	33.73 ± 0.79^{a}	34.03 ± 1.45^{a}
	GalLDH	4.22 ± 0.27^{ab}	4.43 ± 0.09^{a}	4.28 ± 0.15^{ab}	4.34 ± 0.18^{ab}	4.06 ± 0.15^{b}	4.11 ± 0.19^{ab}	4.26 ± 0.17^{ab}
Fruits	DHAR	5.01 ± 1.10^{b}	5.28 ± 0.26^{b}	6.36 ± 0.53^{a}	5.57 ± 0.08^{ab}	5.07 ± 0.17^{b}	5.33 ± 0.27^{b}	5.20 ± 0.62^{b}
	MDHAR	3.82 ± 0.23^{a}	3.99 ± 0.21^{a}	3.82 ± 0.48^{a}	4.17 ± 0.74^{a}	3.87 ± 0.09^{a}	4.10 ± 0.20^{a}	3.88 ± 0.14^{a}
	APX	26.33 ± 0.34^{a}	27.05 ± 1.35^{a}	26.78 ± 1.21^{a}	26.19 ± 1.98^{a}	26.81 ± 1.18^{a}	26.08 ± 1.22^{a}	25.97 ± 1.28^{a}
	GalLDH	3.51 ± 0.21^{a}	3.65 ± 0.35^{a}	3.45 ± 0.09^{a}	3.41 ± 0.19^{a}	3.35 ± 0.13^{a}	3.64 ± 0.22^{a}	3.43 ± 0.15^{a}

Table 2. Contents of AsA, DHA, AsA+DHA and AsA redox status (AsA/DHA) in functional leaves and mature fruits (in mg/g FW) of *DHAR1* and *DHAR2* transgenic lines and wild-type plants (WT); *DHAR1* transgenic lines are represented by D1-2, D1-4 and D1-5, and *DHAR2* transgenic lines are represented by D2-3, D2-5 and D2-6; data are expressed as means ± SD of three replicates; values within the same row followed by the same letter are not statistically different at the $P < 0.05$ probability level

		WT	D1-2	D1-4	D1-5	D2-3	D2-5	D2-6
Functional leaves	AsA	0.64 ± 0.08^{d}	0.77 ± 0.03^{c}	1.22 ± 0.07^{a}	0.89 ± 0.04^{b}	0.73 ± 0.02^{cd}	1.18 ± 0.04^{a}	0.73 ± 0.06^{cd}
	DHA	0.43 ± 0.03^{a}	0.42 ± 0.007^{ab}	0.44 ± 0.02^{a}	0.42 ± 0.01^{ab}	0.40 ± 0.02^{b}	0.40 ± 0.01^{ab}	0.41 ± 0.02^{ab}
	AsA+DHA	1.07 ± 0.09^{d}	1.19 ± 0.03^{c}	1.66 ± 0.07^{a}	1.31 ± 0.06^{b}	1.13 ± 0.008^{cd}	1.58 ± 0.03^{a}	1.14 ± 0.04^{cd}
	AsA/DHA	1.49 ± 0.16^{d}	1.84 ± 0.06^{c}	2.82 ± 0.19^{a}	2.12 ± 0.04^{b}	1.85 ± 0.15^{c}	2.93 ± 0.16^{a}	1.77 ± 0.21^{c}
Fruits	AsA	0.33 ± 0.04^{c}	0.38 ± 0.03^{bc}	0.45 ± 0.06^{a}	0.40 ± 0.03^{ab}	0.32 ± 0.02^{c}	0.37 ± 0.02^{bc}	0.35 ± 0.01b^{c}
	DHA	0.22 ± 0.005^{ab}	0.22 ± 0.004^{ab}	0.23 ± 0.008^{a}	0.23 ± 0.01^{ab}	0.22 ± 0.008^{ab}	0.21 ± 0.002^{b}	0.22 ± 0.006^{ab}
	AsA+DHA	0.55 ± 0.04^{c}	0.60 ± 0.03^{bc}	0.68 ± 0.05^{a}	0.63 ± 0.02^{ab}	0.54 ± 0.03^{c}	0.58 ± 0.02^{bc}	0.57 ± 0.007^{bc}
	AsA/DHA	1.50 ± 0.21^{b}	1.70 ± 0.12^{ab}	1.96 ± 0.31^{a}	1.78 ± 0.17^{ab}	1.48 ± 0.11^{b}	1.71 ± 0.10^{ab}	1.60 ± 0.11^{b}

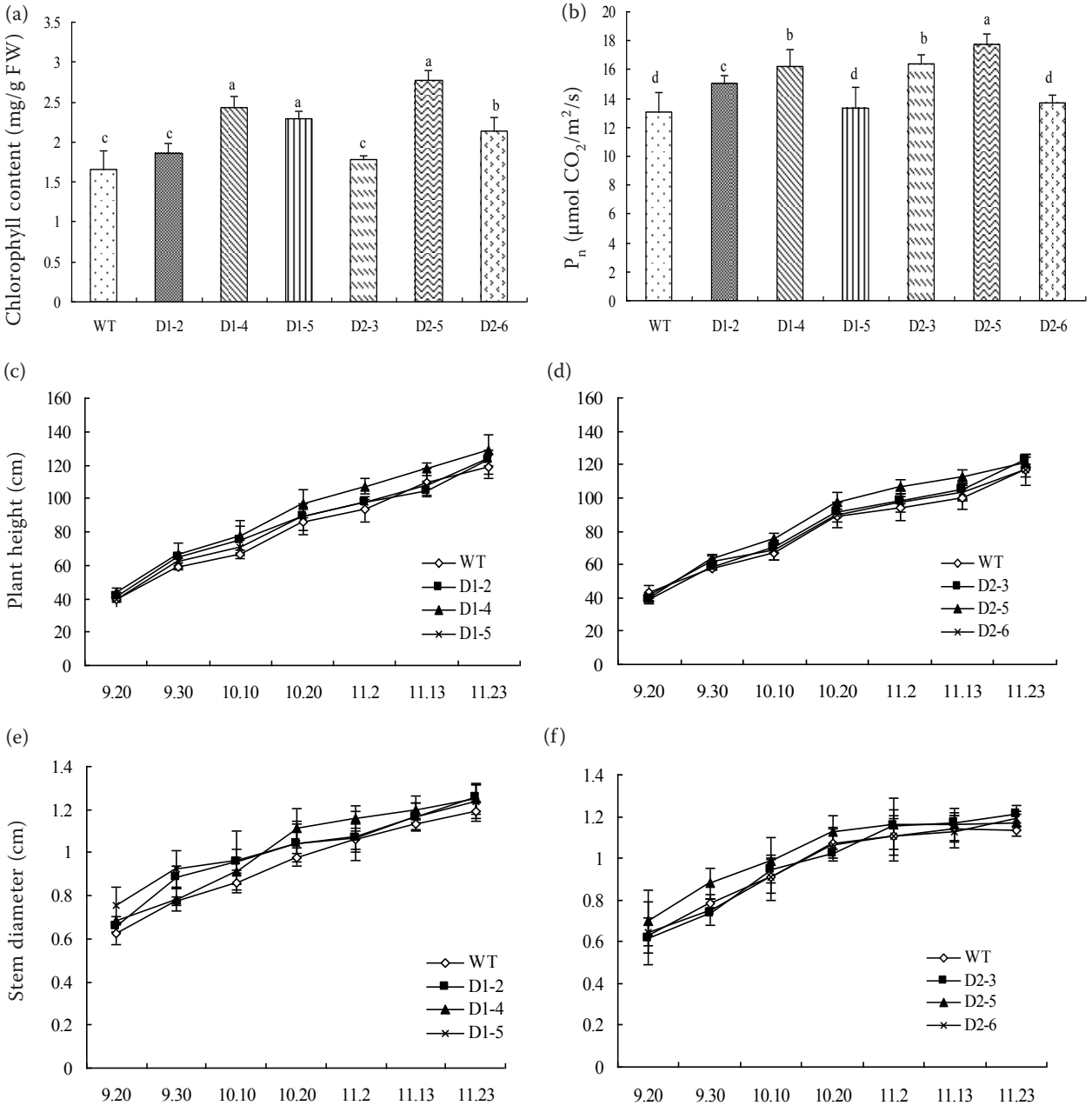

Figure 2. Chlorophyll content, net photosynthetic rate (P_n) and plant growth parameters in *DHAR1* and *DHAR2* transgenic lines and wild-type plants (WT); (a) chlorophyll content; (b) P_n; (c) plant height of *DHAR1* transgenic lines; (d) plant height of *DHAR2* transgenic lines; (e) stem diameter of *DHAR1* transgenic lines; (f) stem diameter of *DHAR2* transgenic lines; *DHAR1* transgenic lines are represented by D1-2, D1-4 and D1-5; *DHAR2* transgenic lines are represented by D2-3, D2-5 and D2-6; data are expressed as means ± SD of three replicates; the same letter above bars indicates a non-significant difference at the $P < 0.05$ probability level

of tomato plants, plant growth, net photosynthetic rate and chlorophyll content of transgenic plants were measured.

The transgenic lines showed higher chlorophyll contents than WT. D1-2, D1-4, D1-5, D2-3, D2-5 and D2-6 showed higher chlorophyll contents of 12.7, 45.5, 33.3, 7.9, 67.9, and 29.7%, respectively, in functional leaves compared to WT (Figure 2a). The chlorophyll level was correlated with the leaf photosynthetic capacity to some extent. In the transgenic lines P_n showed a similar change to that of chlorophyll content: P_n of D1-2, D1-4 and D1-5 increased in functional leaves by 14.5, 23.7 and 1.5%, respectively; in D2-3, D2-5 and D2-6

P_n increased in functional leaves by 24.4, 35.1 % and 3.8%, respectively, compared to WT (Figure 2b).

Plant height and stem diameter were slightly altered in transgenic lines relative to the control at early developmental stages (Figures 2c—f). However, plant height and stem diameter of the six transgenic lines were similar to those of WT plants at the end of the experiment.

Overexpression of *DHAR1* and *DHAR2* enhanced tolerance of tomato leaves to MV stress

Because DHAR activity and AsA content of the D1-4 and D2-5 lines were significantly different from those of WT plants, the D1-4 and D2-5 lines were chosen as representative *DHAR1* and *DHAR2* transgenic lines to analyse the effect of *DHAR* overexpression on the tolerance of tomato to MV and salt stress.

It is reported that AsA contributes to plant tolerance to oxidative stress caused by a variety of abiotic stresses. Leaf disks treated with distilled water remained green in both WT and transgenic lines. However, severe necrosis was observed in the leaf disks of WT plants treated with 50 or 100 μmol/l MV, while only partial necrosis was observed in leaf disks of the transgenic plants at the same MV concentration (Figure 3a).

The observed necrosis was a result of chlorophyll degradation, so the chlorophyll content of leaf disks was determined. With the increasing MV concentration, the chlorophyll content of D1-4, D2-5 and WT decreased gradually. However, the chlorophyll content in the leaf disks of D1-4 and D2-5 plants was higher than that of disks from WT plants. For example, compared to WT plants, chlorophyll content was 85% and 107% higher in leaf disks of D1-4 and D2-5, respectively, in 100 μmol/l MV solution (Figure 3b).

Overexpression of *DHAR1* and *DHAR2* enhanced tolerance of tomato to salt stress

To test whether the increased DHAR activity and AsA content increased the salt stress resistance of tomato, the effects of salt stress on seed germination and seedling growth of transgenic and WT plants were examined. In the controls, the germination rates of D1-4, D2-5 and WT seeds were similar (Figure 4a). However, the germination of WT seeds was severely inhibited in a medium containing 100 mmol/l NaCl, whereas the transgenic lines were much less severely affected (Figure 4a).

The growth of *DHAR* transgenic plants under NaCl stress was less severely affected compared to that of WT (Figures 4b—e). D1-4 and D2-5 trans-

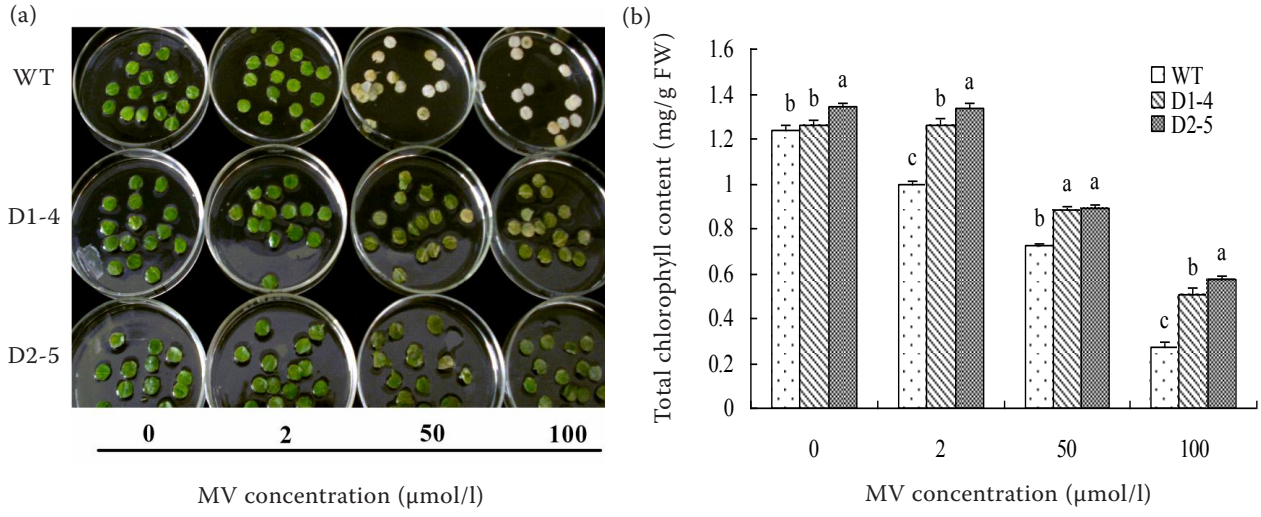

Figure 3. Methyl viologen (MV) induced oxidative damage to leaf disks from wild-type (WT) plants and the D1-4 and D2-5 transgenic lines; (a) phenotypic differences in leaf disks from transgenic and WT plants after MV treatment for 24 h; (b) chlorophyll content in leaf disks of transgenic lines and WT plants treated with 0, 2, 50 or 100 μmol/l MV solution for 24 h; data are expressed as means ± SD of three replicates; the same letter above bars indicates a non-significant difference at the $P < 0.05$ probability level

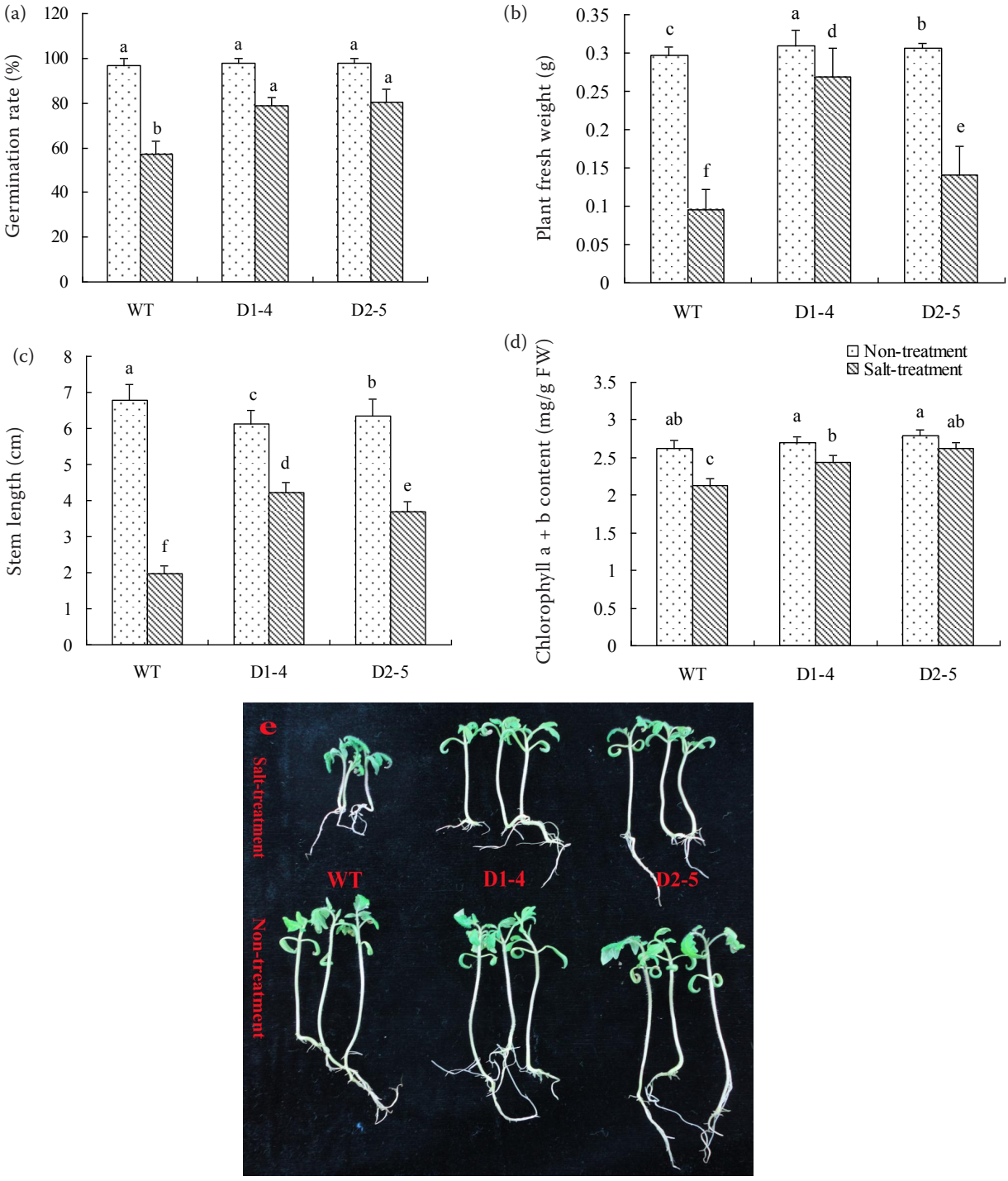

Figure 4. Effects of NaCl on seed germination and seedling growth of *DHAR1* (D1-4) and *DHAR2* (D2-5) transgenic lines and wild-type (WT) plants; (a) germination rate of WT and transgenic tomato seeds; seeds of WT and transgenic plants were germinated on MS with or without 100 mmol/l NaCl; plates were incubated at 25 ± 3°C; (b) plant fresh weight; (c) plant height; (d) chlorophyll content of WT and transgenic tomato plants; seeds of WT and transgenic plants were grown in plant tissue culture containers containing MS basal medium supplemented with 100 mmol/l NaCl at 25°C/22°C (day/night) with a 16 h/8 h (light/dark) photoperiod for 40 days; six independent seedlings of WT and each transgenic line were chosen, and plant fresh weight, plant height and chlorophyll content were measured; (e) phenotypic differences between transgenic and WT seedlings after treatment with 100 mmol/l NaCl for 40 days; data are expressed as means ± SD of six replicates; the same letter above bars indicates a non-significant difference at the $P < 0.05$ probability level

genic plants treated with 100 mmol/l NaCl showed 2.9-fold and 1.5-fold higher plant fresh weights, respectively, as well as 2.1-fold and 1.8-fold higher plant heights than WT plants (Figures 4b, c). Furthermore, the root growth in D1-4 and D2-5 plants was less affected by 100 mmol/l NaCl (Figure 4e). A similar response was observed for chlorophyll content; D1-4 and D2-5 plants treated with 100 mmol/l NaCl showed 14% and 23% higher chlorophyll contents, respectively, than those of WT plants (Figure 4d). These results indicated that the overexpression of potato *DHAR1* and *DHAR2* in tomato plants provided increased protection against oxidative stress caused by high salt.

DISCUSSION

In plants, AsA plays an important role in antioxidant defence, photoprotection, regulation of cell division and growth, as well as delaying senescence (Smirnoff 1996; Davey *et al*. 2000; Smirnoff & Wheeler 2000). DHAR is one of the key enzymes in the AsA-GSH cycle and allows the plant to recycle DHA before it undergoes irreversible spontaneous hydrolysis to 2,3-diketogulonic acid (Potters *et al*. 2002). In this study, an expression vector containing potato *DHAR1* or *DHAR2* cDNA driven by the CaMV 35S promoter was transferred into tomato plants. Transgenic expression of *DHAR1* and *DHAR2* was proved by the real-time RT-PCR analysis (Bustin 2000, 2002; Gachon *et al*. 2004).

The fact that the AsA content and its redox state are closely related with the *DHAR* expression level has been confirmed in many plants by different methods (Kwon *et al*. 2001; Chen & Gallie 2006; Qin *et al*. 2011). *DHAR* overexpression enhanced AsA levels and the AsA/DHA ratio in *Arabidopsis thaliana* (Wang *et al*. 2010). Suppression of *DHAR* expression resulted in less efficient AsA recycling and in a lower AsA redox state (Chen & Gallie 2006). In the present study, overexpression of *DHAR1* distinctly increased *DHAR* transcription, DHAR activity and AsA content in both functional leaves and mature fruits in the T_1 generation (Figure 1, Tables 1 and 2), which was consistent with results reported in tobacco and maize (Chen *et al*. 2003; Eltayeb *et al*. 2006). Overexpression of *DHAR2* also significantly increased DHAR activity and AsA content in functional leaves. However, the differences in DHAR activity and AsA content between transgenic and WT mature fruits were much smaller (Figure 1, Table 1, 2). These results indicated that expression levels of *DHAR1* and *DHAR2* have different effects on DHAR activity and AsA content in different tissues, which was consistent with previous studies (Zou *et al*. 2006; Qin *et al*. 2011). Although increased enzyme activities in the AsA biosynthetic pathway or AsA-GSH cycle might account for the increase in AsA content, no detectable increase in GalLDH, MDHAR, and APX activities was observed. Taken together, these results indicated that the increase in AsA content in *DHAR1*- and *DHAR2*-overexpressing plants may result from an enhanced rescue of DHA.

It is reported that *DHAR1* is constitutively expressed in all plant organs. *DHAR2* is clearly expressed in photosynthetic organs, and the expression level is low in mature fruits which lack chloroplasts (Figure 1d) (Qin *et al*. 2011). Our results showed that *DHAR1* might be expressed mainly in both leaves and mature fruits of tomato, whereas *DHAR2* is expressed mainly in leaves. These expression patterns conform to the assumed corresponding isozyme location (Qin *et al*. 2011). Moreover, the increase in *DHAR2* transcription levels did not result in enhancement of DHAR activity and AsA content in tomato fruits, which might be because of DHAR inactivation resulting from the absence of normal posttranslational processing in chloroplasts (Bruce 2000).

Increasing *DHAR* expression maintains higher levels of chlorophyll, Rubisco large subunit, light-harvesting complex (LHC) II, and photosynthetic function (Chen & Gallie 2006). In the present study, increasing *DHAR* expression resulted in higher chlorophyll content and net photosynthetic rate (Figures 2a, b), which indicated that the level of DHAR activity can affect the leaf function. A possible explanation may be that the overexpression of *DHAR* can sustain the stability of Rubisco, LHCII, and chlorophyll and decrease chlorophyll degradation (Forti & Elli 1995; Chen & Gallie 2006). However, the increasing *DHAR* expression did not substantially increase plant height and stem diameter (Figures 2c, d), which indicated that the endogenous *DHAR* expression may be sufficient for AsA recycling to support maximum growth (Chen *et al*. 2003). Curiously, the higher chlorophyll content and net photosynthetic rate did not result in a significant difference in plant growth between transgenic and WT plants. Similarly, in a previous study on tobacco the increasing *DHAR* expression

resulted in a higher chlorophyll level and a higher rate of CO_2 assimilation, but did not affect the plant growth (Chen *et al*. 2003). Suppression of *DHAR* expression resulted in a preferential loss of chlorophyll, a lower rate of CO_2 assimilation as well as a reduced growth rate (Chen & Gallie 2006). We therefore hypothesize that under normal conditions the tomato plant growth is not correlated with the increasing AsA content, but with a decreasing AsA redox state, or perhaps the AsA redox state mechanisms contributing to cell division or elongation and photosynthesis are different. This possibility requires further investigations.

Abiotic stress, such as MV and salt stress, causes molecular damage to plant cells through ROS formation. As a very effective electron acceptor, MV can suppress the cyclic electron transfer around photosystem I (PSI) (Mano *et al*. 2001). MV catalyzes the photoreduction of O_2 in PSI, thereby accelerating the production of H_2O_2 (Cornic *et al*. 2000). Regeneration of AsA is necessary for the reductive detoxification of H_2O_2, because the H_2O_2-scavenging capacity of chloroplasts and cytosols largely depends on the AsA concentration (Hossain & Asada 1984; Ushimaru *et al*. 2006). In the present study, *DHAR* transgenic lines showed distinctive leaf morphology compared with the control when exposed to MV (Figure 3a). Chlorophyll content was less affected by MV treatment in *DHAR*-overexpressing leaf disks compared with that of the WT. Consistent with previous results (Chen & Gallie 2006), these findings clearly demonstrated that overexpressing *DHAR* confers protection against oxidative stress in tomato, as reflected in relieving oxidative stress and protecting plants from oxidative damage.

One of the main effects of salt stress is the destruction of chlorophyll molecules by ROS, which results in reduction of photosynthetic capacity and growth (Munns & Tester 2008). In the present study, the germination rate, fresh weight, plant height and chlorophyll content of transgenic lines were higher than those of WT plants under salt stress conditions (Figure 4). These results are consistent with those of previous studies (Noctor & Foyer 1998; Chen & Gallie 2005; Huang *et al*. 2005; Pukacka & Ratajczak 2006). Our results indicated that the increased AsA content through overexpression of the two *DHAR* genes may be responsible for the higher tolerance of transgenic tomato under stress conditions.

In conclusion, *DHAR1* overexpression increased AsA content in both leaves and mature fruits of tomato, whereas *DHAR2* overexpression increased mainly AsA content in leaves. Overexpression of *DHAR1* and *DHAR2* could enhance the tolerance of tomato to salt and MV treatments.

Acknowledgments. This study was supported by the National Basic Research Program of China (2009CB119000), China Agriculture Research System (CARS-25-C-1), China-Czech Inter-government Scientific and Technological Cooperation Project (40-1) and funded by the Key Laboratory of Horticultural Crop Biology and Germplasm Innovation, Ministry of Agriculture of China.

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Received for publication August 17, 2011
Accepted after corrections March 15, 2012

Corresponding author:

Xianchang Yu, Ph.D., Chinese Academy of Agricultural Sciences, Institute of Vegetables and Flowers, Beijing 100081, China
e-mail: xcyu1962@163.com