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Transgenic tomato plants overexpressing chloroplastic monodehydroascorbate reductase are resistant to salt- and PEG-induced osmotic stress

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Abstract

RNA gel hybridization showed that the expression of monodehydroascorbate reductase (MDHAR) in the wild type (WT) tomato was decreased firstly and then increased under salt- and polyethylene glycol (PEG)-induced osmotic stress, and the maximum level was observed after treatment for 12 h. WT, sense transgenic and antisense transgenic tomato plants were used to analyze the antioxidative ability to cope with osmotic stresses. After salt stress, the fresh mass (FM) and height of sense transgenic lines were greater than those of antisense lines and WT plants. Under salt and PEG treatments, sense transgenic plants showed a lower level of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA), a higher net photosynthetic rate (P_N), and the maximal photochemical efficiency of PSII (F_v/F_m) compared with WT and antisense transgenic plants. Moreover, sense lines maintained higher ascorbate peroxidase (APX) activity than WT and antisense plants under salt- and PEG-induced osmotic stress. These results indicate that chloroplastic MDHAR plays an important role in alleviating photoinhibition of PSII by elevating ascorbate (AsA) level under salt- and PEG-induced osmotic stress.

Additional key words: ascorbate; monodehydroascorbate reductase; osmotic stresses; reactive oxygen species; tomato.

Introduction

Drought and salinity are two of the serious environmental stresses which occur simultaneously in arid regions (Wang *et al.* 2003, Slama *et al.* 2008). Consequently, physiological and biochemical mechanisms operating under these stresses generate considerable interest (Pagter *et al.* 2009, Slama *et al.* 2007, 2008, Suriyan and Chalempol 2009). Reduced plant growth and malformed developmental processes linked with drought and salt stress have been reported earlier in many plant species (Lutts *et al.* 2004, Tonon *et al.* 2004, Slama *et al.* 2007, 2008, Suriyan and Chalempol 2009). However, there is a variety of defense mechanisms operating in plants subjected to these stresses. One of the important pathways to enhanced stress tolerance is through scavenging of reactive oxygen species (ROS) by antioxidants and antioxidative enzymatic systems upregulated under those stresses (Sharma and Dubey 2005). Although ROS affect many cellular functions, they also play an important role in the oxidative signaling pathway and are an important link between environmental changes and plant tolerance. Thus, it is important to note that whether ROS will act as a damaging, protective or signaling mechanism depends on the delicate equilibrium between ROS production and scavenging (Gratao *et al.* 2005).

Under drought and salinity stress, ROS are responsible for damage to membranes and other essential macro

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Abbreviations: APX – ascorbate peroxidase; AsA – ascorbate; CAT – catalase; DHA – dehydroascorbate; DHAR – dehydroascorbate reductase; FM – fresh mass; F_v/F_m – the maximal photochemical efficiency of PSII; GSH – glutathione; GR – glutathione reductase; MDA – malondialdehyde; MDHA – monodehydroascorbate radical; MDHAR – monodehydroascorbate reductase; MS – Murashige-Skoog agar medium; PEG – polyethylene glycol; PPFD – photosynthetic photon flux density; P_N – net photosynthetic rate; PS – photosystem; ROS – reactive oxygen species; SOD – superoxide dismutase.
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molecules such as photosynthetic pigments, proteins, DNA, and lipids (Gueta-Dahan *et al.* 1997). Previous studies indicated that drought and salinity stress can accelerate the formation of ROS at the cellular level (Foyer *et al.* 1994a, Asada 1999), which are associated with the photoinhibition of photosystem (PS) I (Sonoike 1996, Li *et al.* 2004). Using thylakoids or intact chloroplasts of spinach, Jakob and Heber (1996) found that PSI photoinhibition occurred concomitant with the accumulation of ROS. Moreover, under environmental stress, accelerated ROS generation could enhance the extent of PSII photoinhibition by inactivating the elongation factor of the D1 protein and inhibiting the repair of PSII as the degree of photoinhibition *in vivo* depends on the balance between the rate of photodamage and the rate of repair (Yang *et al.* 2007). The chloroplast, which is the compartment associated with the highly energetic reactions of photosynthesis and has a generous supply of oxygen, is the most likely source of ROS in plant tissues (Asada 1997). The efficient removal of ROS is important for stress tolerance in plants under stress.

Plants develop a combination of antioxidative enzymes such as superoxide dismutase (SOD), APX, catalase (CAT), glutathione (GR), dehydroascorbate reductase (DHAR) and antioxidant**s** including ascorbate (AsA), glutathione (GSH), tocopherols and carotenoids *etc*. to detoxify ROS. Antioxidative enzymes are effective mechanisms for scavenging ROS caused by various environmental stresses. The chloroplast is especially sensitive to damage caused by ROS because electrons that escape from the photosynthetic electron transfer system are able to react with a relatively high concentration of O_2 (Foyer *et al.* 1994b, Kwon *et al.* 2002). The efficient removal of ROS from the chloroplast is a key factor for normal metabolism in plants (Badawi *et al.* 2004). In the chloroplast the AsA-GSH pathway is thought to be the most important antioxidative mechanism. As a major antioxidant, AsA plays a central function in protecting plant cells from oxidative stress. AsA can scavenge free radicals directly and act as an electron donor for H_2O_2 detoxification *via* APX in plant cells (Noctor and Foyer 1998). The primary oxidation product monodehydroascorbate radical (MDHA) can be reduced to AsA through reactions with photoreduced ferredoxin or by monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) in chloroplasts.

MDHAR catalyses the reduction of the MDHA to AsA, using NADH or NADPH as an electron donor, and is believed to be involved in maintaining the ROS scavenging capability of plant cells, as regeneration of AsA from its primary oxidation product MDHA is thought to be an essential process and is obviously indispensable. With its ability to directly regenerate AsA, MDHAR probably plays an important role in the plant antioxidant system by maintaining the pool of AsA.

Tomatoes are an important vegetable crop, which suffers from high salt- and drought stresses in Chinese saline areas. These osmotic stresses are an important limiting factor for production. Therefore, it is important to investigate the effect of overexpressing chloroplastic MDHAR in tomatoes under osmotic stresses. In a previous study, a tomato chloroplastic MDHAR was cloned and MDHAR overexpressing or underexpressing transgenic tomato plants were constructed. Physiological experiments indicated that overexpression of MDHAR increased the tolerance to extreme temperature and methyl viologen-induced oxidative stress (Li *et al*. 2010). In the current study, the role of MDHAR under NaCl and PEG-induced osmotic stress was further analyzed. Results suggest that chloroplastic MDHAR plays an important role in protecting the PSII by maintaining higher AsA levels under osmotic stresses.

Materials and methods

Plant material and treatment: Seeds of tomato cultivar (*Lycopersicon esculentum* Mill. cv. Zhongshu 4) and T_1 transgenic plants were germinated between moistened filter paper at 25°C for 3 d. Sprouted seedlings were transplanted into sterilized soil and grown at 25/20°C (day/night) with a 16-h photoperiod (300–400 μmol m^{-2} s^{-1} PPFD), 50–60% relative humidity in the greenhouse. The 5-week-old WT and transgenic plants were exposed to NaCl- and PEG-induced osmotic stresses, respectively. For osmotic stress the plants were watered with 200 mM NaCl or 20% (w/v) PEG_{6000}, respectively, once a day for 7 d. All the measurements on physiological and biochemical parameters were carried out on the youngest fully expanded leaves.

Northern blot analysis: Total MDHAR RNA was isolated as described by Li *et al.* (2010). 20 μg of total RNA were separated on a 1.2% agarose formaldehyde gel and transferred to nylon membrane as described by Sambrook *et al.* (1989). RNA was fixed on the membrane by a cross-linking with ultraviolet light. Prehybridization was performed at 65°C for 12 h. The 3′ partial cDNA 0.5 kbp of MDHAR was used as gene-specific probe and labeled with [α-^{32}P]-dCTP by the random prime labeling method (*Prime-a-Gene-Labeling System, Promega*, USA). After 24 h of hybridization, filters were washed subsequently in 2 × standard saline citrate (SSC) (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) with 0.2% SDS and 0.2×SSC with 0.2% SDS at 42°C. Autoradiography was performed at –80°C.

Enzyme activity assay and determination of reduced and oxidized AsA: MDHAR activity was assayed according to the method of Hossain and Asada (1984) with little modification. After the salt- and PEG treatments, leaf samples (0.2 g) from MDHAR transgenic and

WT plants were frozen in liquid nitrogen, ground to powder in precooled mortars and homogenized with 2 mL extraction buffer (1 mM AsA in 50 mM potassium phosphate buffer pH = 7.8). The homogenate was centrifuged at 12,000 × *g* for 15 min at 4°C and the crude extract was used in enzyme assay immediately. The reaction mixture contained 50 M Tris-HCl (pH 7.5), 0.2 mM NADH, 2.5 mM AsA, 0.15 unit of ascorbate oxidase and the crude extract. Activity was measured as the ascorbate oxidase-induced oxidation of NADH. The reaction was monitored at 340 nm (extinction coefficient for NADH = 6.2 L $mmol^{-1}$ cm^{-1}).

The reduced and oxidized levels of AsA were determined according to Kampfenkel *et al.* (1995). After the salt and PEG treatments, leaf samples (0.4–1.2 g) from transgenic and WT plants were ground to powder in liquid nitrogen, 2 mL 6% TCA were added and the slurry was then centrifuged for 5 min at 13,000 × *g* (4°C). The supernatant was immediately assayed for AsA and DHA. The following solutions were used: 0.2 mL sample (6% TCA for blank), 0.2 M phosphate buffer (pH 7.4), 0.2 mL ddH_2O, 1 mL 6% TCA, 0.8 mL 42% H_3PO_4, 0.8 mL 4% 2, 2'-bipyridyl and 0.4 mL 3% $FeCl_3$. The assay tube was incubated at 42°C for 1 h and the absorbance was read at 525 nm. For assay the total AsA, 0.2 mL 10 mM DTT was added to reduce DHA to AsA. DHA was calculated as the difference between the total AsA and reduced AsA.

Growth performance of WT and transgenic plants under salt stress: To ensure humidity remained constant, the seeds of WT and the transgenic plants were grown on Murashige-Skoog (MS) agar media in closed Petri dishes. After the plants had germinated (7 d) at 25°C, the seedlings were transferred to the new MS agar media containing 200 mM NaCl in an illuminated incubation chamber. After the plants had been cultivated for another 7 d, FM of their aerial parts was determined.

Measurement of net P_N and chlorophyll (Chl) fluorescence: P_N was measured with a portable photosynthetic system (*CIRAS-2*, *PP Systems*, UK) at 25°C under ambient CO_2 (360 μL L^{-1}), a PPFD of 800 μmol m^{-2} s^{-1} and the relative humidity was 80%.

Chl fluorescence was measured with a portable fluorometer (*FMS2*, *Hansatech*, England) according to the protocol described by Van Kooten and Snel (1990). The minimal fluorescence (F_o) with all PSII reaction centers open was determined by modulated light which was low enough not to induce any significant variable fluorescence (F_v). The maximal fluorescence (F_m) with all reaction centers closed was determined by a pulse of 0.8-s saturating light of 7,000 μmol m^{-2} s^{-1} on a dark-adapted (15 min) leaf. The maximal photochemical efficiency (F_v/F_m) of PSII was expressed as: $F_v/F_m = (F_m - F_o)/F_m$.

Measurement of H_2O_2 and MDA content: H_2O_2 concentration was measured colorimetrically according to the procedure of Lin and Kao (2000) with slight modifications. The leaf samples (0.2 g) were homogenized with 3 mL phosphate buffer (50 mM, pH = 6.5). The homogenate was centrifuged at 6,000 × *g* for 25 min. 3 mL of extracted solution was mixed with 1 mL of 0.1% titanium sulfate in 20% (v/v) H_2SO_4 and the mixture was then centrifuged at 6,000 × *g* for 15 min. The intensity of the yellow colour of the supernatant was measured at 410 nm with a 1 cm path length cuvette. H_2O_2 level was calculated using the extinction coefficient 0.28 L μmol^{-1} cm^{-1} and was expressed as μmol g^{-1} initial FM.

The level of lipid peroxidation was determined by measuring MDA content according to the method of Chen and Gallie (2006). A total of 0.5 g leaf was ground in liquid nitrogen in a 1.5 mL microfuge tube using a micropestle. Then 0.5 mL 0.5% (w/v) thiobarbituric acid in 20% (w/v) trichloroacteic acid and 0.5 mL of buffer (50 mM Tris-HCl, pH = 8.0, 175 mM NaCl) was added. Following heating at 95°C for 25 min, pelleting the cell debris, the absorbance of the supernatant was measured at 532 nm, with the A600 subtracted to account for nonspecific turbidity.

Following heating at 95°C for 25 min, and after pelleting of the cell debris, the absorbance of the supernatant was measured at 532 nm, with the A_{600} subtracted to account for nonspecific turbidity.

APX activity assay: APX was extracted according to the method of Kwon *et al.* (2002). Leaf tissue (0.5 g) was ground in liquid nitrogen, suspended in 5 mL homogenization buffer containing 50 mM Hepes (pH = 7.0), 1 mM ascorbate and 1% (v/v) Triton X-100. After centrifugation 13,000 × *g* for 10 min at 4°C, the supernatants were used to determine enzyme activity and protein concentration (Bradford 1976). Ascorbate peroxidase activity was measured by monitoring the decrease in absorbance at 290 nm (Nakano and Asada 1981). The assay mixture (1 mL) contained 50 mM Hepes (pH = 7.0), 0.1 mM ethylenediaminetetraacetic acid, 0.2 mM H_2O_2, 0.5 mM AsA and enzyme extract. The reaction was initiated by adding H_2O_2. One unit of enzyme was the amount of APX catalyzing the oxidation of 1 μmol AsA per minute.

Statistic analysis: All measurements were subjected to analysis of variance (*ANOVA*) to discriminate the significant difference. The significance in this paper refers to statistic significance at the $P \leq 0.05$ level.

Results

Expression analysis of MDHAR in tomato: The expression of MDHAR was intense in both leaves and stems which have high Chl content (Fig. 1*A*). The transcripts were relatively more abundant in leaves than in stems, thus leaves were used as a material for the northern blot under stress treatment. The expression of MDHAR in WT leaves was detected by northern blot after treatment with 200 mM NaCl or 20% PEG_{6000} at different times (Fig. 1*B,C*). When plants were treated, the relative MDHAR expression in leaves changed. The quantification of mRNA suggests that the MDHAR expression level was decreased firstly and then increased, the maximum level was observed after treatment for 12 h (Fig. 1*D*). The reason may be that the stressful treatment accelerated degradation of mRNA at the early stage, then with the duration of treatment the plants responded to the stress and the synthesis of MDHAR increased to protect the plant from stressful damage.

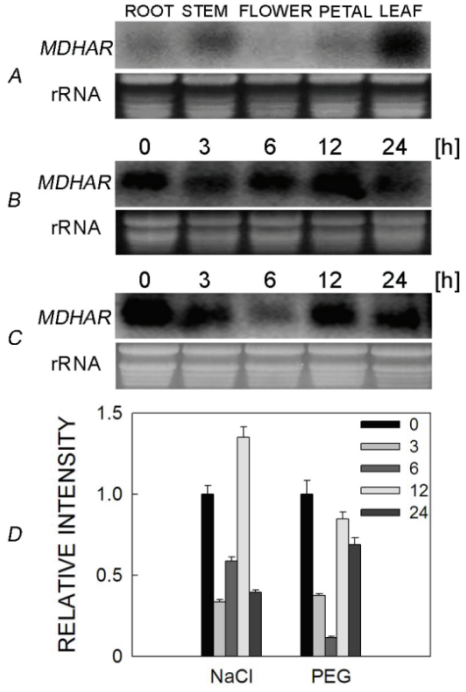

Fig. 1. The expression of MDHAR in different organs (*A*), under 200 mM NaCl (*B*) or PEG-induced osmotic stress (*C*) for different time in the leaves of WT tomato plants. Total RNA was extracted from WT plants. About 20 μg of total RNA was analyzed by RNA gel blot using 3′ partial cDNA of MDHAR as a gene-specific probe. The probe was labeled with [α-^{32}P]-dCTP by the random prime labeling method (*Prime-a-Gene-Labeling System, Promega*). The ethidium bromide staining of the RNA gel is shown as the control for loading (rRNA). Fig. 1*D* represents the relative quantification of Fig. 1*B* and Fig. 1*C* by quantity one software. Each point represents the means ± SD of three measurements on each of the three plants. *P* values were calculated by using *t*-test and are indicated by asterisks (*) when significantly different from WT (P<0.05).

Growth analysis under salt stress: In order to study the tolerance to salt stress, two sense transgenic lines (S17 and S4), two antisense lines (A1 and A14), and WT plants were selected for growth analysis. No differences were observed in the growth of the transgenic and WT plants grown in MS media for 15 d (Fig. 2*A*). However, after treatment with 200 mM NaCl for 7 d, the growth of all plants was suppressed but it was more serious in antisense transgenic lines (Fig. 2*B*). When treated with NaCl for 30 d, the WT and antisense lines defoliated seriously but not the sense lines. Moreover, the root growth of WT and antisense lines was patently more suppressed compared with sense transgenic lines (Fig. 2*C*). The FM of all plants under salt stress decreased compared with those grown under normal conditions but it was more severe in antisense transgenic lines (Table 1). The FM of the sense transgenic plants was about 1.5 fold higher than that of WT plants, while the FM of the antisense plants was approximately 55% that of the WT plants.

Overexpression of MDHAR enhances tolerance to NaCl stress: P_N and F_v/F_m were analyzed to assess the effect of salt stress on the photosynthetic apparatus. No significant difference in P_N between WT and transgenic plants was observed under normal growth conditions. The P_N of all plants decreased with the salt treatment. After salt treatment for 7 d, the P_N of WT and transgenic plants decreased by 39.1%, 42.4%, 50.9%, 62.0% and 64.0% in S17, S4, WT, A14, and A1, respectively (Fig. 3*A*). The decrease of F_v/F_m in sense transgenic plants was slower

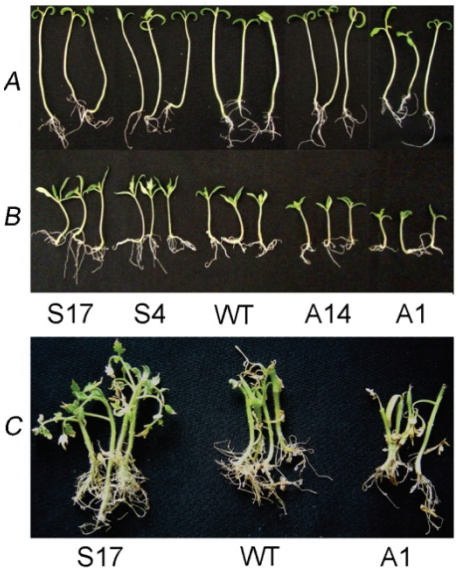

Fig. 2. Growth analysis of tomato plants under 200 mM NaCl stress. 7-day-old wild type (WT) and transgenic seedlings were transferred to glass bottles containing MS agar media (*A*) for 7 d and (*B*) MS agar media 200 mM NaCl for 7 day, (*C*) MS agar media supplemented with 200 mM NaCl for 30 d.

Table 1. Fresh mass (FM) of wild type (WT) and transgenic plants growing on MS media without NaCl or with 200 mM NaCl. Data are expressed as mean values ± SD (n = 6; three measurements on each of the six plants). * – significant difference compared to WT using the Student t-test at $P \leq 0.05$.

Plants	FM [g] 0 mM NaCl	200 mM NaCl
S17	0.1036 ± 0.013	0.0707 ± 0.005*
S4	0.1009 ± 0.012	0.0643 ± 0.007*
WT	0.0993 ± 0.012	0.0480 ± 0.006
A14	0.1012 ± 0.012	0.0306 ± 0.007*
A1	0.1002 ± 0.011	0.0263 ± 0.006*

than that of WT and antisense transgenic line, and the F_v/F_m of antisense lines decreased sharply (Fig. 3*B*).

The content of H_2O_2 in the leaves of WT and transgenic plants was also examined (Fig. 3*C*). No differences were observed in the content of H_2O_2 between WT and transgenic plants under normal growth conditions. After salt treatment, H_2O_2 in all plants increased and the increase was greater in antisense lines and lower in sense lines than that in WT plants. After treatment for 7 d, the level of H_2O_2 in antisense lines is about 4 times the before-treatment one. As a scavenger of H_2O_2, the activity of APX was also determined in order to explain the difference in H_2O_2 content between WT and transgenic

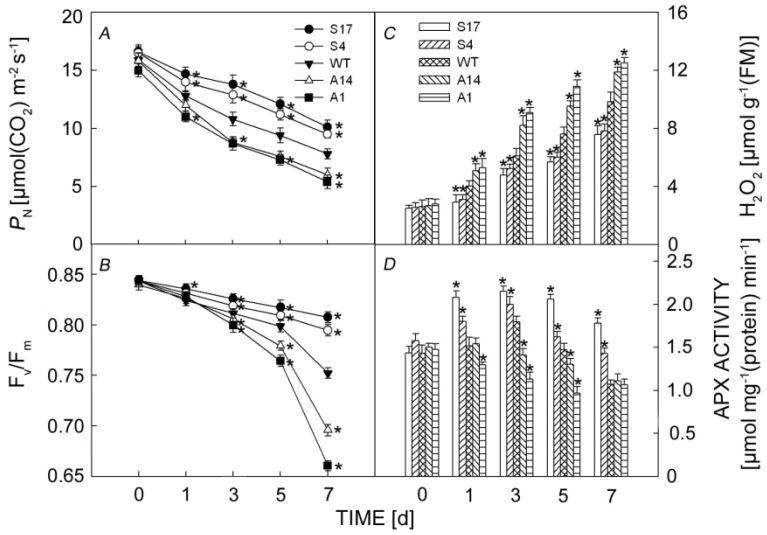

Fig. 3. Changes of net photosynthetic rate (P_N) (*A*), the maximal photochemical efficiency of PSII (F_v/F_m) (*B*), H_2O_2 (*C*) and ascorbate peroxidase (APX) (*D*) in wild type (WT) and transgenic plants during 200 mM NaCl treatment. Plants were irrigated with 200 mM NaCl once a day. P_N (*A*) and F_v/F_m (*B*) were measured at 0, 1, 3, 5, and 7 days at the PPFD of 800 μmol m^{-2} s^{-1}. Means ± SD (n = 5) of five measurements on each of the five types of plants. The content of H_2O_2 and the activity of APX of WT and transgenic plants were measured under salt stress. Each point represents the means ± SD of five measurements on each of the five plants. P values were calculated by using t-test and are indicated by asterisks (*) when significantly different from WT (P<0.05).

plants (Fig. 3*D*). The APX activity in sense lines and WT plants increased firstly and then decreased. But its activity in sense lines was higher than that in WT, while the APX activity in antisense lines decreased continuously during NaCl stress. Moreover the decrease was greater in antisense lines than that in WT plants. There was no evident difference in the extent of lipid peroxidation in all plants without salt treatment (Fig. 4). After 200 mM NaCl treatment for 7 d, the MDA in S17, S4, WT, A14, and A1 increased by 20.9%, 25.3 %, 29.7%, 36.8%, and 37.9%, respectively (Fig. 4).

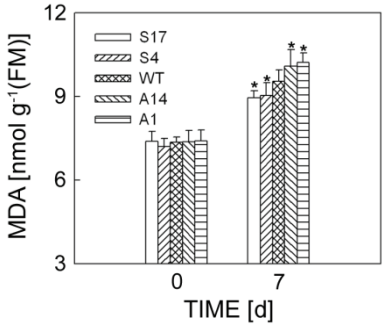

Fig. 4. Changes of malondialdehyde (MDA) in wild type (WT) and transgenic plants under salt stress. Plants were irrigated with 200 mM NaCl once a day. The content MDA of WT and transgenic plants were measured under salt stress after 7 days. Each point represents the means ± SD of five measurements on each of the five plants. P values were calculated by using t-test and are indicated by asterisks (*) when significantly different from WT (P<0.05).

The activity of MDHAR and content of reduced AsA were analyzed after salt treatment (Table 2). Compared with the results under control conditions (Li *et al.* 2010), both the activity of MDHAR and content of reduced AsA were decreased significantly in WT and transgenic plants under salt stress (data not shown, *see* Li *et al.* 2010). However, the MDHAR activity of S4 and S17 was about 1.7–1.8 times that of WT plants, while the MDHAR activities of A1 and A14 were only 59.5% and 61.4% of WT, respectively (Table 2). In accordance with the higher MDHAR activity of sense lines, the content of reduced AsA in S4 and S17 was higher, but the DHA was lower than in WT and antisense transgenic plants. As a result, the redox status of AsA (AsA/DHA) in S4 and S17 are 2.2 and 2.5 times of WT plants, while the ratio in A1 and A14 lines was maintained only 50% and 70% of WT values.

Table 2. MDHAR activity, content of AsA, DHA, and the redox status of ascorbate (AsA/DHA) in transgenic and wild type (WT) plants after treatment with 200 mM NaCl or 20% PEG $_{6000}$ for 7 d. Data are expressed as mean values ± SD (n = 3; three measurements on each of the three plants) * – significant difference compared to WT using the Student t-test at $P \leq 0.05$.

Plants	MDHAR [nmol(NADH oxidized) min^{-1} g^{-1}(FM)]		AsA [μmol g^{-1}(FM)]		DHA [μmol g^{-1}(FM)]		AsA/DHA	
	NaCl	PEG	NaCl	PEG	NaCl	PEG	NaCl	PEG
S17	6.54 ± 0.08*	5.85 ± 0.06*	3.23 ± 0.16*	2.67 ± 0.30*	1.34 ± 0.11*	1.44 ± 0.16*	2.55 ± 0.11*	1.98 ± 0.20*
S4	6.45 ± 0.07*	5.74 ± 0.05*	3.13 ± 0.18*	2.35 ± 0.21*	1.44 ± 0.08*	1.36 ± 0.09*	2.24 ± 0.12*	1.85 ± 0.18*
WT	3.75 ± 0.11	2.63 ± 0.11	2.04 ± 0.22	1.77 ± 0.22	2.05 ± 0.13	1.83 ± 0.09	1.08 ± 0.12	0.94 ± 0.15
A14	2.33 ± 0.12*	2.25 ± 0.10*	1.75 ± 0.10*	1.54 ± 0.13*	2.62 ± 0.10*	2.75 ± 0.08*	0.79 ± 0.04*	0.69 ± 0.09*
A1	2.26 ± 0.10*	2.16 ± 0.07*	1.58 ± 0.08*	1.48 ± 0.15*	2.93 ± 0.11*	3.06 ± 0.12*	0.74 ± 0.03*	0.55 ± 0.12*

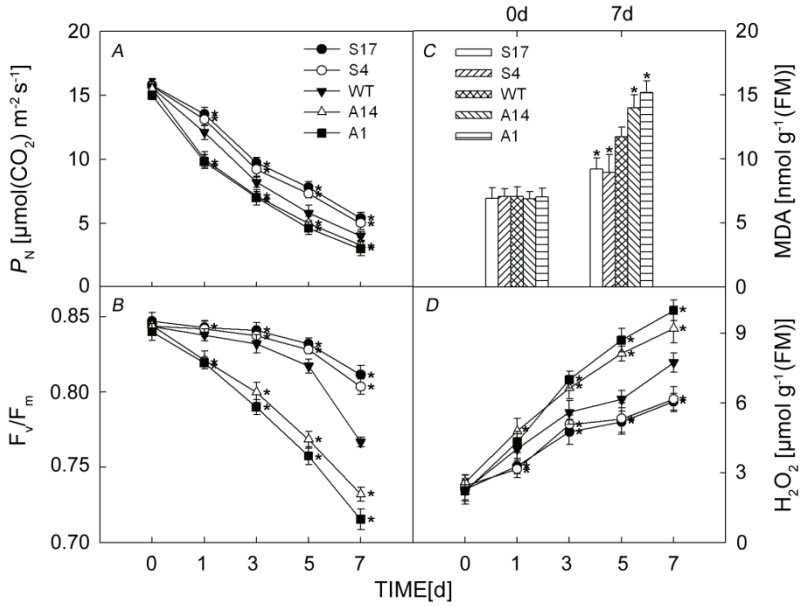

Fig. 5. Changes of net photosynthetic rate (P_N) (*A*), the maximal photochemical efficiency of PSII (F_v/F_m) *(B)*, malondialdehyde (MDA) (*C*) and H_2O_2 (*D*) in wild type (WT) and transgenic plants during 20% PEG_{6000} treatment. Plants were irrigated with 20% PEG_{6000} once a day. P_N and F_v/F_m were measured at 0, 1, 3, 5, and 7 days at the PPFD of 800 μmol m^{-2} s^{-1}. Means ± SD (n = 5) of five measurements on each of the five types of plants under PEG stress. The content of MDA of WT and transgenic plants were measured before and after PEG treatment and the content of H_2O_2 of WT and transgenic plants were measured at 0, 1, 3, 5, and 7 days under PEG stress. Means ± SD (n = 5) of five measurements on each of the five plants. P values were calculated by using t-test and are indicated by asterisks (*) when significantly different from WT (P<0.05).

Overexpression of MDHAR enhanced tolerance to osmotic stress: Although P_N decreased in both transgenic and WT plants, the P_N of sense transgenic lines was higher than that of WT and antisense transgenic plants (Fig. 5*A*). After osmotic stress for 7 d, the P_N in S17, S4, WT, A14, and A1 decreased to 34.2%, 31.8%, 25.6 %, 21.2%, and 20.0%, respectively. The changes of F_v/F_m in the WT and transgenic plants showed the same trend as P_N (Fig. 5*B*). When treated with 20% PEG for 7 d, the F_v/F_m in sense and antisense transgenic lines was decreased about 5% and 15% compared with 10% for WT plants.

Under osmotic stress, the content of MDA increased in all plants (Fig. 5*C*) and the accumulation was the highest in antisense lines but lowest in sense lines. The content of H_2O_2 was also determined during osmotic stress (Fig. 5*D*). Prior to treatment, the content of H_2O_2 in all plants was low and there was no significant difference between WT and transgenic plants. After osmotic stress, the content of H_2O_2 was clearly increased and it was about 2.8, 2.8, 3.4, 4.2, and 4.5 times the original content in S17, S4, WT, A14, and A1 plants, respectively.

The activity of MDHAR and content of reduced AsA were also analyzed under PEG treatment for 7 d (Table 2). Both the activity of MDHAR and the content of reduced AsA were decreased under PEG treatment, compared with plants under control conditions (Li *et al.* 2010). However, the sense transgenic lines had a higher MDHAR activity and reduced AsA content compared to those of WT plants after PEG treatment.

Discussion

Oxidative stress by excessive production of ROS under environmental stress reduces yields of crop plants. Antioxidative mechanisms are an effective system for removal of ROS. Another efficient approach is to manipulate the genes that regulate the expression of these antioxidative enzymes. Since the transgenic products affect a critical pathway associated with stress tolerance, maintenance of redox potential and cell signaling, a high level of activity of an introduced transgene should induce protective mechanisms and play an important role in the protection of plants against various environmental stresses (Kwon *et al.* 2002, Tang *et al.* 2006, Lim *et al.* 2007). Ascorbate plays a key role in protecting plants from environmental stress by scavenging ROS directly or indirectly. Therefore overexpressing enzymes participating in biosynthesis and regeneration of AsA or suppressing the expression of genes related to oxidation of AsA will enhance the tolerance to abiotic stress. Chloroplastic MDHAR is localized in the stroma of the chloroplast (Sano *et al.* 2005) and catalyzes the conversion of MDHA to AsA. The importance of maintaining higher levels of reduced AsA has been reported in previous studies, and overexpressing cucumber ascorbate oxidase in tobacco plants increased ozone sensitivity (Sanmartin *et al.* 2003) and expressing the ascorbate oxidase gene in antisense orientation in tobacco and *Arabidopsis* plants increased salt tolerance (Yamamoto *et al.* 2005). Furthermore, a higher AsA content resulting from overexpression of DHAR (Kwon *et al.* 2003, Eltayeb *et al.* 2006, Ushimaru *et al.* 2006), MDHAR in the cytosol (Eltayeb *et al.* 2007), or MDHAR in chloroplasts (Li *et al.* 2010, Kavitha *et al.* 2010) resulted in enhanced tolerance to different stresses.

Previous studies indicated that the expression of cytosolic MDHAR was induced by many kinds of oxidative stresses (Yoon *et al.* 2004) and by wounding or mechanical stimulation (Grantz *et al.* 1995). A recent study found that the expression of chloroplastic MDHAR was induced by salt stress, H_2O_2, high light intensity and iron overload (Kavitha *et al.* 2010). In this study, northern blot analysis showed that the expression of chloroplastic MDHAR was different in different organs of WT plant. Chloroplast MDHAR was expressed highly in tissues full of Chl and the expression level in leaves was higher than that in other organs (Fig. 1*A*). The expression of chloroplast MDHAR was also induced by salt and PEG-induced osmotic stress (Fig. 1*B*,*C*) and MDHAR appeared to be regulated at the transcriptional level in order to detoxify increased ROS under stressful conditions. The upregulation of MDHAR under these stress conditions and its localization in the chloroplast (Li *et al.* 2010) points towards a key role for this enzyme in stress tolerance.

Drought and salinity stress are two of the most serious environmental stresses limiting the productivity of some economic plants. Under these stresses ROS generated and photodamage and protein degradation are accelerated (Wang *et al.* 2003, Slama *et al.* 2008). Moreover, the accelerated formation of ROS at cellular level under environmental stress leads to photoinhibition by interfering with electron transfer in PSI or by inhibiting the replacement of D1 protein with newly synthesized D1 protein in PSII, respectively (Foyer *et al.* 1994b, Sonoike 1996, Asada 1999, Li *et al.* 2004). Under these stresses, plants also decrease drought and salinity stress-induced damage by accelerating the regeneration of antioxidants or enzymatic activity to eliminate excessive ROS generation (Apel and Hirtm 2004). AsA is involved in free radical scavenging in plants, both enzymatically and nonenzymatically. This plays a key role in photoprotection in chloroplasts (Smirnoff 2000). Previous studies on AsA-deficient mutants indicated that AsA contributes to a plant's tolerance to oxidative stress (Conklin *et al.* 1996, Munné-Bosch and Alegre 2002, Huang *et al.* 2005). AsA regeneration is necessary for the reductive detoxification of H_2O_2 (Kampfenkel *et al.* 1995). Excess accumulation of H_2O_2 is one of the mechanisms by which plants are damaged under salt- and drought stress (Eltayeb *et al.* 2006, Takahashi and Murata 2008). The H_2O_2-scavenging capacity of chloroplasts depends both on the AsA concentration and APX activity (Mano *et al.* 2001). The chloroplastic isoforms of APX were rapidly inactivated below 20 μM AsA (Hossain *et al.* 1984, Miyake and Asada 1994). The regeneration of AsA by MDHAR in chloroplasts is thus essential for maintaining chloroplastic APX activity. Under salt stress the APX activity was higher (Fig. 3*D*) and the content of H_2O_2 (Fig. 3*C*) and MDA (Fig. 4) was lower at all times in sense lines than in the WT and antisense plants. The MDHAR activity and AsA level of reduction state analysis also suggests that the sense transgenic lines still sustain relatively higher MDHAR activity and AsA level after salt treatment (Table 2). These results indicate that overexpression of MDHAR enhances the regeneration of AsA from MDHA which can sustain the activity of APX, detoxify the H_2O_2 effectively and reduce the damage to the lipid membrane. The enhanced tolerance to salt stress in term of higher P_N, F_v/F_m (Fig. 3*A*, *B*) and higher FM (Fig. 2, Table 1) in sense transgenic plants could be understood through the higher activity of MDHAR and higher levels of AsA compared with WT and antisense plants (Table 2). Previous studies found that the AsA-deficient *Arabidopsis* mutant was more sensitive to salt stress, while the addition of exogenous AsA increased resistance to salt stress and reduced lipid peroxidation (Huang *et al.* 2005, Shalata and Neumann 2001). Overexpression of MDHAR in transgenic plants made the recycling of AsA more efficient (Table 2), which should sustain APX activity, detoxify ROS under stress conditions and alleviate the accumulation of ROS further, hence decreasing oxidative damage.

It is reported that an increase in the level of total AsA decreased cellular damage caused by ROS under drought stress in the plant (Adriano *et al.* 2005). Previous studies suggested that the activity of APX and H_2O_2-scavenging were dependent on the AsA content. Under drought stress the oxidation of AsA was increased and so excessive H_2O_2 could not be eliminated resulting in a damage to the plant (Takahashi and Murata 2008). Therefore, the higher P_N (Fig. 5*A*) and F_v/F_m (Fig. 5*B*) in MDHAR transgenic plants under PEG treatment could be attributed to the higher levels of AsA (Table 2). Moreover, the lower content of MDA and H_2O_2 in sense lines than WT and antisense plants (Fig. 5*C*,*D*) suggests that overexpression of MDHAR facilitates the regeneration of AsA, while the higher AsA concentration plays a key role in H_2O_2 elimination and sustains the function of PSII (Table 2). A previous study suggested that DHAR overexpression on transgenic tobacco plants also induces resistance to PEG-induced osmotic stress by enhancing the AsA level and APX activity (Eltayeb *et al.* 2006). Combined with the above results, one can deduce that the overexpression of chloroplast MDHAR should elevate AsA levels which could thus sustain APX activity and accelerate the reductive detoxification of H_2O_2 and finally enhance plant tolerance to salt- and PEG-induced osmotic stress. The increased regeneration of the AsA is able to remove the ROS efficiently and alleviate photoinhibition of PSII caused by osmotic stress by enhancing the repair of photodamaged PSII.

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