Effect of salt stress on tomato fruit antioxidant systems depends on fruit development stage

Ramzi Murshed · Félicie Lopez-Lauri · Huguette Sallanon

Received: 29 May 2013 / Revised: 20 September 2013 / Accepted: 7 October 2013 / Published online: 4 December 2013
© Prof. H.S. Srivastava Foundation for Science and Society 2013

Abstract The effects of different levels of salt stress on the oxidative parameters (H_2O_2 and MDA), the total pool sizes of ascorbate, the activities of antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT), as well as the activities and relative transcript levels of the enzymes of ascorbate-glutathione cycle; ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) were studied in fruits of tomato. Plants were treated by three concentrations of NaCl (50, 100 and 150 mM) and fruits at different development stages were harvested after 3 and 6 days of stress. The concentrations of ascorbate (AsA) and dehydroascorbate (DHA) generally changed with salt stress treatments. Moreover, changes in SOD and CAT activities and DHAR, MDHAR, APX and GR activities and relative transcript levels were dependent on the fruit development stage and the intensity and duration of salt stress. These results suggest that the response of antioxidant systems of tomato fruits to oxidative stress induced by salt stress treatments was different depending on the fruit development stage.

Keywords Antioxidant enzymes · Ascorbate · Salt stress · Oxidative stress · *Solanum lycopersicum* L

Introduction

The increased salinity of agricultural land, due to human activities, agricultural practises and natural processes, is a

common environmental problem (Epstein et al. 1980). It is a major factor limiting crop production because it affects almost all plant functions (Bohnert and Jensen 1996). Adverse effects of salt stress stem from two characteristics: (1) the increased osmotic potential of the soil solution with salinity makes the water in the soil less available for plants and (2) specific effects of some elements (Na, Cl, B, etc.) present in excess concentrations (Yamaguchi and Blumwald 2005; Munns 2005). It is evident from the growing body of experimental evidence that salt stress affects plant growth, the development processes, integrity of cellular membranes and the functioning of the plant photosynthetic apparatus (Serrano et al. 1999; Sairam and Tyagi 2004). It is well documented that salt stress exert their effect at least in part by causing oxidative damage. This damage is caused by increased production of reactive oxygen species (ROS) (Smimoff 1995). Which are routinely generated during normal plant metabolic processes. Chloroplasts, mitochondria and peroxisomes are the most important intracellular generators of ROS. Under salt stress, accumulation of ROS was reported in several plant species such as potato (Queirós et al. 2011), wheat (Sairam et al. 2005), and tomato (Al-Aghabary et al. 2005). Excess production of ROS during stress results from impaired electron transport processes in chloroplast and mitochondria as well as from pathways such as photorespiration (Sánchez-Rodríguez et al. 2012). In the absence of a protective mechanism in plants, ROS can cause serious damage to different aspects of cell structure and function such as initiating lipid peroxidation and damaging DNA, proteins and other small molecules (Arora et al. 2002; Gill and Tuteja 2010; Ahmad et al. 2011). ROS-mediated membrane damage has been demonstrated to be a major cause of the cellular toxicity by salinity in rice, tomato and citrus (Gueta-Dahan et al. 1997; Dionisio-Sese and Tobita 1998; Mittova et al. 2004).

ROS need to be maintained under sublethal levels for normal plant growth. Hence, plants are equipped with enzymatic and non-enzymatic antioxidant systems to alleviate cellular damage

R. Murshed (✉)
Department of Horticultural Sciences, Faculty of Agriculture,
University of Damascus, Damascus, P. O. Box: 30621, Syria
e-mail: rammur76@yahoo.fr

F. Lopez-Lauri · H. Sallanon
Laboratoire de Physiologie des Fruits et Légumes, Campus
agrosociétés, 84916 Avignon Cedex 9, France

caused by ROS (Foyer and Noctor 2000; Apel and Hirt 2004). Multiple antioxidant enzymes are involved in the enzymatic scavenging of ROS. Superoxide dismutases (SOD) react with the superoxide radical to produce H_2O_2 . However, H_2O_2 is also toxic to the cells and has to be further scavenged by catalases (CAT) or ascorbate peroxidase (APX) or both, to water and oxygen (Zhu et al. 2004; Sairam et al. 2005). In the ascorbate-glutathione cycle, APX reduces H_2O_2 using ascorbate (AsA) as an electron donor. The first product of enzymatic action of APX, namely monodehydroascorbate (MDHA), is reduced to AsA by an NAD(P)H-dependent monodehydroascorbate reductase (MDHAR). MDHA that escapes this route of recycling rapidly disproportionates to dehydroascorbate (DHA) (Foyer et al. 1994). DHA is converted back to AsA by the action of dehydroascorbate reductase (DHAR). Reduced glutathione (GSH) functions as an electron donor for DHAR, oxidized glutathione (GSSG) produced in DHA reduction is reconverted to GSH by an NADPH-dependent glutathione reductase (GR) (Mittler 2002; Apel and Hirt 2004). The non-enzymatic systems consist of antioxidant molecules such as α -tocopherol, carotenoids, glutathione (GSH) and ascorbate (AsA) (Salin 1987). AsA and GSH can directly interact with and detoxify $^1\text{O}_2$, $\text{O}_2^{\cdot-}$, and $\text{OH}^{\cdot}$ (Noctor and Foyer 1998; Asada 1999), and AsA acts as a secondary antioxidant during reductive recycling of the oxidized form of α -tocopherol (Noctor and Foyer 1998; Smirnov and Wheeler 2000).

A number of studies have concentrated on the link between salt stress and antioxidant systems in plants (Dat et al. 2000; Van Breusegem et al. 2001; Arora et al. 2002; Borsani et al. 2003). Studies have also established the link between increased antioxidant capacity and salt tolerance in different plants like pea, tomato and citrus (Hernández et al. 1993; Gueta-Dahan et al. 1997; Shalata and Tal 1998). Several workers have shown a positive correlation between resistance to salt stress and more efficient antioxidant systems. Salt tolerance is generally attributed to up-regulated activities of antioxidant enzymes. In tomato, salt-tolerance is attributed to the increased activities of SOD, APX, and CAT (Mittova et al. 2004), and most salt-tolerance studies have explored antioxidant status in leaves. This is not surprising considering the fact that salt stress aggravates ROS mainly in the chloroplastic compartments of leaf cells. However, relatively little information is available regarding the response mechanisms in fruits of tomato subjected to salt stress, in addition, there are conflicting results probably due to fruit age or stress intensity. Therefore, the present study was conducted to evaluate the effects of different levels of salt stress on the antioxidant systems in the fruits of tomato variety Micro-Tom. We thus measured the total pool sizes of ascorbate, the activities of some antioxidant enzymes as well as, the activities and the relative transcript levels of the enzymes of ascorbate-glutathione cycle. Data show that the antioxidant defence systems were altered in tomato fruits subjected to salt stress.

Materials and methods

Plant material and treatments

Tomato (*Solanum lycopersicum* L., cv. Micro-tom) seeds were germinated in boxes filled with peat. When the first real leaf appeared, seedlings were transplanted to 4 L plastic containers containing a mixture of peat and vermiculite (1:1). Plants were grown in a growth room at a relative humidity of $60 \pm 5\%$ and with day/night temperature of $25/20^\circ\text{C}$ using a 16/8 h light/dark cycle under $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ fluorescent light.

Plants were irrigated every day with water except twice a week with a nutrient solution containing (in μM): 8 MnCl_2 , 0.5 $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1.4 ZnSO_4 , 46 H_3BO_3 , 0.25 $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.6 Fe-EDDHA chelate and (in mM): 4.1 KNO_3 , 3.4 $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 0.9 K_2SO_4 , 1 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1.5 KH_2PO_4 .

At the time of anthesis, flowers were tagged for determining fruit age. Plants were grouped into four sets and subjected to water stress treatments: First set (C), control plants were irrigated daily with distilled water. Second set (S50), plants were irrigated with 50 mM NaCl solution. Third set (S100), plants were irrigated with 100 mM NaCl solution. And fourth set (S150), plants were irrigated with 150 mM NaCl solution. The fruits were harvested at 3 and 6 days of treatment, between 9 and 10 AM were grouped into following 5 stages of development: small green (25, 30, 35 days after flowering; DAF), mature green (fully developed green fruit, 40 DAF), breaker (2–3 days after mature green stage), orange (2–3 days after breaker stage) and red (4–6 days after orange stage). For each stage and treatment, fruits were taken from three plants and fresh harvested fruit was immediately frozen in liquid nitrogen, ground and the powder stored at -80°C .

Determination of H_2O_2 content

Hydrogen peroxide levels were determined as described by Murshed et al. (2008a). Frozen fruit powder (0.25 g) was homogenized in an ice bath with 1 mL 0.1 % (w/v) TCA. The homogenate was centrifuged at $12,000 g$ for 15 min at 4°C . Aliquots of 100 μL from each tube were placed in 96-well plates and 50 μL of 10 mM potassium phosphate buffer (pH 7.0) and 100 μL of 1 M KI were added to each well. Commercial H_2O_2 was used to generate a standard curve. The plate was briefly vortexed, incubated at room temperature for 30 min and the absorbance readings were taken at 390 nm in a micro-plate reader. The content of H_2O_2 was determined using the standard curve.

Determination of the malonyldialdehyde content

For the measurement of lipid peroxidation in fruits, the thiobarbituric acid (TBA) test, which determines malonyldialdehyde (MDA) as an end product of lipid peroxidation

(Murshed et al. 2008a), was used. Frozen fruit powder (0.25 g) was homogenized in 1 mL 0.1 % (w:v) TCA solution. The homogenate was centrifuged at 12,000 *g* for 15 min and 0.5 mL of the supernatant was added to 1 mL 0.5 % (w:v) TBA in 20 % TCA. The mixture was incubated in boiling water for 30 min, and the reaction stopped by placing the reaction tubes in an ice bath. Tubes were briefly vortexed, triplicate, 200 μ L aliquots from each tube were placed in 96-well plates, and the absorbance of supernatant was read at 532 nm in a micro-plate reader. The value for non-specific absorption at 600 nm was subtracted. The amount of MDA–TBA complex (red pigment) was calculated using an extinction coefficient of $155 \text{ mM}^{-1} \cdot \text{cm}^{-1}$.

Determination of ascorbate and dehydroascorbate contents

Total ascorbate (AsA plus DHA) and AsA contents were measured according to the method of Kampfenkel et al. (1995), scaled down for micro-plates (Murshed et al. 2008a). A 0.5 g sample of frozen ground tomato fruit was homogenized in 1 mL of cold 6 % (w:v) trichloroacetic acid (TCA). The homogenate was centrifuged at 16,000 *g* at 4 °C for 15 min. The supernatant was used for total ascorbate and AsA determination.

For measurements of total ascorbate, 10 μ L of extract were added to 10 μ L of 10 mM DL-dithiothreitol (DTT) and 20 μ L of 0.2 mM phosphate buffer (pH 7.4). After incubation for 15 min at 42 °C, 10 μ L of 0.5 % (w:v) *N*-ethylmaleimide (NEM) was added with additional incubation for 1 min at room temperature to remove excess DTT. This was followed by adding 150 μ L of a reagent prepared just before use by mixing 50 μ L of 10 % TCA, 40 μ L of 42 % (v:v) of orthophosphoric acid (H_3PO_4), 40 μ L of 4 % (w:v) 2,2-bipyridyl dissolved in ethanol (70 %) and 20 μ L of 3 % (w:v) ferric chloride. After further incubation for 40 min at 42 °C, the absorbance was measured at 525 nm in a micro-plate reader. For AsA determination, the same reaction was used but 0.2 M phosphate buffer (pH 7.4) was used in place of DTT and NEM. The amount of DHA was estimated from the difference of total ascorbate and AsA. Commercial L-Ascorbic acid was used to generate a standard curve.

Antioxidant enzyme assays

Extraction of enzymes

Protein extraction was performed according to the Murshed et al. (2008b) method. Frozen fruit powder (0.20 to 0.4 g) was homogenized in 1 mL of 50 mM MES/KOH buffer (pH 6.0), containing 40 mM KCl, 2 mM CaCl_2 , and 1 mM AsA. Extracts were centrifuged at 4 °C for 15 min at 16,000 *g*, and the supernatants were analysed immediately for enzyme activities. Protein was quantified using Bradford's method (Bradford 1976).

Enzyme assays

All enzyme activities were measured in 200 μ L volume kinetic reactions at 25 °C, using a micro-plate reader. APX, DHAR, MDHAR and GR activities were measured as described in the method of Murshed et al. (2008b). SOD activity was assayed in a 1.0 mL reaction mixture containing 50 mM potassium phosphate (pH 7.8) buffer, 13 mM methionine, 75 μ M nitro blue tetrazolium (NBT), 0.1 mM EDTA, 10 μ L of sample supernatant and 2 μ M riboflavin. Tubes were briefly vortexed and triplicate, 200 μ L aliquots from each tube, were placed in plastic 96-well plates. Commercial SOD in amounts of 0.6, 1.2, 1.8, 2.4 and 3 units was used to generate a standard curve. The plates were then placed under white light provided by a commercial overhead-transparency projector (Horizon, Model Apollo, Lincolnshire, IL, USA) for 5 min. After the light treatment, absorbance readings were taken at 560 nm in a plate reader. SOD in the extract inhibited the photochemical reduction of NBT to blue formazan. Activity was expressed in units of SOD from the standard curve of activity units versus absorbance. This method was modified from that of Dhindsa et al. (1981). CAT activity was measured using a method adapted from that of Aebi (1984) in a reaction mixture containing 50 mM phosphate buffer (pH 7.0), 15 mM H_2O_2 and 20 μ L of sample supernatant. Activity was determined by measuring the decrease in the reaction rate at 240 nm and calculated using a $43.6 \text{ M}^{-1} \cdot \text{cm}^{-1}$ extinction coefficient.

RNA extraction, cDNA synthesis and quantitative RT-PCR detection

Total RNA was isolated from 50 to 100 mg of frozen fruit powder using Tri Reagent (MRC, Molecular Research Center, INC) following to manufacturer's instructions. RNAs were quantified spectrophotometrically measuring the absorbance at 260 and 280 nm. RNA integrity was verified on 1 % agarose gels.

For RT-PCR experiments, first-strand cDNA was synthesized from 4 μ g of total RNA using the Masterscript™ RT-PCR system (5 PRIME) following to manufacturer's instructions. After the initial template RNA denaturation at 65 °C for 5 min, the first-strand cDNA was synthesized using Oligo d(T) primer at 42 °C for 60 min, and then the reaction was interrupted by enzyme inactivation at 85 °C for 5 min.

The quantitative assessment of mRNA levels was performed using the RealMasterMix SYBR ROX (5 PRIME) in Mastercycler ep Realplex (Eppendorf). The quantification of the accumulation of the target transcript relative to the actin (*SlActin*) transcript, taken as the control gene for the experimental conditions, was performed with specific primers for each gene. Specific primers pairs of *SlActin*, *SlAPXcyto*, *SlAPXt*, *SIDHAR1*, *SIDHAR2*, *SIMDHAR*, *SIGRcyto* and *SIGRcp* were

designed based on sequences present in the GenBank database (<http://www.ncbi.nlm.nih.gov>) using DNAMAN software (Table 1). Three independent RNA isolations, with two replications for each of the RNA isolations, were performed for each stage and treatment. Amplified fragments of PCR product were purified using the QIAquick PCR Purification Kit (QIAGEN) following to manufacturer's instructions, and sequenced using Genome Express services. Sequences data have been deposited with the GenBank database under accession numbers referenced in Table 1.

Statistic analysis

The experiment has been repeated three times, and the values presented here are the means of the three experiments. The results are expressed as means with standard error ($\pm$ SE). Statistical significance was determined by analysis of variance ($p < 0.05$) using MINITAB Release 14 Statistical Software.

Results

H₂O₂ content and lipid peroxidation

The effects of salt stress on H₂O₂ content and lipid peroxidation in fruits are shown in Tables 2 and 3. Salt stress increased the H₂O₂ content in fruits at 25, 30 and 35 DAF of plants stressed for 3 days, however, H₂O₂ content not changed in fruits of plants stressed for 6 days. In fruits at the mature green stage, H₂O₂ content decreased in all S100 and S150 fruits. In fruits at the breaker stage, the decrease in H₂O₂ content was only found in S100 and S150 fruits of plants stressed for 6 days. In fruits at orange and red stages, H₂O₂ content was decreased by all salt stress treatments (Table 2). Malonyldialdehyde (MDA) content

increased in S100 and S150 fruits at 25, 30 and 35 DAF of plants stressed for 3 days, and in all fruits at 35 DAF of plants stressed for 6 days. The increase in MDA content was also found in all fruits at the mature green stage of stressed plants. In fruits at the breaker stage, MDA increased in S100 and S150 fruits of plants stressed for 3 days and only in S100 fruits in plants stressed for 6 days. In fruits at the orange stage, MDA was increased by all salt stress treatments. In fruits at the red stage, MDA increased in S100 fruits of plants stressed for 3 days and in S50 and S100 fruits of plants stressed for 6 days, but it decreased in S150 fruits of plants stressed for 6 days (Table 3).

Non-enzymatic antioxidants

The effects of salt stress on ascorbate (AsA) and dehydroascorbate (DHA) concentrations in fruits are shown in Figs. 1 and 2. In fruits at 25 and 30 DAF, AsA concentration increased only in S100 and S150 fruits of plants stressed for 6 days. The increase in AsA concentration was also found in S150 fruits at 35 DAF of plants stressed for 3 days (Fig. 1a) and in all fruits at 35 DAF of plants stressed for 6 days (Fig. 1b). In fruits at the mature green and breaker stages, AsA concentration increased in all fruits of plants stressed for 3 days, and only in S50 and S100 fruits at the mature green stage and S50 fruits at breaker stage of plants stressed for 6 days. In fruits at the orange stage, AsA concentration increased in S150 fruits of plants stressed for 3 days and in all fruits at the orange stage of plants stressed for 6 days. In fruits at the red stage, AsA concentration increased only in S50 fruits of stressed plants (Fig. 1).

DHA concentration was not changed in fruits at 25 DAF of plants stressed for 3 days (Fig. 2a), but it decreased in all fruits at 25 DAF of plants stressed for 6 days (Fig. 2b). In fruits at 30 DAF, DHA concentration increased in S50 fruits and

Table 1 Sets of primers used to amplify gene-specific regions, corresponding size and accession number of the amplified product

PCR fragment	Encoded protein	Primer sequence (5'→3')	Size (bp)	Accession number
<i>SLAPXt</i>	Thylakoid-bound ascorbate peroxidase	Sense: TTCACCCAATGACTTCCCT Antisense: TATCATTTAGTCCCATTCTGT	699	FJ532352
<i>SLAPXcyto</i>	Cytosolic ascorbate peroxidase	Sense: GTTGAAGGTCGCTTGCCG Antisense: CCAAGGTATGGGCACCAG	118	FJ532353
<i>SIDHAR1</i>	Dehydroascorbate reductase	Sense: TGCCTCTGTGGGCTCGAA Antisense: ACCACCCTGCGATGACGT	335	FJ532354
<i>SIDHAR2</i>	Dehydroascorbate reductase	Sense: ACAACTCCTAACAAGCTCGG Antisense: GTCCAAGCGACAAATCAGCA	430	FJ532355
<i>SIMDHAR</i>	Monodehydroascorbate reductase	Sense: GGAGAAGTTTCGTTGCTGCT Antisense: TGAGCAGCTTTCTCTGAATTGT	371	FJ544908
<i>SIGRcp</i>	Chloroplastic glutathione reductase	Sense: TGGAGGCGTGGTGGCAC Antisense: CTATAGCATACTCGCTTCCAG	362	FJ544906
<i>SIGRcyto</i>	Cytosolic glutathione reductase	Sense: GCAAAGAATTATGGATGGGA Antisense: CACAGCACGCTTTGGTAA	333	FJ544907
<i>SLActin</i>	Actin	Sense: ATGACTCAAATCATGTTTGAG Antisense: TACCTTAATCTTCATGCTGCT	633	FJ532351

Table 2 H₂O₂ (nmol. g⁻¹ FW) content in fruits of control plants (C) and plants stressed (S50, S100 and S150) for 3 and 6 days. Values are the mean ($\pm$ S.E.) of five replicates, and different letters within lines indicate significant differences ($P < 0.05$)

	3D				6D			
	C	S50	S100	S150	C	S50	S100	S150
25 DAF	368.26 $\pm$ 10.3 ^a	695.86 $\pm$ 29.5 ^b	683.03 $\pm$ 25.3 ^b	687.43 $\pm$ 26.4 ^b	368.26 $\pm$ 10.3 ^a	358.46 $\pm$ 12.4 ^a	341.91 $\pm$ 11.3 ^a	348.87 $\pm$ 28.4 ^a
30 DAF	172.50 $\pm$ 6.7 ^a	246.68 $\pm$ 14.6 ^b	290.75 $\pm$ 11.3 ^c	196.39 $\pm$ 7.2 ^d	172.50 $\pm$ 6.7 ^a	180.49 $\pm$ 8.7 ^a	182.32 $\pm$ 6.5 ^a	173.90 $\pm$ 9.7 ^a
35 DAF	145.01 $\pm$ 8.6 ^a	203.65 $\pm$ 13.7 ^b	261.46 $\pm$ 11.7 ^c	178.55 $\pm$ 9.4 ^d	145.01 $\pm$ 8.6 ^a	150.25 $\pm$ 11.6 ^a	149.62 $\pm$ 8.7 ^a	146.88 $\pm$ 9.6 ^a
Mature green	30.48 $\pm$ 2.6 ^a	32.29 $\pm$ 1.6 ^a	4.85 $\pm$ 0.3 ^b	4.43 $\pm$ 0.5 ^b	30.48 $\pm$ 2.6 ^a	31.69 $\pm$ 2.2 ^a	3.72 $\pm$ 0.3 ^b	9.07 $\pm$ 0.6 ^c
Breaker	4.40 $\pm$ 0.6 ^a	4.25 $\pm$ 0.3 ^a	4.03 $\pm$ 0.4 ^a	4.49 $\pm$ 0.3 ^a	4.40 $\pm$ 0.6 ^a	3.92 $\pm$ 0.6 ^a	2.38 $\pm$ 0.4 ^b	1.59 $\pm$ 0.2 ^b
Orange	17.36 $\pm$ 1.9 ^a	4.28 $\pm$ 0.8 ^b	2.05 $\pm$ 0.9 ^c	4.21 $\pm$ 0.2 ^b	17.36 $\pm$ 1.9 ^a	4.73 $\pm$ 1.0 ^b	6.42 $\pm$ 1.4 ^b	5.73 $\pm$ 1.4 ^b
Red	50.32 $\pm$ 2.7 ^a	8.25 $\pm$ 1.8 ^b	7.03 $\pm$ 0.4 ^b	1.91 $\pm$ 0.4 ^c	50.32 $\pm$ 2.7 ^a	6.61 $\pm$ 0.4 ^b	4.31 $\pm$ 0.3 ^c	3.25 $\pm$ 0.8 ^c

decreased in S100 and S150 fruits of plants stressed for 3 days. Nevertheless, it increased in all fruits at 30 DAF of plants stressed for 6 days. In fruits at 35 DAF, DHA concentration increased only in S100 and S1500 fruits of plants stressed for 3 days and in S50 and S100 fruits of plants stressed for 6 days. In fruits at the mature green stage, the increase in DHA was only found in fruits of plants stressed for 6 days. In fruits at the breaker stage, DHA concentration increased only in S100 fruits of plants stressed for 3 days, but decreased in S50 and S150 fruits of all stressed plants. In fruits at the orange stage, DHA concentration decreased only in S150 fruits of plants stressed for 3 days, but the decrease in DHA concentration was found in all fruits of plants stressed for 6 days. DHA concentration was also decreased in fruits at the red stage by all salt stress treatments (Fig. 2).

Antioxidant enzyme activities

Changes in the activity of antioxidant enzymes (SOD, CAT, APX, DHAR, MDHAR and GR) in fruits of plants subjected to salt stress treatments are shown in Figs. 3 and 4.

SOD activity was not affected by salt stress treatments in fruits at 25, 30 and 35 DAF of plants stressed for 3 days

(Fig. 3a1). However, it increased in fruits at these stages of plants stressed for 6 days (Fig. 3a2). In fruits at the mature green stage, an increase in SOD activity was found in S50 and S100 fruits of plants stressed for 3 days and in all fruits of plants stressed for 6 days. Moreover, SOD activity increased in all fruits at the breaker and orange stages of plants stressed for 3 days, in S100 and S150 fruits of at breaker stage and S50 and S100 fruits at orange stage of plants stressed for 6 days. SOD activity was not changed by salt stress treatments in fruits at the red stage (Fig. 3a1 and a2).

CAT activity increased in S50 and S100 fruits at 25 DAF of plants stressed for 3 days (Fig. 3b1) and in S100 fruits at 25 DAF of plants stressed for 6 days (Fig. 3b2), however, it decreased in all S150 fruits at 25 DAF. In fruits at 30 DAF, CAT activity increased only in S50 fruits. In fruits at 35 DAF, the increase in CAT activity was found in S50 fruits of plants stressed for 3 days and in S50 and S100 fruits of plants stressed for 6 days. In fruits at the mature green stage, CAT activity increased in S150 fruits of plants stressed for 3 and in S100 fruits of plants stressed for 6 days, but it decreased in S50 fruits of plants stressed for 6 days. In fruits at the breaker stage, CAT activity was increased by all salt stress treatments. In fruits at the orange stage, CAT activity increased in all fruits

Table 3 Malonyldialdehyde (MDA; nmol. g⁻¹ FW) content in fruits of control plants (C) and plants stressed (S50, S100 and S150) for 3 and 6 days. Values are the mean ($\pm$ S.E.) of five replicates, and different letters within lines indicate significant differences ($P < 0.05$)

	3D				6D			
	C	S50	S100	S150	C	S50	S100	S150
25 DAF	29.00 $\pm$ 1.3 ^a	30.50 $\pm$ 1.1 ^a	36.45 $\pm$ 1.7 ^b	34.37 $\pm$ 1.1 ^b	29.00 $\pm$ 1.3 ^a	28.04 $\pm$ 1.8 ^a	31.97 $\pm$ 1.9 ^a	33.32 $\pm$ 2.7 ^a
30 DAF	30.23 $\pm$ 1.8 ^a	33.81 $\pm$ 2.4 ^a	35.32 $\pm$ 1.6 ^b	36.97 $\pm$ 1.8 ^b	30.23 $\pm$ 2.8 ^a	31.32 $\pm$ 2.8 ^a	31.47 $\pm$ 2.8 ^a	34.50 $\pm$ 2.7 ^a
35 DAF	26.14 $\pm$ 1.1 ^a	28.79 $\pm$ 0.7 ^a	35.10 $\pm$ 2.5 ^b	36.55 $\pm$ 2.7 ^b	26.14 $\pm$ 1.1 ^a	31.08 $\pm$ 2.0 ^b	45.20 $\pm$ 2.6 ^c	37.00 $\pm$ 1.3 ^d
Mature green	31.19 $\pm$ 2.2 ^a	37.27 $\pm$ 0.9 ^b	37.71 $\pm$ 2.8 ^b	37.76 $\pm$ 1.3 ^b	31.19 $\pm$ 2.2 ^a	39.37 $\pm$ 1.5 ^b	46.36 $\pm$ 1.7 ^c	45.49 $\pm$ 2.3 ^c
Breaker	33.79 $\pm$ 1.0 ^a	32.97 $\pm$ 1.6 ^a	44.33 $\pm$ 2.3 ^b	39.24 $\pm$ 2.2 ^b	33.79 $\pm$ 1.0 ^a	36.30 $\pm$ 2.8 ^a	40.24 $\pm$ 2.2 ^b	35.20 $\pm$ 2.5 ^a
Orange	36.08 $\pm$ 2.7 ^a	42.42 $\pm$ 2.1 ^b	43.00 $\pm$ 2.9 ^b	56.85 $\pm$ 3.1 ^c	36.08 $\pm$ 2.7 ^a	43.48 $\pm$ 1.5 ^b	60.18 $\pm$ 2.4 ^c	43.12 $\pm$ 2.8 ^b
Red	47.50 $\pm$ 1.9 ^a	45.16 $\pm$ 2.7 ^a	55.50 $\pm$ 2.7 ^b	47.93 $\pm$ 2.9 ^a	47.50 $\pm$ 1.9 ^a	54.46 $\pm$ 3.9 ^b	54.46 $\pm$ 3.9 ^b	41.50 $\pm$ 2.6 ^c

Fig. 1 Ascorbate (AsA) concentration (nmol g⁻¹ FW) in fruits of control plants (C) and plants stressed (S50, S100 and S150) for 3 days (a) and 6 days (b). Values are the mean ($\pm$ S.E.) of five replicates. DAF: Days after flowering

of plants stressed for 3 days; however, it decreased in S50 fruits of plants stressed for 6 days. In fruits at the red stage, CAT activity not changed in fruits of plants stressed for 3 days, but it increased in S100 fruits of plants stressed for 6 days (Fig. 3b1 and b2).

APX activity increased in S50 and S100 fruits at 25 DAF of plants stressed for 3 days (Fig. 4a1) and in all fruits at 25 DAF of plants stressed for 6 days (Fig. 4a2). In fruits at 30 DAF, APX activity not changed except in S100 and S150 fruits of plants stressed for 6 days, where it increased. The increase in APX activity in fruits at 35 DAF was only found in S50 fruits of plants stressed for 6 days. In fruits at the mature green stage, APX activity increased only in S100 fruits of plants stressed for 6 days. In fruits at the breaker stage, an increase in APX activity was found in all S50 and S100 fruits of plants stressed for 3 and 6 days. In fruits at the orange stage, APX activity was increased by all salt stress treatments. In fruits at the red stage, APX activity not changed except an increase in S100 fruits and a decrease in S150 fruits of plants stressed for 6 days (Fig. 4a1 and a2).

DHAR activity decreased in S100 and S150 fruits at 25 DAF of plants stressed for 3 days (Fig. 4b1) and this decrease was found in all fruits at 25 DAF of plants stressed for 6 days (Fig. 4b2). In fruits at 30 DAF, DHAR activity increased only in

Fig. 2 Dehydroascorbate (DHA) concentration (nmol g⁻¹ FW) in fruits of control plants (C) and plants stressed (S50, S100 and S150) for 3 days (a) and 6 days (b). Values are the mean ($\pm$ S.E.) of five replicates. DAF: Days after flowering

S50 fruits. In fruits at 35 DAF, DHAR activity decreased in all fruits of plants stressed for 6 days. In fruits at the mature green stage, an increase in DHAR activity was found in S100 and S150 fruits of plants stressed for 3 days and in all fruits of plants stressed for 6 days. In fruits at the breaker stage, DHAR activity increased only in S50 fruits of plants stressed for 3 and 6 days. In fruits at the orange stage, DHAR activity decreased in S50 and S100 fruits of plants stressed for 3 days and in S150 fruits of plants stressed for 6 days, however, it was increased in S100 fruits of plants stressed for 6 days. In fruits at the red stage, DHAR activity not changed except in S100 fruits of plants stressed for 6 days, where it increased (Fig. 4b1 and b2).

MDHAR activity increased in S50 and S100 fruits at 25 DAF of plants stressed for 3 days (Fig. 4c1) and in all fruits at 25 DAF of plants stressed for 6 days (Fig. 4c2). In fruits at 30 DAF, MDHAR activity increased in S50 fruits of plants stressed for 3 days and in S50 and S100 fruits of plants stressed for 6 days, however, it decreased in S150 fruits of plants stressed for 3 days. In fruits at 35 DAF, MDHAR activity decreased in S50 fruits and increased in S150 fruits of plants stressed for 3 days, but it not changed in fruits of plants stressed for 6 days. A marked increase in MDHAR activity was found in all fruits at the mature green stage of

Fig. 3 Activity of superoxide dismutase (SOD; a) and catalase (CAT; b) in fruits of control plants (C) and plants stressed (S50, S100 and S150) for 3 days (a1 and b1) and 6 days (a2 and b2). Values are the mean ($\pm$ S.E.) of five replicates. DAF: Days after flowering

plants stressed. In fruits at the breaker stage, MDHAR activity increased only in S100 and S150 fruits of plants stressed for 3 days and in S150 fruits of plants stressed for 6 days. In fruits at the orange stage, MDHAR activity increased in all fruits of plants stressed for 3 days, but it increased only in S50 and S100 fruits of plants stressed for 6 days. In fruits at the red stage, MDHAR activity decreased in all fruits of plants stressed for 3 days and in S100 and S150 fruits of plants stressed for 6 days, but it increased in S50 fruits of plants stressed for 6 days (Fig. 4c1 and c2).

GR activity was not changed in fruits at 25 DAF of plants stressed for 3 days (Fig. 4d2); however, it increased in all fruits at 25 DAF of plants stressed for 6 days (Fig. 4d1). In fruits at 30 DAF, GR activity was not affected by salt stress treatments. In fruits at 35 DAF, GR activity increased in S100 and S150 fruits of plants stressed for 3 days and in all fruits of plants stressed for 6 days. In contrast, in fruits at the mature green stage, GR activity decreased in S100 and S150 fruits of plants stressed for 3 and 6 days. The decrease in GR activity in S100 and S150 fruits was also found in fruits at the breaker stage, but the GR activity increased in S50 fruits at this stage of plants stressed for 6 days. In fruits at the orange stage, GR activity not changed in fruits of plants stressed for 3 days; however, it increased in S50 and S100 fruits and decreased in S150 fruits of plants stressed for 6 days. In fruits at the red

stage, a decrease in GR activity was found in all fruits of plants stressed for 3 days and in S50 and S150 fruits of plants stressed for 6 days (Fig. 4d1 and d2).

Gene expression of antioxidant enzymes

The relative transcript levels of some genes encoding the enzymes associated with the ascorbate-glutathione cycle: cytosolic *APX* (*SLAPX_{cyto}*), thylakoid-bound *APX* (*SLAPX_t*), *SIDHAR1*, *SIDHAR2*, *SIMDHAR*, cytosolic *GR* (*SIGR_{cyto}*) and chloroplastic *GR* (*SIGR_{cp}*) were measured in fruits of control and treated plants. Data were expressed as relative values with respect to the value found in the control fruits (Fig. 5).

SLAPX_{cyto} transcript decreased in S50 and S100 fruits at 25 DAF of plants stressed for 3 days (Fig. 5a1) and in S100 and S150 fruits at 25 DAF of plants stressed for 6 days (Fig. 5a2). In fruits at 30 DAF, *SLAPX_{cyto}* transcript increased in all fruits of plants stressed for 3 days, however, it not changed in fruits of plants stressed for 6 days. In fruits at 35 DAF, *SLAPX_{cyto}* transcript increased in all fruits of plants stressed. In fruits at the mature green stage, *SLAPX_{cyto}* transcript increased in S50 and S100 fruits of plants stressed for 3 days and in S100 fruits of plants stressed for 6 days, but it decreased in S150 fruits of plants stressed for 6 days. In fruits

at the breaker stage, a decrease in *SLAPXcyto* transcript was found in S100 and S150 fruits of plants stressed for 3 days and in all fruits of plants stressed for 6 days. In fruits at the orange stage, the decrease in *SLAPXcyto* transcript was only found in S50 fruits, however, *SLAPXcyto* transcript was increased by other salt stress treatments except in S150 fruits of plants stressed for 6 days, where it not changed. In fruits at the red stage, *SLAPXcyto* transcript increased in S100 fruits of plants stressed for 3 days and in S50 fruits of plants stressed for 6 days, but it decreased in all S150 fruits (Fig. 5a1 and a2).

SLAPXt transcript increased in all fruits of plants stressed for 3 days except in S100 fruits at 25 DAF, in S50 fruits at the mature green stage and in S50 fruits at the orange stage, where it not changed and in fruits at the breaker stage, where a decrease in *SLAPXt* transcript was found (Fig. 5b1). However, in plants stressed for 6 days, *SLAPXt* transcript decreased in all fruits except in S50 fruits at 25 DAF, in S100 and S150 fruits at 30 DAF and in S50 and S100 fruits at 35 DAF and red stage, where it increased, and in S150 fruits at 25 and 35 DAF and in S50 fruits at 30 DAF, where it not changed (Fig. 5b2).

SIDHAR1 transcript increased in all fruits of plants stressed for 3 days except in S50 and S100 fruits at 25 DAF, where it decreased, and in S150 fruits at 25 DAF, in S50 and S100

fruits at 30 DAF, in S100 fruits at breaker stage and in S50 fruits at mature green, orange and red stages, where it not changed (Fig. 5c1). In plants stressed for 6 days, an increase in *SIDHAR1* transcript was found in all fruits except in all fruits at 25 DAF and mature green stage and in S150 fruits at 35 DAF, orange and red stages, where a decrease in *SIDHAR1* transcript was found, and in S50 fruits at 30 DAF and orange stage and in S100 fruits at 35 DAF, where *SIDHAR1* transcript was not changed (Fig. 5c2).

SIDHAR2 transcript, in plants stressed for 3 days, increased in S150 at 25 and 35 DAF and orange stage, in S50 at the mature green stage and in all fruits at the red stage, but it decreased in S150 fruits at 30 DAF, in S100 and S150 fruits at the mature green stage, in all fruits at the breaker stage and in S50 and S100 fruits at the orange stage (Fig. 5d1). In plants stressed for 6 days, *SIDHAR2* transcript decreased in all fruits of plants stressed except in all fruits at 35 DAF and in S50 and S100 fruits at red stage, where it increased and in S150 fruits at 25 DAF and in S50 fruits at the breaker stage, where it was not changed (Fig. 5d2).

SIMDHAR transcript, in plants stressed for 3 days, increased only in S150 fruits at 25 DAF, in S100 at 30 DAF, in S100 and S150 fruits at 35 DAF and orange stage and in S100 at the red

Fig. 4 Activity of ascorbate peroxidase (APX; a), dehydroascorbate reductase (DHAR; b), monodehydroascorbate reductase (MDHAR; c) and glutathione reductase (GR; d) in fruits of control plants (C) and plants

stressed (S50, S100 and S150) for 3 days (**a1**, **b1**, **c1** and **d1**) and 6 days (**a2**, **b2**, **c2** and **d2**). Values are the mean ($\pm$ S.E.) of five replicates. DAF: Days after flowering

Fig. 4 (continued)

stage, but it decreased in S100 and S150 fruits at the breaker stage and in S150 fruits at the red stage (Fig. 5e1). In plants stressed for 6 days, *SIMDHAR* transcript increased only in S150 fruits at 25 DAF, in S100 at the breaker stage and S50 and S100 at the orange and red stages, but it decreased in S100 and S150 fruits at 35 DAF, in all fruits at the mature green stage and in S150 fruits at the red stage (Fig. 5e2).

SIGRcyto transcript increased in all fruits of plants stressed for 3 days except in S50 fruits at 25 DAF, 35 DAF, breaker and orange stages and in S50 and S100 fruits at 30 DAF and red stage, where it was not changed and in S100 and S150 at the breaker stage and in S150 fruits at the red stage, where it decreased (Fig. 5f1). In plants stressed for 6 days, *SIGRcyto* transcript decreased in all fruits except in S150 fruits at 25 DAF and mature green stage, in S50 and S100 fruits at the orange stage and in S100 fruits at the red stage, where it increased, and in S50 fruits at 25 DAF and red stage, in S100 fruits at mature green stage and in S150 at the orange stage, where it not changed (Fig. 5f2).

Moreover, *SIGRcp* transcript increased in all fruits of plants stressed for 3 days except in fruits at the breaker stage and in S150 fruits at the red stage, where it decreased, and in S50 fruits at 25 DAF, 35 DAF and orange and red stages, where it was not changed (Fig. 5g1). In plants stressed for 6 days, *SIGRcp* transcript decreased in all fruits except in S100 fruits

at the red stage, where it increased and in S50 and S150 fruits at 25 DAF, in S150 fruits at 30 DAF and in S50 fruits at the red stage, where it was not changed (Fig. 5g2).

Discussion

Exposure of plants to abiotic stresses such as high salinity, drought, extreme light and temperature leads to major loss in crop productivity worldwide. Among the various abiotic stress factors, salinity is the biggest threat to plant agriculture. Salt stress affects almost every aspect of the physiology and biochemistry of plants and significantly reduces yield. Therefore, a concerted effort to understand the effects of salinity on plants is essential to combat the world's salinity problem (Rengasamy 2006). In the present study tomato plants were subjected to three different levels of salt stress to evaluate the effects of salt stress on antioxidant defence mechanisms in fruits. The fruit oxidative parameters (H_2O_2 and MDA) were determined, and the fruit mechanisms that confer protection to oxidative stress were investigated. For this purpose, AsA and DHA concentrations, and the activities of antioxidant enzymes: SOD and CAT, as well as the activities and relative transcript levels of the enzymes of ascorbate-glutathione cycle (APX, DHAR, MDHAR and GR) were analysed.

Increased H_2O_2 levels as a result of salt stress have been reported in different plant species (Agarwal and Pandey 2004; Sairam et al. 2005; Kukreja et al. 2005). In tomato, Mittova et al. (2002) observed a significant increase in H_2O_2 under salt stress. In our study, an increase in H_2O_2 content during fruit growth was observed only with a short period of salt stress (3 days); however, during fruit ripening, H_2O_2 content was decreased with salt stress treatments (Table 2). These different responses of H_2O_2 content to salt stress between fruit growth and ripening stages could be explained, in part, by a lower

formation of H_2O_2 in fruits of control plants during ripening compared to that during fruit growth (Table 2). Also the decrease in H_2O_2 in fruits stressed at ripening stages can be related to the increase in the antioxidant enzymes in these fruits, suggested that salt stress-induced H_2O_2 formation was associated with a parallel increase in activities of CAT and APX occur and may contribute to lower H_2O_2 content. Therefore, the H_2O_2 accumulation under high salinity conditions may be a signal for adaptive response to the stress (Van Breusegem et al. 2001).

Fig. 5 Changes in the levels of mRNA encoding the enzymes associated with the ascorbate-glutathione cycle: cytosolic APX (*SLAPXcyto*; a), thylakoid-bound APX (*SLAPXt*; b), *SIDHAR1* (c), *SIDHAR2* (d), *SIMDHAR* (e), cytosolic GR (*SIGRcyto*; f) and chloroplastic GR (*SIGRcp*; g) in fruits of

control plants (C) and plants stressed (S50, S100 and S150) for 3 days (a1, b1, c1, d1, e1, f1 and g1) and 6 days (a2, b2, c2, d2, e2, f2 and g2). Data were expressed as relative values with respect to the value found in the control fruits. Values are the mean ($\pm$ S.E.) of nine replicates. DAF: Days after flowering

Fig. 5 continued.

Excess amounts of ROS are harmful to many cellular components, including membrane lipids. ROS cause peroxidation of polyunsaturated fatty acids in the membranes (Smimoff 1995). It is well known that ROS-induced lipid peroxidation of membranes is a reflection of stress-induced damage at the cellular level (Jain et al. 2001). Therefore, the level of MDA, a decomposition product of polyunsaturated fatty acids produced during peroxidation of membrane lipids, is often used as an indicator of oxidative damage (Mittler 2002). It has been reported that salt treatment increases lipid peroxidation in plant tissues (Mittal and Dubey 1991; Hernández et al. 1993). Moreover, ROS-mediated membrane damage has been demonstrated to be a major cause of the cellular toxicity by salt stress in rice, tomato and citrus (Gueta-Dahan et al. 1997; Dionisio-Sese and Tobita 1998; Mittova et al. 2004). The results reported in this study show that MDA content increased in fruits of plants exposed to salt stress (Table 3). This increase of MDA content in tomato fruits suggests that antioxidant systems were not efficient enough to counter the oxidative damage caused by salt stress. These results do not agree with those of previous studies where a lower lipid peroxidation resulted from elevated activities of antioxidants under salt stress was reported in rice (Dionisio-Sese and Tobita 1998), sugar-beet (Bor et al. 2003) and tomato (Shalata et al. 2001).

Numerous studies indicate that acquisition of salt tolerance may be a consequence of improving resistance to oxidative stress (Shalata and Tal 1998; Hernández et al. 2001; Bor et al. 2003). Most of these studies show a positive correlation between resistance to salt stress and more efficient antioxidant systems. Plants employ both enzymatic and non-enzymatic antioxidant defence systems against oxidative damage. The AsA–GSH cycle plays a key role in these defence systems (Jiménez et al. 1997; Noctor and Foyer 1998), and the components of this cycle are present in different organelles in plant cells (Nakano and Asada 1981; Jiménez et al. 1997; Mittova et al. 2004). One of these components is ascorbate (AsA), a non-enzymatic antioxidant, which associated with H_2O_2 scavenging via APX (Sairam et al. 1998), which also reacts with superoxide radicals ($O_2^{\cdot-}$), hydroxyl radicals ($OH^{\cdot}$) and lipid hydroperoxidases (Reddy et al. 2004). A role for increased AsA content in the amelioration of oxidative stress tolerance has been reported by Sairam et al. (1998, 2005) and Panda and Upadhyay (2003). The AsA pool is generally determined by its rates of not only synthesis but also regeneration (Song et al. 2005). Therefore, the higher AsA concentration in fruits with salt stress treatments that was observed in our study (Fig. 1) can be explained by the increase of the stimulation of AsA synthesis and/or the AsA regeneration from MDHA and DHA. A significant increase in total ascorbate was found in some plants subjected to salinity condition (Shalata et al. 2001). Under salinity stress, AsA is mainly regenerated from MDHA (Shalata et al. 2001; Mittova et al. 2000) or DHA

(Hernández et al. 2000; Meneguzzo et al. 1998). DHA accumulation is generally considered a negative event for the cell metabolism (De Gara et al. 2000). Our results show different responses of DHA to salt stress depending on the fruit development stage and the intensity and duration of salt stress (Fig. 2). Suggesting that conversion rate of DHA to AsA via DHAR under salt stress can be different depending on the fruit development stage. Various associations between salt stress and components of the AsA–GSH cycle have been reported in numerous plant species (Noctor and Foyer 1998; Hernández et al. 2001; Mittova et al. 2003, 2004; Amor et al. 2006).

Antioxidant enzymes are key elements in defence mechanisms. Several antioxidant enzymes participate in the detoxification of ROS such as superoxide dismutase (SOD), catalase (CAT) and ascorbate-glutathione cycle enzymes. SOD is the first line of defence against oxidative stress in plants (Salin 1987). It plays an important part in determining the concentration of $O_2^{\cdot-}$ and H_2O_2 , it causes dismutation of $O_2^{\cdot-}$ to produce H_2O_2 (Bowler et al. 1992). In agreement with the report by Gómez et al. (2004), who found an increase in all SOD isoenzymes of pea under salt stress, our results show that SOD activity was increased only by long-term salt stress treatments (6 days) during fruit growth; however, the increase in SOD activity was found with both salt stress periods (3 and 6 days) during fruit ripening (Fig. 3a). This increase in SOD activity can increase the ability of the fruits to scavenge $O_2^{\cdot-}$ radicals, which might lessen membrane damage. Several studies have revealed increased SOD activity in salt-tolerant genotypes of pea, cotton and tomato, and this induction of SOD activity was suggested as a reason for improved tolerance to salinity in these cases (Gossett et al. 1994; Hernández et al. 2000; Mittova et al. 2004).

H_2O_2 produced by SOD is itself a potent oxidizing agent. Therefore, increase in CAT and APX activity is supposed to be an adaptive trait possibly helping to overcome the damage to the tissue metabolism by reducing toxic levels of H_2O_2 and protection against oxidative stress (Sudhakar et al. 2001; Bor et al. 2003). CAT is involved in scavenging of H_2O_2 during salt stress and other abiotic stress conditions (Willekens et al. 1997). Our results show that the activity of CAT was changed under salt stress and these changes depended on fruit age as well as on the salinity level and the duration of stress (Fig. 3b). The increase in CAT activity under salt stress may indicate that CAT is a major enzyme detoxifying H_2O_2 in tomato fruits. This increased activity of CAT upon salt stress was often related to the enhanced tolerance to salt stress (Mittova et al. 2004).

Ascorbate peroxidase (APX) is antioxidant performing the same general function as catalase. However, unlike catalase, it catalyses removal of H_2O_2 by using ascorbate as a reductant. APX plays an important role in regulation of intracellular level of H_2O_2 in higher plants (Van Breusegem et al. 2001; Shigeoka et al. 2002). APX is a family of isozymes with

different characteristics. APX is localized in all the four major cellular compartments: stromal, thylakoid-membrane-bound APX in chloroplasts (APX_t), microbody (including glyoxysomes and peroxisomes) membrane-bound APX, cytosolic APX (APX_{cyto}) and lastly a mitochondrial-membrane-bound form (Shigeoka et al. 2002). Several studies have reported a large increase in APX activity under salinity stress (Gossett et al. 1994; Hernández et al. 2000; Lee et al. 2001; Mittova et al. 2004). In our study, APX activity was increased by some salt stress treatments especially with the long period of stress (6 days) (Fig. 4a). These results were consistent with the results of Lopez et al. (1996) who described an increase in APX activity in salt tolerant wild tomato and radish plants. The increase in APX activity suggest that this is an adaptation to remove the excess H₂O₂ generated due to increase in SOD activity under salt stress. Our results also show that changes in *SLAPX_{cyto}* and *SLAPX_t* transcript levels in fruits varies according to fruit development stage as well as to the intensity and duration of salt stress (Figs. 5a and b). The changes in APX activity were not correlated with that in *SLAPX_{cyto}* and *SLAPX_t* transcripts, this suggests that there is a post-transcription regulation of APX. Over expression of genes encoding APX in plants has been reported to increase protection against oxidative stress (Wang et al. 1999). Cytosolic *APX* transcript level was strongly induced in the salt-tolerant variety of peas but not in the salt-sensitive one, suggesting that the induction of antioxidant defences is one component of the tolerance mechanisms to salt treatment (Hernández et al. 2000). A recent transcriptome-based analysis also confirms the susceptibility of *APX_{cyto}* to environmental factors (Kim et al. 2007). *APX_t* transcript levels change also in response to environmental stress (Tanabe et al. 2007). Ishikawa and Shigeoka (2008) reported that the expression of chloroplastic *APX* is post-transcriptionally regulated by alternative splicing.

DHAR, with the participation of GSH, catalyzes the reduction of DHA to AsA and thus contributes to the regulation of ascorbate redox state (Foyer and Noctor 2003). The increase in DHAR activity ensures efficient regeneration of AsA, which can scavenge higher levels of H₂O₂ under stress conditions. In the current study, changes in DHAR activity (Fig. 4b) as well as in *SIDHAR1* and *SIDHAR2* transcript levels were found (Figs. 5c and d) in response to salt stress. These changes depend on fruit development stages and the intensity and duration of salt stress. The changes in DHAR activity are not directly related to changes in *SIDHAR1* and *SIDHAR2* genes expression, suggested a post-transcriptional regulation or the existence of other genes coding for DHAR. The increase in DHAR activity in response to various environmental stresses that promote ROS generation was reported in several studies (Ushimaru et al. 1992; Urano et al. 2000), suggesting that DHAR is important for maintenance of the cellular ascorbate pool and protection against such stresses.

The second enzyme implicate in AsA regeneration is MDHAR, it is one of the major components of the AsA–GSH cycle (Noctor and Foyer 1998). It has been suggested that regeneration of AsA is regulated in this cycle mainly by NAD(P)H-dependent MDHAR activity (Shalata and Tal 1998; Mittova et al. 2000; Shalata et al. 2001). The increase in MDHAR activity increases AsA regeneration, which can then be utilized as a reductant by APX for detoxifying H₂O₂. Results in this study revealed that the response of MDHAR activity (Fig. 4c) and *SIMDHAR* transcript level (Fig. 5e) to different salt stress treatments varied according to fruit development stage. We also noted a marked increase in MDHAR activity in fruits at mature green stage of plants stressed (Fig. 4c). This suggested that the AsA regeneration by MDHAR in fruits at this stage becomes more important under salt stress. Moreover, we didn't found a correlation between MDHAR activity and *SIMDHAR* transcript accumulation under salt stress, suggested a post-transcription regulation. Mittova et al. (2003) shown an increase in MDHAR activity in tomato leaf under salt stress; however, decreased MDHAR activity is responsible for lower regeneration of AsA in root of salt-sensitive tomato species under salt-stress conditions (Mittova et al. 2004).

Oxidized glutathione (GSSG) produced during AsA regeneration by DHAR is reduced by NADPH-dependent GR to reduced glutathione (GSH) to allow the chain reactions of scavenging H₂O₂ by APX to be completed and continued (Mittler 2002; Apel and Hirt 2004). GR is present in several isoforms located in different cell compartments. Over-expression of GR increases antioxidant activity and improves tolerance to oxidative stress (Noctor et al. 1998; Potters et al. 2004). Results obtained in this investigation reveal that salt stress treatments modified GR activity (Fig. 4d) as well as *SIGR_{cyto}* and *SIGR_{cp}* transcript levels (Figs. 5f and g) according to the fruit development stage. Our results also showed no between GR activity and *SIGR_{cyto}* and *SIGR_{cp}* transcripts, suggesting a post-transcription regulation. In tomato plants, salt treatment did not affect GR activity in root; however, GR activity in leaf decreased under salt stress (Mittova et al. 2003, 2004). Hernández et al. (2000) reported that cytosolic GR was strongly induced in the NaCl-tolerant variety of pea.

In conclusion, our present results indicate that salt stress modulates the antioxidative responses of tomato fruits. A perusal of the results shows many changes in the ascorbate pool and the antioxidant enzymes under salt stress, and these changes vary according to level of salt stress and the fruit development stage. Our results highlight the capacity of tomato to withstand salt stress conditions by regulating the ascorbate–glutathione cycle, and the important role of antioxidant enzymes and compounds in protecting cellular apparatus under these conditions which may be important for the selection for salinity resistance in tomato.

References

- Aebi H (1984) Isolation, purification, characterization, and assay of antioxygenic enzymes: catalase in vitro. *Methods Enzymol* 105: 121–126
- Agarwal S, Pandey V (2004) Antioxidant enzyme responses to NaCl stress in *Cassia angustifolia*. *Biol Plant* 48:555–560
- Ahmad P, Nabi G, Jeel CA, Umar S (2011) Free radical production, oxidative damage and antioxidant defense mechanisms in plants under abiotic stress. In: Ahmad P, Umar S (eds) *Oxidative stress: role of antioxidants in plants*. Studium Press, New Delhi, pp 19–53
- Al-Aghabary K, Zhu Z, Shi Q (2005) Influence of silicon supply on chlorophyll content, chlorophyll fluorescence, and antioxidative enzyme activities in tomato plants under salt stress. *J Plant Nutr* 12:2101–2115
- Amor NB, Jiménez A, Megdiche W, Lundqvist M, Sevilla F, Abdelly C (2006) Response of antioxidant systems to NaCl stress in the halophyte *Cakile maritima*. *Physiol Plant* 126:446–457
- Apel K, Hirt H (2004) Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. *Annu Rev Plant Biol* 55:373–399
- Arora A, Sairam RK, Srivastava GC (2002) Oxidative stress and antioxidant systems in plants. *Curr Sci* 82:1227–1238
- Asada K (1999) The water–water cycle in chloroplasts: scavenging of active oxygen and dissipation of excess photons. *Annu Rev Plant Physiol Plant Mol Biol* 50:601–609
- Bohnert HJ, Jensen RG (1996) Metabolic engineering for increased salt tolerance: the next step. *Aust J Plant Physiol* 23:661–667
- Bor M, Ozdemir F, Turkan I (2003) The effect of salt stress on lipid peroxidation and antioxidants in leaves of sugar beet *Beta vulgaris* L. and wild beet *Beta maritima* L. *Plant Sci* 164:77–84
- Borsani O, Valpuesta V, Botella MA (2003) Developing salt tolerant plants in a new century: a molecular biology approach. *Plant Cell Tissue Organ Cult* 73:101–115
- Bowler C, Van Montagu M, Inzé D (1992) Superoxide dismutase and stress tolerance. *Annu Rev Plant Physiol Plant Mol Biol* 43:83–116
- Bradford MM (1976) A rapid and sensitive method for the quantitation of micrograms quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* 72:248–254
- Dat JF, Vandenabeele E, Vranova M, Mantagu V, Inzé D, Breusegem FV (2000) Dual action of the active oxygen species during plant stress responses. *Cell Mol Life Sci* 57:779–795
- De Gara L, Paciolla C, De Tullio MC, Motto M, Arrigioni O (2000) Ascorbate-dependent hydrogen peroxide detoxification and ascorbate regeneration during germination of a highly productive maize hybrid: evidence of an improved detoxification mechanism against reactive oxygen species. *Physiol Plant* 109:7–13
- Dhindsa RS, Plumb-Dhindsa P, Thorpe TA (1981) Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. *J Exp Bot* 32:93–101
- Dionisio-Sese ML, Tobita S (1998) Antioxidant responses of rice seedlings to salinity stress. *Plant Sci* 135:1–9
- Epstein E, Norlyn JD, Rish DW, Kingsbury RW, Kelley DB, Cunningham GA, Wrona AF (1980) Saline culture of crops: a genetic approach. *Science* 210:399–404
- Foyer CH, Noctor G (2000) Oxygen processing in photosynthesis: regulation and signaling. *New Phytol* 146:359–388
- Foyer CH, Noctor G (2003) Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. *Physiol Plant* 119:355–364
- Foyer CH, Lelandais M, Kunert KJ (1994) Photooxidative stress in plants. *Physiol Plant* 92:696–717
- Gill S, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol Biochem* 48:909–930
- Gómez JM, Jiménez A, Olmas E, Sevilla F (2004) Location and effects of long-term NaCl stress on superoxide dismutase and ascorbate peroxidase isoenzymes of pea (*Pisum sativum* cv. Puget) chloroplasts. *J Exp Bot* 55:119–130
- Gossett DR, Millhollon EP, Lucas MC (1994) Antioxidant response to NaCl stress in salt-tolerant and salt-sensitive cultivars of cotton. *Crop Sci* 34:706–714
- Gueta-Dahan Y, Yaniv Z, Zilinskas BA, Ben-Hayyim G (1997) Salt and oxidative stress: similar and specific responses and their relation to salt tolerance in citrus. *Planta* 203:460–469
- Hernández JA, Corpas FJ, Gómez M, del Río LA, Sevilla F (1993) Salt-induced oxidative stress mediated by activated oxygen species in pea leaf mitochondria. *Physiol Plant* 89:103–110
- Hernández JA, Jiménez A, Mullineaux PM, Sevilla F (2000) Tolerance of pea (*Pisum sativum* L.) to long-term salt stress is associated with induction of antioxidant defenses. *Plant Cell Environ* 23:853–862
- Hernández JA, Ferrer MA, Jiménez A, Ros-Barcelo A, Sevilla F (2001) Antioxidant system and O_2^-/H_2O_2 production in the apoplast of *Pisum sativum* L. leaves: its relation with NaCl-induced necrotic lesions in minor veins. *Plant Physiol* 127:817–831
- Ishikawa T, Shigeoka S (2008) Recent advances in ascorbate biosynthesis and the physiological significance of ascorbate peroxidase in photosynthesizing organisms. *Biosci Biotechnol Biochem* 72: 1143–1154
- Jain M, Mathur G, Koul S, Sarin NB (2001) Ameliorative effects of proline on salt stressed lipid peroxidation in cell lines of groundnut (*Arachis hypogea* L.). *Plant Cell Rep* 20:463–468
- Jiménez A, Hernández JA, del Río LA, Sevilla F (1997) Evidence for the ascorbate–glutathione cycle in mitochondria and peroxisomes of pea leaves. *Plant Physiol* 114:275–284
- Kampfenkel K, Van Montagu M, Inzé D (1995) Extraction and determination of ascorbate and dehydroascorbate from plant tissue. *Anal Biochem* 225:165–167
- Kim DW, Shibato J, Agrawal GK, Fujihara S, Iwahashi H, Kim DH, Shim IS, Rakwal R (2007) Gene transcription in the leaves of rice undergoing salt-induced morphological changes (*Oryza sativa* L.). *Mol. Cells* 24:45–59
- Kukreja S, Nandwal AS, Kumar N, Sharma SK, Sharma SK, Unvi V, Sharma PK (2005) Plant water status, H_2O_2 , scavenging enzymes, ethylene evaluation and membrane integrity of *Cicer arietinum* roots as affected by salinity. *Biol Plant* 49:305–308
- Lee DH, Kim YS, Lee CB (2001) The inductive responses of the antioxidant enzymes by salt stress in rice (*Oryza sativa* L.). *J. Plant Physiol* 158:737–745
- Lopez F, Vansuyt G, Casse-Delbart F, Fourcroy P (1996) Ascorbate peroxidase activity not the mRNA level, is enhanced in salt stressed *Raphanus sativus* plants. *Physiol Plant* 97:13–20
- Meneguzzo S, Sgherri C, Navari-Izzo F, Izzo R (1998) Stromal and thylakoid-bound ascorbate peroxidase in NaCl treated leaves. *Physiol Plant* 104:735–740
- Mittal R, Dubey RS (1991) Behaviour of peroxidases in rice: changes in enzyme activity and isoforms in relation to salt tolerance. *Plant Physiol Biochem* 29:31–40
- Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci* 7:405–410
- Mittova V, Volokita M, Guy M, Tal M (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
- Mittova V, Guy M, Tal M, Volokita M (2002) Response of the cultivated tomato and its wild salt-tolerant relative *Lycopersicon pennellii* to salt-dependent oxidative stress: increased activities of antioxidant enzymes in root plastids. *Free Radic Res* 36:195–202
- Mittova V, Tal M, Volokita M, Guy M (2003) Up-regulation of the leaf mitochondrial and peroxisomal antioxidative systems in response to

- salt-induced oxidative stress in the wild salt-tolerant tomato species *Lycopersicon pennellii*. Plant Cell Environ 26:845–856
- Mittova V, Guy M, Tal M, Volokita M (2004) Salinity upregulates the antioxidative system in root mitochondria and peroxisomes of the wild salt-tolerant tomato species *Lycopersicon pennellii*. J Exp Bot 55:1105–1113
- Munns R (2005) Genes and salt tolerance: bringing them together. New Phytol 167:645–663
- Murshed R, Lopez-Lauri F, Keller C, Monnet F, Sallanon H (2008a) Acclimation to drought stress enhances oxidative stress tolerance in *Solanum lycopersicum* L. fruits. Plant Stress 2:145–151
- Murshed R, Lopez-Lauri F, Sallanon H (2008b) Microplate quantification of enzymes of the plant ascorbate–glutathione cycle. Anal Biochem 383:320–322
- Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol 22:867–880
- Noctor G, Foyer CH (1998) Ascorbate and glutathione: keeping active oxygen under control. Annu Rev Plant Physiol Plant Mol Biol 49:249–279
- Noctor G, Arisi ACM, Jouanin L, Kunert KJ, Rennenberg H, Foyer CH (1998) Glutathione: biosynthesis, metabolism and relationship to stress tolerance explored in transformed plants. J Exp Bot 49:623–647
- Panda SK, Upadhyay RK (2003) Salt stress injury oxidative alterations and antioxidative defence in the roots of *Lemna minor*. Biol Plant 48:249–253
- Potters G, Horemans N, Bellone S, Caubergs RJ, Trost P, Guisez Y, Asard H (2004) Dehydroascorbate influences the plant cell cycle through a glutathione-independent reduction mechanism. Plant Physiol 134:1479–1487
- Queirós F, Rodrigués JA, Almeida JM, Almeida DPF, Fidalgo F (2011) Differential responses of the antioxidant defence system and ultrastructure in a salt-adapted potato cell line. Plant Physiol Biochem 49:1410–1419
- Reddy AR, Chaitanya KV, Vivekanandan M (2004) Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. J Plant Physiol 161:1189–1202
- Rengasamy P (2006) World salinization with emphasis on Australia. J Exp Bot 57:1017–1023
- Sairam RK, Tyagi A (2004) Physiology and molecular biology of salinity stress tolerance in plants. Curr Sci 86:407–421
- Sairam RK, Deshmukh PS, Saxena DC (1998) Role of antioxidant systems in wheat genotypes tolerance to water stress. Biol Plant 41:387–394
- Sairam RK, Srivastava GC, Agarwal S, Meena RC (2005) Differences in antioxidant activity in response to salinity stress in tolerant and susceptible wheat genotypes. Biol Plant 49:85–89
- Salin ML (1987) Toxic oxygen species and protective systems of the chloroplast. Physiol Plant 72:681–689
- Sánchez-Rodríguez E, Rubio-Wilhelmi M, Blasco B, Leyva R, Romero L, Ruiz JM (2012) Antioxidant response resides in the shoot in reciprocal grafts of drought-tolerant and drought-sensitive cultivars in tomato under water stress. Plant Sci 188–189:89–96
- Serrano R, Mulet J, Rios G, Marquez J, de Larrinoa I, Leube M, Mendizabal I, Pascual-Ahuir A, Proft M, Ros R, Montesinos C (1999) A glimpse of the mechanisms of ion homeostasis during salt stress. J Exp Bot 50:1023–1036
- Shalata A, Tal M (1998) The effects of salt stress on lipid peroxidation and antioxidants in the leaf of the cultivated tomato and its wild salt-tolerant relative *Lycopersicon pennellii*. Physiol Plant 104:169–174
- Shalata A, Mittova V, Volokita M, Guy M, Tal M (2001) Response of the cultivated tomato and its wild salt-tolerant relative *Lycopersicon pennellii* to salt-dependent oxidative stress: the root antioxidative system. Physiol Plant 112:487–494
- Shigeoka S, Ishikawa T, Tamoi M, Miyagawa Y, Takeda T, Yabuta Y, Yoshimura K (2002) Regulation and function of ascorbate peroxidase isoenzymes. J Exp Bot 53:1305–1319
- Smimoff N (1995) Antioxidant systems and plant response to the environment. In: Smimoff N (ed) Environment and Plant Metabolism. Bios Scientific Publishers, Oxford, pp 217–243
- Smimoff N, Wheeler GL (2000) Ascorbic acid in plants: biosynthesis and function. Crit Rev Plant Sci 19:267–290
- Song XS, Hu WH, Mao WH, Ogwen JO, Zhou YH, Yu JQ (2005) Response of ascorbate peroxidase isoenzymes and ascorbate regeneration system to abiotic stresses in *Cucumis sativus* L. Plant Physiol Biochem 43:1082–1088
- Sudhakar C, Lakshmi A, Giridarakumar S (2001) Changes in the antioxidant enzyme efficacy in two high yielding genotypes of mulberry (*Morus alba* L.) under NaCl salinity. Plant Sci 161:613–619
- Tanabe N, Yoshimura K, Kimura A, Yabuta Y, Shigeoka S (2007) Differential expression of alternatively spliced mRNAs of Arabidopsis SR protein homologs, atSR30 and atSR45a, in response to environmental stress. Plant Cell Physiol 48:1036–1049
- Urano J, Nakagawa T, Maki Y, Masumura T, Tanaka K, Murata N, Ushimaru T (2000) Molecular cloning and characterization of a rice dehydroascorbate reductase. FEBS Lett 466:107–111
- Ushimaru T, Shibasaki M, Tsuji H (1992) Development of the O₂⁻ detoxification system during adaptation to air of submerged rice seedlings. Plant Cell Physiol 33:1065–1071
- Van Breusegem F, Vranova E, Dat JF, Inzé D (2001) The role of active oxygen species in plant signal transduction. Plant Sci 161:405–414
- Wang J, Zhang H, Allen RD (1999) Overexpression of an Arabidopsis peroxisomal ascorbate gene increase protection against oxidative stress. Plant Cell Physiol 40:725–732
- Willekens H, Chamnongpol S, Davey M, Schraudner M, Langebartels C, Van Montagu M (1997) Catalase is a sink for H₂O₂ and is indispensable for stress defence in C3 plants. EMBO J 16:4806–4816
- Yamaguchi T, Blumwald E (2005) Developing salt-tolerant crop plants: challenges and opportunities. Trends Plant Sci 10:615–620
- Zhu Z, Wei G, Li J, Qian Q, Yu J (2004) Silicon alleviates salt stress and increases antioxidant enzymes activity in leaves of salt-stressed cucumber (*Cucumis sativus* L.). Plant Sci 167:527–533