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Original Paper

Overexpression of apple spermidine synthase 1 (*MdSPDS1*) leads to significant salt tolerance in tomato plants

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Abstract The aim of this research is to study the effects of salt stress during different stages of development in transgenic tomato plants overexpressing the apple spermidine synthase gene (*MdSPDS1*) compared to wild type (WT) plants. Under salt treatment (100 and 150 mM NaCl), tomato plants clearly displayed several stress symptoms such as impaired seedling growth, decreased chlorophyll content, reduction in fruit yield and increased electrolyte leakage (EL) in leaves. These changes were more prominent in WT plants compared to *MdSPDS1* transgenic plants which accumulate significantly more polyamines, namely spermine and spermidine. The response of ascorbate peroxidase (APX) isoenzymes in tomato leaves under saline conditions was also investigated. The transcript levels of *SlApx* genes were significantly up regulated under 100 mM NaCl either in wild type or in transgenic plants. Under 150 mM NaCl, only transgenic plants were capable to maintain high expression of *SlApx* genes, whereas in WT plants the expression declined after one month treatment. As a consequence, APX activity was significantly higher in *MdSPDS1* transgenic plants compared to WT under saline conditions. These results suggest that elevated APX activity might contribute largely to the protection against oxidative stress generated by NaCl treatments and thus confer salinity tolerance in tomato overexpressing *MdSPDS1*.

Key words: Acorbate peroxidase, polyamines, salt tolerance, spermidine synthase, tomato.

Salinity represents a real stress and serious threat for plant growth, agriculture and environment. It is considered to be among the major constraints limiting plant growth, productivity and distribution in large areas worldwide (Ashraf and Foolad 2007). According to UNESCO Water Portal in 2007, more than 6% of land and 30% of the irrigate areas in the world already suffer from salinity problems. Moreover, the salt-affected areas are annually increasing at a rate of 10%. Low precipitations, high surface evaporation, weathering of native rocks, irrigation with saline water, and poor cultural practices are among the major contributors to the increase of soil salinity (Foolad 2004). In plants, the excessive exposure of cells to high concentrations of NaCl induces imbalance of the cellular ions and osmotic potential which are deleterious to plant cells (Apse and Blumwald 2002). High concentrations of sodium disrupt potassium, iron and other mineral nutrition, create hyperosmotic stress, and cause secondary problems such as oxidative stress (Zhu 2000).

Tomato (*Solanum lycopersicum* L.) is one of the most important crops in the world. Although the cultivated tomato is widely adapted to different climates (Bolarin et al. 2001), its growth and development are rather moderately sensitive to salinity, and as a result, economic yield is substantially reduced under salt stress (Bolarin et al. 1993). Unlike in *Arabidopsis*, direct studies on salinity effects, adaptation, and molecular changes in tomato can be assessed for crop yield (Borsani et al. 2001). The engineering of salt tolerance of crop has been a long-held and intensively sought objective (Apse and Blumwald 2002). Despite the salinity tolerance of tomato plant is known to be a complex trait controlled and regulated by multiple factors, very encouraging results have been reported even though when using single genes as target (AbuQamar et al. 2009; Qi et al. 2010). Advances in molecular genetics and plant transformation techniques have made it feasible to assess

Abbreviations: APX, ascorbate peroxidise; chl, chlorophyll; EL, electrolyte leakage; PA, polyamines; Put, putrescine; Spd, spermidine; Spm, spermine; SPDS, spermidine synthase; WT, wild type.
This article can be found at http://www.jspcmb.jp/

biotechnological strategies such as activated signal cascades responsive to stress, or engineering of biosynthetic pathways by modification of targeted gene and protein expression (Hasegawa et al. 2000).

Polyamines have long been implicated in plant growth and development, as well as adaptation to abiotic and biotic stress (reviewed by Alcázar et al. 2010). The increase in polyamine levels may be an integral self-protecting response to salinity environments (Zhao et al. 2007). In a previous study, we generated transgenic tomato lines overexpressing apple spermidine synthase (*MdSPDS1*, Zhang et al. 2003) under the control of 35S promoter (Neily et al. 2010). These lines were particularly characterized by a significant metabolic alteration during the phase of fruit ripening, especially high accumulation in carotenoid content. Carotenoids are not only widely accepted as one of major antioxidants (Schindler et al. 1994) but also suggested to be a required factor for salt tolerance in plants (Hernandez et al. 1995). Therefore, a generation of a new tomato variety with higher carotenoid content would benefit to improve agricultural practices in salt-affected areas as well as its nutritional value as a source of health-promoting antioxidants in human diet. In the present study, we evaluated the level of salt tolerance of the transgenic tomato lines accumulating polyamines by analyzing plant biomass, chlorophyll content, electrolyte leakage, and also antioxidant activity which is linked to the accumulation of carotenoids, under severe salt stress. We discussed the possible physiological mechanisms underlying the tolerance provided by polyamine accumulation.

Material and methods

Plant materials

The transgenic tomato (*Solanum lycopersicum* L., cv. Micro-tom) lines overexpressing *MdSPDS1,* (GenBank accession number AB072915) (Zhang et al. 2003) were submitted to this work, which were generated by *Rhizobium radiobactor* mediated transformation method in previous work (Neily et al. 2010). At the start of the present work, two transgenic lines P_{35S}:*Mdspds1*^{OE}: 36A.12 and 70A.3 with single copy of the transgene were selected. T_3 and T_4 plant generations of each line and wild-type (WT) plants were used in the experiments.

Growth conditions and salinity treatments

The tomato seeds were sown on distilled, saturated filter paper in Petri dishes at room temperature. Seedlings were hydroponically cultivated in a commercial nutrient solution (Otsuka A; Otsuka Chemical Co. Ltd., Osaka, Japan) adjusted for N, P, K, Ca and Mg at 18.6, 5.1, 8.6, 8.2 and 3.0 mequiv. per liter respectively. Plants were cultivated in growth chamber under controlled environmental conditions (16 h light/8 h dark with approximately 250 μmol photons m^{-2} s^{-1}) and the temperature was set to 25ºC. When the seedlings were 4 weeks-old, sodium chloride (NaCl) was added to the nutrient solution to reach the saline concentration of 0, 100 and 150 mM NaCl in a single step and the cultures were maintained for 1 month under the same growth conditions. Plants were used to analyze root and stem-leaf-shoot weight after drying in an oven at 70ºC for 72 h.

To evaluate the effect of salt stress on fruit yield (weight and number), additional plants were cultivated with the same conditions. Seedlings were transplanted into the 5×5×5 cm rock wool pots (Toyobo, Osaka, Japan). At flowering time, the salt stress was started by adding NaCl to the nutrient solution to 100 and 150 mM. The culture was maintained in these conditions up to the harvest of red-ripe fruits at approximately 65 days after flowering. The hydroponic solution was renewed every 5 days during salt exposure to avoid possible pH changes and nutrient depletion. After harvest, half of the plants was used to analyze root and stem-leaf-shoot weight after drying in an oven at 70ºC for 72 h. For each treatment, biomass data was obtained from 6 individual plants.

Polyamines measurement

Measurement of free polyamine contents was performed by homogenizing the tomato fruits with 5% (w/v) perchloric acid. After centrifugation, the supernatant was preserved and pellet was resuspended in 5% perchloric acid after several washes. After dansylation of the supernatant, polyamines were quantified with HPLC as described by Burtin et al. (1989); 1,6 hexanediamine was used as an internal standard.

Electrolyte leakage (EL)

Measurement of EL was performed according to the method of Liu et al. (2004) with slight modifications. From the transgenic and WT tomato plants, 0.3 g of leaf at the same position was sampled and incubated in 25 ml of distilled water at 25°C for 2 h. After the incubation, the initial conductivity (Ci) was checked by a conductivity meter (Cyberscan PC1, Singapore). The samples were boiled for 30 min to trigger a complete leakage, and then cooled at 25°C for 12 h before measuring the final electrolyte conductivity (Cf). The relative conductivity (C) was expressed as a percentage of the total ion leakage and calculated as follow: C (%)=100×Ci/Cf.

Measurement of chlorophyll content in leaves

The total chlorophyll content in developing leaf tissues was measured according to Porra et al. (1989). The leaf samples were harvested from the transgenic and WT plants exposed to 0, 100 and 150 mM NaCl conditions for 15 days. Ten mg of leaf sections were placed in glass tube containing 2 ml of N, N-dimethylformamide (DMF) and incubated for overnight at 4°C under absolute dark condition. The absorbances at 663.8 nm and 646.8 nm were measured, respectively and the total chlorophyll contents were calculated with the following formula, chlorophyll content (μg ml^{-1})=7.17×$A_{663.8}$+17.67×$A_{646.8}$. Total chlorophyll content was subsequently converted to leaf chlorophyll concentration and expressed as μg of chlorophyll per g fresh weight (FW).

Quantitative real-time PCR analysis of APX genes

We used real-time PCR to examine and to quantify the expression of seven APX genes in leaf samples from transgenic lines and WT. Leaves were collected from 4–5 weeks plants grown for 1 month under 0 (control), 100 and 150 mM NaCl. Total RNA were extracted and treated with DNase from each

Table 1. Sets of PCR primers used for qRT-PCR analysis of *SlApx* genes

cDNA		Primer sequence (5′? 3′)	Size (bp)	Accession no.
SlApx 1	Sense	ACGATGATATTGTGACACTCTTCCA	71	DQ099420*
	Antisense	AAGCGATGAAACCACAAAAACA		
SlApx 2	Sense	TGGGAGGGTGGTGACATATTTT	190	DQ099421*
	Antisense	TTGAAGTGCATAACTTCCCATCTTT		
SlApx 3	Sense	TTCAACAGCAACTACTCCAGCC	151	DQ131129*
	Antisense	GGAACAGTTCCCAATCCTATCC		
SlApx 4	Sense	GGAACAGTTCCCAATCCTATCC	207	DQ131130*
	Antisense	CATAGGTTCCTGCATCATGCCACC		
SlApx 5	Sense	AGTAGATGCAGAGTATCTGAAGGA	199	DQ131131*
	Antisense	CATAGGTTCCTGCATCATGCCACC		
SlApx 6	Sense	TTCACCCAATCCACAAATTCCG	146	DQ131132*
	Antisense	GTAAGACAGTAGGGCCTTGTCC		
SlApx 7	Sense	CTTTCTTCAATGGCTTCTCTCACCG	169	DQ131133*
	Antisense	CAACCTGGTAGCGAAACACATGGG		
ubi 3	Sense	CACCAAGCCAAAGAAGATCA	120	X58253
	Antisense	TCAGCATTAGGGCACTCCTT		

*These Primer sequences was cited by Najami et al. (2008).

sample using the RNeasy Plant mini kit (Qiagen, Maryland, USA). The RNA concentration and quality were checked using a Agilent Bioanalyzer 2100 (Agilent RNA 6000 Nano Kit, Germany). Total RNA (1.5 µg) were used to synthesize the first-strand cDNA using the SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). The first-strand cDNA was diluted 500 fold and 1 µl of the dilution was used for quantitative RT-PCR with SYBR Premix Ex Taq (Takara BioInc. Otsu, Japan). The PCR thermal cycling conditions were performed following the manufacturer's instructions. The tomato ubiquitin gene (*ubi3*) (accession number: X58253) was used as an internal standard. Specific amplifications were confirmed by single transcript amplification in agarose gel, single dissociation peak, and calibration curves. The primer sequences for the seven *SlApx* genes (Najami et al. 2008) used in our study are described in Table 1.

Assay of APX activity

Firstly, 0.1 g of fresh leaf tissue was homogenized in three fold volume of ice-cold 50 mM potassium phosphate buffer (pH 7.5) containing 2% (w/v) PEG 6000, 1 mM PMSF, 8% (w/v) insoluble polyvinylpolypyrrolidone and 0.01% (v/v) Triton X-100. After centrifugation at 15000 *g* for 20 min at 4°C, the supernatant was collected and APX activity was immediately assayed by following the oxidation of ascorbate at 290 nm according to Nakano and Asada (1981) with minor changes. The reaction mixture (1 ml) consisting of 80 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, and 100 µl of extract was incubated for 2 min at 25°C. The reaction started by adding 0.2 mM H_2O_2. The rate was corrected by subtracting the non-enzymatic oxidation of ascorbate in the absence of extract.

Results

Effect of salt stress on biomass

Prior to this work, we tested the intensity, duration of the salinity stress treatment and suitable growth stage for tomato plant for subsequent experiments. We thus determined that 100 and 150 mM NaCl at the stage with 4th true leaves emergence for one month treatment were the optimum conditions to observe clear phenotypic and physiological responses (Figure 1). Lines 36A.12 and 70A.3 behaved similarly under control conditions.

In order to evaluate the degree of tolerance in P_{35S}:*Mdspds1*^{OE} lines 36A.12 and 70A.3 compared to WT, plant biomass after 1 month treatment was measured in leaves, stem, flowers and root on FW-and dry-weight (DW) bases (Figure 2). In the control condition (0 mM NaCl), the plant biomass showed no significant change between transgenic lines and WT plants. Under salt treatment, the biomass of the aerial parts was reduced proportionally with the stress intensity on the basis of the FW and DW; FW biomass was 26–44% under 100 mM and 13–27% under 150 mM NaCl (Figure 2A), and DW biomass was 33–72% under 100 mM and 16–40% under 150 mM NaCl (Figure 2B) compared to the control condition. Except for the FW biomass of 36A.12 under 100 mM NaCl, the biomass of the P_{35S}:*Mdspds1*^{OE} lines was significantly higher than that of WT in both on the base of the FW and DW. On the other hand, the level of biomass reduction by salt stress was much limited in the roots even though the P_{35S}:*Mdspds1*^{OE} lines tended to exhibit a larger root biomass.

Effect of salt stress on fruit yield

To further dissect the level of tolerance in *MdSPDS1* overexpression tomato plants, we evaluated the influence of salt stress on the reproductive stage, namely fruit yield (Figure 3). The fruit number per plant was greatly reduced by increasing the salinity intensity. It decreased by 78%, 62% and 69% in WT, 36A.12 and 70A.3 respectively at 100 mM NaCl compared to the control, and up to 94% in WT and 12% in both of P_{35S}:*Mdspds1*^{OE} lines at 150 mM NaCl (Figure 3A). Regarding the fruit weight, no significant difference was observed in all the genotypes in the absence of NaCl. Salinity negatively

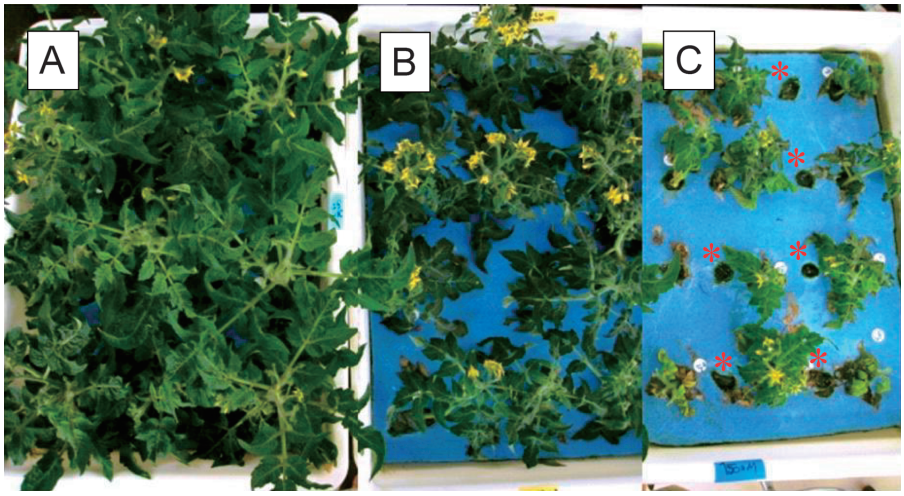

Figure 1. Response of WT and P_{35S}:*Mdspds1*^{OE} plants to salt stress after 1 month treatment. Four-week-old plants were watered with nutritive solution (A), the second group was watered with 100 mM NaCl (B), the third group was watered with 150 mM NaCl (C). WT plants were marked with red asterisks.

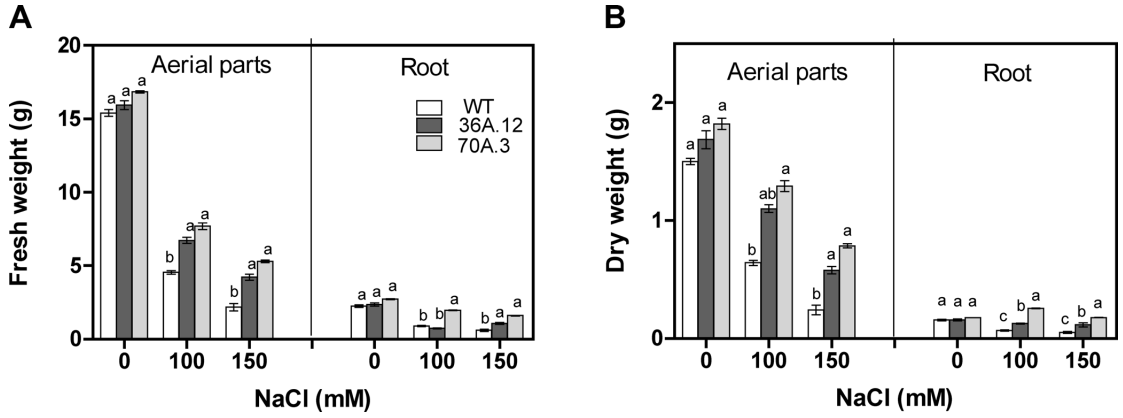

Figure 2. Biomass of WT and P_{35S}:*Mdspds1*^{OE} plants exposed to the salt stress. Four-week-old plants were submitted to the hydroponic-cultivation for 1 month with nutrient solution without/with 100 and 150 mM NaCl, respectively. (A) Fresh and (B) dry weight. Values (g) are averages from 6 plants in each line and are shown as means±SD (n=6). Different letters indicate significant differences by Scheffe's F-test, $p \leq 0.05$.

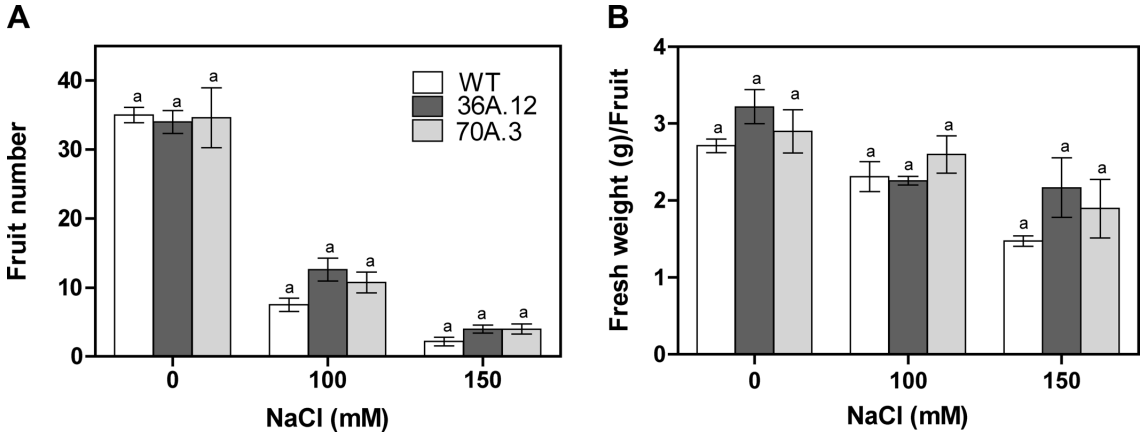

Figure 3. Fruit yield of WT and P_{35S}:*Mdspds1*^{OE} plants exposed to the salt stresses. (A) Fruit number and (B) fresh weigh (g)/fruit. At flowering time, the salt stress was started by adding NaCl to the nutrient solution up to 100 and 150 mM and continued by the harvest of red-ripe fruits for approximately 65 days. Values are averages from 6 plants in each line and are shown as means±SD (n=6). Different letters indicate significant differences by Scheffe's F-test, $p \leq 0.05$.

affected the fruit weight as shown by the decrease of 15% in WT, 29% in 36A.12 and 10% in 70A.3 lines at 100 mM NaCl. The effect was stronger at 150 mM NaCl, as the fruit weight decrease was 55% in WT and only 32% and 34% in 36A.12 and 70A.3 lines (Figure 3B).

Effect of salt stress on free polyamine accumulation

Polyamine content was measured in the leaf tissue of WT and P_{35S}:*Mdspds1*^{OE} lines treated with 0, 100 and 150 mM NaCl during one month (Figure 4). Among the free polyamines, spermidine and spermine were present at higher levels whereas putrescine content was the lowest in all the genotypes and conditionss. In control condition (0 mM NaCl), spermidine contents were almost 1.4 to 1.5 times higher in 36A.12 and 70A.3 than in WT. In the presence of 100 mM NaCl a significant increase of putrescine content was observed notably in the WT with a 2.9 folds increase. In the WT, the putrescine content remained high at 150 mM NaCl contrary to the P_{35S}:*Mdspds1*^{OE} lines. In contrast to putrescine, in all plants spermidine content considerably decreased in correlation with the increase of stress intensity (Figure 4B and C). In WT, it dropped to 50% at 100 mM NaCl and 26% at 150 mM NaCl compared to control condition. Showing similar tendency, in the P_{35S}:*Mdspds1*^{OE} spermidine content decreased to 75% at 100 mM and 52% at 150 mM in 36A.12, and in a lower manner in 70A.3 to 50% at 100 mM and 25% at 150 mM. Regarding spermine, the content in WT and the P_{35S}:*Mdspds1*^{OE} lines were similar under control (Figure 4A). Unlike spermidine, spermine was not negatively affected by 100 mM NaCl treatment; it was rather increased to 1.7 and 1.3 folds in WT and the P_{35S}:*Mdspds1*^{OE} lines respectively compared to the control condition (Figure 4B). In plants treated with 150 mM NaCl, spermine contents decreased in WT and remained in 36A.12 to the level of the control, whereas it was the highest level in all the treatment in 70A.3 (Figure 4C).

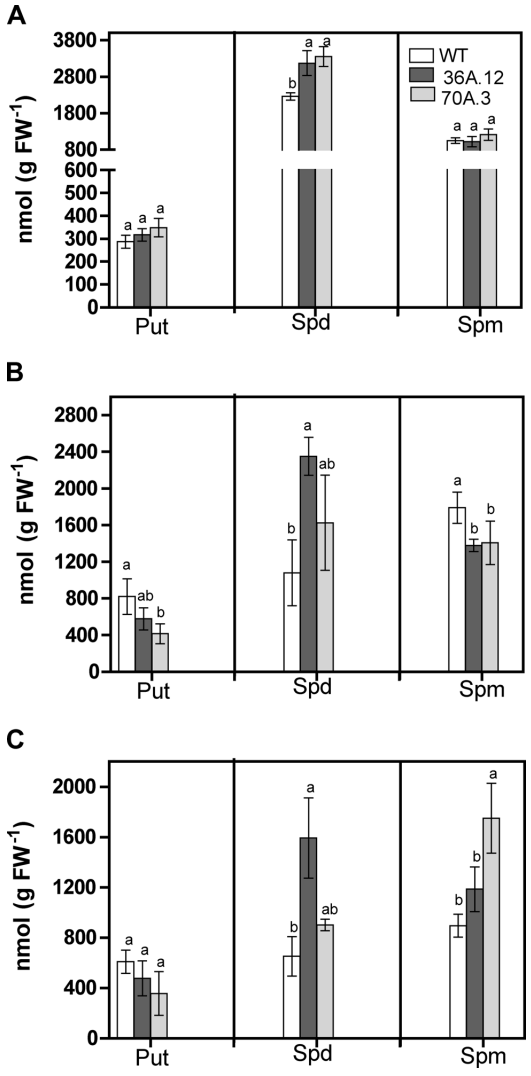

Figure 4. Accumulation of free polyamines in the leaf tissue of WT and P_{35S}:*Mdspds1*^{OE} lines, 36A.12 and 70A.3. Four-week-old plants were submitted to the hydroponically-cultivation with nutrient solution without NaCl (A) or with 100 (B) and 150 mM NaCl (C), respectively. Put; Putrescine, Spd; spermidine and Spm; spermine. Values (nmol gFW^{-1}) are averages from 4 plants in each line and are shown as means ± SD (n=4). Different letters indicate significant differences by Scheffe's F-test, $p \leq 0.05$.

Effect of salt stress on chlorophyll content in leaves

Although the chlorophyll contents in leaves decreased in all the genotypes with increasing intensity of salt stress, there were clear differential responses between WT and the P_{35S}:*Mdspds1*^{OE} lines (Table 2). With 100 mM of NaCl treatment, chlorophyll *a* (chl *a*) and *b* (chl *b*) were reduced by 22%; and 30%; of the control in WT leaves whereas there was almost no effect on chlorophyll contents in 36A.12 and 70A.3 lines. The effect of salt stress on leaf chlorophyll content was more remarkable on P_{35S}:*Mdspds1*^{OE} plants in 150 mM NaCl treatment. Indeed, chl *a* is reduced by 45% in 36A.12 and 70A.3 while chl *b* decreased by 50 to 65% compared to levels of the control condition. In WT plants, chl *a* and chl *b* contents were reduced by 76% and 80%. As a consequence, total chlorophyll content in WT was reduced by 21% in 100 mM NaCl and by 77% under 150 mM NaCl treatments. Such reduction was only observed at 150 mM NaCl in P_{35S}:*Mdspds1*^{OE} lines which displayed a decrease of 47% in both lines.

Effect of salt stress on electrolyte leakage from leaves

The EL was measured in leaf tissues from plants grown under control (0 mM NaCl) and salt stress conditions at 100 and 150 mM NaCl (Figure 5). Under control condition, the EL values were almost at the same levels

Table 2. Effect of increasing salt concentrations on chlorophylls accumulation in WT and P_{35S}:*Mdspds1*^{OE}

	Treatments	Genotypes		
		WT	36A.12	70A.3
Chl *a* (μg ml^{-1})	0 mM NaCl	16.09 ± 0.46 a	17.87 ± 1.22 a	15.74 ± 0.85 a
	100 mM NaCl	12.61 ± 1.22 b	18.57 ± 0.48 a	16.09 ± 0.46 a
	150 mM NaCl	3.9 ± 0.57 b	9.87 ± 0.50 a	8.7 ± 1.31 a
Chl *b* (μg ml^{-1})	0 mM NaCl	5.32 ± 0.16 ba	6.11 ± 0.46 a	4.98 ± 0.32 a
	100 mM NaCl	3.7 ± 0.46 b	5.82 ± 0.23 a	5.32 ± 0.16 a
	150 mM NaCl	1.05 ± 0.17 b	2.89 ± 0.15 a	2.14 ± 0.15 a
Total Chl (μg mgFW^{-1})	0 mM NaCl	4.29 ± 0.12 a	4.81 ± 0.33 a	4.09 ± 0.19 a
	100 mM NaCl	3.4 ± 0.02 a	4.89 ± 0.14 a	4.29 ± 0.12 a
	150 mM NaCl	0.99 ± 0.15 b	2.56 ± 0.13 a	2.18 ± 0.47 a

Chl *a*, chlorophyll *a*, Chl *b*, chlorophyll *b*. Different letters indicate significant differences by Scheffe's F test, $p \leq 0.05$

in WT (8%), 36A.12 (7.7%) and 70A.3 (7%) lines. Increase of stress intensity resulted in higher EL values in all genotypes. However, the electrolyte leakage at 100 mM NaCl was significantly lower in P_{35S}:*Mdspds1*^{OE} lines, as shown by the 14% and 16% increase for 36A.12 and 70A.3 respectively, compared to 24% in WT. At 150 mM NaCl, the EL values reached 48% in WT while it remained at relatively low levels in 36A.12 (34%) and 70A.3 (30%) (Figure 5).

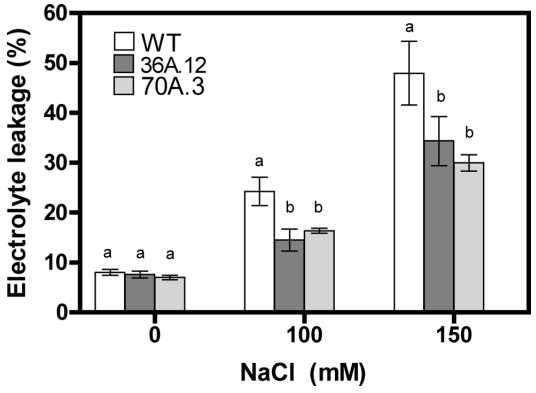

Figure 5. Change on EL of WT and P_{35S}:*Mdspds1*^{OE} lines after 1 month culture under 0, 100, 150 mM NaCl stress. Values (nmol gFW^{-1}) are averages of independent leaf data from each line (n=5) and are shown as means±SD. Different letters indicate significant differences by Scheffe's F-test, $p \leq 0.05$.

Effect of salt stress on expression of APX genes

Transcription levels of seven genes *SlApx* encoding APX were evaluated by quantitative real time PCR (Figure 6). Among all *SlApx* genes, the highest expression level in leaf tissue was observed for *SlApx1*, followed by *SlApx7*, *SlApx4* and *SlApx6* (Figure 6A, G, D and F) while *SlApx2, SlApx3 and SlApx5* showed relatively low expression (Figure 6B, C and E). Under increasing salinity the group of highly expressed genes tended to be up regulated or maintained at higher levels in P_{35S}:*Mdspds1*^{OE} lines compared to WT. For instance, the expression level of *SlApx1* displayed a 2 fold increase in WT and P_{35S}:*Mdspds1*^{OE} lines under 100 mM NaCl, At 150 mM NaCl, *SlApx1* expression remained close to this level in WT while in P_{35S}:*Mdspds1*^{OE} lines it continued to raise by 2 to 2.5 fold in 36A.12 and 70A.3 respectively.

Effect of salt stress on APX activity

The levels of APX activity in leaf tissues increased with the increasing stress intensity in both WT, 36A.12 and 70A.3 lines (Figure 7). However, at 100 mM the augmentation of APX activity was more prominent in the P_{35S}:*Mdspds1*^{OE} lines, as shown by the 3- to 4-fold in comparison with the 1.6-fold increase observed in the WT. This tendency was maintained at 150 mM as the APX activity was increased by 1.5-fold in the P_{35S}:*Mdspds1*^{OE} lines compared to those at 100 mM, whereas it remained at the same level for the WT. It is worth mentioning that activity changes perfectly correlated with those observed at transcription level for the most expressed enzyme SlAPX1.

Discussion

Polyamine accumulation induces salt tolerance in tomato plants overexpressing MdSPDS1

Tomato transgenic lines P_{35S}:*Mdspds1*^{OE} genes have previously been generated in cv. Micro-Tom (Neily et al. 2010). Two P_{35S}:*Mdspds1*^{OE} lines, 36A.12 and 70A.3 displaying significant increase in polyamine content were selected to evaluate the response to salt tolerance in tomato. In the two salt conditions, 100 and 150 mM, tomato plants including transgenic lines and WT clearly exhibit stress symptoms as ascertained by the low productivity with reduced plant biomass and fruit yield, the increase of EL, and the decrease of chlorophyll content in leaves. However, the degree of change in these physiological parameters was much different between WT and P_{35S}:*Mdspds1*^{OE} lines. Hence, prolonged exposure to salinity resulted in a substantial reduction of biomass and fruit yield and these traits were particularly more pronounced in the WT (Figures 2, 3).

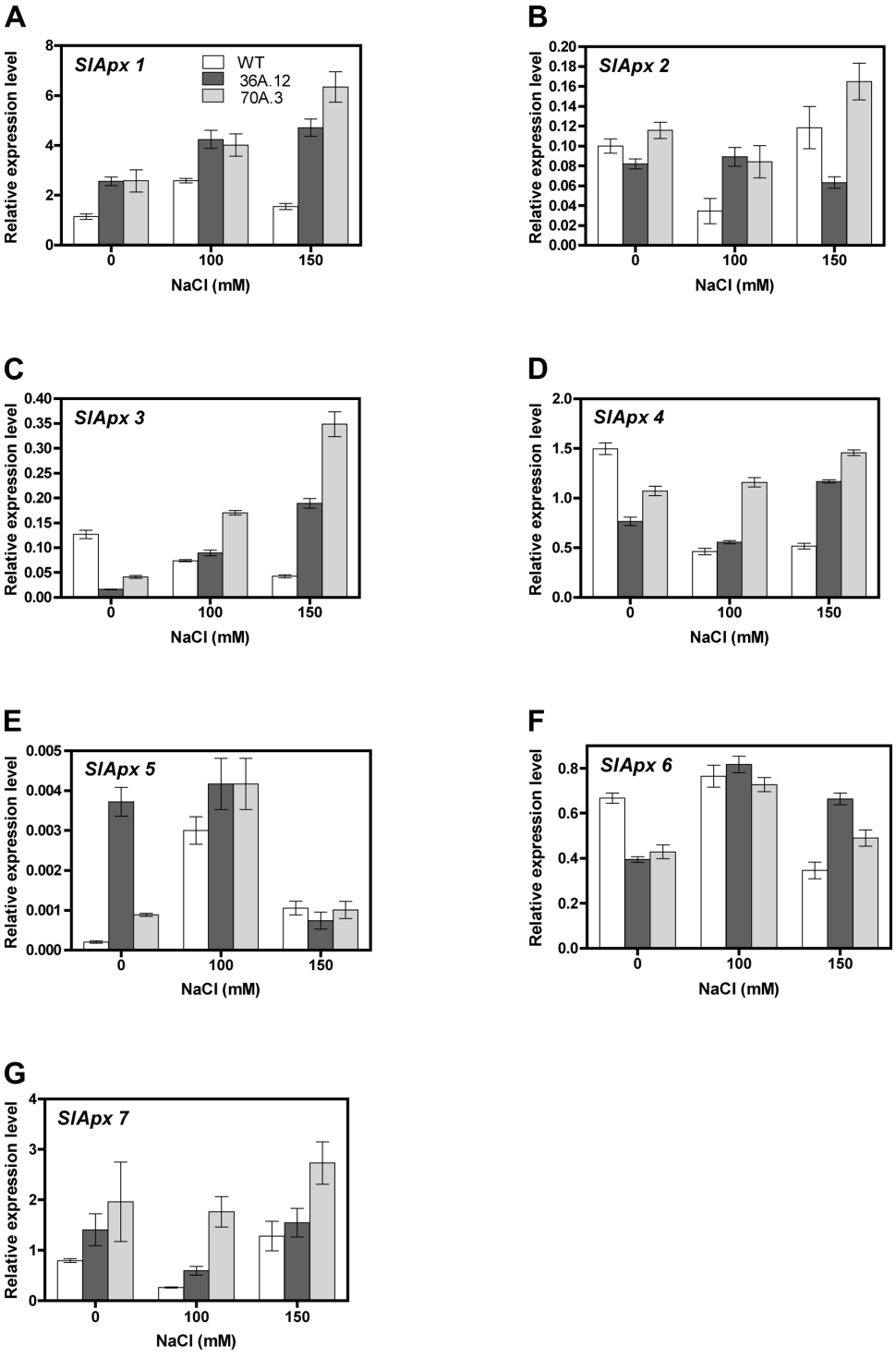

Figure 6. Transcriptional levels of tomato *APX* genes (*SlApx1-7*) in leaves of WT and P_{35S}:*Mdspds1*^{OE} lines in response of 1month of salinity treatment. Relative expression levels were normalized with ubiquitin (*ubi3* gene) expression and represented as relative expression levels. Each transcriptional level was analyzed in quadruplet and repeated two times and are shown as means±SD (n=4).

Photosynthesis is one of the most important processes inhibited under salinity (Demetriou et al. 2007). Regarding chlorophyll content, it is well known that prolonged salt stress results on the degradation of chlorophyll and photosynthesis related protein complexes. In tomato leaves, Khavarinejad and Mostofi (1998) showed that the contents of total chlorophyll (Chl $a+b$), chl a, and chl b declined considerably under NaCl stress. Accordingly, in our study the total chlorophyll content was reduced in WT by 24% after 15 days of treatment at 100 mM NaCl while no change was observed in the P_{35S}:*Mdspds1*^{OE} plants. This effect was

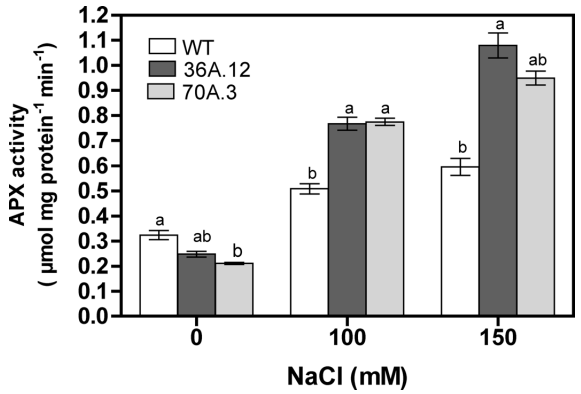

Figure 7. APX activities in leaves of WT and P_{35S}:*Mdspds1*^{OE} lines in response to 1 month salinity treatments. Values are averages from 6 plants in each line and are shown as means±SD (n=6). Different letters indicate significant differences by Scheffe's F-test, $p \leq 0.05$.

exacerbated under more severe salt stress at 150 mM NaCl, with a drop of total chlorophyll content by 77% in WT and only 47% in 36A.12 and 70A.3 lines (Table 2). Kotzabasis et al. (1993) reported that the main polyamines, putrescine, spermidine and spermine are associated with the thylakoid membranes and, especially, at the level of light-harvesting complex II as well as PSII complex. Several reports have attributed a key role of polyamines in the regulation of photosynthetic apparatus under adverse conditions like salinity and UV light (Demetriou et al. 2007; Sfichi et al. 2004). The EL reflects the damage of cell membranes. Under salt stress, WT tomato plants displayed higher EL values whereas in P35: Mdspds10E lines the increase of EL was significantly lessened (Figure 5). Tiburcio et al. (1994) reported that polyamines play a role in reducing the EL of cellular membrane under osmotic stress conditions. It is thus tempting to suggest that the higher spermidine accumulation observed in the P_{35S}: *Mdspds1*^{OE} lines cultivated under salt stress (Figure 4) may function to preserve thylakoid membrane integrity, and consequently in the reduction of the EL and chlorophyll degradation (Table 2, Figure 5).

The limited growth and fruit yield (Figures 2 and 3) have been already reported in tomato plant exposed to salinity (Adams and Ho 1989, Yin et al. 2010). Although photosynthesis activity was not measured in the present work, it would be reasonable to expect that under salt stress, the reduction of chlorophyll content (Table 2) could directly impair the photosynthetic efficiency. As a consequence that would affect plant growth and fruit yield. Such a trait clearly observed in WT could be thus diminished in the P_{35S}:*Mdspds1*^{OE} lines as a result of the excessive accumulation of polyamines (Figure 4).

Changes in polyamine accumulation under salt stress

Spermidine synthase is a key enzyme in the biosynthesis of spermidine from putrescine, and also affects the spermine level. In the present study, the P_{35S}:*Mdspds1*^{OE} lines showed higher accumulation of spermidine and spermine compared to WT under both control and salt stress conditions (Figure 4). Actually, the levels of polyamines were lower under salt condition than in the control. A plausible explanation could be that under high salinity either the polyamine biosynthesis pathway is repressed and/or the degradation is up-regulated. In the P_{35S}:*Mdspds1*^{OE} lines, an enhanced spermidine synthesis should compensate the decrease of polyamine pool in the salinity stressed leaves. The positive correlation between salt-induced changes in polyamine levels (putrescine, spermidine, spermine) and salt tolerance in transgenic tomato indicates the possible involvement of polyamines precisely (spermidine and spermine) in adaptation to NaCl stress. Such observation was previously reported in tomato, where the (spermidine+spermine/putrescine) ratios increased with salinity in the salt tolerant species but not in the salt sensitive species (Santa-Cruz et al. 1997). Furthermore, apple shoots treated *in vitro* with salt displayed an up regulation of *MdSPDS1* gene expression (Liu et al. 2008) and the overexpression of this gene in pear confers multiple abiotic stress tolerance (Wen et al. 2008). At last, it was also reported that spermine and spermidine significantly prevented the EL and amino acids from roots and shoots of rice subjected to salinity (Chattopadhayay et al. 2002). According to those reports, the main role of polyamines in plant cells is to maintain the cation-anion balance during long term salinity (Santa-Cruz et al. 1997; Tonon et al. 2004).

Increase of APX activity under NaCl treatments

Plant response to salinity stress involves major changes in various physiological and biochemical processes. APX activity, which is important component of the antioxidant system, plays a key role in eliminating H_2O_2 molecules and in the modulation of its steady-state levels in various plant subcellular compartments (Najami et al. 2008). Our data reveals that the three most expressed *SlApx* genes are up-regulated in WT and P_{35S}:*Mdspds1*^{OE} lines exposed to salt treatment (Figure 6). Such an increase was more pronounced in 36A.12 and 70A.3 lines compared to WT, especially when treatment was changed from 100 to 150 mM NaCl. This was largely reflected at the enzyme activity level, as the APX activity was significantly higher in 36A.12 and 70A.3 at 150 mM NaCl compared to WT (Figure 7). These results are consistent with those from Mittova et al. (2002) who demonstrated a salt-induced up regulation of the APX activity in the wild salt-tolerant tomato species but not in the sensitive cultivated species. It is more likely that one

of the mechanisms present in the P_{35S}:*Mdspds1*^{OE} lines that participate in salt tolerance is associated with the capacity to maintain high APX activity in order to prevent chlorophyll degradation (Table 2), functional photosynthetic apparatus and maintain cell membrane integrity (Figure 5).

In conclusion, the present study suggests that it is possible to improve significantly the salt tolerance in tomato plants by overexpressing *MdSPDS1*. As a result, the maintenance of high levels of polyamines spermidine and spermine under prolonged salt stress, leads in the prevention of chlorophyll degradation, biomass and fruit yield reduction under salt stress. It also leads to an up regulation of *SlApx* genes that coincides with enhanced APX activity. Such activity has largely contributed to the protection against oxidative stress generated by NaCl treatments.

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