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Exogenous sucrose can enhance tolerance of *Arabidopsis thaliana* seedlings to salt stress

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Abstract

To investigate the physiological mechanisms of salt stress mitigated by exogenous sucrose, *Arabidopsis thaliana* seedlings grown on Murashige and Skoog medium were treated with 3 % (m/v) sucrose combined with 75, 150, and 225 mM NaCl for 3 d. Our results show that increased salinity significantly decreased the survival rate, fresh mass, content of proteins, chlorophyll *a* (Chl *a*), and chlorophyll *b* (Chl *b*), and activities of antioxidant enzymes, whereas enhanced the content of malondialdehyde. However, the treatment with sucrose significantly enhanced salt stress tolerance in the *Arabidopsis* seedlings by decreasing lipid peroxidation and increasing the activities of superoxide dismutase, peroxidase, and catalase, the content of proteins, Chl *a*, Chl *b*, anthocyanins, and the transcription of genes involved in anthocyanin biosynthesis. Thus, sucrose might reduce ROS-induced oxidative damage by enhancing activities of antioxidant enzymes and the content of anthocyanins, thereby preventing membrane peroxidation and denaturation of biomolecules.

Additional key words: anthocyanins, acorbate peroxidase, chlorophyll, catalase, gene expression, malondialdehyde, NaCl, peroxidase, superoxide dismutase.

Introduction

Salinity is one of the major abiotic stresses that adversely affect crop productivity and quality (Lei *et al.* 2014). Exposure of plants to salt stress results in changes in many physiological and biochemical processes resulting in a disturbance of normal growth and development (Flowers and Yeo 1995). Increasing salinity also leads to oxidative stress through an increase in reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide, and hydroxyl radicals, which result in cellular damage (Miller *et al.* 2010, Qiu *et al.* 2011). To cope with oxidative damage, plants possess a complex antioxidant defense system including antioxidative enzymes, such as superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), peroxidase (POD; EC 1.11.1.7), ascorbate peroxidase (APX; EC 1.11.1.11), and non-enzymatic antioxidants, such as ascorbic acid, glutathione, phenolic compounds, and anthocyanins. SOD is major superoxide radical scavenger and its enzymatic action results in H_2O_2 and O_2 formation. H_2O_2 is still toxic and must be eliminated by CAT or APX (Mittler 2002). Anthocyanins are not only colour-makers but also important antioxidants protecting plants against various biotic and abiotic stresses (Neill and Gould 2003, Gould 2004, Daiponmak *et al.* 2010). The high content of anthocyanins in transgenic tobacco plays an important role in sustaining a relatively low accumulation of ROS under chilling stress (Meng *et al.* 2014). Increasing evidence has demonstrated that the antioxidant systems play important roles in protecting plants against oxidative damage induced by salt stress (Mittler 2002, Xie *et al.* 2008).

Sucrose, which plays a central role in plant structure and metabolism, is also involved in responses to a number of stresses and act as signalling molecule (Smeekens *et al.* 2010, Wind *et al.* 2010). Treatment with sucrose confers a tolerance to atrazine in seedlings of

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Abbreviations: APX - ascorbate peroxidase; CAT - catalase; Chl - chlorophyll; *DFR* - gene encoding dihydroflavonol 4-reductase; *LDOX* - leucoanthocyanidin dioxygenase; MDA - malondialdehyde; MS - Murashige and Skoog; *MYB* - gene encoding transcription factor; NBT - nitroblue tetrazolium; POD - peroxidase; ROS - reactive oxygen species; SOD - superoxide dismutase; TBA - thiobarbituric acid; *TT8* - gene encoding transparent testa 8.

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Arabidopsis or mustard (Ramel *et al.* 2007, Sulmon *et al.* 2007). By using *Affymetrix ATH1 GeneChip* arrays, Loreti *et al.* (2005) reported that exogenous sucrose greatly enhances anoxia tolerance in *Arabidopsis* seedlings. However, it is not known if sucrose treatment also induces salt stress tolerance.

Therefore, the aim of this study was *1*) to determine the effects of sucrose and NaCl on *Arabidopsis* seedlings and *2*) to evaluate whether exogenous sucrose mitigated salt stress by regulation of antioxidant system.

Material and methods

Plants and treatments: Seeds of *Arabidopsis thaliana* L. (ecotype Columbia, Col-0) were surfaced-sterilized with diluted bleach [5 min incubation in 0.1 % (m/v) $HgCl_2$, rinsing and washing in sterile water 7 times] and allowed to germinate and grow on a half-strength Murashige and Skoog (MS) solid medium containing 0.8 % (m/v) agar and 1 % (m/v) sucrose (pH 6.0) at a temperature of 23 °C, a 16-h photoperiod, and an irradiance of 400 μmol m^{-2} s^{-1} for 14 d before treatments. NaCl was dissolved in a half-strength Hoagland solution to obtain 75, 150 and 225 mM concentrations. Sucrose (3 %) was added to the Hoagland solutions with or without NaCl, and this 3 % sucrose treatment was chosen according to our preliminary experiment (data not shown). After the treatment at those final concentrations for 3 d, the seedlings were sampled for various analyses.

Malondialdehyde (MDA) content was assessed by the thiobarbituric acid (TBA) reaction as described by Predieri *et al.* (1995). Samples of fresh leaves (0.2 g) were homogenized with a mortar and pestle in a 50 mM phosphate buffer (pH 7.8) and then centrifuged at 8 000 *g* for 15 min. The supernatant (1 cm^3) was combined with 2.5 cm^3 of TBA, incubated in boiling water for 30 min, and then quickly cooled in an ice-bath. The mixture was centrifuged at 10 000 *g* for 5 min and the absorbance of supernatant was monitored spectrophotometrically (*Lambda35*, *Perkin Elmer,* USA). The MDA content was calculated from the difference of absorbances at 532 and 600 nm using the coefficient of absorbance of 155 mM^{-1} cm^{-1}.

Determination of chlorophyll, anthocyanin, and protein content: Photosynthetic pigments were extracted from fresh leaves (0.2 g) with 10 cm^3 of 80 % (v/v) aqueous acetone. The content of chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) were measured according to the method of Arnon (1949). Absorbances were recorded at 663 and 645 nm, respectively. Anthocyanin was extracted using the method of Ronchi *et al.* (1997) with some modifications. Samples were placed in 100 cm^3 Erlenmeyer flasks containing ethanol, HCl and distilled H_2O (79:1:20, v/v/v) for 24 h in darkness. The total content of anthocyanins was estimated by measuring the absorbance at 535 nm with a UV/visible spectrophotometer (*Lambda35*, *Perkin Elmer*, USA). The protein content was assessed using the method of Bradford (1976).

Enzyme activity determination: Frozen leaves (0.2 g) were homogenized with 3 cm^3 of a 50 mM ice-cold phosphate buffer (pH 7.8) containing 1 mM Na_2EDTA and 1 % (m/v) polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 15 000 *g* and 4 °C for 15 min. The supernatant was used for assays of enzyme activities. The activity of SOD was assayed according to its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) using the method of Giannopolitis and Ries (1977). The reaction mixture (3 cm^3) contained 0.1 cm^3 of the enzyme homogenate, 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 130 mM methionine, 0.75 mM NBT, and 0.02 mM riboflavin. The absorbance of the reaction mixture was read at 560 nm and one unit of SOD activity (U) was defined as the amount of enzyme required to cause a 50 % inhibition of the reduction of NBT. The CAT activity was determined by following the comsumption of H_2O_2 at 240 nm for 3 min according to Cakmak and Marschner (1992). The reaction mixture (3 cm^3) contained a 50 mM phosphate buffer (pH 7.0), 15 mM H_2O_2, and 0.1 cm^3 of the enzyme homogenate. One unit of catalase activity was defined as the change of absorbance of 0.01 min^{-1}. The analysis of POD activity was based on the oxidation of guaiacol using H_2O_2 according to the method of Zhang and Kirham (1994) as described in Qiu *et al.* (2011). The reaction mixture (3 cm^3) contained 0.02 cm^3 of guaiacol, 0.01 cm^3 of H_2O_2, a 50 mM phosphate buffer (pH 7.0), and 0.02 cm^3 of the enzyme homogenate. The addition of the enzyme homogenate started the reaction, and the increase in absorbance was recorded at 470 nm for 5 min. The enzyme activity was quantified by the amount of tetraguaiacol formed using the coefficient of absorbance of 26.6 mM^{-1} cm^{-1}. The APX activity was assayed according to the method of Nakano and Asada (1981) by monitoring the rate of hydrogen peroxide-dependent oxidation of ascorbate at 290 nm (the coefficient of absorbance of 2.8 mM^{-1} cm^{-1}). The reaction mixture (3 cm^3) contained a 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 0.5 mM ascorbate, 0.1 mM H_2O_2, and 0.1 cm^3 of the enzyme homogenate. The reaction was started by the addition of H_2O_2, and ascorbate oxidation was measured at 290 nm for 3 min.

Reverse transcription polymerase chain reaction (RT-PCR): Total RNA from leaves was isolated using a *TRIZOL* reagent (*TaKaRa*, Dalian, China), following the

supplied protocol. RNA was treated with DNase I (*Promega*, Beijing, China) to remove contaminant DNA traces before reverse transcription. The first-strand cDNA synthesis was performed according to the manufacturer's instructions (a *TransScript II* first-strand cDNA synthesis supermix, *Promega*). Gene-specific primers were designed using *Primer 5.0* (Table 1) and *Arabidopsis actin8* as internal reference gene. Expressions of *Arabidopsis* genes *DFR, LDOX, TT8*, and *MYB* encoding dihydroflavonol reductase, leucoanthocyanidin dioxygenase, and transcription factors (transparent testa 8 and avian myeloblastosis oncogene homolog), respectively, were carried out using the primers and PCR conditions as mentioned in Table 1.

Statistical analysis: The experiment was arranged in a random complete design with three replications. All data obtained were subjected to the two-way analysis of variance (*ANOVA*), and the mean differences were compared by the Duncan's multiple range test at the 5 % probability level using the *SPSS* software *v. 16.0*.

Table 1. Sequences of PCR primers and PCR conditions used in the present study.

Gene	Accession No.	Primer sequences	Size [bp]	PCR conditions
Actin8	XM002882721	F 5′-TCGTTGGTCGTCCTCGAC R 5′- GGGTTGAGAGGAGCCTCA	235	94 °C for 5 min; 30 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s; 72 °C for 10 min
DFR	NM123645	F 5′-ATGGTTAGTCAGAAAGAGACCG R 5′- GTCTTATGATCGAGTAATGCGC	673	94 °C for 5 min; 30 cycles of 94 °C for 30 s, 60 °C for 45 s, 72 °C for 1 min; 72 °C for 10 min
LDOX	NM001036623	F 5′-AGACCATGGTTGCGGTTGAAAGAGTTG R 5′-ATCATTTTTCTCGGATACCAATTCCTC	1073	94 °C for 5 min; 30 cycles of 94 °C for 30 s, 58 °C for 45 s, 72 °C for 1 min; 72 °C for 10 min
TT8	NM117050	F 5′-ATGGATGAATCAAGTATTATTCCGG R 5′- CTCCACGTGGCAAACGATGATTGG	953	94 °C for 5 min; 30 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 1 min; 72 °C for 10 min
MYB	DQ222405	F 5′- GCTCTGATGAAGTCGATCTTC R 5′- CTACCTCTTGGCTTTCCTCT	530	94 °C for 5 min; 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min; 72 °C for 10 min

Results

The MDA content significantly increased in the *Arabidopsis* seedlings with increasing salinity. In contrast, exogenous sucrose combined with the 150 and 225 mM NaCl treatments kept the MDA content at a lower level indicating a significant decrease in lipid peroxidation. In addition, the sucrose treatment alone did not affect the content of MDA (Table 2). The increased salinity stress caused a significant reduction in the content of proteins and photosynthetic pigments as compared with the control. However, a higher content of proteins, Chl *a*, and Chl *b* was observed in the sucrose + NaCl treatments as compared to seedlings treated with NaCl alone with an exception of the Chl *a* content at 225 mM NaCl. The sucrose treatment alone significantly

Table 2. The effects of exogenous sucrose (3 %) on the content of MDA [μmol g^{-1}(f.m.)], proteins [mg g^{-1}(f.m.)], and photosynthetic pigmenst [mg g^{-1}(f.m.)] in *Arabidopsis* seedlings grown on a half strength Hoagland solution (control) with different concentrations of NaCl for 3 d. Means ± SE, $n = 3$; different letters within the same column indicate significant differences at the 0.05 level.

Treatments	MDA content	Protein content	Chl *a* content	Chl *b* content
Control	67.23 ± 8.88ef	18.91 ± 0.01d	14.55 ± 1.54c	13.56 ± 1.27bc
Sucrose	59.61 ± 6.02f	70.30 ± 7.51a	25.86 ± 1.87a	14.74 ±0.52b
75 mM NaCl	76.74 ± 4.41e	15.29 ± 0.01de	9.15 ± 1.12d	10.79 ± 0.20cd
75 mM NaCl + sucrose	71.77 ± 10.80e	61.64 ± 5.90b	22.76 ± 0.24b	20.69 ± 1.67a
150 mM NaCl	114.94 ± 11.73b	13.79 ± 0.02e	7.18 ± 0.01d	8.63 ± 0.30de
150mM NaCl + sucrose	86.71 ± 6.79d	24.89 ± 5.77c	17.24 ± 1.19c	10.90 ±1.19cd
225 mM NaCl	142.93 ± 16.44a	7.74 ± 0.01f	8.80 ± 0.82d	5.38 ± 0.31e
225 mM NaCl + sucrose	102.93 ± 19.65c	12.06 ± 1.73e	5.24 ± 0.41e	8.19 ± 0.22de

Table 3. The effects of exogenous sucrose on the activities of SOD, POD, CAT and APX [U g^{-1}(f.m.)] in *Arabidopsis* seedlings grown on a half strength Hoagland solution (control) with different concentrations of NaCl for 3 d. Means ± SE, n = 3; different letters within the same column indicate significant differences at the 0.05 level.

Treatments	SOD	POD	CAT	APX
Control	9.75 ± 0.06b	13.81 ± 1.38c	2.38 ± 0.15c	13.20 ± 1.88c
Sucrose	15.38 ± 1.58a	28.00 ± 1.67a	21.96 ± 2.70a	31.20 ± 6.23a
75 mM NaCl	6.19 ± 0.24d	10.79 ± 2.29cd	0.78 ± 0.13c	5.60 ± 1.68d
75 mM NaCl + sucrose	9.17 ± 0.06bc	24.30 ± 1.67a	8.18 ± 1.51b	19.20 ± 2.09b
150 mM NaCl	3.13 ± 0.07e	7.40 ± 0.91de	0.37 ± 0.05d	3.00 ± 1.01de
150mM NaCl + sucrose	6.62 ± 0.84c	19.40 ± 0.23b	3.48 ± 0.53c	5.06 ± 1.51de
225 mM NaCl	1.85 ± 0.08e	6.45 ± 0.70e	0.23 ± 0.01d	1.20 ± 0.08e
225 mM NaCl + sucrose	4.27 ± 0.72de	12.30 ± 0.13c	0.94 ± 0.05c	3.80 ± 0.46de

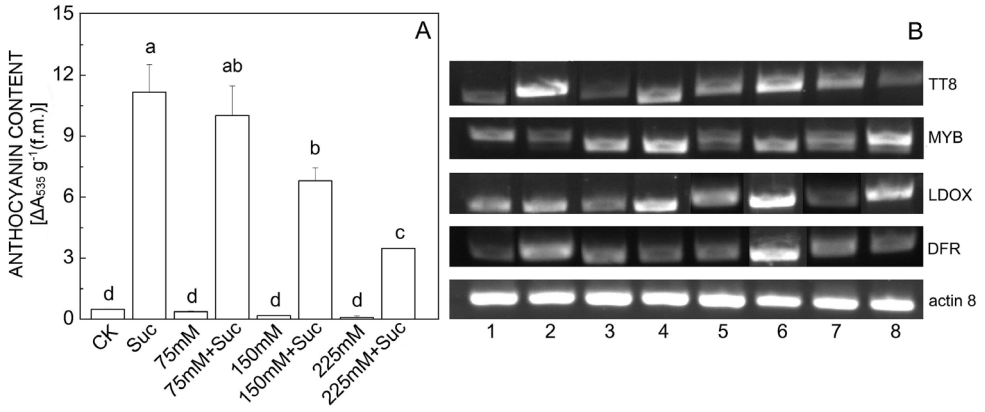

Fig. 1. The effect of 3 % sucrose and 75, 150, and 225 mM NaCl on anthocyanin content (*A*) and expressions of genes (semi-quantitative RT-PCR) related to anthocyanin biosynthesis (*B*) in *Arabidopsis* seedlings grown under different concentrations of NaCl for 3 d. *Lanes*: 1 - CK, 2 - 3 % sucrose, 3 - 75 mM NaCl, 4 - 3 % sucrose + 75 mM NaCl, 5 - 150 mM NaCl, 6 - 3 % sucrose + 150 mM NaCl, 7 - 225 mM NaCl, and 8 - 3 % sucrose + 225 mM NaCl. *Actin 8* was amplified as internal control. The experiment was repeated three times with similar results.

enhanced the content of proteins and Chl *a* as compared with the control.

The salt stress (150 and 225 mM NaCl) caused a significant inhibition of SOD, POD, APX, and CAT activities as compared with the control (Table 3). In contrast, the treatment with sucrose alone significantly increased the activities of SOD, POD, CAT, and APX in the seedlings compared with the control. The enzyme activities in the seedlings treated with NaCl and sucrose were mostly higher that in the seedlings treated with NaCl alone.

The content of anthocyanin was higher in the seedlings treated with sucrose than in the control seedlings and the seedlings treated with NaCl and sucrose (Fig. 1). To investigate the effect of sucrose on anthocyanin biosynthesis in the *Arabidopsis* seedlings grown on the medium supplemented with the different NaCl concentrations, the mRNA content of genes involved in the anthocyanin biosynthesis were investigated by RT-PCR (Fig. 1). The transcriptions of *DFR*, *LDOX*, *MYB*, and *TT8* were highest in the sucrose treated seedlings exposed to 75 and 150 mM NaCl with an exception of *DFR* at 75 mM NaCl. Furthermore, the transcriptions of *DFR*, *LDOX*, and *TT8* in the seedlings treated with sucrose alone was much higher than in the control seedlings suggesting that sucrose could induce anthocyanin biosynthesis genes in the seedlings grown on the medium supplemented either with NaCl or without it.

Morphological observations were carried out to investigate whether the edges of leaves were soft and wilting, and the percentage of wilting leaves in a total number of leaves was calculated. The survival rate of seedlings treated with 3 % sucrose at the various concentrations of NaCl was significantly greater than that of those treated with NaCl alone (Fig. 2). In the presence of 75 or 150 mM NaCl and 3 % sucrose, more than 90 % of the seedlings were alive after 3 d, and even at 225 mM NaCl + 3 % sucrose, 80 % of the seedlings survived. Also the fresh mass of seedlings treated with the combination of NaCl and sucrose was higher that of those treated only with NaCl (Fig. 2). In addition, exogenous sucrose caused a red coloration in the hypocotyl area, but the red coloration declined gradually with the increasing NaCl concentration (Fig. 2).

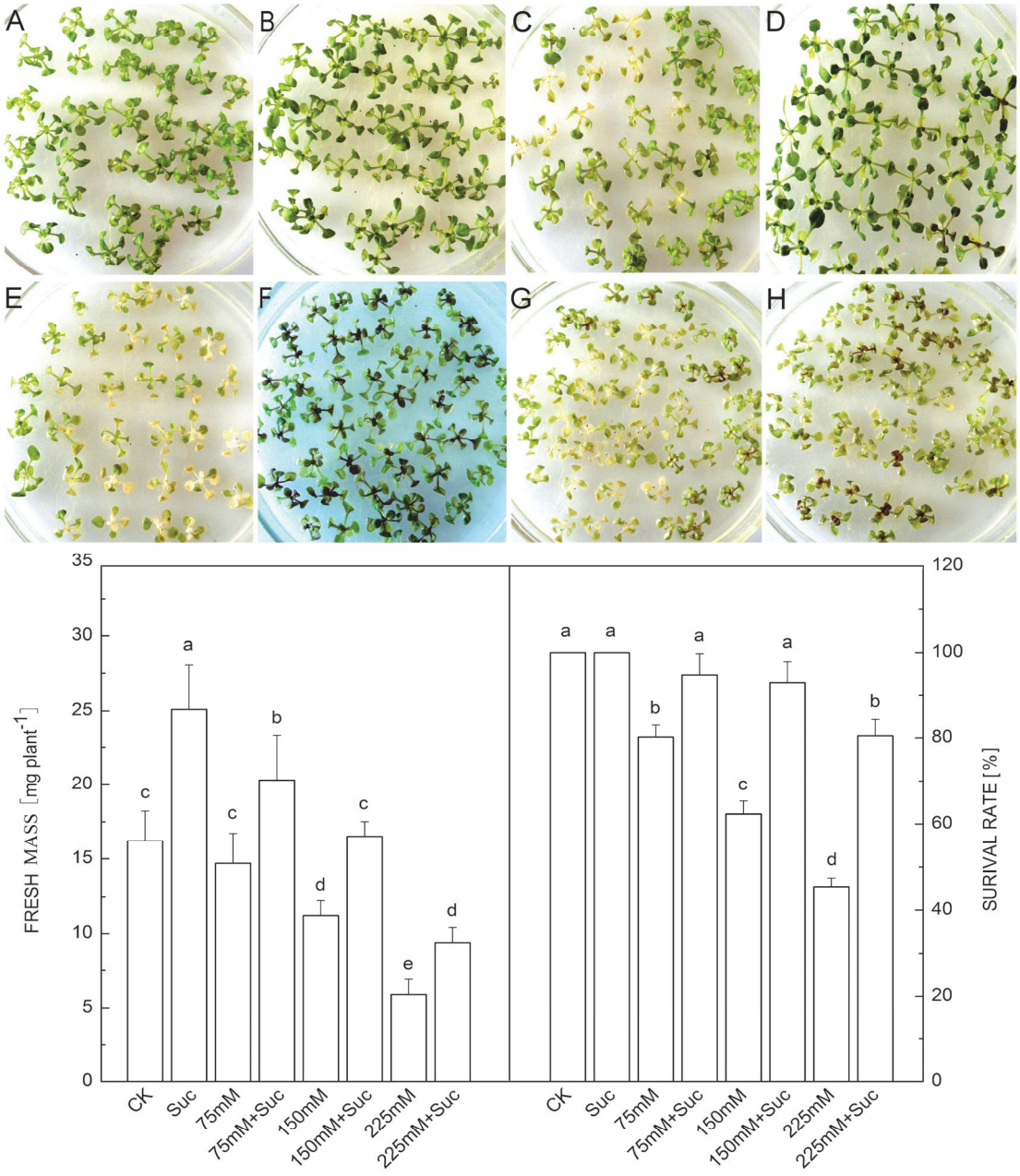

Fig. 2. The effect of 3 % sucrose (Suc) and 75, 150, and 225 mM NaCl on phenotype, fresh mass, and survival rate of *Arabidopsis* seedlings grown under different concentrations of NaCl for 3 d. Means ± SE, n = 20. Different letters indicate significant differences at the 0.05 level. *A*, *B*, *C*, *D*, *E*, *F*, *G*, *H* correspond to treatments mentioned on the x-axes.

Discussion

Reduced plant growth is common phenomenon under increased salinity (Xie *et al.* 2008). The NaCl treatment of the *Arabidopsis* seedlings resulted in wilting leaves, a low fresh mass, and survival rate. However, exogenous sucrose improved plant growth and resulted in the higher survival rate and fresh mass of the seedlings. Ramel *et al.* (2007) reported that sucrose protects atrazine-treated *Arabidopsis* seedlings and maintains their active growth by alleviating oxidative stress and increasing photosynthesis. The content of MDA, a product of lipid peroxidation, is generally an indicator of oxidative damage of cell membranes (Li *et al.* 2011). Also in our experiments, the improved tolerance of the *Arabidopsis* seedlings to the NaCl stress due to the sucrose treatment

was correlated with a decrease in the MDA content suggesting that exogenous sucrose could prevent lipid peroxidation under the salt stress and thus protect cells.

Salt tolerance requires the coordinated action of several gene families that regulate an array of physiological processes leading to adaptation. It is now well evident that salt tolerance in most crop plants is associated with an efficient antioxidant system (Li *et al.* 2011, Qiu *et al.* 2011). It is vital for plants to adjust enzymatic and nonenzymatic antioxidants to control the ROS overproduction (Daiponmak *et al.* 2010). To test if antioxidant enzymes have a role in the protection against salt induced oxidative damage, the activities of key antioxidant enzymes were assayed. We found a remarkable increase in the activities of SOD, CAT, and POD in the seedlings treated with 3 % sucrose combined with the different NaCl concentrations, which improved the survival rate of the *Arabidopsis* seedlings under the salt stress. It is well known that the antioxidant enzymes, such as SOD, CAT and POD, play a significant role in scavenging ROS in salt-stressed plants (Li *et al.* 2011, Qiu *et al.* 2013). Therefore, sucrose can exert a protective function, at least in part, by regulating these antioxidant enzyme activities under salt stress.

Chl content is widely used as index of plant tolerance to abiotic stresses. Reduction in Chl content due to salt stress was found in *Arabidopsis* seedlings and also in many crops, such as maize, wheat, *etc*. (Li *et al.* 2011, Qiu *et al.* 2011). However, 3 % sucrose considerably enhanced the content of the photosynthetic pigments under the salinity. The increased Chl content due to exogenous sucrose might increase photosynthesis and growth. In our experiment, the exogenous sucrose also increased the soluble protein content in the seedlings grown on the medium supplemented with different concentrations of NaCl.

Recently, Sperdouli and Moustakas (2012) reported an accumulation of anthocyanins which maintained a high antioxidant protection of *Arabidopsis* leaves under drought stress. Sucrose can induce an efficient anthocyanin accumulation in plants (Teng *et al.* 2005). Moreover, a sucrose-specific induction of anthocyanin biosynthesis has been also recently demonstrated in *Arabidopsis* seedlings (Solfanelli *et al.* 2006). Anthocyanin production in cotyledons or leaves increases when *Arabidopsis* seedlings are grown in a sugar-containing medium (Ohto *et al.* 2001, Das *et al.* 2010), as revealed by a purple coloration of the tissue. Our results demonstrate that 3 % sucrose significantly increased the anthocyanin content and caused a red pigmentation in the hypocotyl area in the seedlings grown on the medium supplemented with different concentrations of NaCl. Our results are in agreement with Oh *et al.* (2011), who suggested that an enhanced anthocyanin content induced by sucrose treatment significantly increases the chance of survival of *Arabidopsis* plants exposed to a high salinity. Hossain *et al.* (2009) also reported that an anthocyanin content increased in *Melissa officinalis* leaves by a sucrose application and this confers an increased ROS scavenging activity. Thus, our results indicate that the enhanced anthocyanin content induced by exogenous sucrose likely contributed to the enhanced ROS scavenging activity of the *Arabidopsis* seedlings under the salt stress.

To examine the effect of sucrose on anthocyanin biosynthesis, the expressions of some structural genes (*DFR* and *LDOX*) and regulatory genes (*MYB* and *TT8*) in the *Arabidopsis* seedlings grown on the medium with the different concentrations of NaCl were examined using RT-PCR. Our results demonstrate that the transcriptions of these genes were higher in the sucrose treated seedlings than in those treated with NaCl alone. It is in agreement with results of Solfanelli *et al.* (2006) who reported that exogenous sucrose increases the transcriptions of the *DFR* and *LDOX* considerably in *Arabidopsis*. Park *et al.* (2011) also reported that the *RsMYB* overexpression may regulate the transcription of anthocyanin-biosynthetic genes to increase an anthocyanin accumulation. These results suggest that the enhanced expression of the anthocyanin biosynthetic related gene contributed to the increase in the anthocyanin content, and thus resulting in the protection against oxidative damage.

In conclusion, the results obtained in the present study clearly indicate that exogenous sucrose could enhance tolerance of the *Arabidopsis* seedlings to the salt stress through raising the activities of SOD, POD, and CAT, the content of proteins, Chl *a*, Chl *b*, anthocyanins, and the transcription of anthocyanin biosynthetic genes. Hence, it can be concluded that the supplementation of sucrose proved to be beneficial for the plant system in combating salt stress.

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