

Exogenous salicylic acid-triggered changes in the glutathione transferases and peroxidases are key factors in the successful salt stress acclimation of *Arabidopsis thaliana*

Edit Horváth^{A,C}, Szilvia Brunner^A, Krisztina Bela^A, Csaba Papdi^B, László Szabados^B, Irma Tari^A and Jolán Csiszár^A

^ADepartment of Plant Biology, Faculty of Science and Informatics, University of Szeged, Közép fasor 52, 6726 Szeged, Hungary.

^BInstitute of Plant Biology, Biological Research Centre, Hungarian Academy of Sciences, Temesvári körút 62, 6726 Szeged, Hungary.

^CCorresponding author. Email: horvathedo@yahoo.com

Abstract. Salicylic acid (SA) applied exogenously is a potential priming agent during abiotic stress. In our experiments, the priming effect of SA was tested by exposing *Arabidopsis thaliana* (L.) Heynh. plants to 2-week-long 10^{-9} – 10^{-5} M SA pretreatments in a hydroponic medium, followed by 1 week of 100 mM NaCl stress. The levels of reactive oxygen species and H₂O₂, changes in antioxidant enzyme activity and the expression of selected glutathione transferase (GST) genes were investigated. Although 10^{-9} – 10^{-7} M SA pretreatment insufficiently induced defence mechanisms during the subsequent salt stress, 2-week pretreatments with 10^{-6} and 10^{-5} M SA alleviated the salinity-induced H₂O₂ and malondialdehyde accumulation, and increased superoxide dismutase, guaiacol peroxidase, GST and glutathione peroxidase (GPOX) activity. Our results indicate that long-term 10^{-6} and 10^{-5} M SA treatment mitigated the salt stress injury in this model plant. Enhanced expression of *AtGSTU19* and *AtGSTU24* may be responsible for the induced GST and GPOX activity, which may play an important role in acclimation. Modified GST expression suggested altered signalling in SA-hardened plants during salt stress. The hydroponic system applied in our experiments proved to be a useful tool for studying the effects of sequential treatments in *A. thaliana*.

Additional keywords: antioxidant enzyme activity, NaCl stress, priming, reactive oxygen species.

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Introduction

Salicylic acid (SA) is known to regulate diverse physiological and biochemical processes in plants, including seed germination, growth and productivity, photosynthesis, senescence and water relations (Rivas-San Vicente and Plasencia 2011). Elevated SA levels were shown to correlate with enhanced resistance to pathogen infection (Raskin 1992; Shirasu *et al.* 1997; Vlot *et al.* 2009). SA mediates the oxidative burst that leads to cell death in the hypersensitive response. At the site of infection, a rapid change in ion flux and reactive oxygen species (ROS) occurs, which leads to the induction of defence responsive genes, including those which are directly or indirectly involved in SA synthesis (Dangl and Jones 2001; Métraux 2001; Ashraf *et al.* 2010; Xia *et al.* 2015). SA acts as a signal for the development of the systemic acquired resistance, preventing further infection of the plant by the pathogen, but it was also shown to provide tolerance against various environmental stresses (Shirasu *et al.* 1997).

SA signalling has been studied intensively. One of the main pathways is associated with the reduction of the intermolecular

disulfide bonds of the cytosolic oligomer Nonexpressor of Pathogenesis-Related genes 1 (NPR1) protein. The resulting monomers are then able to translocate to the nucleus and activate the expression of defence genes in the NPR1-dependent pathway (Mou *et al.* 2003). A novel and interesting feature of NPR1, besides being a metalloprotein acting as a transcription regulator, is that it acts as a SA receptor (Wu *et al.* 2012; Kuai *et al.* 2015). However, recent evidence suggests that H₂O₂-dependent changes in the glutathione pool can activate SA-dependent defence responses independently of NPR1 (Han *et al.* 2013). SA signalling transcriptional factors, such as NPR1, TGACG motif-binding protein (TGA) factors, TGA box and *as-1*-like elements were suggested to act as redox sensors for temporal control of gene expression modulated by SA, whereas early NPR1-independent and SA-activated gene products may have antioxidant and detoxifying activity (Blanco *et al.* 2009).

Although involvement of SA in plant defence against pathogen attack is well documented, recent articles demonstrate that this regulator can be implicated in responses to abiotic stresses,

including high salinity (Jayakannan *et al.* 2015). Exogenous application of SA has been used as a priming or hardening compound to enhance the resistance of plants to biotic and abiotic stresses (Hayat *et al.* 2010; Joseph *et al.* 2010). SA was shown to protect several plant species against injuries of salinity including *Arabidopsis thaliana* (L.) Heynh. (Lee and Park 2010; Jayakannan *et al.* 2013), tomato (*Solanum lycopersicum* L.) (Tari *et al.* 2002; Stevens *et al.* 2006), mungbean (*Vigna radiata* (L.) R. Wilczek) (Khan *et al.* 2010), maize (*Zea mays* L.) (Gunes *et al.* 2007), barley (*Hordeum vulgare* L.) (El-Tayeb 2005), sunflower (*Helianthus annuus* L.) (Noreen *et al.* 2009) and mustard (*Brassica juncea* L.) (Syed *et al.* 2011). It was suggested that SA treatment alleviates the damage of salt stress through strengthening the antioxidant capacity (Szepesi *et al.* 2008; Palma *et al.* 2009; Khan *et al.* 2010; Rivas-San Vicente and Plasencia 2011; Syed *et al.* 2011). Nevertheless, some controversy regarding the involvement of SA in salt stress responses still exists. The results of experiments using *A. thaliana* mutants with modified SA contents suggest that SA is directly involved in the NaCl-induced growth inhibition and disturbance of metabolism (Hao *et al.* 2012). Hao *et al.* (2012) reported that SA deficiency or signalling blockage in *A. thaliana* plants was favourable to salt adaptation whereas *sid2 A. thaliana* mutants, which were impaired in SA biosynthesis, were shown to be hypersensitive to salt stress (Alonso-Ramírez *et al.* 2009).

Salinity affects plant growth and development in a complex manner. On the one hand, salt reduces the soil water potential and causes osmotic stress; on the other hand, it imposes ionic stress by excessive uptake of Na^+ and Cl^- ions (Munns 2005). Salt stress leads to the accumulation of ROS, such as $^1\text{O}_2$, $\text{O}_2^{\cdot-}$, OH^{\cdot} and H_2O_2 , through the disruption of photosynthetic electron transport, generation of H_2O_2 in the peroxisome, an increase of respiration, and the activation of membrane-bound NADPH oxidase and apoplastic diamine oxidase (Munns and Tester 2008; Abogadallah 2010). ROS are natural byproducts of normal metabolism and have important roles in cell signalling and control of redox homeostasis. Unbalanced generation of these oxygen species, however, induces detrimental oxidation of macromolecules, such as DNA, proteins and lipids. ROS-mediated membrane damage is among the major causes of the cellular toxicity provoked by salinity (Kim *et al.* 2005). In order to keep ROS levels tightly regulated and to minimise ROS-derived damage, different nonenzymatic antioxidants (such as ascorbate, glutathione, carotenoids and tocopherols) and enzymatic systems (superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase; ascorbate peroxidase, glutathione peroxidase (GPOX) and glutathione reductase) have evolved in aerobic organisms. Zhang *et al.* (2012) evaluated the results of proteomic studies conducted with 34 salt-treated plant species (including *A. thaliana* and *Oryza sativa* L. model plants, 7 agricultural crops and 12 economic crops, 11 halophytes and 2 tree species) and revealed 184 protein identities as ROS scavenging-related proteins, 143 were induced by salinity (for more details, see the review of Zhang *et al.* 2012). In *A. thaliana* plants, the abundance of SOD, peroxidases, ascorbate peroxidase, glutathione reductase, GST and other enzymes were affected by salt treatment (Zhang *et al.* 2012).

GSTs are induced by diverse environmental stimuli and were proposed to contribute to protection against various stress conditions that promote oxidative stress (Marrs 1996). The *A. thaliana* genome contains 55 GST genes, which can be divided into eight classes, including seven soluble (tau, phi, zeta, theta, lambda, dehydroascorbate reductase and tetrachlorohydroquinone dehalogenase) and one membrane-bound (microsomal) class (Dixon *et al.* 2010). The plant-specific tau (GSTU) and phi (GSTF) classes of GSTs have important roles in protection against cytotoxic and xenobiotic compounds (Dixon *et al.* 2002). They are the two largest GST classes in *A. thaliana*, comprising 28 and 13 members, respectively (Dixon *et al.* 2010). Both the GSTU and GSTF classes have members with high glutathione-conjugating (GST) and glutathione-dependent peroxidase (GPOX) activities (Dixon *et al.* 2009), and are known to be essential in alleviating oxidative damages (Roxas *et al.* 2000). Gene expression and protein abundance of GSTs can be altered by a wide variety of plant growth regulators and stress factors, including SA, and also by NaCl treatments used in different concentrations and durations (Wagner *et al.* 2002; Sappl *et al.* 2004; Sappl *et al.* 2009; Zhang *et al.* 2012). The spatial and temporal changes in the levels of ROS and NO were shown to have a central role in the crosstalk of different hormones, developmental regulation and stress responses (Kocsy *et al.* 2013).

Previously, we found that priming tomato plants with SA was able to mitigate salt stress injury in a concentration-dependent manner. Pretreatment of tomato plants with 10^{-4} M SA increased the efficiency of enzymatic and nonenzymatic antioxidant systems, and provided protection against 100 mM NaCl stress in a hydroponic culture system (Szepesi *et al.* 2008; Szepesi *et al.* 2009; Gémes *et al.* 2011). More recent results suggest that GSTs are important in SA-induced acclimation to high salinity in tomato (Csiszár *et al.* 2014). In this work, we investigated the effect of SA on *A. thaliana* plants' overall oxidative state by measuring the reactive oxygen content and the antioxidant activity. Our aim was to characterise the effects of a long-term SA treatment on 5-week-old *A. thaliana* plants and evaluate the possibility of using SA as a priming compound in this model plant. Here, we report that applying 10^{-6} – 10^{-5} M SA to the nutrient solution for two weeks successfully alleviates the deleterious effects of the subsequent salt stress. We show that SA priming may contribute to the fine-tuning of the H_2O_2 levels in *A. thaliana* plants and reduce peroxides by increased guaiacol peroxidase, GST and GPOX activity.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana (L.) Heynh. (ecotype Columbia) plants were grown in Hoagland solution in a growth chamber (Fitoclima S 600 PLH, Aralab, Rio de Mouro, Portugal) at 21°C under $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity with a 10:14 h day:night photoperiod; the relative humidity was 70%. After being kept under control conditions for 5 weeks, the plants were treated with 10^{-9} – 10^{-4} M SA solutions for 2 weeks and were subsequently exposed to salinity, imposed by adding 100 mM NaCl directly to the medium, for 1 week. Hydroponic application

of SA on 5-week-old *A. thaliana* plants revealed that 10^{-4} M SA was lethal after 2 weeks of treatment (data not shown). Samples were taken from fully expanded leaves and roots 1 and 2 weeks after SA exposure, and 1 week after the 100 mM NaCl treatment. The experiments were repeated at least three times and the measurements were performed with three replicates unless indicated otherwise.

Investigation of ROS using fluorescent microscopy

A Zeiss Axiovert 200M microscope (Carl Zeiss, Jena, Germany) equipped with a high-resolution digital camera and suitable filter sets was used for the fluorescent detection of ROS in 10-mm diameter leaf disks and in the root tips of *A. thaliana* plants. To detect ROS, 2'-7'-dichlorodihydrofluorescein diacetate (Sigma-Aldrich, St Louis, MO, USA) was used at 37°C for 15 min, then the samples were washed four times in 20 min with buffer containing 10 mM MES (2-(N-Morpholino)ethanesulfonic acid hydrate) and 50 mM potassium chloride (pH 6.15), according to Petó *et al.* (2013). The intensity of ROS-dependent fluorescence was measured on digital images with the help of Axiovision ver. 4.8 software (Carl Zeiss Inc., Munich, Germany). Fluorescence intensity values were determined in 200- μ m diameter circles 300 μ m from the root tip in roots and 600- μ m diameter circles in leaves. The diameter of circles was not modified during the experiments. The measurements were performed in 10 replicates; mean \pm s.e. are given on the figures.

Determination of the H₂O₂ level

The H₂O₂ level was measured spectrophotometrically as described in Gémes *et al.* (2011). After homogenisation of 400 mg of shoot or root tissue on ice with 750 μ L of 0.1% trichloroacetic acid (TCA), the samples were centrifuged at 10 000g for 20 min at 4°C. The reaction contained 0.25 mL of a 10-mM phosphate buffer (pH 7.0), 0.5 mL of 1-M KI and 0.25 mL of the supernatant. The absorbance of the samples was measured after 10 min at 390 nm. The amount of H₂O₂ was calculated using a standard curve prepared with 0.1–5 μ mol mL⁻¹ H₂O₂ concentrations.

Malondialdehyde determination

Malondialdehyde (MDA) formation was followed by using the thiobarbituric acid method (Ederli *et al.* 1997). In this step, 100 mg shoot or root tissue was homogenised with 0.1% TCA; 100 μ L of 4% butylhydroxytoluene was added to avoid further lipidperoxidation. The extracts were centrifuged at 10 000g for 20 min at 4°C and after that, 0.25 mL of supernatant was added to 1 mL of 20% TCA containing 0.5% thiobarbituric acid. The mixture was incubated in 96°C water for 30 min. The absorbance was measured at 532 nm and adjusted for nonspecific absorbance at 600 nm. MDA concentration was calculated using an extinction coefficient of 155 mM⁻¹ cm⁻¹.

Determination of antioxidant enzyme activity

Enzyme activity was determined as published earlier (Csizsár *et al.* 2004) with some modifications. To analyse the enzyme activity, 0.2 g tissue was homogenised on ice in 1 mL of a 100-mM phosphate buffer (pH 7.0) containing 1 mM phenylmethylsulfonyl

fluoride and 1% polyvinyl-pyrrolidone. The homogenate was centrifuged for 20 min at 10 000g at 4°C and the supernatant was used for enzyme activity assays.

SOD (EC 1.15.1.1) activity was determined by measuring the ability of the enzyme to inhibit the photochemical reduction of p-Nitro-Blue tetrazolium chloride (Sigma-Aldrich) in the presence of riboflavin in the light. One enzyme unit (U) of SOD was calculated as the amount causing a 50% inhibition of p-Nitro-Blue tetrazolium chloride reduction in light. The enzyme activity was expressed as U g⁻¹ FW. CAT (EC 1.11.1.6) activity was determined by the decomposition of H₂O₂ and was measured spectrophotometrically by following the decrease in absorbance at 240 nm. One U was equal to the amount of H₂O₂ (in μ mol) decomposed in 1 min. Peroxidase (EC 1.11.1.7) activity was determined by monitoring the increase in absorbance at 470 nm during the oxidation of guaiacol (molar extinction coefficient, $\epsilon_{470} = 26.6 \text{ mM}^{-1} \text{ cm}^{-1}$). The amount of enzyme producing 1 μ mol min⁻¹ of oxidised guaiacol was defined as 1 U. GST (EC 2.5.1.18) activity was determined spectrophotometrically by using an artificial substrate, 1-chloro-2,4-dinitrobenzene (CDNB, Sigma-Aldrich). The reaction was initiated by the addition of CDNB and the increase in absorbance at 340 nm was determined. One U was the amount of the enzyme producing 1 μ mol of conjugated product in 1 min ($\epsilon_{340} = 9.6 \text{ mM}^{-1} \text{ cm}^{-1}$). GPOX (EC 1.11.1.9) activity was measured with cumene hydroperoxide (Sigma-Aldrich) as a substrate. The reaction mixture contained 4 mmol L⁻¹ GSH, 0.2 mmol L⁻¹ NADPH, 0.05 U of glutathione reductase (from baker's yeast, Sigma-Aldrich), 100 μ L enzyme extract and 0.5 mmol L⁻¹ substrate in a phosphate buffer (0.1 mol L⁻¹, pH 7.0) in a total volume of 1 mL. The decrease in NADPH was followed by measuring the absorbance at 340 nm. The nonspecific NADPH decrease was corrected for by using additional measurements without the substrate ($\epsilon_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$). One U was equal to μ mol converted NADPH min⁻¹.

RNA extraction, expression analyses with quantitative real-time reverse transcription-PCR

The expression rate of *A. thaliana* GST genes was determined by quantitative real-time reverse transcription-PCR (RT-qPCR) after the purification of RNA from 100 mg of plant material according to Chomczynski and Sacchi (1987), as described in Csizsár *et al.* (2014). The primers used for the RT-qPCR can be found in Table S1, available as Supplementary Material to this paper. Representative amplified products of RT-qPCR were confirmed by sequencing. The expression rate of GST genes was monitored as published earlier in Gallé *et al.* (2009). The 18S rRNA (*At3g41768* and *At2g01010*) and *actin2* (*At3g18780*) genes were used as high and low internal controls, respectively (Masclaux-Daubresse *et al.* 2007; Papdi *et al.* 2008). The *actin2* gene exhibited constant expression in our experiments, so it was used for data normalisation. Data from the RT-qPCR were calculated using the $2^{(-\Delta\Delta C_t)}$ formula (Livak and Schmittgen 2001). To demonstrate the differences between changes in the expression levels of different GSTs, the relative transcript level in the control root samples was arbitrarily considered to be 1 for each gene.

Statistical analysis

Statistical analysis was carried out with SigmaPlot ver. 11.0 software (Systat Software Inc., Erkrath, Germany) by Duncan's test and differences were considered significant at $P \leq 0.05$. Data presented here are the means \pm s.d. of at least three measurements unless indicated otherwise.

Results

The effect of SA and NaCl on plant growth

We applied 10^{-9} – 10^{-5} M SA to 5-week-old *A. thaliana* plants grown hydroponically, and the effects of SA treatment on plant growth and different physiological parameters were measured at weekly intervals between the fifth and eighth weeks. SA slightly promoted the growth of rosette size, shoot and root weight (Fig. S1). Data on the changes in growth parameters, ROS accumulation, ROS-triggered damage, and the activity of SOD, CAT, guaiacol peroxidase, GST and GPOX during the 3-week SA treatment are documented in the Figs S1–S4.

The priming effect of SA on salt tolerance was investigated by measuring these parameters on plants sequentially treated with 10^{-9} – 10^{-5} M SA and 100 mM NaCl. Although the FW of shoots was significantly higher in salt-stressed plants after SA treatments, the FW of roots was higher only in plants pretreated with 10^{-6} and 10^{-5} M SA, and the length of roots did not change significantly (Fig. 1). The improved growth parameters of SA-treated plants indicate that successful priming took place in salt-stressed plants in a concentration-dependent manner.

ROS accumulation and oxidative damage in SA- and salt-treated plants

Total ROS levels transitionally increased in the leaf disks and root tips of SA-treated plants but were reduced to constitutive levels after 2 and 3 weeks of SA treatments (Fig. S2).

The results show that 100 mM NaCl stress caused a two- to threefold increase in the total ROS (especially H_2O_2) levels in roots and leaves. Fluorescence microscopy investigations revealed that SA pretreatment significantly reduced ROS accumulation in leaf discs and root tips after the 1-week salt treatment. However, the H_2O_2 content (measured by a photometric method) was further enhanced by 10^{-9} and 10^{-8} M SA pretreatment in the leaves but was lowered by most SA concentrations in the roots during salt stress. A similar tendency was observed in the MDA accumulation in leaves. Interestingly, H_2O_2 and MDA contents were less elevated in plants pretreated with 10^{-7} – 10^{-5} M SA. In roots, the H_2O_2 content was enhanced by salt stress but it was not affected significantly by simultaneous SA treatment, except for treatment with 10^{-8} M SA (Fig. 2).

The effect of SA pretreatment and salt stress on the activity of selected antioxidative enzymes

The main enzymatic antioxidants in plants include SOD, which converts $O_2^{\cdot-}$ to the less toxic H_2O_2 ; CAT, which takes part in removing the H_2O_2 , and guaiacol peroxidases, which oxidise various substrates in the presence of H_2O_2 but may also produce ROS, such as $O_2^{\cdot-}$, OH^{\cdot} or HOO^{\cdot} via the hydroxylic cycle (Passardi *et al.* 2004). SA treatment slightly reduced CAT activity in a time-dependent manner, whereas the activity of

SOD did not change. Guaiacol peroxidase activity was not affected by most SA concentrations during the 2-week treatment (Fig. S3).

Adding 100 mM NaCl to the hydroponic solution for 1 week enhanced the activity of these antioxidant enzymes in roots but did not affect or reduce them in shoots. Pretreatment with SA reduced SOD in roots, but not in leaves, where 10^{-6} and 10^{-5} M SA enhanced it. Salt stress caused a threefold induction in SOD activity of the roots without pretreatment but the enhancement was smaller in SA-pre-treated roots. CAT activity was either not affected or reduced by SA pretreatment in salt-stressed plants. Guaiacol peroxidase activity was higher in roots of plants treated with 10^{-6} and 10^{-5} M SA, but they were only moderately affected by other SA treatments. Under salt stress, guaiacol peroxidase activity was elevated in several cases compared with plants without SA pretreatments (Fig. 3).

GST and glutathione-dependent peroxidase activity in SA- and salt-treated plants

In leaves, GST activity was induced by SA treatment in a concentration-dependent manner; in roots, GST was only moderately affected by SA (Fig. S4). By the end of the 3 weeks of treatment, 10^{-9} - to 10^{-5} -M SA concentrations elevated the total GST activity in leaves; in roots, enhancement was significant only in plants treated with 10^{-6} – 10^{-5} M SA. GPOX was induced by 10^{-5} M SA in roots and 10^{-6} – 10^{-5} M SA in leaves (Fig. 4). In both leaves and roots, 100 mM NaCl increased the GST activity but inhibited the GPOX enzyme activity. SA pretreatment resulted in enhanced GPOX activity in salt-stressed plants (Fig. 4).

Transcript amounts of selected GST genes after SA pretreatment and salt stress

To investigate whether SA and salt modulates GST activity by affecting the expression of these genes, transcript levels of selected salt- or SA-inducible *A. thaliana* GST genes were investigated in 8-week-old *A. thaliana* plants, which were subjected to sequential SA (10^{-5} and 10^{-7} M) and salt (100 mM NaCl) treatments as described above. Real-time RT-qPCR was used to determine the expression of selected GST genes after 1 week of salt stress with or without 10^{-5} and 10^{-7} M SA pretreatments. Considerable variation was detected in transcript levels of individual GST genes. In control conditions, *AtGSTF8*, *AtGSTF9* and *AtGSTU19* expression was higher in leaves than roots, whereas *AtGSTU24* and *AtGSTU25* had higher transcription in roots than in leaves (Fig. 5). Salt stress considerably enhanced the transcription of *AtGSTU19* and *AtGSTU24* in both leaves and roots, whereas expression of the other three GST genes was reduced in both organs. Pretreatment with 10^{-7} M SA enhanced transcription of *AtGSTU19* and *AtGSTU24* genes in leaves, but did not affect the expression of *AtGSTF8*, *AtGSTF9* and *AtGSTU25* genes in salt-stressed plants. Pretreatment with a higher SA concentration (10^{-5} M SA) had a negative effect on the expression of the investigated genes (Fig. 5).

The significant up- or downregulation of selected GST genes, which were induced by 100 mM NaCl treatment after 1 week, was still detected in plants pretreated with 10^{-7} M SA; however, these changes did not appear in plants treated with 10^{-5} M SA after salt

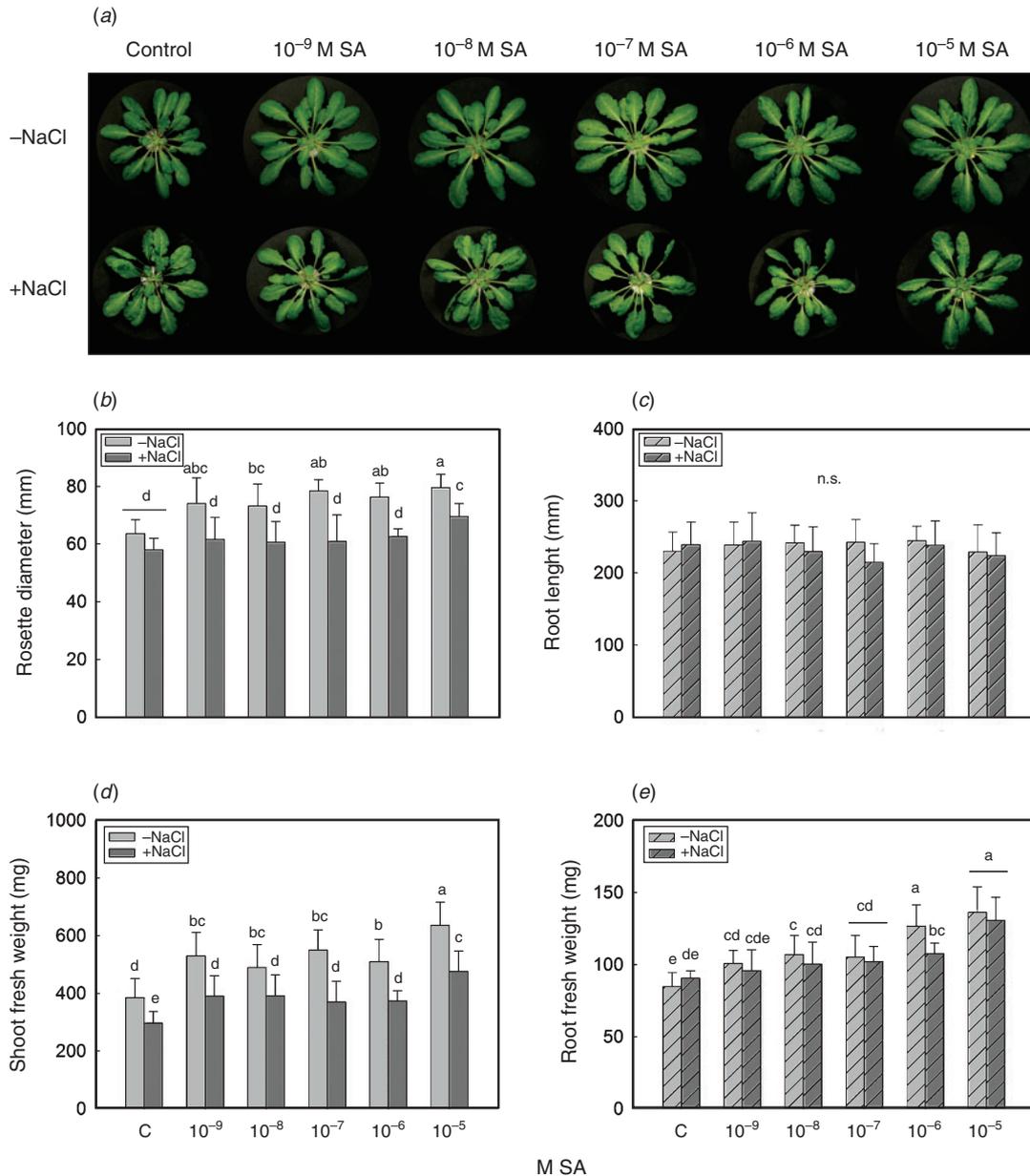


Fig. 1. Effects of 2-week pretreatments with 10^{-9} – 10^{-5} M salicylic acid (SA) on leaf and root growth and FW of *Arabidopsis thaliana* plants after a 1-week exposure to 100 mM NaCl. (a) Rosette morphologies of typical plants. (b) Rosette diameters, (c) root lengths, (d) shoot FW and (e) root FW of SA and salt-treated plants (means \pm s.d., $n=8$ –12). Columns with different letters are significantly different at $P < 0.05$, determined by Duncan's test. n.s., not significant.

stress, which indicates the more effective priming effect of the higher SA concentration in alleviating the NaCl-induced stress (Fig. 5).

Discussion

Although SA is a plant hormone mainly associated with the induction of defence mechanisms against biotic stresses, an increasing amount of evidence suggests that SA can influence responses to abiotic stresses. Exogenous application of SA in a

suitable concentration exerts diverse physiological effects on plants, like the activation of antioxidants, which, in turn, can lead to a better stress tolerance (Horváth *et al.* 2007; Ashraf *et al.* 2010). Looking for clues to understand the role of SA in defence to salt stress, we focussed our attention on long-term priming, followed by extended salt stress (1 week of 100 mM NaCl stress) in *A. thaliana* plants.

Our earlier results showed that similar SA pretreatments of tomato plants significantly improved tolerance against high salinity (triggered with 100 mM NaCl for 1 week). In tomato,

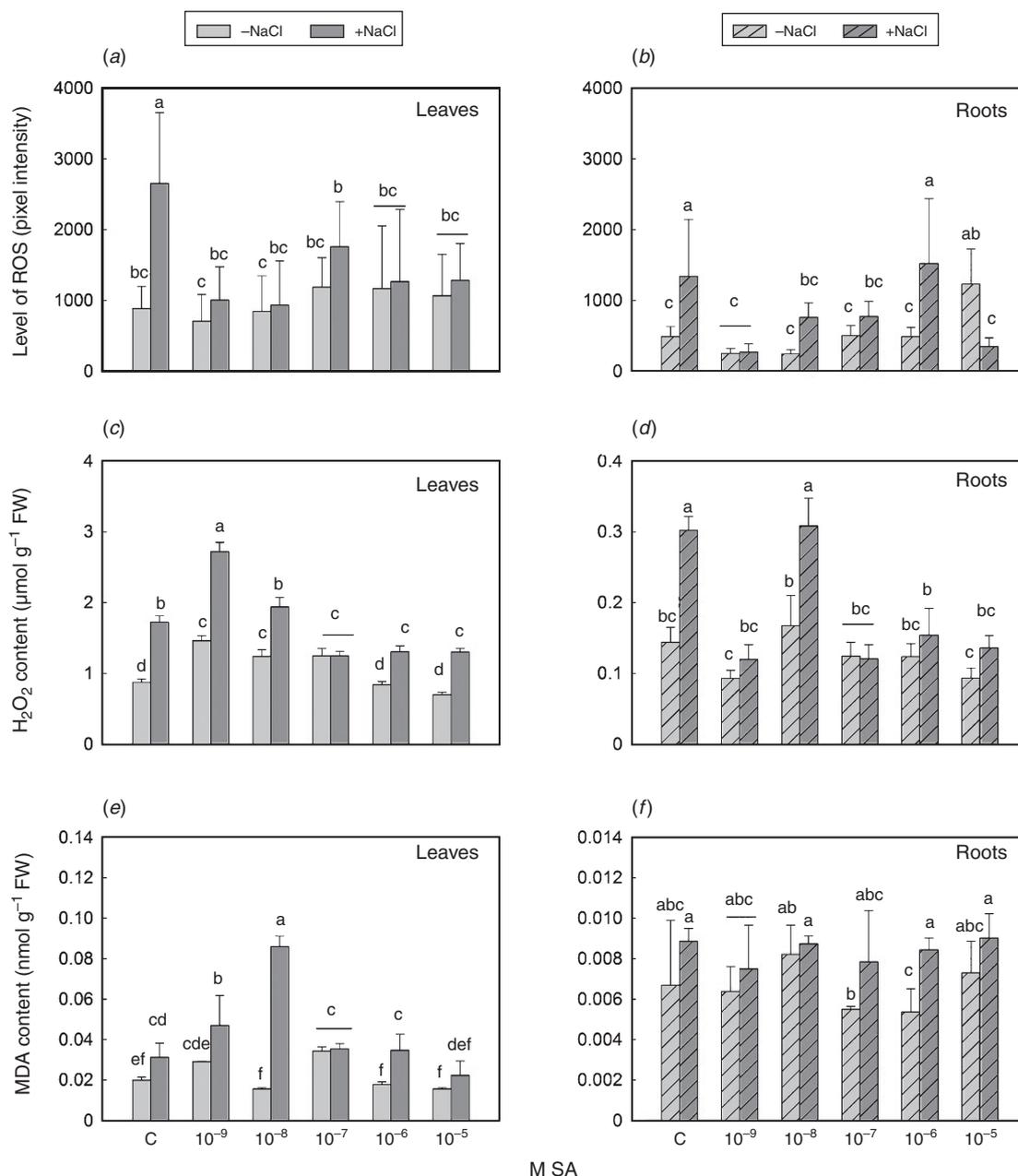


Fig. 2. Changes in (a, b) reactive oxygen species (ROS), (c, d) H_2O_2 and (e, f) malondialdehyde (MDA) levels in the (a, c, e) leaves and (b, d, f) roots of 8-week-old *Arabidopsis thaliana* plants pretreated with 10^{-9} – 10^{-5} M salicylic acid (SA) and subsequently stressed with 100 mM NaCl. The ROS levels were determined using 2'-7'-dichlorodihydrofluorescein diacetate. Means \pm s.d. or means \pm s.e.; $n=9$. Columns with different letters are significantly different at $P < 0.05$, determined by Duncan's test.

10^{-4} M SA could stimulate the acclimation processes and alleviate the deleterious effects of subsequently applied salt stress. SA pretreatment of salt-stressed tomato plants reduced the ratio of $Na^+ : K^+$ content; enhanced ABA levels; improved water relations and osmotic adaptation (Szepesi *et al.* 2009; Horváth *et al.* 2015), prevented the decline of photosynthetic parameters (Poór *et al.* 2011); decreased ROS, nitric oxide and MDA contents (Szepesi *et al.* 2008; Gémes *et al.* 2011); and increased GST and GPOX activity (Szepesi *et al.* 2008; Csizsár

et al. 2014). This study was designed to evaluate the use of SA priming in *A. thaliana* model plants, to gain deeper insights into the molecular events behind the acclimation process.

The effect of exogenously applied SA was previously shown to depend on the dose and the plant species tested (reviewed by Rivas-San Vicente and Plasencia 2011). High SA doses can induce an oxidative burst by increasing the plasma membrane-localised NADPH oxidase activity, and by decreasing the activity of CAT and APX (Vlot *et al.* 2009; Hayat *et al.* 2010). In contrast,

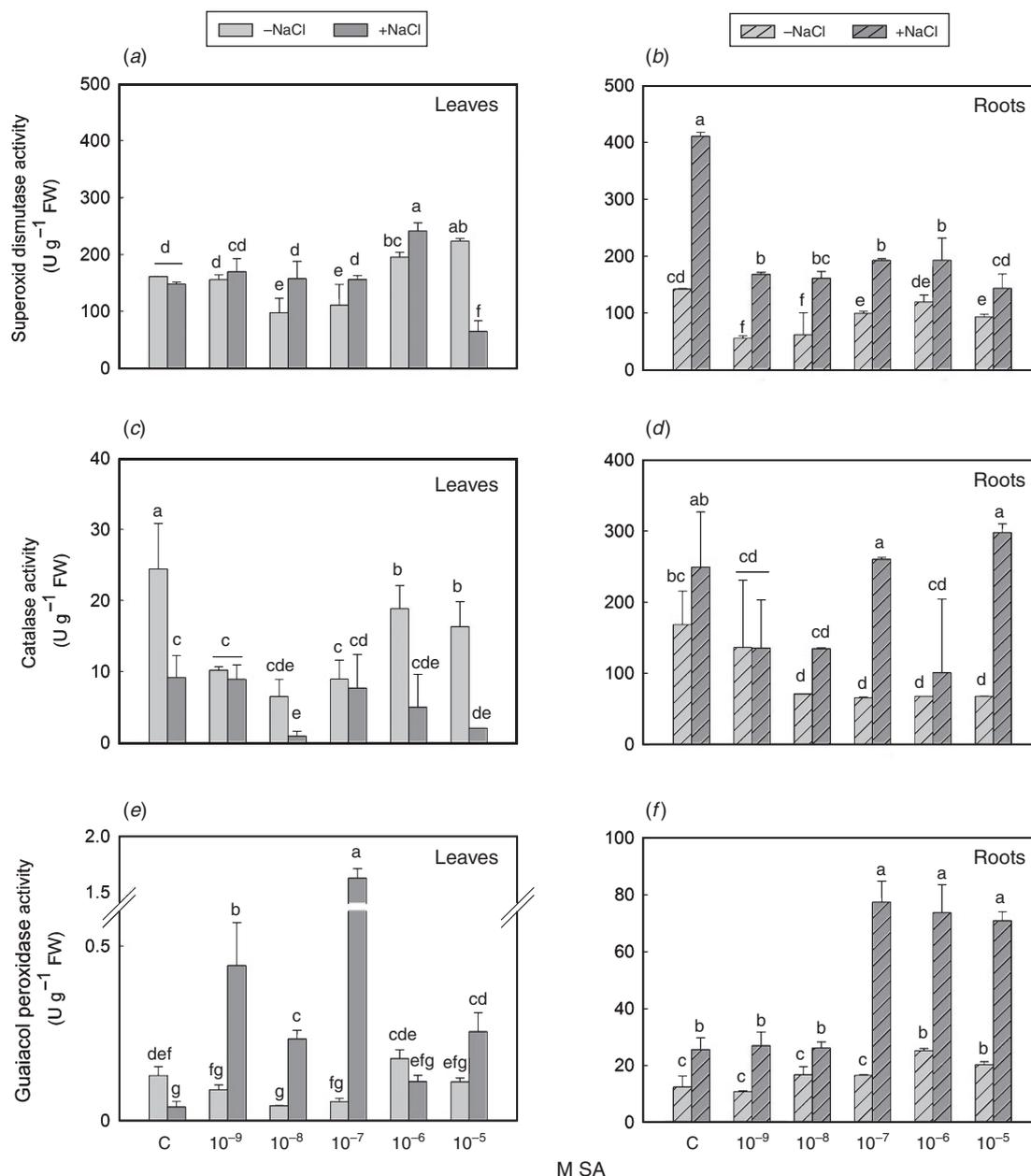


Fig. 3. Effect of 2-week salicylic acid (SA) pretreatments on the activity of (a, b) superoxide dismutase, (c, d) catalase and (e, f) guaiacol peroxidase enzymes in the (a, c, e) leaves and (b, d, f) roots of *Arabidopsis thaliana* plants exposed to 100 mM NaCl for 1 week. Data are means \pm s.d. Means denoted by different letters indicate a significant difference between the treatments ($P < 0.05$, Duncan's test).

low doses of exogenously applied SA increase the antioxidant enzyme activity in plants and alleviate abiotic stress-induced damage (Alonso-Ramírez *et al.* 2009). Addition of 10^{-9} – 10^{-5} M SA to Hoagland solution for 3 weeks did not have any deleterious effect on *A. thaliana* (Fig. S1). However, some changes in the levels of ROS and H₂O₂ could be observed in most of the SA concentrations used in this study. Although SA treatment alone in most cases did not significantly alter the activity of antioxidant enzymes after 3 weeks, with the 10^{-6} and 10^{-5} M SA treatments, SOD, guaiacol peroxidase and GST activity were comparable to

the control or were even higher (Figs S2–4). The elevated SOD, CAT, guaiacol peroxidase and GST activity in plants may participate in the salt stress response in this experimental system.

The damaging and signalling effect of ROS is an important consequence of NaCl stress and the antioxidant mechanism is a key component of salt stress tolerance in plants (Munns and Tester 2008). ROS accumulation is partially controlled by an enzymatic detoxification system, which is usually induced upon stress exposure (Gill and Tuteja 2010). Three days of 100 mM NaCl treatment increased H₂O₂ and MDA content, and SOD, CAT and

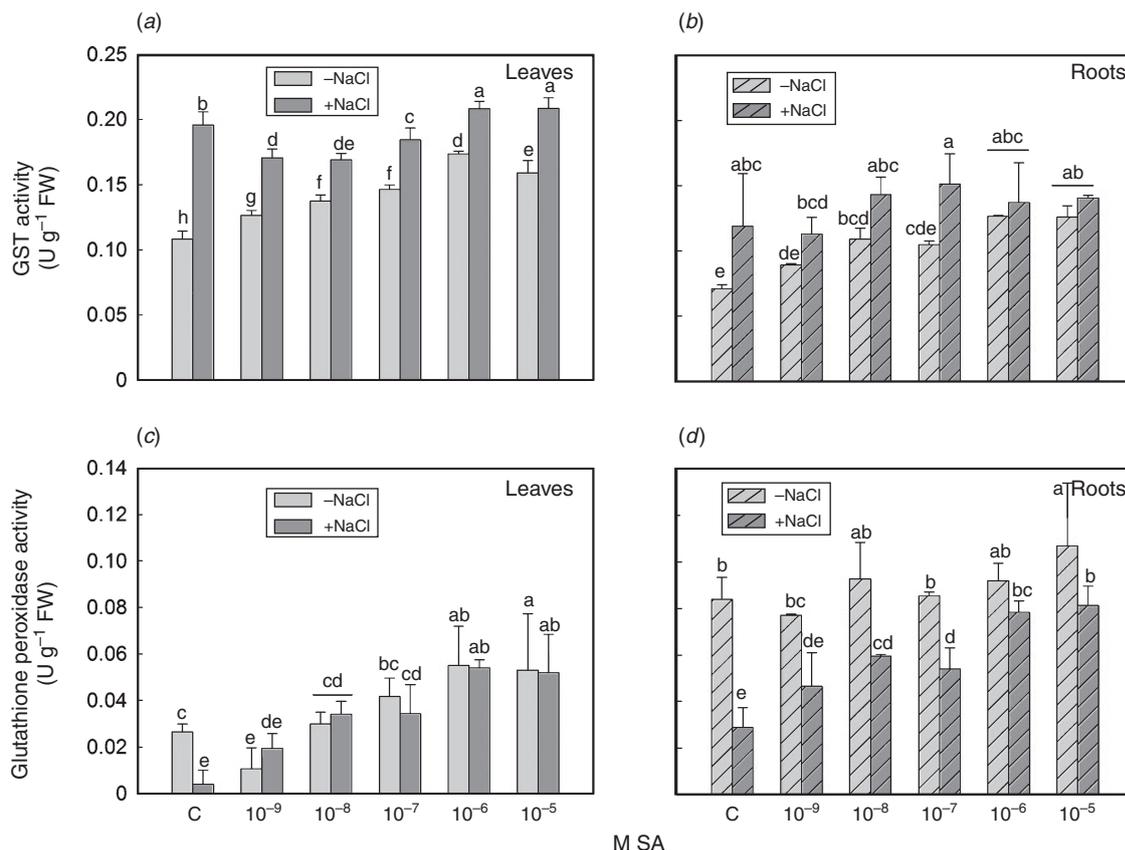


Fig. 4. Effect of 2-week salicylic acid (SA) pretreatment and subsequent 100 mM NaCl treatment for 1 week on (a, b) GST and (c, d) glutathione peroxidase activity in the (a, c) leaves and (b, d) roots of *Arabidopsis thaliana* plants. Data consist of means \pm s.d. obtained from at least three measurements. Means denoted by different letters indicate a significant difference between the treatments ($P < 0.05$, Duncan's test).

peroxidase activity in *A. thaliana* leaves (Ellouzi *et al.* 2011). Proteomic analysis of *A. thaliana* roots subjected to the 150 mM NaCl treatment revealed an increase in the amount of important ROS-scavenging and detoxifying proteins, including ascorbate peroxidase, glutathione peroxidase, Class III peroxidases, GST and SOD (Jiang *et al.* 2007). However, Attia *et al.* (2008) could not detect changes in SOD activity after 2 weeks of 50 mM NaCl treatment in *A. thaliana* plants. In our experiments, the 1-week-long treatment with 100 mM NaCl increased the intracellular ROS and H_2O_2 contents and MDA accumulation in *A. thaliana* plants, suggesting enhanced oxidative stress. Although SOD, CAT and guaiacol peroxidase activities were enhanced by salt stress in roots, these activities were reduced or did not change in leaves. GST activity was enhanced but GPOX was reduced by salt stress in both organs. Nevertheless, induction of antioxidant capacity was insufficient to prevent the accumulation of ROS and lipid peroxides. Differences in our results and those reported in other studies can be explained by different experimental conditions, the strength and length of salt stress, and differences in the plant genotypes used.

In contrast to the enhanced ROS levels of salt-stressed *A. thaliana* plants, the leaves of plants treated with 10^{-7} – 10^{-5} M SA had lower levels of ROS and H_2O_2 after 1 week of 100 mM

NaCl treatment. Plants pretreated with 10^{-7} – 10^{-5} M SA had higher guaiacol peroxidase activity even after applying 100 mM NaCl for a week. Similarly, in sunflower, Noreen *et al.* (2009) found that SA alleviated the effect of 120 mM NaCl, mainly due to enhanced peroxidase activity. Guaiacol peroxidases were implicated in the responses to different biotic and abiotic stresses, including pathogen attack, heavy metals, cold, dehydration and salt stress, and in various physiological processes such as auxin catabolism, biosynthesis of secondary metabolites, lignification, suberisation and senescence (De Gara 2004; Cosio and Dunand 2009; Csiszár *et al.* 2012; Guo *et al.* 2014). These enzymes catalyse the reduction of H_2O_2 using electrons from various donor molecules (Passardi *et al.* 2004). Guaiacol peroxidase is suggested to be involved in fine regulation of H_2O_2 content, because it has a higher affinity to H_2O_2 than CAT, whereas CAT may be implied in mass scavenging of H_2O_2 (Abogadallah 2010). Our results suggest that SA-triggered acclimation during salt stress can at least partially be explained by enhanced guaiacol peroxidase activity in SA-pretreated plants. Moreover, enhanced GST and GPOX activity in SA-treated plants could also contribute to salt tolerance.

Although pretreatment with 10^{-9} – 10^{-8} M SA significantly increased the MDA content in leaves after applying salt stress, in

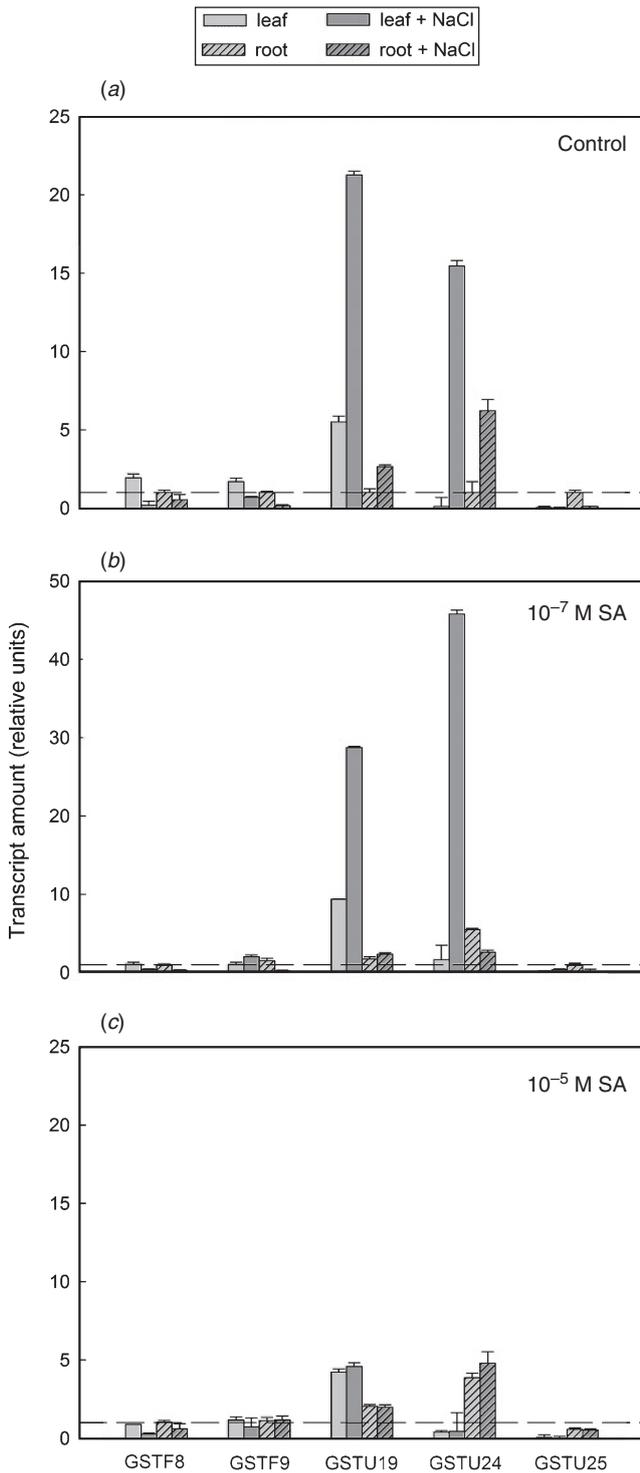


Fig. 5. Effect of three-week salicylic acid (SA) pretreatment on the transcript levels of selected *Arabidopsis thaliana* GST genes in leaves and roots of 8-week-old *A. thaliana* plants after applying 100 mM NaCl for 1 week. (a) control treatment; (b) pretreatment with 10^{-7} M SA; (c) pretreatment with 10^{-5} M SA. Data were normalised using the *A. thaliana actin2* gene as an internal control. The relative transcript level in control root samples was arbitrarily considered to be 1 for each gene (indicated with a dashed line). Data consist of means \pm s.d.; $n = 3$.

the case of higher SA concentrations, the level of MDA was similar to the control both in leaves and roots. Lower amounts of thiobarbiturate-reactive lipid peroxidation products were reported under salt stress in SA-pretreated tomato and bean (*Phaseolus vulgaris* L.) plants (Tari *et al.* 2002; Palma *et al.* 2009). GSTs were suggested to play a pivotal role in protecting of plants from oxidative damage under salt stress by preventing the degradation of organic hydroperoxides to cytotoxic aldehyde derivatives (Zhang *et al.* 2012). Our earlier results showed that GSTs participate in the SA-induced priming in tomato (Csiszár *et al.* 2014). Furthermore, some GSTs were identified as SA-binding proteins (AtGSTF2, AtGSTF8, AtGSTF10 and AtGSTF11) and thus they may be direct targets of SA (Tian *et al.* 2012).

To test whether the alteration in GST activity is controlled at the transcription level, expression of selected GST genes were tested by RT-qPCR in *A. thaliana* plants subjected to SA and salt treatments. GST genes with relative high affinity towards the used substrates (CDNB and cumene hydroperoxide) were chosen (Dixon *et al.* 2009). AtGSTU19 provides high GST activity and was the most abundant protein identified in *A. thaliana* cell culture (Sappl *et al.* 2004). The expression of the *AtGSTU19* gene was induced by compatible pathogen interactions (Wagner *et al.* 2002), SA and H₂O₂ (Sappl *et al.* 2009). In a proteomic study, AtGSTU24 proved to be SA-inducible (Sappl *et al.* 2004). The overexpression of either *AtGSTU24* or *AtGSTU25* resulted in elevated CDNB conjugating activity in *A. thaliana* plants under control conditions, and these two genes exhibited a significantly enhanced ability to withstand and detoxify 2,4,6-trinitrotoluene (Gunning *et al.* 2014). In our experiments, the expression of most GST genes was not altered significantly by SA treatments, except for *AtGSTU19* and *AtGSTU24*, for which the expression was higher in plants treated with 10^{-7} M SA. Salt stress-induced transcription of *AtGSTU19* and *AtGSTU24* was further enhanced by 10^{-7} M SA pretreatment in leaves. Higher SA, however, reduced salt induction of these genes. These data suggest that alteration of GST activity in salt- and SA-treated *A. thaliana* plants can be at least partially derived from differential transcriptional activation of *AtGSTU24* and *AtGSTU19*.

Based on the results obtained in this study, the protective effects of exogenously applied SA depend on the concentration used and on the affected plant tissue. We demonstrated that the proper SA concentrations in *A. thaliana* plants are 10^{-6} – 10^{-5} M SA pretreatments for the induction of priming, which enhances SOD, guaiacol peroxidase, GST and GPOX activity, and reduced H₂O₂ and MDA accumulation compared with the salt-treated control plants. These results suggest that SA-mediated acclimation can reduce the oxidative damage caused by salt stress through modulating the activity of some of the key ROS and peroxide detoxifying enzymes. At least some of the alterations in enzyme activity derive from modulation of the transcriptional control of key detoxification genes, such as GSTs. Our results show that long-term SA treatment on 5-week-old *A. thaliana* plants resulted in priming and mitigated salt stress injury in this model plant. The applied hydroponic experimental system can be a useful tool to study the effect of sequential treatments in *A. thaliana* and to gain deeper insights into the regulatory

mechanism that controls all aspects of SA-mediated stress acclimation in higher plants.

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