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

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Running title: GSTs and SA-triggered hardening in *Arabidopsis*

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Using chemicals, such as salicylic acid (SA) as pre-treatment agent on plants may alleviate subsequently applied salt stress-triggered damages in *Arabidopsis*. Exogenous SA fine-tunes the production of reactive oxygen species and, in a proper concentration, increases the antioxidant peroxidase and glutathione transferase (GST) activities, enhances the transcript amount of several GST genes. Induction of *AtGSTU24* and *AtGSTU19* genes by SA can be an important part of priming and salt stress acclimation.

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.) plants to 2-week-long 10^{-9}-10^{-5} M SA-pre-treatments in hydroponic medium, followed by a one-week-long 100 mM NaCl stress. The levels of reactive oxygen species and hydrogen peroxide (H_2O_2), changes in antioxidant enzyme activities and the expression of selected glutathione transferase (GST) genes were investigated. While 10^{-9}–10^{-7} M SA pre-treatment insufficiently induced the defense mechanisms during subsequent salt stress, two-week-long pre-treatments with 10^{-6} and 10^{-5} M SA alleviated the salinity-induced H_2O_2 and malondialdehyde accumulation and increased, superoxide dismutase, guaiacol peroxidase, GST and glutathione peroxidase (GPOX) activities. Our results indicate that the 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 activities, which may play an important role in the acclimation. Modified GST expressions suggest an altered signaling in SA-hardened plants during salt stress. The hydroponic system applied in our experiments was proved to be a useful tool to study the effects of sequential treatments in *Arabidopsis*.

Key words: antioxidant enzyme activity; NaCl stress; priming; reactive oxygen species; salicylic acid

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 defense responsive genes, including those which are directly or indirectly involved in SA synthesis (Dangl and Jones 2001; Metraux 2001; Ashraf *et al.* 2010; Xia *et al.* 2015). SA acts as a signal for the development of the systemic acquired resistance (SAR) 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 signaling has been studied intensively. One of the main pathways is associated with the reduction of intermolecular disulfide bonds of the cytosolic oligomer NPR1 (non-expressor of pathogenesis-related genes 1) protein. The resulting monomers are then able to translocate to the nucleus and activate the expression of defense genes in the NPR1-dependent pathway (Mou *et al.* 2003). Novel and interesting feature of NPR1, besides being a metalloprotein acting as a transcription regulator, is that it acts as an SA-receptor (Wu *et al.* 2012; Kuai *et al.* 2015). However, recent evidence suggests that H_2O_2-dependent changes in the glutathione pool can activate SA-dependent defense responses independently of NPR1 (Han *et al.* 2012). SA signaling transcriptional factors, such as NPR1, 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, while NPR1-independent early SA activated gene products may have antioxidant and detoxifying activities (Blanco *et al.* 2009).

While involvement of SA in plant defense 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* (Lee and Park 2010; Jayakannan *et al.* 2013), tomato (Tari *et al.* 2002; Stevens *et al.* 2006), mungbean (Khan *et al.* 2010), maize (Gunes *et al.* 2007), barley (El-Tayeb 2005), sunflower (Noreen *et al.* 2009) and mustard (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; Syeed *et al.* 2011). Nevertheless, some controversy regarding the involvement of SA in salt stress responses still exists. Results of experiments using *Arabidopsis* 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 signaling blockage in *Arabidopsis* plants was favorable to salt adaptation while *sid2 Arabidopsis* mutants, impaired in SA biosynthesis, were shown to be hypersensitive to salt stress (Alonso-Ramirez *et al.* 2009).

Salinity affects plant growth and development in a complex manner. On the one hand, salt reduces 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 singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2) – through the disruption of photosynthetic electron transport, generation of H_2O_2 in the peroxisome, 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 signaling 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 cause of the cellular toxicity provoked by salinity (Kim *et al.* 2005). In order to keep ROS levels tightly regulated and to minimize ROS-derived damage, different non-enzymatic antioxidants (such as ascorbate, glutathione, carotenoids, tocopherols) and enzymatic systems (superoxide dismutase, SOD; catalase, CAT; guaiacol peroxidase; ascorbate peroxidase, APX; glutathione peroxidase, GPOX; glutathione reductase, GR) have evolved in aerobic organisms. Zhang et al. (2012) evaluated the results of proteomic studies conducted with 34 salt-treated plant species (including *Arabidopsis thaliana* and *Oryza sativa* model plants, 7 agricultural and 12 economic crops, 11 halophytes and 2 tree species) and revealed 184 protein identities (IDs) as ROS scavenging-related proteins, of which 143 IDs were induced by salinity (for more details, see the review of Zhang et al. 2012). In *Arabidopsis* plants, the abundance of SOD, peroxidases, APX, GR, 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 *Arabidopsis* 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 (Edwards *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 *Arabidopsis* comprising of 28 and 13 members, respectively (Edwards *et al.* 2010). Both, 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 duration (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 of tomato plants with SA was able to mitigate salt stress injury in a concentration dependent manner. Pre-treatment of tomato plants with 10^{-4} M SA increased the efficiency of enzymatic and non-enzymatic 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 glutathione transferases (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 *Arabidopsis thaliana* L. plants' overall oxidative state by measuring the reactive oxygen content and the antioxidant activities. Our aim was to characterize the effects of a long-term SA treatment on 5-week-old *Arabidopsis 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 *Arabidopsis* plants and reduce peroxides by increased guaiacol peroxidase, GST and GPOX activities.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana L. (ecotype Columbia) plants were grown in Hoagland solution in growth chamber (Fitoclima S 600 PLH, Aralab, Portugal) at 21°C under 100 μmol m^{-2} s^{-1} light intensity with 10/14 h day/night photoperiod, and the relative humidity was 70%. After being kept under control conditions for five weeks, the plants were treated with 10^{-9}–10^{-4} M salicylic acid solutions for two weeks and were subsequently exposed to salinity – by adding 100 mM NaCl directly to the medium – for one week. Hydroponic application of SA on 5-week-old *Arabidopsis* plants revealed that 10^{-4} M SA was lethal after two weeks of treatment (data not shown). Samples were taken from fully expanded leaves and roots, one and two weeks after the SA exposure and one week after the 100 mM NaCl treatment. The experiments were repeated at least three times, the measurements were performed in three replicates unless indicated otherwise.

Investigation of reactive oxygen species 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 reactive oxygen species in 10 mm diameter leaf disks and in the root tips of *Arabidopsis* plants. To detect ROS, 2'-7'-dichlorodihydrofluorescein diacetate (H_2DCF DA; Sigma-Aldrich) was used at 37°C for 15 min, then the samples were washed 4 times in 20 min with 2-(N-morpholino)ethanesulfonic acid/potassium chloride buffer (10 mM/50 mM, pH 6.15), according to Pető *et al.* (2013). The intensity of ROS-dependent fluorescence was measured on digital images with the help of Axiovision Rel. 4.8 software. Fluorescence intensity values were determined in 200 μm diameter circles 300 μm from the root tip in roots and 600 μm diameter circles in leaves. The diameter of circles was not modified during the experiments. The measurements were performed in 10 replicates, mean ± SE are given on the figures.

Determination of H_2O_2 level

H_2O_2 level was measured spectrophotometrically as described earlier in Gémes *et al.* (2011). After homogenization of 400 mg shoot or root tissue on ice with 750 μL 0.1% trichloroacetic acid (TCA), the samples were centrifuged at 10 000 *g* for 20 min at 4°C. The reaction contained 0.25 mL 10 mM phosphate buffer (pH 7.0), 0.5 mL 1M KI and 0.25 mL supernatant. The absorbance

of samples was measured after 10 min at 390 nm. The amount of H_2O_2 was calculated using a standard curve prepared with 0.1-5 μmol mL^{-1} H_2O_2 concentrations.

Malondialdehyde determination

Malondialdehyde (MDA) formation was followed by using the thiobarbituric acid method (Ederli *et al.* 1997). 100 mg shoot or root tissue was homogenized with 0.1% TCA and 100 μL 4% butylhydroxytoluene was added to avoid further lipidperoxidation. The extracts were centrifuged at 10 000 *g* 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 excitation coefficient of 155 mM^{-1} cm^{-1}.

Determination of antioxidant enzyme activities

The enzyme activities were determined as published earlier (Csiszár *et al.* 2004) with some modifications. To analyze the enzyme activities, 0.2 g tissue was homogenized on ice in 1 mL 100 mM phosphate buffer (pH 7.0) containing 1 mM phenylmethylsulfonyl fluoride and 1% polyvinyl-polypirrolidone. The homogenate was centrifuged for 20 min at 10 000 *g* at 4°C and the supernatant was used for enzyme activity assays.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined by measuring the ability of the enzyme to inhibit the photochemical reduction of nitro blue tetrazolium chloride (NBT; Sigma-Aldrich) in the presence of riboflavin in the light. One unit (U) of SOD was calculated as an amount causing a 50% inhibition of NBT reduction in light. The enzyme activity was expressed as enzyme units per gram fresh weight (U g^{-1} FW). Catalase (CAT, EC 1.11.1.6) activity was determined by the decomposition of H_2O_2 and was measured spectrophotometrically by following the decrease in absorbance at 240 nm. One U = the amount of H_2O_2 (in μmol) 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. ε470=26.6 mM^{-1} cm^{-1}. The amount of enzyme producing 1 μmol min^{-1} of oxidized guaiacol was defined as 1 U. Glutathione transferase (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 A_{340} was determined. One U is the amount of the enzyme producing 1 μmol conjugated product in 1 min, ε_{340}=9.6 mM^{-1} cm^{-1}. Glutathione peroxidase (GPOX, EC 1.11.1.9) activity was measured with cumene hydroperoxide (CHP; Sigma-Aldrich) as a substrate. The reaction mixture contained 4 mmol L^{-1} GSH, 0.2 mmol L^{-1} NADPH, 0.05 U of GR (from baker's yeast, Sigma-Aldrich), 100 μL enzyme extract, and 0.5 mmol L^{-1} substrate in phosphate buffer (0.1 mol L^{-1}, pH 7.0) in a total volume of 1 mL. The decrease of NADPH was followed by measuring the absorbance at 340 nm. The nonspecific NADPH decrease was corrected for by using additional measurements without substrate, ε_{340}=6.22 mM^{-1} cm^{-1}. One U = μmol converted NADPH min^{-1}.

*RNA **extraction**, expression analyses with quantitative real-time RT-PCR*

The expression rate of *Arabidopsis* GST genes was determined by quantitative real-time RT-PCR (RT-qPCR) after the purification of RNA from 100 mg plant material according to Chomczynski and Sacchi (1987) as was described in Csiszár *et al.* (2014). The primers used for the RT-qPCR can be found in Table S1. 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 ribosomal RNA (*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 exhibited constant expression in our experiments, thus it was used for data normalization. Data of RT-qPCR was calculated using the $2^{(-\Delta\Delta Ct)}$ 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 as one for each gene.

Statistical analysis

Statistical analysis was carried out with SigmaPlot 11.0 software by Duncan's test and differences were considered significant at $P \leq 0.05$. Data presented here are the means ± SD of at least 3 measurements unless indicated otherwise.

Results

The effect of SA and NaCl on plant growth

10^{-9}–10^{-5} M SA was applied to 5-week-old Arabidopsis plants grown hydroponically, and the effect of SA treatment on plant growth and different physiological parameters were measured at weekly intervals between the 5^{th} and 8^{th} weeks. SA slightly promoted the growth of rosette size, shoot and root weight [Supplementary Material – Figs. S1]. (Data on changes of growth parameters, ROS accumulation, ROS-triggered damage, activities of SOD, CAT, guaiacol peroxidase, GST, GPOX during the totally 3-week-long SA treatment are documented in the Supplemetary Material [Supplementary Material – Figs. S1-4].)

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. While the fresh weight of shoots was significantly higher in salt-stressed plants after SA treatments, the fresh weight of roots was higher only in plants pre-treated by 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 two and three weeks of SA treatments [Supplementary Material – Fig. S2].

100 mM NaCl stress caused a 2-3-fold increase in the total ROS (especially H_2O_2) levels in roots and leaves. Fluorescence microscopy investigations revealed that SA pre-treament significantly reduced the ROS accumulation in leaf discs and root tips after the one-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 pre-treatment in the leaves but was lowered by most SA concentrations in the roots during salt stress. Similar tendency was observed in the MDA accumulation in leaves.

Interestingly, H_2O_2 and MDA contents were less elevated in plants pre-treated 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 10^{-8} M SA (Fig. 2).

The effect of SA pre-treatment and salt stress on the activities of selected antioxidative enzymes

The main enzymatic antioxidants in plants include superoxide dismutases (SOD), which converts the $O_2^{\bullet -}$ to the less toxic H_2O_2; catalases (CAT), which take part in removing the H_2O_2; and guaiacol peroxidases, which oxidize various substrates in the presence of H_2O_2, but may also produce ROS, such as $O_2^{\bullet -}$, $OH^{\bullet}$ or $HOO^{\bullet}$ via the hydroxylic cycle (Passardi *et al.* 2004). SA treatment slightly reduced CAT activities in a time-dependent manner, while the activity of SOD did not change. Guaiacol peroxidase activities were not affected by most SA concentrations during the two-week-long treatment [Supplementary Material – Fig. S3].

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

Glutathione transferase and glutathione-dependent peroxidase activities in SA and salt-treated plants

In leaves, GST activities were induced by SA-treatment in a concentration-dependent manner, while in roots GST was only moderately affected by SA [Supplementary Material – Fig. S4]. By the end of the 3 weeks of treatment, 10^{-9}–10^{-5} M SA concentrations elevated the total GST activities in leaves, while in roots enhancement was significant only in 10^{-6}–10^{-5} M SA-treated plants. GPOX was induced by 10^{-5} M SA in roots and 10^{-6}–10^{-5} M SA in leaves (Fig. 4). 100 mM

NaCl increased the GST activity both in leaves and roots, but inhibited the GPOX enzyme activities in these organs. SA pre-treatment resulted in enhanced GPOX activities in salt stressed plants (Fig. 4).

Transcript amounts of selected glutathione transferase genes after SA pre-treatment and salt stress

To investigate whether SA and salt modulates GST activities by affecting the expression of these genes, transcript levels of selected salt- and/or SA-inducible *Arabidopsis* GSTs were investigated in 8-week-old *Arabidopsis* plants, which were subjected to sequential SA (10^{-5} and 10^{-7} M) and salt (100 mM NaCl) treatments as described above. Quantitative real-time RT-PCR was used to determine the expression of selected GST genes after one week of salt stress with or without 10^{-5} and 10^{-7} M SA pre-treatments. Considerable variation was detected in transcript levels of individual GST genes. In control conditions *AtGSTF8, AtGSTF9, AtGSTU19* expression was higher in leaves than roots, while *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, while expression of the other three GST genes was reduced in both organs. Pre-treatment 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. Pre-treatment with higher SA concentration (10^{-5} M SA) had a negative effect on the expression of the investigated genes (Fig. 5).

The significant up- or down-regulations of selected AtGST genes, which were induced by 100 mM NaCl treatment after one week, was still detected in plants pre-treated with 10^{-7} M SA, however, these changes did not appear in 10^{-5} M SA-pre-treated plants after salt stress, which indicates a more effective priming effect of the higher SA concentration to alleviate the NaCl-induced stress (Fig. 5).

Discussion

Although SA is a plant hormone mainly associated with the induction of defense mechanisms against biotic stresses, increasing number of evidence suggest 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 defense to salt stress, we focused our attention on the long-term priming, followed by extended salt stress (one-week-long 100 mM NaCl) in *Arabidopsis* plants.

Our earlier results showed that similar SA pre-treatments of tomato plants significantly improved tolerance against high salinity (triggered with 100 mM NaCl for one week). In tomato, 10^{-4} M SA could foment the acclimation processes and alleviate the deleterious effects of subsequently applied salt stress. SA pre-treatment 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 activities (Szepesi *et al.* 2008; Csiszár *et al.* 2014). This study was designed to evaluate the use of SA priming in *Arabidopsis* model plants, to gain deeper insights into the molecular events behind the acclimation process.

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-localized NADPH oxidase activity and by decreasing the activity of CAT and APX (Vlot *et al.* 2009; Hayat *et al.* 2010). In contrast, low doses of exogenously applied SA increase the antioxidant enzyme activities in plants and alleviate the abiotic stress-induced damages (Alonso-Ramirez *et al.* 2009). Addition of 10^{-9}–10^{-5} M SA to the Hoagland solution for 3 weeks did not have any deleterious effect on *Arabidopsis* [Supplementary Material – Fig. S1]. However, some changes in the levels of ROS and H_2O_2 could be observed in most of the SA concentrations used in this study. While SA treatment alone in most cases did not significantly alter the activities of antioxidant enzymes after three weeks; with the 10^{-6} and 10^{-5} M SA treatment, SOD, guaiacol peroxidase and GST activities were comparable to the control or were even higher [Supplementary Material – Fig. S2-4]. The elevated SOD, CAT, guaiacol peroxidase and GST activities in plants may participate in the salt stress response in this experimental system.

The damaging and signaling 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 H_2O_2 and MDA content and SOD, CAT and peroxidase activities in *Arabidopsis* leaves (Ellouzi *et al.* 2011). Proteomic analysis of *Arabidopsis* 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 activities after 2 weeks of 50 mM NaCl treatment in *Arabidopsis* plants. In our experiments, the one-week-long treatment with 100 mM NaCl increased the intracellular ROS and H_2O_2 contents and MDA accumulation in *Arabidopsis* plants, suggesting enhanced oxidative stress. While SOD, CAT and guaiacol peroxidase activities were enhanced by salt stress in roots, these activities were reduced or did not change in leaves. GST activities were 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 reported ones can be explained by different experimental conditions, strength and length of salt stress and differences in plant genotypes used.

In contrast to the enhanced ROS levels of salt stressed Arabidopsis plants, the 10^{-7}–10^{-5} M SA pre-treated plant leaves had lower level of ROS and H_2O_2 after one week 100 mM NaCl treatment. 10^{-7}–10^{-5} M SA pre-treated plants had higher guaiacol peroxidase activity even after applying 100 mM NaCl for a week. Similarly, Noreen *et al.* (2009) found in sunflower that SA alleviated the effect of 120 mM NaCl mainly due to enhanced peroxidase activity. Guaiacol peroxidases were implicated in responses to different biotic and abiotic stresses including pathogen attack, heavy metal, cold, dehydration, salt stress, and in various physiological processes such as auxin catabolism, biosynthesis of secondary metabolites, lignification, suberization and senescence (De Gara 2004; Cosio *et al.* 2009; Csiszár *et al.* 2012; Guo *et al.* 2014). These enzymes catalyze 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 activities in SA pre-treated plants. Moreover, enhanced GST and GPOX activities in SA-treated plants could also contribute to salt tolerance.

While pre-treatment with 10^{-9}–10^{-8} M SA significantly increased the MDA content in leaves after applying salt stress, in the case of higher SA concentrations its level 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-pre-treated tomato and bean plants (Tari *et al.* 2002; Palma *et al.* 2009). GSTs were suggested to play a pivotal role in protection 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 AtGSTs were identified as SA-binding proteins (AtGSTF2, AtGSTF8, AtGSTF10 and AtGSTF11), thus they may be direct targets of SA (Tian *et al.* 2012).

To test whether alteration of GST activities is controlled at transcription level, expression of selected GST genes were tested by RT-qPCR in *Arabidopsis* plants subjected to SA and salt treatments. AtGST genes with relative high affinity toward the used substrates (CDNB and CHP) were chosen (Dixon *et al.* 2009). AtGSTU19 provides high GST activity and was the most abundant protein identified in *Arabidopsis* cell culture (Sappl *et al.* 2004). The expression of *AtGSTU19* gene was induced by compatible pathogen interaction (Wagner *et al.* 2002), SA and H_2O_2 (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 *Arabidopsis* 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, expression of most GST genes was not altered significantly by SA treatments, except for *AtGSTU19* and *AtGSTU24*, whose expression was higher in plants treated by 10^{-7} M SA. Salt stress induced transcription of *AtGSTU19* and *AtGSTU24*, which was further enhanced by 10^{-7} M SA pre-treatment in leaves. Higher SA, however, reduced salt induction of these genes. These data suggest that alteration of GST activities in salt- and SA-treated *Arabidopsis* plants can be at least partially derived from differential transcriptional activation of *AtGSTU24* and *AtGSTU19* genes.

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 *Arabidopsis* plants are 10^{-6}–10^{-5} M SA pre-treatments for the induction of priming which enhanced SOD, guaiacol peroxidase, GST and GPOX activities and reduced H_2O_2 and MDA accumulation compared to the salt treated control plants. These results suggest that SA-mediated acclimation can reduce oxidative damage caused by salt stress through modulating activities of some of the key ROS and peroxide detoxifying enzymes. At least some of the alterations in enzyme activities derive from modulation of transcriptional control of key detoxification genes, such as GSTs. Our results show that the long-term SA treatment on 5-week-old *Arabidopsis thaliana* plants resulted in priming and mitigated salt stress injury of this model plant. The applied hydroponic experimental system can be a useful tool to study the effect of sequential treatments in Arabidopsis, and to gain deeper insight into the regulatory mechanism that controls all aspects of SA-mediated stress acclimation in higher plants.

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Figures

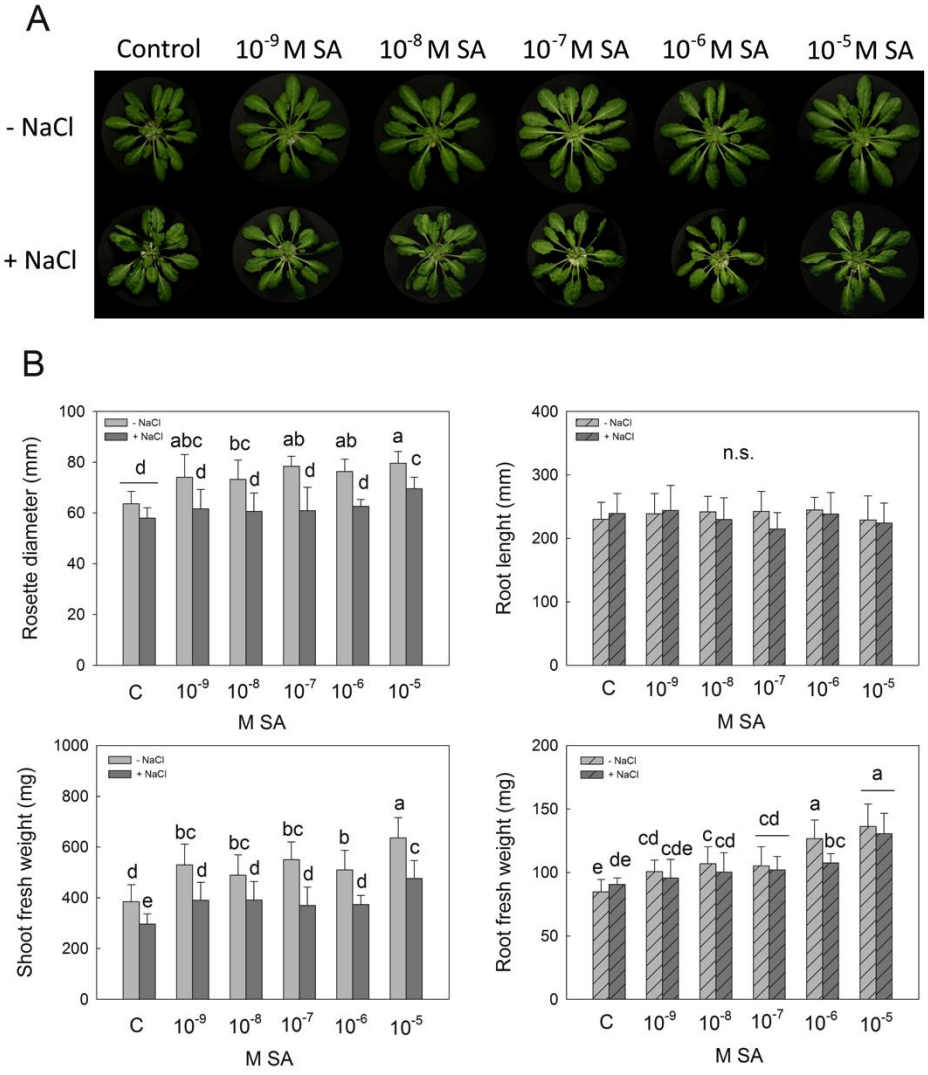


Fig. 1 Effects of 2-week-long pre-treatments with 10^{-9}–10^{-5} M SA on leaf and root growth and fresh weight of *Arabidopsis* plants after a 1-week 100 mM NaCl exposure A) Rosette morphologies of typical plants. B) Rosette diameters, root lengths, shoot and root fresh weight of SA and salt-treated plants (means ± SD, n=8–12). Columns with different letters are significantly different at $P < 0.05$, determined by Duncan's test. n. s.= not significant.

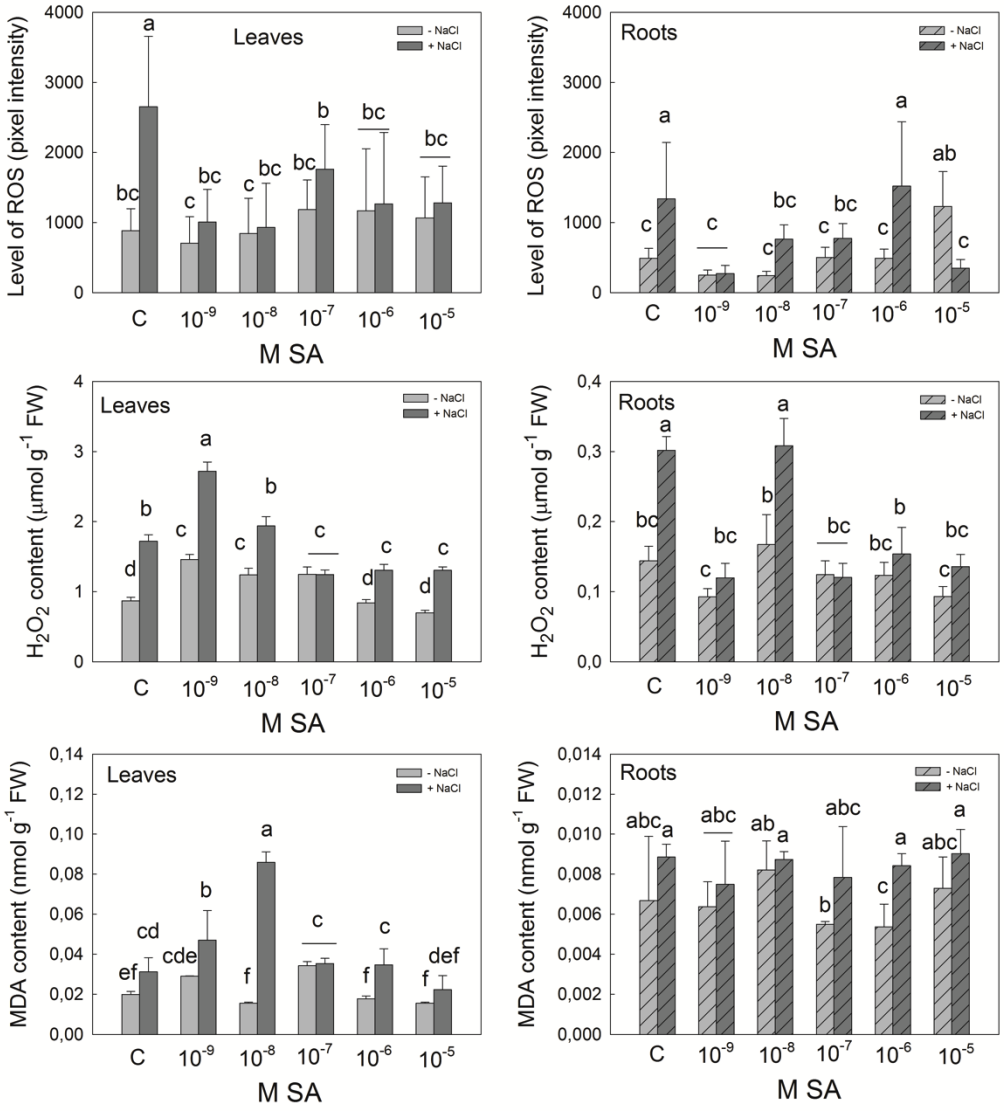


Fig. 2 Changes in ROS, H_2O_2 and MDA levels in the leaves and roots of 8-week-old *Arabidopsis* plants pre-treated with 10^{-9}–10^{-5} M SA and subsequently stressed with 100 mM NaCl. The ROS levels were determined using H_2DC FDA. Means ± SD or Means ± SE, n=9. Columns with different letters are significantly different at $P < 0.05$, determined by Duncan's test.

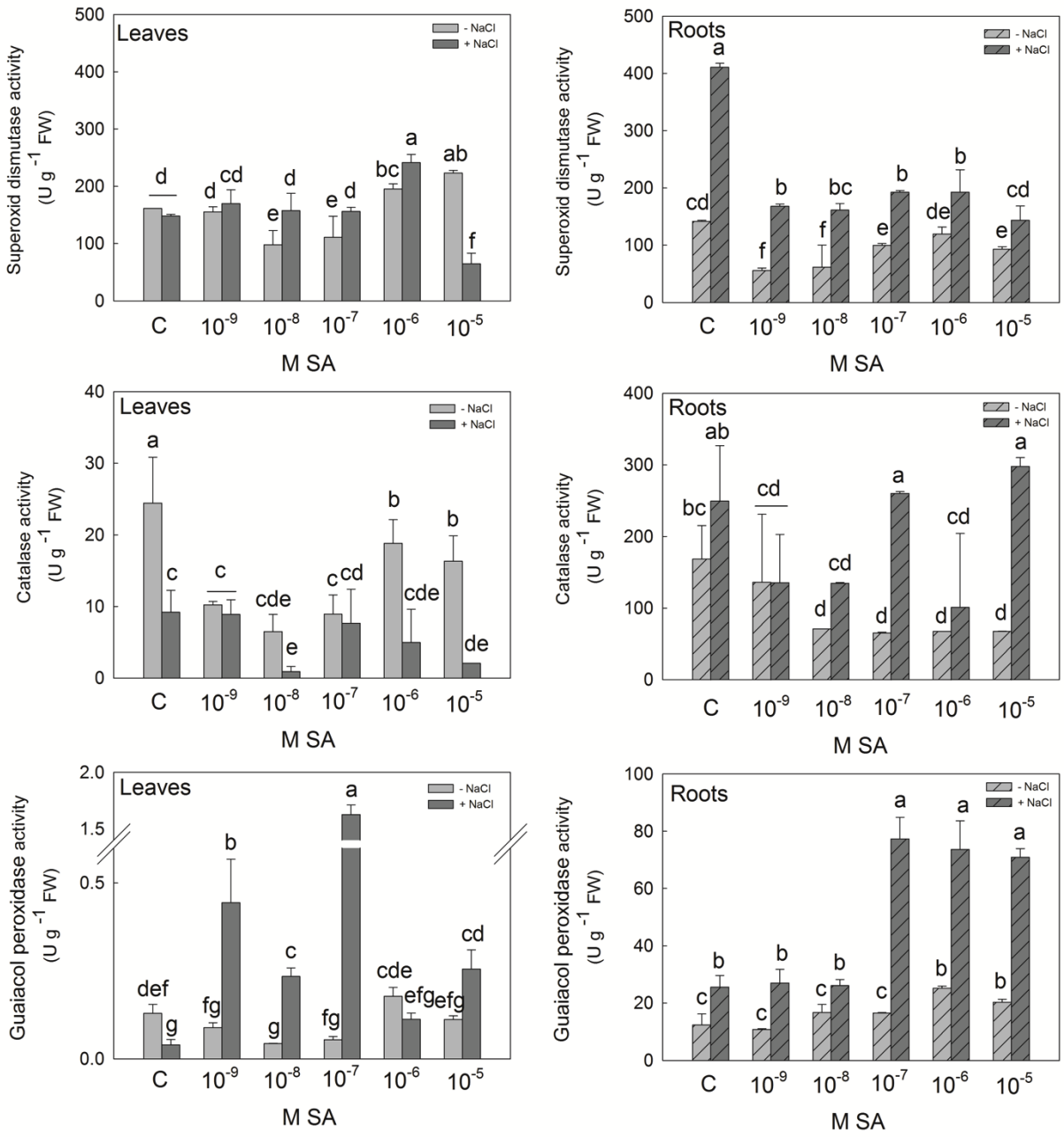


Fig. 3 Effect of two-week SA-pre-treatments on the the activities of superoxide dismutase (SOD), catalase (CAT) and guaiacol peroxidase enzymes in the leaves and roots of *Arabidopsis* plants exposed to 100 mM NaCl for one week. Data are means ± SD. Means denoted by different letters indicate a significant difference between the treatments ($P < 0.05$, Duncan's test).

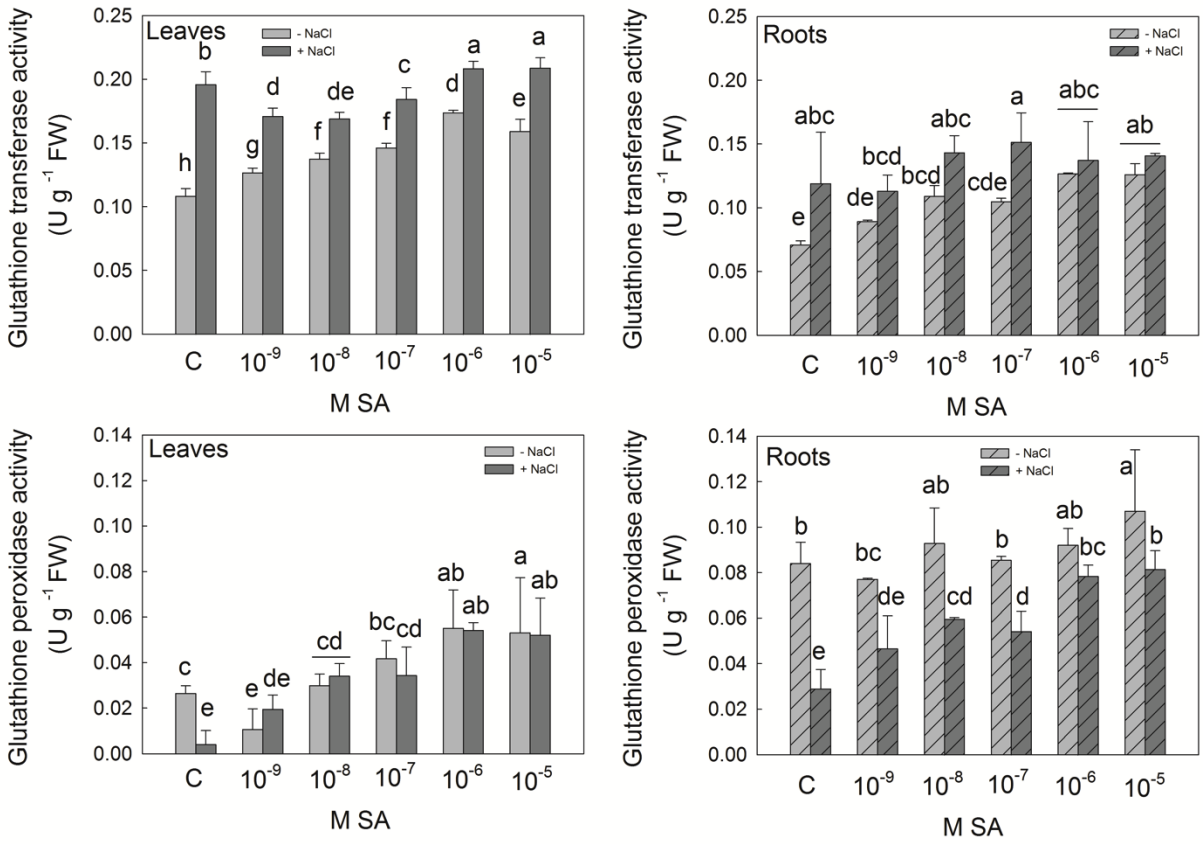


Fig. 4 Effect of two-week salicylic acid (SA) pre-treatment and subsequent 100 mM NaCl treatment for one week on glutathione S-transferase (GST) and glutathione peroxidase (GPOX) activities. Data consist of means ± SD obtained from at least 3 measurements. Means denoted by different letters indicate a significant difference between the treatments ($P < 0.05$, Duncan's test).

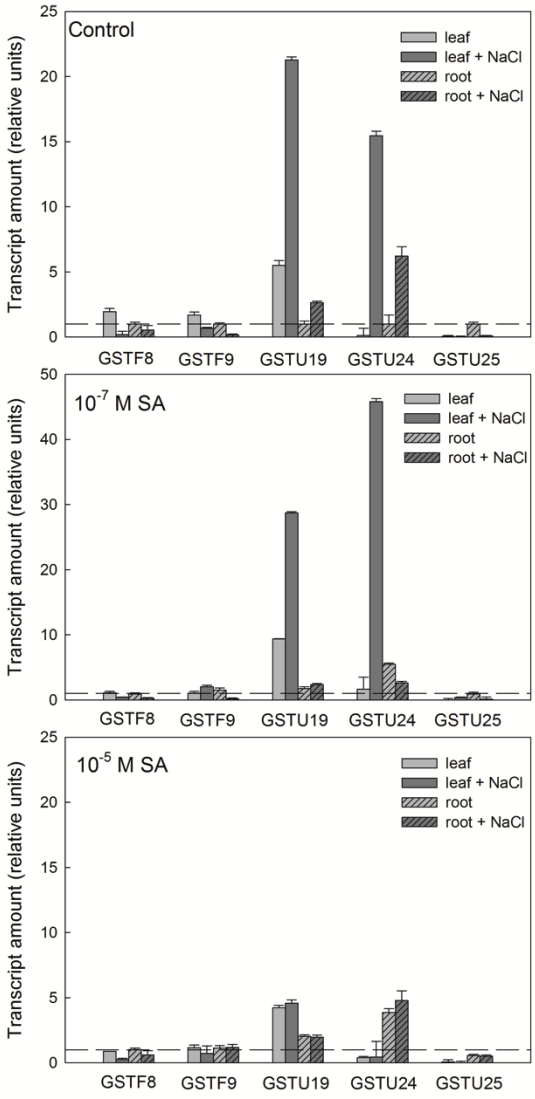
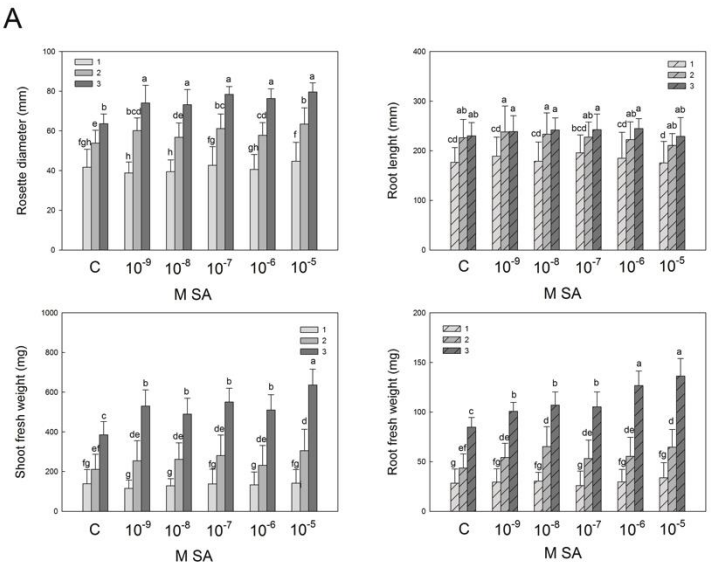


Fig. 5 Effect of 3-week salicylic acid (SA) pre-treatment on the transcript levels of selected *Arabidopsis* glutathione transferase (GST) genes in leaves and roots of 8-week-old *Arabidopsis* plants after applying 100 mM NaCl for one week. Data were normalized using the *Arabidopsis actin2* gene as internal control. The relative transcript level in control root samples was arbitrarily considered as one for each gene (indicated with a dashed line). Data consist of means ± SD, n=3.

Supplementary Material

Table S1: Primer pairs used for RT-qPCR.

Name of Arabidopsis gene	Abbreviation used in the text	Arabidopsis genome locus identifier	Direct and reverse primer pair sequences (5'-3')
Glutathione transferase phi 8	***AtGSTF8***	***At2g47730***	**F: ACGGTGATTTGACGCTTTTC** **R: GGTTGCCTTGACTTTCTTGC**
Glutathione transferase phi 9	***AtGSTF9***	***At2g30860***	**F: GGGAAAACCGTTGAAGACAG** **R: ATGACTGATGCGAACATTATG**
Glutathione transferase tau 19	***AtGSTU19***	***At1g78380***	**F: ATGATGCTCAGAGGAAGGTG** **R: ATAGCCAAAGTCATCGCCAC**
Glutathione transferase tau 24	***AtGSTU24***	***At1g17170***	**F: AAGGTGAGGAGCAAGAAGCA** **R: ACATACCCAAAAGTTTCGTCTC**
Glutathione transferase tau 25	***AtGSTU25***	***At1g17180***	**F: AGCAAAACCCCACTTCTTCC** **R: CTCCCCAAATCAACCTCGC**



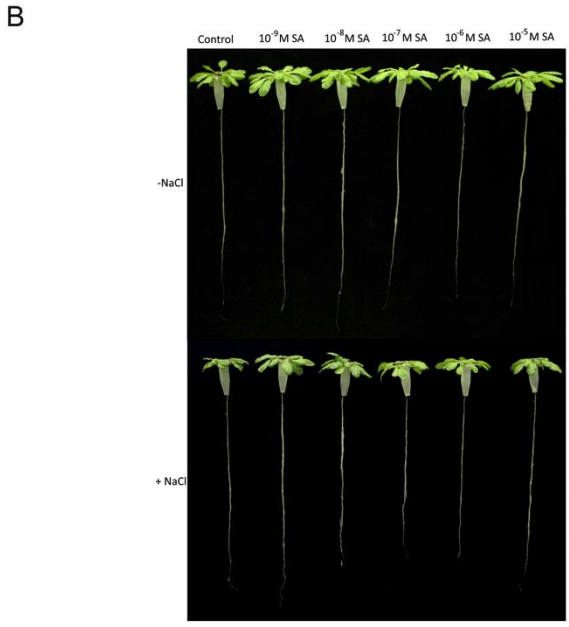


Fig. S1 Effects of 3-week-long pre-treatments with 10^{-9}–10^{-5} M salicylic acid (SA) on leaf and root growth and fresh weight of *Arabidopsis* plants. A) Rosette diameters, root lengths, shoot and root fresh weight of SA treated plants (means ± SD, n=8–12). Means denoted by different letters indicate a significant difference between the treatments ($P < 0.05$, Duncan's test). B) Root and shoot morphologies of typical plants **after 2-week SA pre-treatment and 1-week 100 mM NaCl stress.**

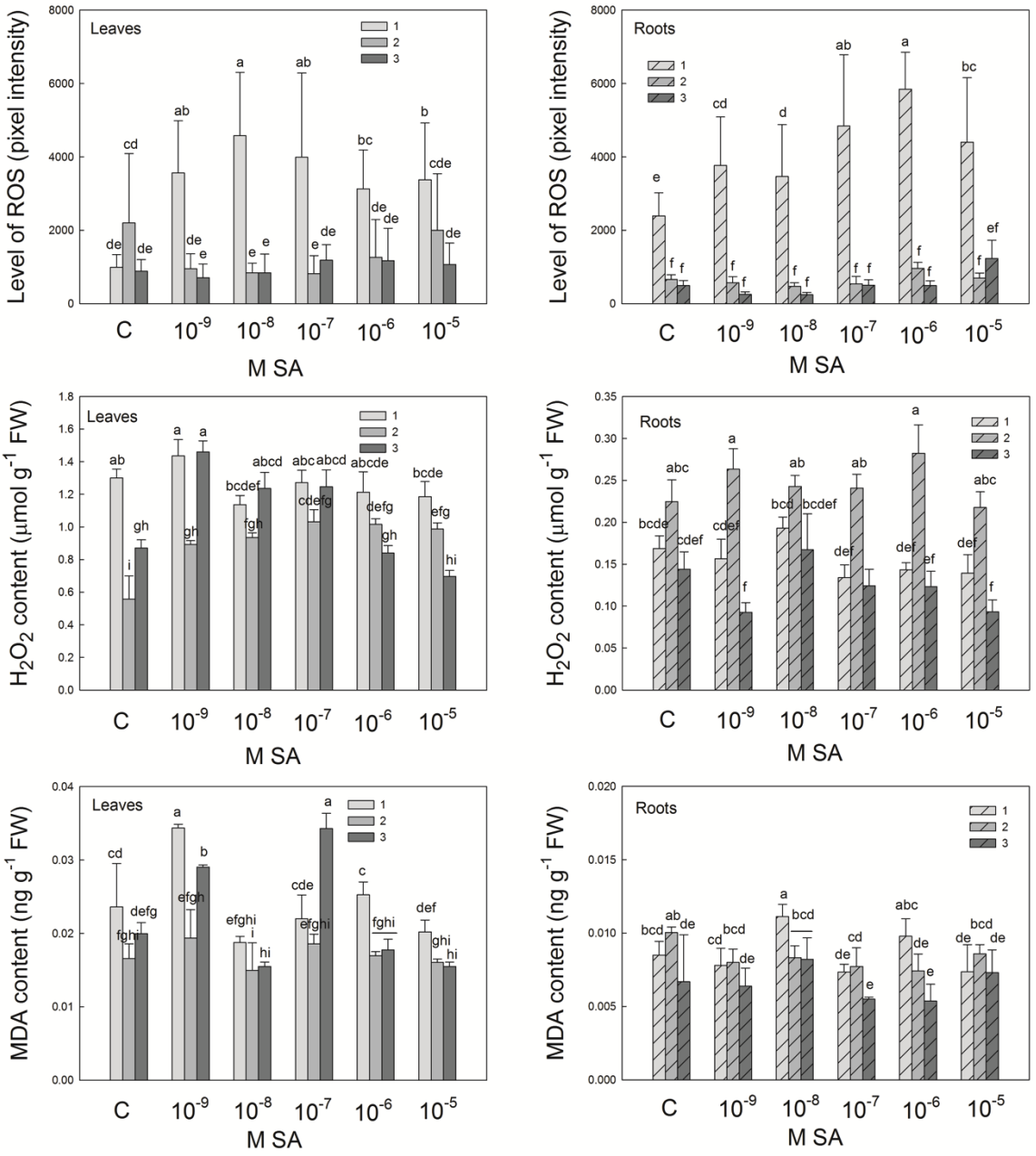


Fig. S2 Changes in ROS, H_2O_2 and MDA levels in the leaves and roots of 8-week-old *Arabidopsis* plants pre-treated with 10^{-9}–10^{-5} M salicylic acid (SA). The ROS levels were determined using H_2DC FDA. Means ± SD or Means ± SE, n=9. Columns with different letters are significantly different at $P < 0.05$, determined by Duncan's test.

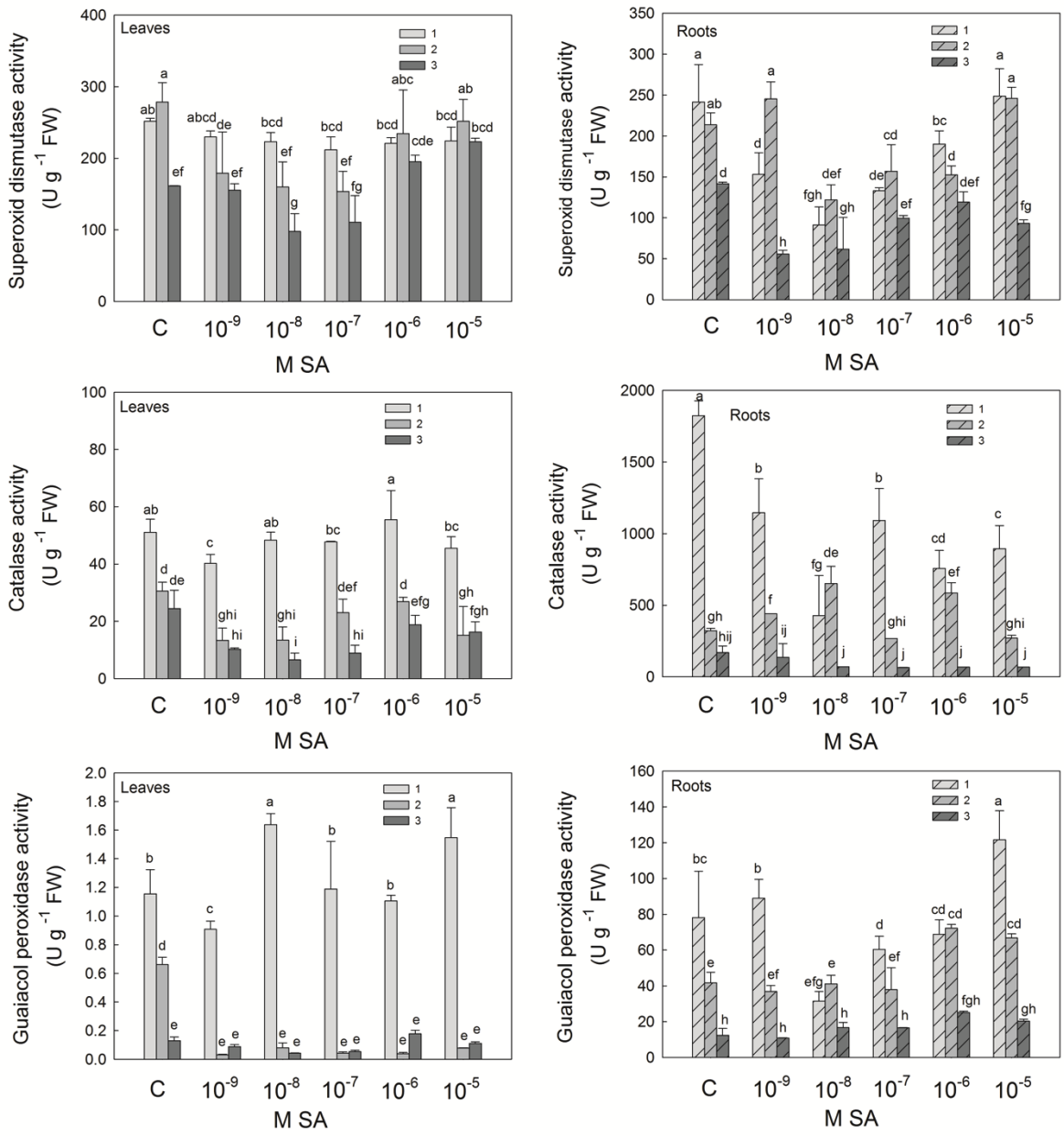


Fig. S3 Effect of three-week salicylic acid (SA) pre-treatments on the the activities of superoxide dismutase (SOD), catalase (CAT) and guaiacol peroxidase enzymes in the leaves and roots of *Arabidopsis* plants. Data are means ± SD. Means denoted by different letters indicate a significant difference between the treatments ($P < 0.05$, Duncan's test).

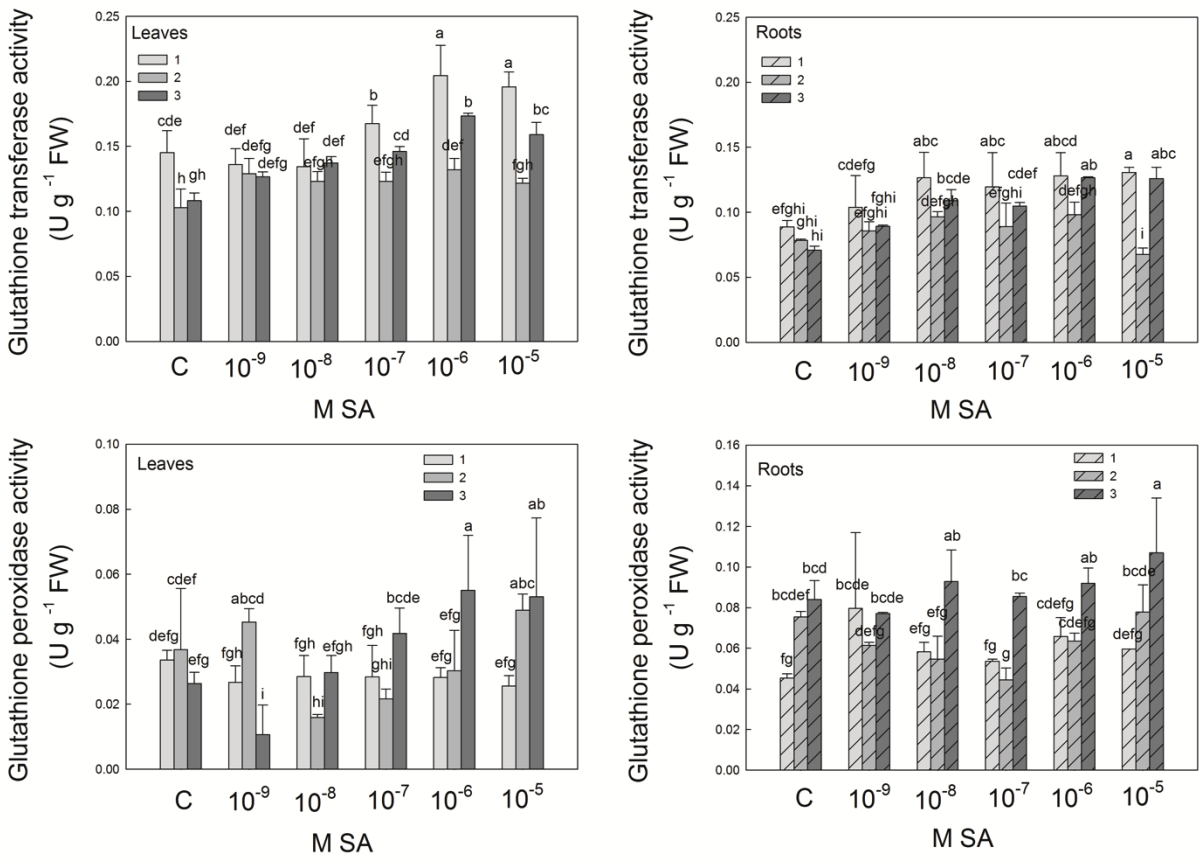


Fig. S4 Effect of three-week salicylic acid (SA) pre-treatment on glutathione transferase (GST) and glutathione peroxidase (GPOX) activity. Data consist of means ± SD obtained from at least 3 measurements. Means denoted by different letters indicate a significant difference between the treatments ($P < 0.05$, Duncan's test).