Lack of Salicylic Acid in *Arabidopsis* Protects Plants against Moderate Salt Stress

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Previous studies showed that salicylic acid (SA)-deficient transgenic *Arabidopsis* expressing the salicylate hydroxylase gene *NahG* had a higher tolerance to moderate salt stress. SA may potentiate the stress response of germination and growth of *Arabidopsis* seedlings by inducing reactive oxygen species (ROS). However, the detailed mechanism for a better adaption of *NahG* plants to moderate salt stress is largely unknown. In the present study we found that a higher GSH/GSSG (glutathione/oxidized glutathione) ratio and ASA/DHA (ascorbic acid/dehydroascorbate) ratio in *NahG* plants during the stress may be the key reason for their stress-tolerance advantage. *NahG* plants actually could not produce more active antioxidant enzymes than the wild-type ones under natural conditions, but maintain higher activities of glutathione reductase (GR) and dehydroascorbate reductase (DHAR) during the stress. Hereby, the reduced glutathione and reduced ascorbic acid contents are higher in *NahG* plants under salt stress. However, *NahG* plants do not adapt better under severe salt stress. All antioxidant enzyme activities, GSH/GSSG ratio and ASA/DHA ratio declined substantively at 400 mM NaCl stress in both *NahG* and wild-type seedlings.

Key words: Ascorbic Acid, Glutathione, Salicylic Acid, Oxidative Damage

Introduction

Salt stress is one of the most important limitations to crop productivity and species distribution. It can affect several physiological processes, from seed germination to plant development. Photosynthesis, a key metabolic pathway in plant development, is a major target for salt stress. During salt stress, reduction of the chloroplast stromal volume and generation of reactive oxygen species (ROS) are thought to play important roles in photosynthesis inhibition (Shi *et al.*, 2006; Luo *et al.*, 2008). ROS can be generated in the chloroplasts by direct transfer of excitation energy from chlorophyll to produce singlet oxygen, or by univalent oxygen reduction at photosystem I in the Mehler reaction (Laloi *et al.*, 2007; Breusegem *et al.*, 2008).

The role of salicylic acid (SA) in defense mechanisms against pathogens' attack has been known for several years. Furthermore, its role in abiotic stresses has been also summarized (Yuan and Lin, 2008). SA mediates pathogenesis-related (PR) gene expression, systemic acquired resistance, and redox homeostasis under environmental stresses. Studies with an *Arabidopsis* transgenic line expressing a salicylate hydroxylase gene, *NahG*, to reduce endogenous SA demonstrated that SA is required for O_3 tolerance by maintaining the cellular redox state and inducing defense responses (Sharma *et al.*, 1996; Yang *et al.*, 2004). However, a high level of SA activates an oxidative burst and a cell death pathway meanwhile leading to O_3 and salt stress sensitivities (Rao and Davis, 1999; Borsani *et al.*, 2001).

Generally, when an environmental stress is initiated, a plant needs a certain level of SA to increase the level of ROS by inhibiting antioxidant enzymes. Then the ROS act as a secondary stress signal to enhance the activities of cellular protective enzymes during the later stress (Yuan and Lin, 2008). Exogenous H_2O_2 or SA pre-treatment

similarly increases the stress tolerance by activating cellular protective enzymes (Shi *et al.*, 2006; Wahid *et al.*, 2007; Lei *et al.*, 2008; Luo *et al.*, 2008). Hereby, it could be deduced that *NahG* plants adapt worse to stress. However, wild-type plants germinated in media supplemented with 100 mM NaCl or 270 mM mannitol showed intensive necrosis in the shoots, while *NahG Arabidopsis* plants germinated under the same conditions remained green and developed true leaves (Borsani *et al.*, 2001). Borsani *et al.* (2001) suggested that SA potentiates the generation of ROS in photosynthetic tissues during salt and osmotic stress. However, *NahG* does not generate SA-induced antioxidant enzymes. Some protective substances other than antioxidant enzymes should be promoted in *NahG* and hampered in wild-type plants, such as reduced glutathione (Borsani *et al.*, 2001).

Here, we investigated the mechanism of higher tolerance to moderate salt stress of *NahG* plants, and found that a higher GSH/GSSG (glutathione/oxidized glutathione) ratio and ASA/DHA (ascorbic acid/dehydroascorbate) ratio in *NahG* plants may be accounted as the reason. *NahG* plants actually did not produce more active antioxidant enzymes than the wild-type ones did under natural conditions, but maintained higher activities of glutathione reductase (GR) and dehydroascorbate reductase (DHAR) during the stress. Correspondingly, the reduced states of the glutathione pool and the ascorbic acid pool were stabilized in *NahG* plants under salt stress. However, the reduced glutathione and ascorbic acid only played a limited role in stress tolerance. *NahG* plants did not adapt better under severe salt stress.

Material and Methods

Plant growth and stress treatments

Seeds of *Arabidopsis* wild-type Columbia (Col) and ecotype Cvi-0, and transgenic *NahG* plants (Friedrich *et al.*, 1995) were surface-sterilized in 20% (v/v) commercial bleach for 20 min, followed by six washes with sterile distilled water. The seeds were sown onto agar plates for germination. The basal agar medium contained Murashige and Skoog (Murashige and Skoog, 1962) salts with 2% (w/v) sucrose and 0.7% (w/v) agar. The various agar plates used in this work were made by adding the appropriate amount of NaCl to the molten basal media. The plates with the seeds were placed at 4°C in the dark for 48 h to improve germination uniformity before transfer to growth chambers with 16 h of light (approx. 100 μmol m^{-2} s^{-1}) at 22 °C, 8 h of dark at 18 °C, and 70% relative humidity for 20 d.

For GSH, GSSG, ASA and DHA determination, and RT-PCR analysis, approx. 150 35-d-old seedlings were transferred from Murashige and Skoog plates to 1,000-mL flasks containing 500 mL of 200 and 400 mM NaCl solutions. The flasks were shaken at 120 rpm at 22 °C with continuous cool fluorescent light illumination (approx. 100 μmol m^{-2} s^{-1}). 8 h later, the seedlings were collected from the flasks and frozen immediately in liquid nitrogen. The samples were ground in liquid nitrogen and kept at −80 °C until use.

Oxidative damage estimation

Electrolyte leakage was measured according to Sun *et al.* (2006). After measuring the conductivity, the barley samples were boiled for 15 min to achieve 100% electrolyte leakage.

The H_2O_2 content of leaves was measured as described by Velikova *et al.* (2002). Approx. 0.5 g of fresh leaves were cut into small pieces and homogenized in an ice bath with 5 mL 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 × *g* for 20 min at 4 °C. 0.5 mL of the supernatant was added to 0.5 mL 10 mM potassium phosphate buffer (pH 7.0) and 1 mL 1 M KI. The absorbance of the supernatant was read at 390 nm.

Lipid peroxidation was estimated by measuring the thiobarbituric acid-reactive substances (TBARS) as previously described (Sun *et al.,* 2006). The lipid peroxides were expressed as nanomoles of malonaldehyde.

Determination of GSH, GSSG, ASA and DHA

Approx. 200 mg of the powder resulting from the procedure described above were resuspended in 0.5 mL of 5% (w/v) sulfosalicylic acid and sonicated over 10 min. Extraction and determination of the GSH and GSSG content were as described previously (Law *et al.,* 1983). ASA and DHA were extracted and determined according to Wang *et al.* (1991).

Determination of antioxidant enzymes

For the enzyme assays, 0.3 g of leaves was ground with 3 mL ice-cold 25 mM HEPES buffer (pH 7.8) containing 0.2 mM EDTA, 2 mM ascor-

bate and 2% PVP. The homogenate was centrifuged at 4 °C for 20 min at 12,000 × *g* and the resulting supernatant was used for the determination of the enzymatic activity (Zhu *et al.,* 2000).

The superoxide dismutase (SOD) activity was assayed by measuring the ability to inhibit the photochemical reduction of nitroblue tetrazolium following the method of Stewart and Bewley (1980). The catalase (CAT) activity was measured as the decline in the absorbance at 240 nm due to the decrease of extinction of H_2O_2 using the method of Patra *et al.* (1978). The guaiacol peroxidase (GPX) activity was measured as the increase in the absorbance at 470 nm due to guaiacol oxidation (Nickel and Cunningham, 1969). The ascorbate peroxidase (APX) activity was measured by the decrease in the absorbance at 290 nm as ascorbate was oxidized (Nakano and Asada, 1981). The DHAR activity was assayed by measuring the increase in the absorbance at 265 nm due to reduced ascorbate formation (Nakano and Asada, 1981). The GR activity was measured according to Foyer and Halliwell (1976) and depends on the rate of the decrease in the absorbance of NADPH at 340 nm.

Transcripts analysis

RNA was extracted from the frozen tissue as described previously (Yuan *et al.*, 2005). The *RD29A* and *GPX* clones, corresponding to the expressed sequence tag accessions nos. 31G2T7 and 139F9T7, were amplified by PCR using the following primers: forward AGGATGTGCCGACGGGATT and reverse AAGGCTTTGTCTTCTTCTTCAGTTGT, forward TGCAAATGGCGGATGA and reverse CCAAACGAAAGTTCTTGAAAC, respectively. The primers for the *PR1* clone were forward CTCTTGTTCTTCCCTCGAA and reverse ATGCTCCTTATTGAAATACTGAT.

Statistics

Values presented are means ± one standard deviation (SD) of three replicates. Statistical analyses were carried out by analysis of variance (ANOVA) using SAS software (SAS Institute, Cary, NC, USA). Differences between treatments were separated by the least significant difference (LSD) test at a 0.05 probability level.

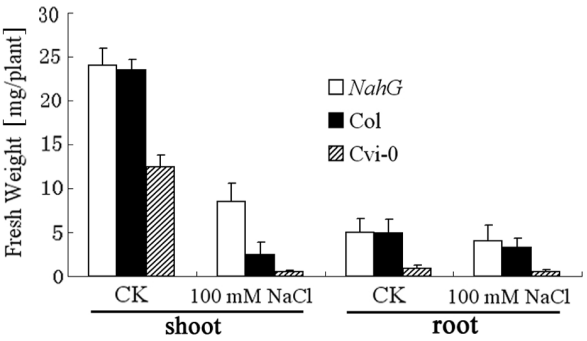

Fig. 1. Fresh weight of Col, *NahG*, and Cvi-0 seedlings after growing in Murashige and Skoog media containing 0 or 100 mM NaCl. Seedlings were germinated and grown on plates under light and after 15 d the seedlings were collected and weighed. The *Arabidopsis* ecotype Cvi-0 showed greater sensitivity to NaCl than Col and *NahG*. Bars represent standard deviations of 3 independent replicates (n = 50). CK, control.

Results

SA inhibits the growth of Arabidopsis under salt stress in the light

To investigate the possible role of SA in salt stress, seeds of wild-type *Arabidopsis* Col and Cvi-0, and SA-deficient transgenic *NahG* plants were germinated in several concentrations of NaCl at moderate light intensity. In the control, a basal agar medium (MS), there were no significant differences in the growth of wild-type plants and *NahG* plants, but at 100 mM NaCl, the growth of wild-type seedlings was severely restrained, whereas *NahG* seedlings germinated and grew normally, as Borsani *et al.* (2001) reported previously. The fresh weights of the shoots of the *NahG* seedlings were around three times higher than those of Col and sixteen times higher than those of Cvi-0 plant grown 20 d in 100 mM NaCl under light. However, no significant differences in terms of fresh weight were found in the roots (Fig. 1). When germinated in the dark, either in the absence or the presence of NaCl in the medium, no differences were found between *NahG* and wild-type plants (data not shown).

SA increases oxidative damages under salt stress

The coupling of salt sensitivity to light exposure in wild-type and Cvi-0 seedlings of *Arabidopsis* suggested that high NaCl concentrations enhanced the production of ROS, and that SA

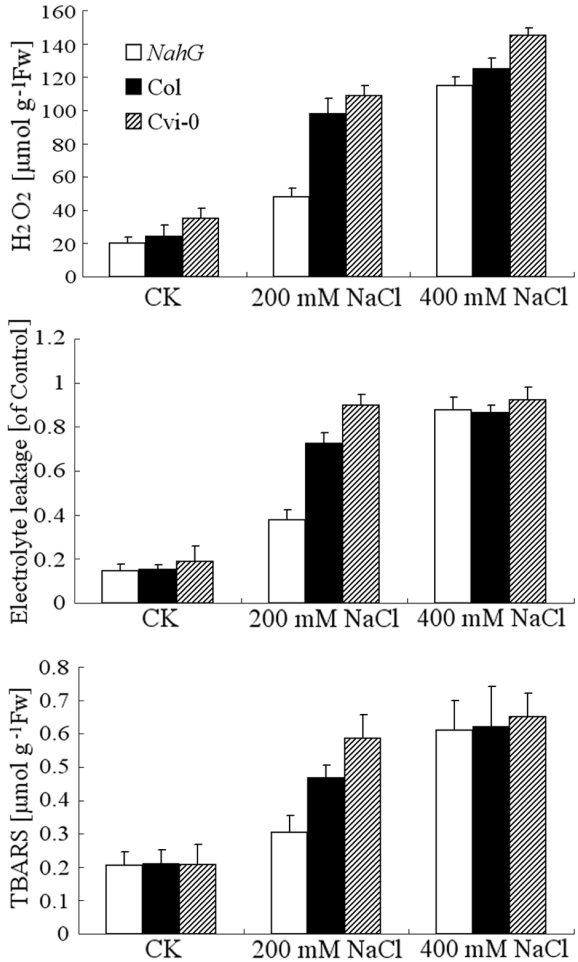

Fig. 2. H_2O_2 accumulation, electrolyte leakage and lipid peroxidation induced by NaCl under light. Bars represent standard deviations of 5 independent replicates (n = 5). CK, control.

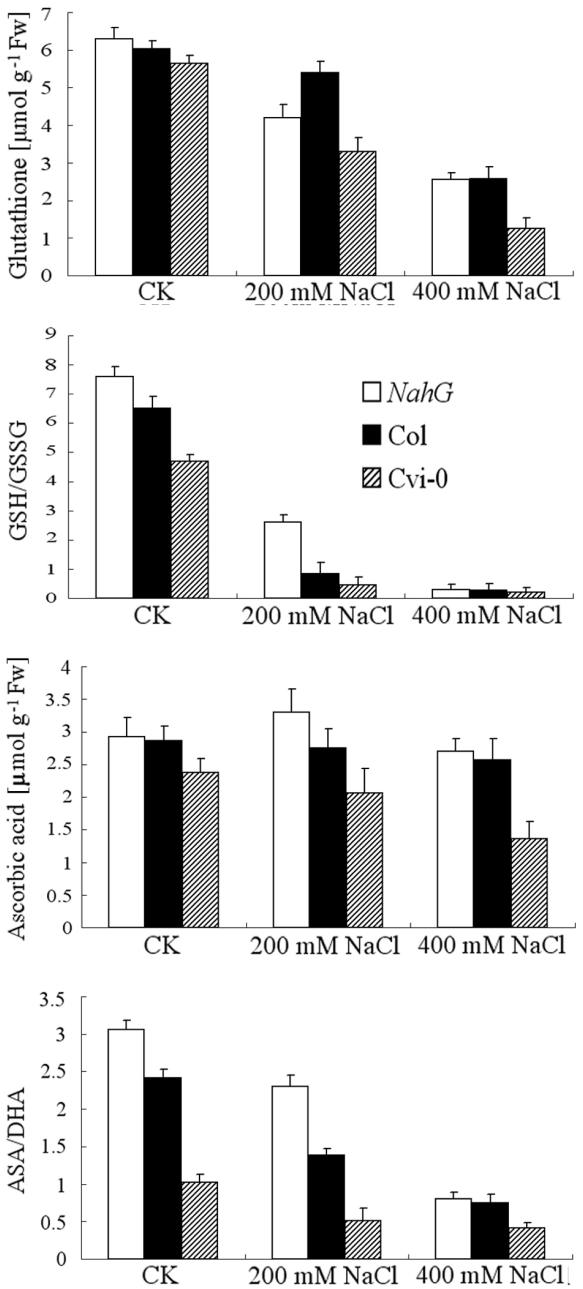

Fig. 3. Glutathione and ascorbic acid levels and GSH/GSSG and ASA/DHA ratios under NaCl stress. Glutathione means (GSH + GSSG) total content; ascorbic acid means (ASA + DHA) total content. Bars represent standard deviations of 3 independent replicates (n = 3). CK, control.

could be involved. During the lack of SA, the decreases in the generation of ROS may explain the increased tolerance of *NahG* seedlings to NaCl.

To test this assumption, 35-d-old seedlings of wild-type, *NahG* and Cvi-0 *Arabidopsis* were transferred from MS plates to 1,000-mL flasks containing 500 mL of 200 mM, 400 mM NaCl solution, respectively, and control (1/2 Hoagland nutrition solution). After 8 h, the H_2O_2 levels were determined. Results showed that at 200 mM and 400 mM NaCl, the H_2O_2 level increased greatly in the wild-type and Cvi-0 seedlings. In contrast, the generation of H_2O_2 increased slightly in *NahG*

plants at 200 mM NaCl, but nearly to the level of to Col and Cvi-0 plants at 400 mM NaCl (Fig. 2).

Oxidative damage also can be assessed by monitoring changes in the lipid peroxidation and electrolyte leakage. Thiobarbituric acid-reactive substances (TBARS) and electrolyte leakages increased similarly to the H_2O_2 contents in Col, *NahG* and Cvi-0 seedlings at 200 mM and 400 mM NaCl. These increases were significantly higher in Col and Cvi-0 plants than in *NahG* plants at 200 mM NaCl. However, there were no significant differences among wild-type, *NahG*, and Cvi-0 plants at 400 mM NaCl (Fig. 2).

NahG plants maintain the reduction of glutathione and ascorbic acid under salt stress

Previous studies demonstrated that the addition of reduced GSH and ASA could decrease the ROS level and reverse the toxic effect caused by NaCl (Borsani *et al.,* 2001). Furthermore, SA is necessary for the induction of antioxidant defenses and maintaining the redox state of the glutathione pool (Sharma *et al.,* 1996). The amounts of GSH, GSSG, ASA and DHA, and the ratios of GSH/GSSG and ASA/DHA were measured. At 200 mM and 400 mM NaCl, the total glutathione and total ascorbic acid content in most plants increased slightly, whereas the ratios of GSH/GSSG and ASA/DHA in Col and Cvi-0 seedlings declined greatly (Fig. 3). In contrast, *NahG* plants maintained significantly higher levels of GSH/GSSG and ASA/DHA ratios (more than tree times to Cvi-0) at 200 mM NaCl, although the ratios declined at 400 mM NaCl (Fig. 3).

NahG plants maintain higher activities of GPX and DHAR under salt stress

Plants are capable of removing ROS using several antioxidant enzymes such as SOD, CAT, GPX, GR, APX and DHAR. Higher activities of several antioxidant enzymes in *NahG Arabidopsis* may partially explain its higher tolerance to moderate salt stress. As shown in Fig. 4, all antioxidant enzyme activities decreased under stress, may be 8 h of stress were not enough to prompt antioxidant enzyme activities. It is noticeable that GR and DHAR activities in *NahG* seedlings almost did not decrease at 200 mM NaCl, but significantly decreased at 400 mM NaCl, corresponding to the higher levels of GSH/GSSG and ASA/DHA ratios at 200 mM NaCl. The decline of SOD, CAT, GPX, and APX activities was slighter in *NahG* plants than in Col seedlings at 200 mM NaCl, what may reflect the slighter damages in *NahG* plants, rather than antioxidant enzymes could be promoted by SA-deficiency.

Expression analysis of RD29A, PR1, and GPX

The transcriptional levels of three representative genes, *RD29A*, *PR1*, and *GPX*, were tested. *RD29A* gene expression is induced by NaCl and osmotic stresses and the gene encodes a protein with potential protective function during desiccation (Yamaguchi-Shinozaki and Shinozaki, 1993). *PR1* gene expression is induced by SA and pathogen attack (Hammond-Kosack and Jones, 1996). Therefore, it can be considered as a molecular marker for SA accumulation. *GPX* expression can be considered as a molecular marker for oxidative stress (Rao and Davis, 1999).

As shown in Fig. 5, all expressions of *RD29A* were induced by NaCl. In *NahG* plants, *RD29A* was also induced, even more intensively, suggesting that this induction was independent of SA. As expected, salt stress did not induce *PR1* expression in *NahG* plants, because SA was actively degraded. NaCl increased the *GPX* expression in all types of plants, and the induction was significant at 400 mM NaCl. It is interesting that *GPX* expression was also induced in *NahG* plants by NaCl, suggesting that NaCl produced an oxidative stress independent of SA. This is consistent with the increased oxidative damages in *NahG* plants caused by NaCl (Fig. 2). Change of *GPX* gene expression were not detected during changes of the GPX enzyme activity, especially at 400 mM NaCl, because plants were severely stressed at this concentration.

Discussion

Salt stress affects the plant metabolism and growth from seed germination to seedling development. The complexity of the plant response to salt stress, on one hand, can be explained by the osmotic stress and the ionic imbalance caused by salinity (Zhu, 2001). On the other hand, oxidative stress resulted from exposure of plants to high NaCl concentration also is a major reason (Borsani *et al*., 2001). The contribution and interaction among these components remain elusive.

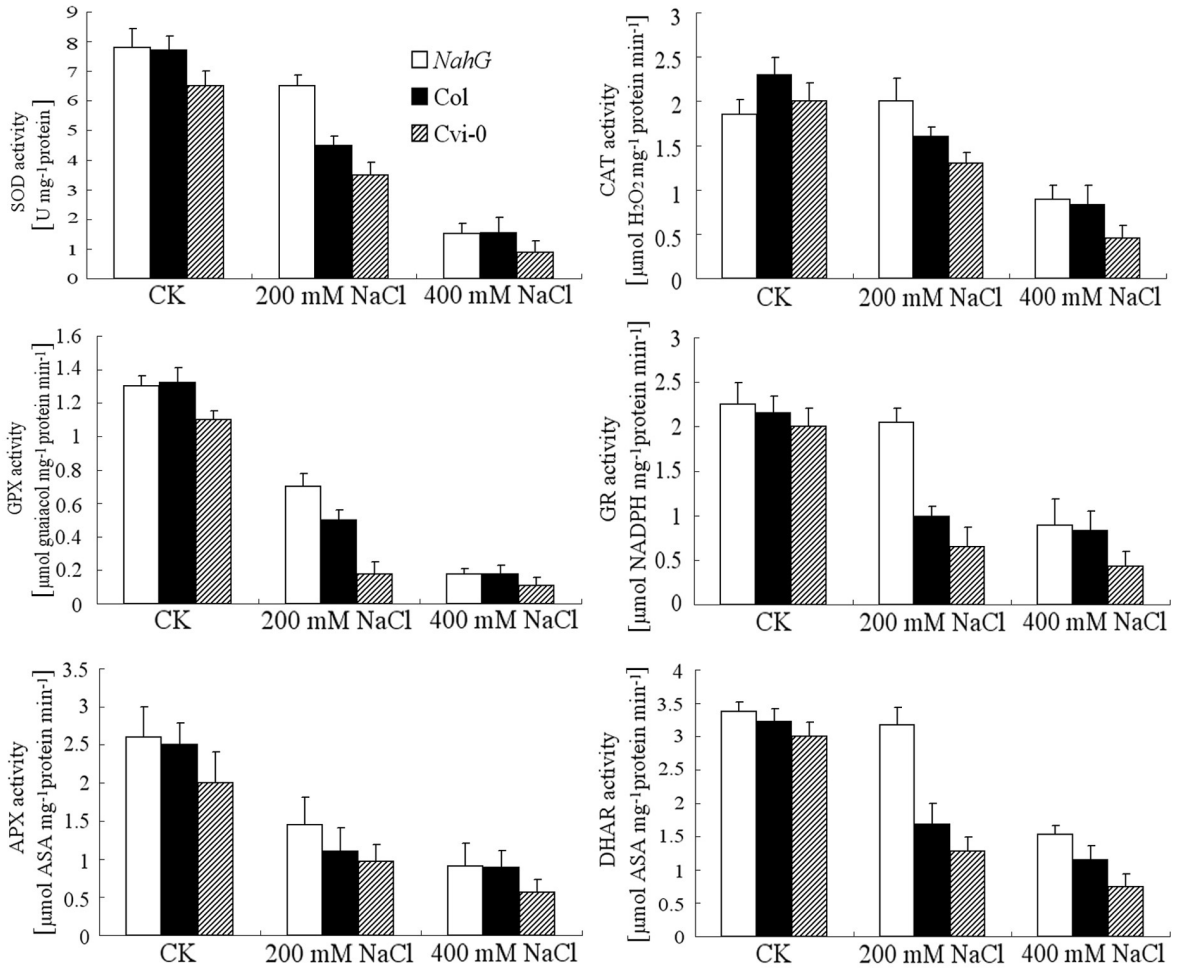

Fig. 4. Effects of different NaCl stresses on the activities of SOD, CAT, GPX, GR, APX, and DHAR in Col, *NahG*, and Cvi-0 seedlings. Bars represent standard deviations of 3 independent replicates (n = 3). CK, control.

Previous studies proposed that SA could be a signaling molecule forming a feedback amplification cycle in concert with ROS during abiotic stresses, just as in plant-pathogen interactions (Shim *et al.*, 2003; Yuan and Lin, 2008). In this way, SA induction is not required but the endogenous SA present amplifies the effects of ROS initial levels. This is supported by our data showing that increased oxidative damages and *GPX* induction occurred in *NahG* seedlings at high NaCl concentrations. Moreover, this indicates that part of the oxidative stress generated during NaCl exposure is independent of the presence of SA.

SA has been proposed to have a dual role in plants. First, SA is necessary for the induction of antioxidant defenses and maintaining the redox state of the cells (Sharma *et al.*, 1996; Clarke *et al.*, 2004). For example, endogenous SA protects rice plants from oxidative damages caused by aging as well as biotic and abiotic stresses (Yang *et al.*, 2004); SA also protects maize from cadmium stress (Metwally *et al.*, 2003; Pal *et al.*, 2005). Second, excessive SA accumulation can induce a programmed cell death pathway, leading to a hypersensitive reaction in response to stresses (Rao and Davis, 1999; Mateo *et al.*, 2006; Yuan and Lin, 2008).

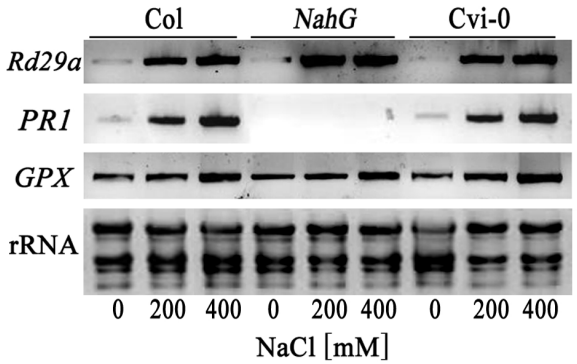

Fig. 5. Effect of NaCl on the expression of *RD29A*, *PR1*, and *GPX* genes in Col, *NahG*, and Cvi-0 seedlings. 10 μg of total RNA from the seedlings were loaded per lane.

Several reports showed that elevated levels of GSH and ASA are associated with increased oxidative stress tolerance (Borsani *et al.*, 2001; Mateo *et al.*, 2006; Shi *et al.*, 2006). Transgenic plants overexpressing glutathione reductase had both elevated levels of GSH and increased tolerance to oxidative stress in leaves (Broadbent *et al.*, 1995). Therefore, the ratios of GSH/GSSG and ASA/DHA may play a major and key role in determining the capability of removing ROS and enhancing oxidative stress tolerance of plants under salt stress. Further investigations showed that higher activities of GR and DHAR in *NahG* plants during moderate salt stress should be the reason for higher GSH/GSSG and ASA/DHA ratios.

Our studies also showed that the growth of several kinds of *Arabidopsis* seedlings containing different levels of SA varies significantly. The fresh weights of shoots of SA-deficient *NahG* seedlings were higher than those of wild-type and Cvi-0 seedlings, whereas the high-level-SA *Arabidopsis* ecotype Cvi-0 was more sensitive to NaCl than the wild-type and *NahG* seedlings, supporting a role of SA in mediating the effects of salt stress.

Advantages of SA-dificient *NahG* seedlings were also presented in many other tests. At 5 °C, *NahG* plants displayed relative growth rates about one-third greater than wild-type plants. In contrast, the *cpr1 Arabidopsis* mutant at 5 °C accumulated very high levels of SA, and its growth was much more inhibited than in wild-type plants (Scott *et al.*, 2004). The phenomenon can be attributed to the growth inhibitory properties of SA. However, the long-term growth at 5 °C caused no detectable injury in wild-type *Arabidopsis*. Therefore, the role of SA suggested by this study is likely distinct from that in severe cold stress. Similarly, lack of SA in *NahG Arabidopsis* cannot protect seedlings at very high levels of NaCl. No matter which ratios of GSH/GSSG and ASA/DHA the *NahG* plant keeps, it cannot weave more effective antioxidant enzymes than the wild types, especially in the severe stress. Thus, the lack of SA in *NahG Arabidopsis* seedlings is not sufficient to protect these seedlings at very high levels of NaCl.

Acknowledgements

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