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Full Length Research

Involvement of nitric oxide synthase-dependent nitric oxide and exogenous nitric oxide in alleviating NaCl induced osmotic and oxidative stress in *Arabidopsis thaliana*

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To elucidate the roles of endogenous nitric oxide on *Arabidopsis thaliana* tolerance to salt stress, a moderate concentration of NaCl was applied to wild-type (WT) and mutant (*Atnoa1*) plants which have an impaired *in vivo* nitric oxide synthase and reduced endogenous nitric oxide content due to T-DNA insertion in the first exon of the NOA1 gene. The exhibited greater inhibition of root growth, higher leaf water loss (LWL), lower contents of chlorophyll, soluble protein, proline, higher activities of peroxidase (POD), ascorbate peroxide (APX), and gluthinone reductase (GR), it also showed lower activities of superoxide dismutase (SOD) and catalase (CAT) than wild-type plants under NaCl stress. The nitric oxide synthase (NOS) inhibitor NG-nitro-L-Arg (L-NNA) enhanced NaCl induced growth inhibition, osmotic stress, and oxidative stress in wild-type plants. Meanwhile the NO donor, sodium nitroprusside (SNP), alleviated the NaCl induced damages in *Atnoa1* plants. These results indicate that both NOS-dependent endogenous NO and exogenous NO were involved in salt resistance in *A. thaliana.*

Key words: Antioxidant enzymes, *Arabidopsis thaliana*, reactive oxygen species.

INTRODUCTION

Earth is a salty planet, with most of its water containing about 30 g of sodium chloride per litre. this salt solution has affected, and continues to affect, the land on which crops are, or might be, grown. Although the amount of salt-affected land (about 900×106 ha) is imprecisely known, its extent is sufficient to pose a threat to agriculture (Flowers and Yeo, 1995; Munns, 2002; Flowers, 2004). Salinity stress affects plant growth, development, and metabolism in many different ways. Excessive salt causes ion toxicity inside the cell. High concentrations of salt in the root medium also create hyperosmotic stress that impedes water absorption and transport. Secondary stresses such as nutritional imbalance and oxidative stress often occur as a consequence of ion toxicity and hyperosmotic stress (Zhu, 2001; Shi et al., 2002; Zhu, 2003). Under NaCl stress, free Na+ competes with K+ for uptake, resulting in the toxic levels of intracellular Na+ and insufficient K+ concentrations for K±dependent enzymatic reactions (Zhu, 2003). Furthermore, plants with high Na+ levels produce and accumulate disproportionate levels of reactive oxygen species (ROS), including hydrogen peroxide, hydroxyl radicals, and superoxide anions. Excessive ROS can damage cellular structures and alter macromolecules. Plant cells can modulate intracellular ROS concentrations by scavenging ROS with antioxidant enzymes (Xiong et al., 2002).

Recently, nitric oxide (NO) has been suggested to be an important signaling molecule in plants (Neill et al.,

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2003; Wendehenne et al., 2004; Crawford and Guo, 2005; Zhao et al., 2007). NO has been shown to affect growth and development of plant tissue (Durner and Klessing, 1999) , induce seed germination instead of red light (Beligni and Lamattina, 2000a), affect plant maturation and senescence (Guo and Crawford, 2005), mediate abscisic acid (ABA) induce stomatal closure, and play a role in the light mediated greening (Zhang et al., 2006). Furthermore, NO has been implicated to be involved in drought stress, salt stress, heat stress, disease resistance and apoptosis (Delledonne et al., 1998; Durner and Klessing, 1999; Mata and Lamattina, 2001; Zhao et al., 2004; Zhang et al., 2006).

Nitric oxide synthase (NOS)-like activity has been widely detected in plants, and NO generation has been inhibited by mammalian NOS inhibitors in plants (Delledonne et al., 1998; Durner and Klessing, 1999; Foissner et al., 2000). The Arabidopsis (*Arabidopsis thaliana*) AtNOS1 gene has been suggested to encode a protein with sequence similarity to a protein that is involved in NO synthesis in the snail Helix pomatia which has been isolated (Guo et al., 2003). And its counterpart homozygous mutant line, Atnos1, was obtained by inserting T-DNA in the first exon of NOS1 gene (Guo et al., 2003). *In vivo* NOS activity was suppressed by 75% in Atnos1 mutant plants (Guo et al., 2003). However, recent findings demonstrated that recombinant AtNOS1 protein showed no NOS activity in vitro (Zemojtel et al., 2006), suggesting that AtNOS1 is involved in NO biosynthesis and accumulation in either indirect or regulatory pathways. In light of these findings, it has been suggested that AtNOS1 be renamed *AtNOA1* (Crawford et al., 2006).

Despite AtNOS1's mechanism of action, the *Atnoa1* mutant has provided a powerful tool for experimentally controlling NOS activity and endogenous NO levels. In the present study, the influence of endogenous NO as well as exogenous NO on NaCl-induced changes of growth, water osmotic status, and antioxidant enzyme activities have been investigated by comparing *Atnoa1* and wild-type Arabidopsis seedlings under salinity stress in the presence and absence of a NOS inhibitor and an NO donor.

MATERIALS AND METHODS

Plant material and growth conditions

Wild-type Arabidopsis (*Arabidopsis thaliana*) L. Heynh. ecotype Columbia and *Atnoa1* plants were grown on pot vermiculite (vermiculite : soil =3:1) in a greenhouse under conditions of 14 h photoperiod with irrandiance of 120 µmol m-2 s-1, temperature of 25±2°C and air humidity 60-70%.

Treatments

Four-week-old wild-type plants were divided into three groups: group 1 was irrigated with 150 mmol L^{-1} NaCl, group 2 was irrigated with 150 mmol L^{-1} NaCl + 300 µmol L^{-1} L-NNA and group 3 was irrigated with water and was used as control. Four-week-old *Atnoa1* plants were also divided into three groups: group 1 was irrigated with 150 mmol L^{-1} NaCl, group 2 was irrigated with 150 mmol L^{-1} NaCl +150 µmol L^{-1} SNP and group 3 was irrigated with water and was used as control. All the treatments were fulfilled with at least three replicates.

Plant physiological parameters determination

Leaf water loss (LWL) was determined according to the method of Xing et al. (2004). Four-week-old wild-type and *Atnoa1* plants were carefully washed out of the vermiculite and cultured for 24 h in distilled water and then transferred to treatment solutions for 4 h. The leaves of the wild-type and *Atnoa1* plants at the same leaf order were cut from the leaf base and their fresh masses (M1) recorded. The leaves were then left to evaporate under room condition for 2 h and were re-weighed (M2). LWL was calculated by (M1 - M2)/M1×100%.

Leaves of wild-type and *Atnoa1* plants were sampled at the same leaf order from the leaf base after 48 h treatment. Pigments were extracted from leaf samples (300 mg) with 5 ml of 80% acetone until complete bleaching. The extracts were subjected to spectrophotometric measurements at 645 and 663 nm (Arnon, 1949)

Free proline content was determined using the ninhydrin method (Bates et al., 1973). After 24 h treatment, 0.5 g plant leaves were homogenized in 10 ml of 3% aqueous sulphosalicylic acid and the homogenate was centrifuged at 2000 g for 5 min. 2 ml of the extract reacted with 2 ml of acid-ninhydrine and was mixed with 2 ml of glacial acetic acid. The mixture was boiled in water bath at 100° for 60 min and the reaction was stopped by cooling the tubes in ice bath for 5 min. The chromophore formed was extracted with 3 ml of toluene by vigorous shaking and the tubes were placed in the dark for 50 min. Absorbance of the resulting organic layer was measured at 520 nm. The concentration of proline was estimated by referring to a standard curve for L-proline.

After 24 h treatment, plant leaf protein content was determined according to Bradford (Bradford, 1976) with bovine serum albumin as standard.

For enzyme extraction, 24 h treated fresh leaves (0.5 g) were homogenized in 5 ml ice-cold 50 mM sodium phosphate buffer (pH 7.8) containing 1% polyvinyl- polypyrrolidone. The homogenate was centrifuged at 10000 g for 20 min, and the supernatant was used for assaying the enzyme activities.

SOD activity was determined by monitoring its ability to inhibit photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm (Beauchamp, 1971). One enzyme unit of SOD activity was defined as the amount of enzyme by which 50% SOD activity was inhibited in reaction solution.

POD activity was determined using the guaiacol oxidation method (Kochba, 1977). Three ml reaction solution containing 100 mmol L-1 phosphate buffer (pH 6.0), 8 mmol L-1 guaiacol, 100 µl enzyme extract and 2.75 mmol L-1 H_2O_2 was fully mixed. The increase in absorbance was recorded at 470 nm within 3 min after the enzyme extract was added.

CAT (EC 1.11.1.6) activity was determined by monitoring the disappearance of H_2O_2 and by measuring the decrease in absorbance at 240 nm of a reaction solution containing 0.3 ml of 3% H_2O_2, 2.5 ml of 50 mM phosphate buffer (pH 7.0), and 0.2 ml of the extract (Aebi, 1983).

APX (EC 1.11.1.11) activity was measured in 1 ml reaction volume containing 50 Mm potassium phosphate buffer (pH 7.0), 0.1 Mm hydrogen peroxide and 0.5 mM ascorbate. The H_2O_2 was

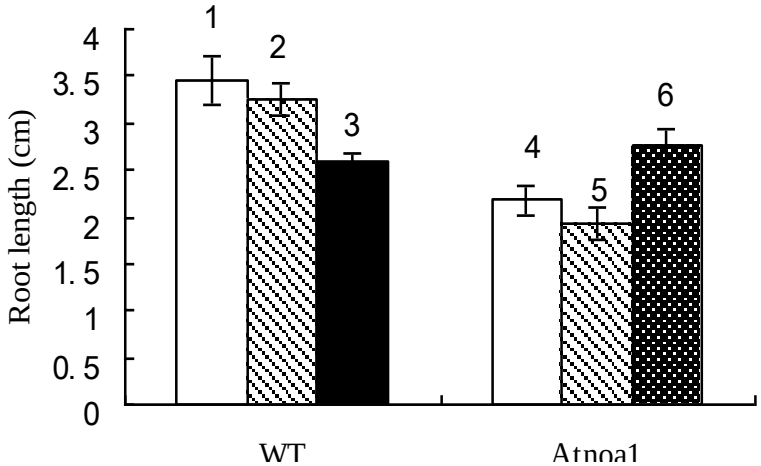

Figure 1. Effect of different treatments on root length in two genotypes, wild type (WT) and *Atnoal* mutant, of *A. thaliana* seedlings (n=8). (1) Control; (2) NaCl; (3) NaCl+L-NNA; (4) Control; (5) NaCl; (6) NaCl +SNP

added to start the reaction, and the decrease in absorbance at 290 nm was recorded for 1 min to determine the oxidation rate for ascorbate (Nakano and Asada, 1981).

GR (EC 1.6.4.2) activity was estimated by measuring the decrease of absorbance at 340 nm and 25° due to the oxidation of NADPH (Carlberg and Mannervik, 1985). The 1 ml reaction mixture containing 100 mM HEPES-NaOH (PH 7.8), 1 mM EDTA, 3 mM $MgCl_2$, 0.5 Mm oxidized glutathione, 150 µl enzyme extract, and 0.2 mM NADPH was mixed thoroughly to begin the reaction.

Plant root growth determination

Root growth was determined by measuring root length. Wild-type plants grown on Murashige and Skoog (MS) solid medium (Murashige and Skoog, 1962) for 20 days were transferred to new MS solid medium. Treatments were made as follows: 1) control; 2) 150 mmol L^{-1} NaCl; 3) 150 mmol L^{-1} NaCl+300 µmol L^{-1} L-NNA. *Atnoa1* plants grown on MS solid medium for 20 days were transferred to new MS solid medium. Treatments were made as follows: 1) control; 2) 150 mmol L^{-1} NaCl; 3) 150 mmol L^{-1} NaCl +150 µmol L^{-1} SNP. L-NNA and SNP after filter sterilization were added on the surface of the solid MS medium containing 150 mmol L-1 NaCl. After 7 days of treatment, plants were carefully moved out of the MS medium and the taproot length of all treatments was measured and at least 8 taproots in each treatment used.

Statistics analysis was conducted by SPSS for windows (version 15.0). Least significant difference (LSD) test was employed to determine differences among the treatments at $p<0.05$. All the values were mean ± SE.

RESULTS

As shown in Figure 1, salt-induced inhibition of root growth was exacerbated in *Atnoa1* plants. Seven days of salt stress (150 mM NaCl) reduced root length by 6.0% in wild-type seedlings and 11.5% in the mutant seedlings. Administration of 300 µM L-NNA inhibited root length by 20.3% beyond salt stress alone in wild-type plants ($P< 0.05$ vs NaCl stressed group) meanwhile, administration of 150 µM SNP increased root length in salt-stressed mutant plants by 42.9% ($P< 0.05$ vs. NaCl stressed group) (Figure 1).

Salt stress treatment inhibited LWL both in wild-type and *Atnoa1* plant leaves. Treatment with L-NNA markedly increased LWL in wild-type plant leaves. Meanwhile, treatment of the mutant plants with SNP decreased LWL compared to the NaCl stressed mutant group (Figure 2).

The salt stress treatment reduced chlorophyll and soluble protein content in both wild-type and mutant plants. However, the decreases were more pronounced in the mutants. Inhibition of NO accumulation decreased chlorophyll and soluble protein content in salt stressed wild-type plants (P's < 0.05 vs. NaCl stressed wild-type group). Meanwhile, exogenous NO increased chlorophyll and soluble protein content in the salt stressed mutant plants (P's < 0.05 vs. NaCl stressed mutant group) (Figures 3 and 4).

Proline content was 87.7% higher in wild-type plants than that in the mutant plants following administration of NaCl ($P < 0.05$). Specifically, salt stress increased proline content by 2212.9% in wild-type plants and by 170.6% in the mutant plants (P's < 0.05 vs. respective baselines). Application of L-NNA increased proline content in salt stressed wild-type plants by 88.1% ($P < 0.05$ vs. NaCl stressed wild-type group). However, application of SNP also significantly increased proline content by 639.2% in salt-stressed wild-type plants ($P < 0.05$ vs. NaCl stressed wild-type group) (Figure 5).

NaCl stress showed different influences on the change of antioxidant enzyme activities in WT and *Atnoa1* Arabidopsis seedlings. CAT activity was inhibited by NaCl

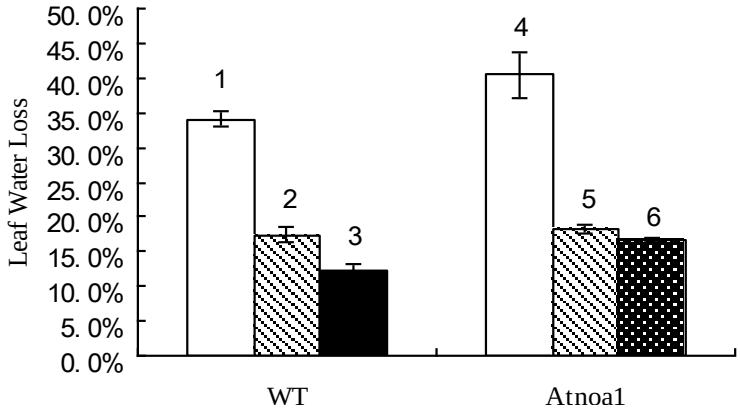

Figure 2. Effect of different treatments on LWL in two genotypes of *Arabidopsis thaliana* seedlings (n=8). (1) Control; (2) NaCl; (3) NaCl+L-NNA; (4) Control; (5) NaCl; (6) NaCl +SNP.

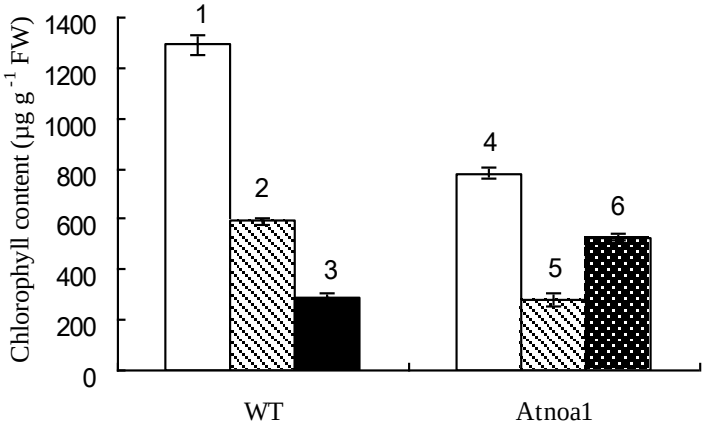

Figure 3. Effect of different treatments on chlorophyll content in two genotypes of *Arabidopsis thaliana* seedling leaves (n=3). (1) Control; (2) NaCl; (3) NaCl+L-NNA; (4) Control; (5) NaCl; (6) NaCl +SNP

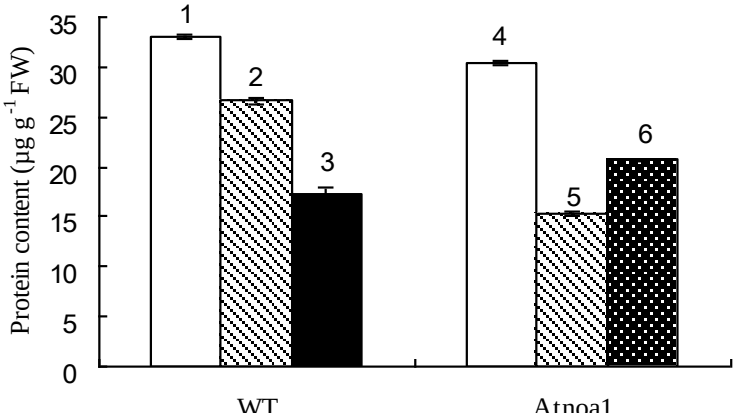

Figure 4. Effect of different treatments on soluble protein content in two genotypes of *Arabidopsis thaliana* seedling leaves (n=3). (1) Control; (2) NaCl; (3) NaCl+L-NNA; (4)Control; (5) NaCl; (6) NaCl +SNP.

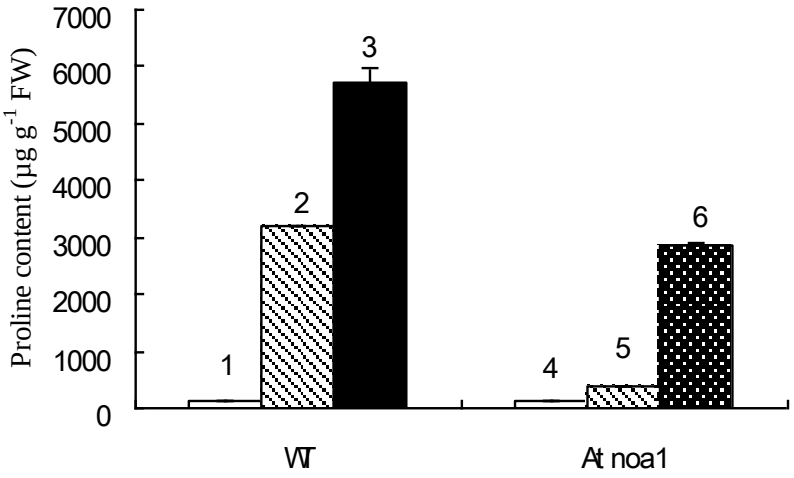

Figure 5. Effect of different treatments on proline content in two genotypes of *Arabidopsis thaliana* seedling leaves (n=3). (1) Control; (2) NaCl; (3) NaCl+L-NNA; (4)Control; (5) NaCl; (6) NaCl +SNP.

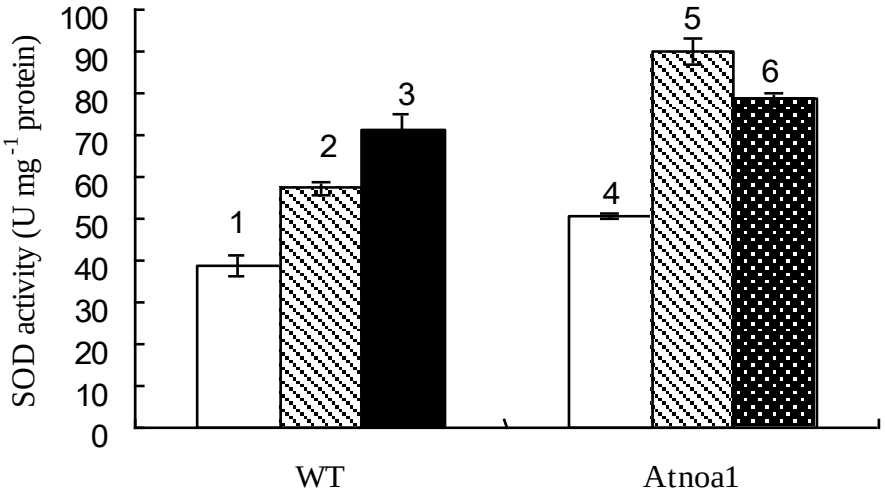

Figure 6. Effect of different treatments on SOD activity in two genotypes of *Arabidopsis thaliana* seedling leaves (n=3). (1) Control; (2) NaCl; (3) NaCl+L-NNA; (4)Control; (5) NaCl; (6) NaCl +SNP

in WT Arabidopsis seedlings and all of the other antioxidant enzymes including SOD, POD, APX, and GR were dramatically stimulated by NaCl stress (P's < 0.05 vs. respective baselines). Impairment of NO production following L-NNA treatment further increased activities of SOD, POD, APX, and GR while inhibiting the CAT activity in salt stressed wild-type plants(P's < 0.05 vs. respective NaCl stressed groups). Meanwhile, application of SNP decreased the activities of SOD, POD, APX, and GR while increasing CAT activity in the mutant plants under NaCl stress (P's < 0.05 vs. respective NaCl stressed groups) (Figures 6 - 10).

DISCUSSION

In the present study, we demonstrated that NO-deficient *Atnoa1* mutant plants were more sensitive to NaCl stress than wild-type plants. Salinity stress causes a number of changes in plant metabolism and growth (Abdin et al., 2002). It inhibits plant growth and protein synthesis (Hernandez et al., 1995; Kahane and Poljakoff, 1968; Kaya et al., 2002). In the present study, the inhibitory effect of NaCl on mutant Arabidopsis root growth was exacerbated. Salt stress induced more root length reduction in the mutant than in wild-type plants. Inhibition

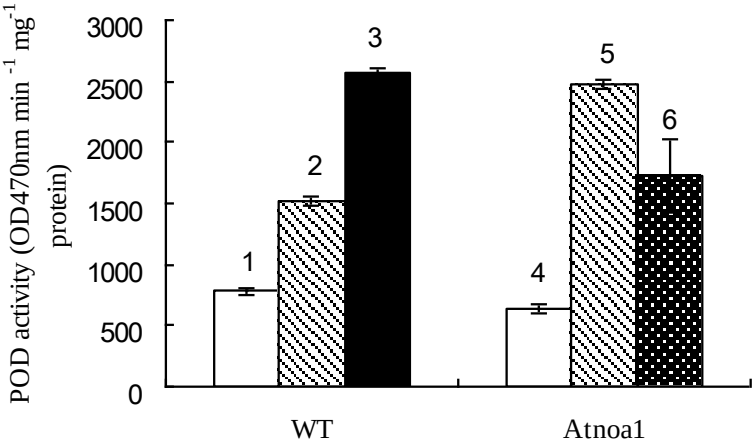

Figure 7. Effect of different treatments on POD activity in two genotypes of *Arabidopsis thaliana* seedling leaves (n=3). (1) Control; (2) NaCl; (3) NaCl+L-NNA; (4)Control; (5) NaCl; (6) NaCl +SNP.

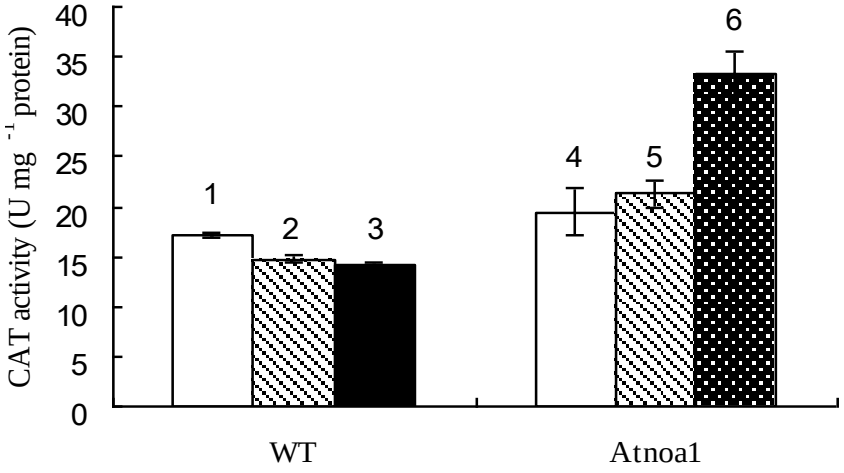

Figure 8. Effect of different treatments on CAT activity in two genotypes of *Arabidopsis thaliana* seedling leaves (n=3). (1) Control; (2) NaCl; (3) NaCl+L-NNA; (4) Control; (5) NaCl; (6) NaCl +SNP.

of NO production by L-NNA resulted in a further decrease of root length in salt stressed wild-type plants while application of SNP increased root length in salt stressed mutant plants. The salt stress treatment reduced soluble protein content in both wild-type and mutant plants. However, the decrease was more pronounced in the mutants. Inhibition of NO accumulation decreased soluble protein content in salt stressed wild-type plants. Meanwhile, exogenous NO increased soluble protein content in the salt stressed mutant plants. The rice soluble protein content was decreased under salt stress and the magnitude of the decrease correlated with the sensitivity of the rice to salt (Zhou et al., 2004).

Plant root is the major tissue of abscisic acid (ABA) biosynthesis in response to water stress and transported ABA plays an important role in stomatal closure as well as leaf water control (Zhang and Davies, 1989). In the present study, both wild-type and mutant plant leaves had decreased LWL after treatment of NaCl when compared with their control. Inhibition of NO accumulation by L-NNA increased LWL in salt stressed wild-type plants, while exogenous NO decreased LWL in salt stressed mutant plants, indicating that both endogenous and exogenous NO could reduce Arabidopsis osmotic stress by regulating leaf water status. NO may participate in modulating Arabidopsis leaf osmotic stress by ABA induced stomatal

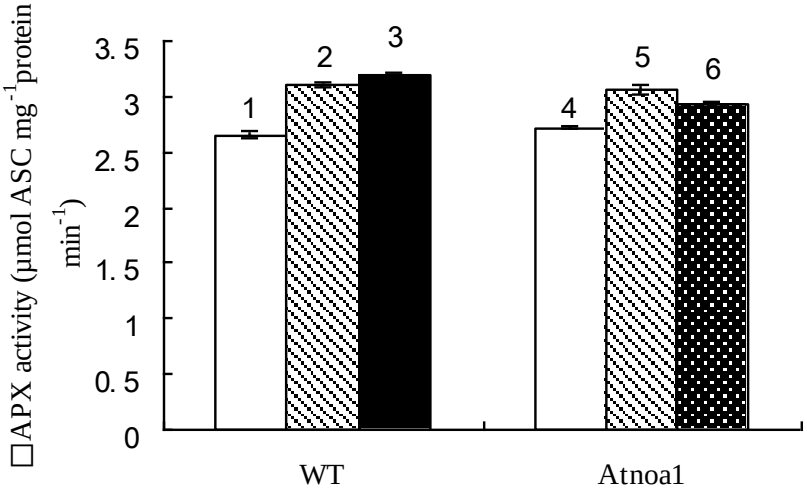

Figure 9. Effect of different treatments on APX activity in two genotypes of *Arabidopsis thaliana* seedling leaves (n=3). (1) Control; (2) NaCl; (3) NaCl+L-NNA; (4) Control; (5) NaCl; (6) NaCl +SNP.

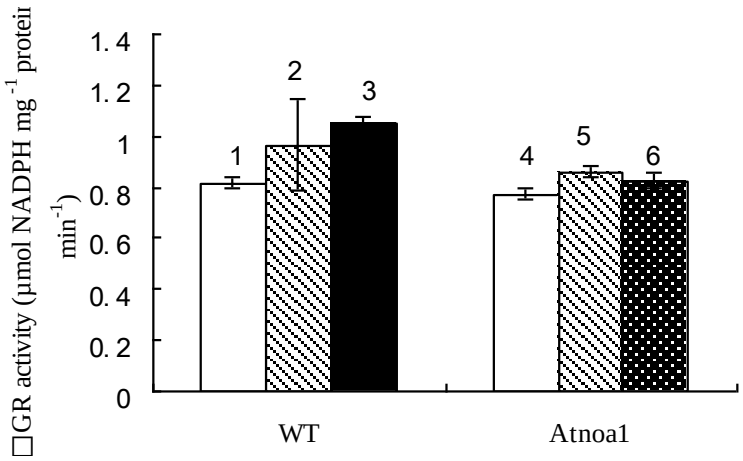

Figure 10. Effect of different treatments on GR activity in two genotypes of *Arabidopsis thaliana* seedling leaves (n=3). (1) Control; (2) NaCl; (3) NaCl+L-NNA; (4)Control; (5) NaCl; (6) NaCl +SNP.

closure. Our results are consistent with previous works (Garcı´a-Mata and Lammattina, 2001; Garcı´a-Mata and Lammattina, 2002; Xing et al., 2004)

Salinity stress also resulted in the degradation of the membranes of cell organelles (Mitsuya et al., 2000), especially the thylakoids of the chloroplasts, which were swollen and showed a wavy shape (Salama et al., 1994) when exposed to high light intensity. The changes of thylakoids have been reported as a typical symptom of oxidative stress (Hernandez et al., 1995) suggesting that the damages in the chloroplasts were induced by a photo-oxidative reaction caused by salt stress and not directly correlated with the salt content in the tissue (Mitsuya et al., 2000). NO is also involved in inducing partial greening of etiolated wheat seedlings (Beligni and Lamattina, 2000). The salt stress treatment reduced chlorophyll content in both wild-type and mutant plants. However, the reduction was more pronounced in the mutants. Inhibition of NO accumulation decreased chlorophyll content in salt stressed wild-type plants. Meanwhile, exogenous NO increased chlorophyll content in the salt stressed mutant plants, suggesting that both endogenous and exogenous NO participated in counteracting salt stress induced oxidative damages in chloroplasts.

The accumulation of free proline in response to a wide range of stressors, including hypersalinity, may play a role in stress adaptation within plant cells (Delauney and

Verma, 1993; Ashraf and Foolad, 2007). Proline improves salt-tolerance by protecting protein turnover machinery against stress-damage and up-regulating stress protective proteins (Khedr et al., 2003) contributes to osmotic adjustment (Perez et al., 1993) and to enzyme protection, it also acts as a major reservoir of energy and nitrogen (Chandrasheker and Sandhyarani, 1996) and as a protein-folding chaperone (Samuel et al., 2000), it improves macromolecules and organelles structure stability.

Proline content was significantly increased in both wild-type and mutant plants when subjected to NaCl stress. There was more increase of proline content in wild- type plants than in mutant plants, indicating that the mutant plants displayed reduced adaptation ability to NaCl stress. In wild-type plants, inhibition of NO production may aggravate salt-induced stress, leading to an increase of the observed proline content. The increased proline content observed in SNP-treated salt stressed mutant plants suggested that the mutant plants suffered greater stress induced by NaCl due to the impaired NO production. The results also indicated that both endogenous and exogenous NO functioned in regulating proline metabolisms under NaCl stress, leading to a relatively stable environment within the cells.

Plants possess an active oxygen-scavenging system. SOD is a key enzymatic antioxidant that converts O_2- into H_2O_2 and can be activated by different stress factors (Hu et al., 2007). NO also reacts with O_2- to produce peroxynitrite, which can then be converted to NO_2- and NO3-. Thus, NO is also an O_2- scavenger in stressful conditions (Shi et al., 2005). Salt stress induced an increase of SOD activity and was augmented in the *Atnoa1* mutants. The impairment of NO production following L-NNA treatment further increased SOD activity in salt stressed wild-type plants. Meanwhile, application of SNP decreased SOD activity in the mutant plants under NaCl stress. A similar pattern was observed for POD activity. The results indicated that both endogenous and exogenous NO acted as an O_2- scavenger in salt stressed Arabidopsis plants. ROS levels were elevated in transgenic tobacco (Nicotiana tobacum) expressing an alfalfa (Medicago sativa) NO-scavenging enzyme hemoglobin during bacterial infection (Seregelyes et al., 2003).

It is well known that ascorbate-glutathione cycle is the key mechanism of scavenging ROS in plant chloroplast and its high efficiency is responsible for the alleviation of oxidative damage to chloroplast under abiotic and biotic stress (Asada 2006; Kuzniak and Sklodowska, 2001). In the cycle, APX plays the most important role in removing H_2O_2, and DHAR and GR are mainly responsible for providing substrate for APX through the reproduction of reduced ascorbate and glutathione.

Salt stress induced increases in APX and GR activities were augmented in wild-type Arabidopsis plants, which indicated that wild-type Arabidopsis plants were more acclimated to salt stress than *Atnoa1* mutant plants. Impairment of NO production following L-NNA treatment further increased activities of APX and GR in NaCl stressed WT plants. Meanwhile, application of SNP decreased the activities of APX and GR in NaCl stressed mutant plants, which indicated that both exogenous and endogenous NO were involved in alleviating salt induced oxidant damages in Arabidopsis plants.

CAT as well as APX and POD were involved in H_2O_2 scavenging under stressful conditions. CATs convert H_2O_2 to water and molecular oxygen in peroxisomes (Noctor and Foyer, 1998). An alternative mode of H_2O_2 destruction is via peroxidases, which are found throughout the cell and have a much greater affinity to H_2O_2 than CAT (Jimenez et al., 1997).The present results indicated that the ascorbate-glutathione pathway and POD were more important than CAT in salt stressed Arabidopsis detoxification.

The present findings provide evidence that both endogenous and exogenous NO are involved in alleviating NaCl induced growth inhibition, osmotic stress and regulating antioxidant enzyme activities under salt stress in Arabidopsis. In conclusion, the present work indicates that both NOS-dependent endogenous and exogenous NO are involved in Arabidopsis salt resistance.

ACKNOWLEDGMENTS

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