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Full Length Research Paper

A potato *NOA* gene increased salinity tolerance in *Arabidopsis thaliana*

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The purpose of this study is to produce recombinant *StNOA1* in transgenic plants and to test its potential role in plant salt stress responses. The newly cloned *StNOA1* gene from *Solanum tuberosum* L. was inserted into *AtnOA1* mutant plant genome by Agrobaterium-mediated floral dip method. Transgene integration was verified by polymerase chain reaction (PCR) in 4 different lines of transgenic *Atnoa1*. Expression of *StNOA1* gene was further analyzed by reverse trancription (RT)-PCR. Physiological analyses indicated that the transgenic line TL9 had higher proline, soluble protein and chlorophyll contents as well as lower content of malondialdehyde (MDA) compared to its receptor, *Atnoa1* mutant, under salt stress condition. Root elongation and survival rate in TL9 were significantly higher than those in *Atnoa1* seedlings under salt stress. Present study proved that *StNOA1* participated in *Arabidopsis thaliana* salt stress responses and increased its salinity tolerance.

Key words: *StNOA1* transformation, *Solanum tuberosum*, *Atnoa1* mutant, salt tolerance.

INTRODUCTION

Nitric oxide (NO) is a pleiotropic reactive nitrogen species controlling many physiological processes in plants (Leshem, 1996; Delledonne et al., 1998; Durner and Klessing, 1999; Beligni and Lamattina, 2000; Mata and Lamattina, 2001; Desikan et al., 2002; Delledonne et al., 2003; Zhao et al., 2004; Guo and Crawford, 2005; Zhang et al., 2006; Song et al., 2006; Zhao et al., 2007a). Increasing studies have indicated that NO synthesis in plants mainly includes nitric oxide synthase (NOS) and nitrite reductase (NR)-dependent pathways (Cueto et al., 1996; Delledonne et al., 1998; Durner et al., 1998; Foissner et al., 2000; Bright et al., 2006; Rokel et al., 2002; Ribeiro et al., 1999; Yamasaki et al., 1999; Yamasaki and Sakihama, 2000). However, the genes encoding NOS proteins in higher plants remain to be identified. Previous result has indicated that the unique gene *AtnOA1* isolated from *Arabidopsis* participates in endogenous NO synthesis by measurement of NO contents and NOS activities in wild type and *Atnoa1* mutant plants with T-DNA insertion in the first exon of *AtnOA1* gene (Guo et al., 2003; Zhao et al., 2007b; Vitecek et al., 2008).

AtNOA1 functions as a GTPase. However, AtNOA1 has some different characteristics from small GTPase like Ras, Rho and Cdc42 (Moreau et al., 2008). Complementation experiments of *AtNOA1* mutant plants with different constructs of *AtNOA1* show that GTP hydrolysis is necessary but not sufficient for the physiological function of *AtNOA1*. Mutant *AtNOA1* lacking the C-terminal domain, although retaining GTPase activity, failed to complement *AtNOA1*, suggesting that this domain plays a crucial role in *planta* (Moreau et al., 2008).

AtNOA1 plays a significant role in hormonal signaling and stress responses (Guo et al., 2003; Zhao et al., 2007a; Zhao et al., 2007b). In this study, we cloned *StNOA1,* a homolog of *AtNOA1* from potato (*Solanum tuberosum*) and examined *StNOA1* function on salt stress response by comparing transgenic *Atnoa1* plants with *Atnoa1* mutant and wild type *Arabidopsis* plants.

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Abbreviations: RT-PCR, Reverse trancription-polymerase chain reaction; **NO,** nitric oxide; **NOS,** nitric oxide synthase; **NR,** nitrite reductase; **RACE,** rapid amplification of cDNA ends; **TCA,** trichloroacetic acid; **TBA,** thiobarbituric acid.

Table 1. Sequences of the primers used in this study.

Primer name	Sequence
P_{3R}	5'-AAGCAGTGGTAACAACGCAGAGTAC d$(T)_{30}$N(AGC)(AGCT)
Stnoa1	5'-ATATCCTTCTTGTTCGAGCTTCC-3'
Stnoa2	5'-TTCGAGCTTCCTTTCACAATTAAC-3'
Stnoa3	5'-TGACCAGGAGACATACGATTTGA-3'
Pnup	5'- AAGCAGTGGTAACAACGCAGAGT-3'
Stnoa-flf	5' -CAATATCCTTCTTGTTCGAGC-3'
Stnoa-flr	5' -CCCGTATATACCTGTTGTAGCA-3'
Stnoa5	5' -GCGGTACCATGGCGCCTAAACTCCTAGCTC-3' *Kpn1*
Stnoa6	5' -GCGGATCCTCAGAAAAACCATTTGGGTCT-3' BamH1
Hpt-F	5'ATGCCTGAACTCACCGCGAC 3'
Hpt-R	5'CTATTCCTTTGCCCTCGGACG 3'

MATERIALS AND METHODS

Plant materials and growth conditions

Solanum tuberosum and *Arabidopsis thaliana* plants (including wild type, *AtnOA1* and transgenic *AtnOA1*) were grown in an environmentally controlled greenhouse under conditions of 16 h photoperiod with temperature of 23 ± 2°C and air humidity 60 - 70%.

StNOA1 full length cDNA isolation from *S. tuberosum*

StNONA1 5' cDNA end sequence obtaining and 3' RACE primer designing

Using *A. thaliana Atnoa1* cDNA sequence as a querying probe to search against *S. tuberosum* expressed sequence tag (EST) database in GenBank, the cDNA 5' end sequence was obtained by EST merging. The 3' rapid amplification of cDNA ends (RACE) primers (*Stnoa1*, Stnoa2, Stnoa3, Stnoa5 and Stnoa-flf) were designed according to *StNOA1* 5' end sequence (Table 1).

Isolation of *StNOA1* full length *cDNA* by *3' RACE*

For reverse transcription polymerase chain reaction (RT-PCR), total RNA was extracted from 100 mg potato fresh leaf and flower mixed sample using Trizol reagent according to manufacture's protocol. The extracted RNA was, precipitated by ethanol and then re-suspended in 30 ul of RNase free sterile distilled water. First strand of cDNA was generated from 5 ug of RNA with the superscript reverse transcriptase using RT primer P_{3R} (Table 1). The *StNOA1* 3' cDNA end was obtained by cassette PCR with primer pairs (*Stnoa1*; Stnoa2 and Pnup; Stnoa3 and Pnup) (Table 1). Subsequently the *StNOA1* 3' cDNA was ligated into Puc-T vector and sequenced. The gene specific primers (Stnoa-flr and Stnoa6) were designed according to the sequence of *StNOA1* 3' cDNA end sequence. The *StNOA1* cDNA full length was amplified by cassette PCR with gene specific primer pairs (Stnoa-flf and Stnoa-flr; Stnoa5 and Stnoa6) (Table 1). Four independent RT-PCR products were sub-cloned into pBluescript II SK (+) vector, which was further used for sequencing. For 3' RACE, PCR were performed under the following conditions: 3 min at 94°C for full denaturalization, 30 s at 94°C, 30 s at 55°C, 1 min at 72°C for 30 circles of amplification and 10 min at 72°C for additional extension. For full length cDNA isolation, PCR procedure was as follows: 3 min at 94°C for full denaturalization, 30 s at 94°C, 30 s at 55°C, 2 min at 72°C for 30 circles of amplification, and 10 min at 72°C for additional extension.

Construction of plant expression vector

A *StNOA1* cDNA was cut from pBSK-*StNOA1* by BamH1 and Kpn1 enzymes and then inserted into pWM-101 after digestion by BamH1 and Kpn1 enzymes. The plasmid was named pWM-101- *StNOA1*.

Plant transformation and growth

Atnoa1 plants were transformed by the floral dip method (Clough and Bent, 1998). Seeds (T1) from these plants were collected and selected on Murashige and Skoog (MS) solid medium (Murashige and Skoog, 1962) containing hygromycin (20 mg/l). Transgenic plants were grown to maturity and selected based on the ratio of hygromycin resistant/sensitive plants of T2 generation.

Analysis of putative transgenic *Arabidopsis* plants with PCR and RT-PCR

The genomic DNA was extracted from leaves of transformed, wild type and *AtnOA1 Arabidopsis* plants. Integration of the transgene into the plant genome was screened by PCR using *StNOA1*-specific primer pair (stnoa5 and stnoa6) and hygromycin phosphotransferase gene-specific primer pair (Hpt-F and Hpt-R) (Table 1).

For RT-PCR, total RNA was extracted from 100 mg control and transgenic plant fresh leaves using trizol reagent according to manufacture's protocol. First strand of cDNA was generated from 5 ug of RNA with the superscript reverse transcriptase. RT-PCR was conducted by *StNOA1*-specific primer pair (stnoa5 and stnoa6) and the ACTIN gene was used as internal standard (Table 1).

Analysis of transgenic plants for tolerance to salt stress

A. thaliana ecotype Colnumbia, *Atnoa1*, and transgenic plants with were used for analysis of salt tolerance.

Plant survival assay

Four-week-old plants grown in potting soil with three repetitions were irrigated with 300 mM NaCl solution every 2 days. The survival plants were counted on day 10 after the first irrigation. Survival rates were calculated from the counted data.

Root elongation assay

Wild type, *Atnoa1*, and transgenic seedlings (14 days old) grown on

MS solid medium were transferred to MS solid medium with or without different concentrations of NaCl for stress treatments. Root elongation was measured after 7 days of treatment. All treatments were repeated at least three times, and photographs were taken on the 7th day.

Determination of chlorophyll and total soluble protein

For chlorophyll extraction, leaves of wild-type, *AtnOA1* and transgenic plants were sampled at the same leaf order from the leaf base after NaCl 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). Total soluble protein contents were determined according to Bradford (Bradford, 1976) with bovine serum albumin as standard.

Measurement of proline

Free proline content was determined using the ninhydrin method (Bates et al., 1973). 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 then 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 concen-tration of proline was estimated by referring to a standard curve for L-proline.

Analysis of leaf lipid peroxidation

Plant lipid peroxidation was expressed as equivalents of malon-dialdehyde (MDA). The MDA content was determined as described by Dhindsa et al. (1981). Fresh leaves (about 0.5 g) were homogenized in 10 ml of 10% trichloroacetic acid (TCA), and centrifuged at 12,000 × g for 10 min. Then, 2 ml 0.6% thiobarbituric acid (TBA) in 10% TCA was added to 2 ml of the supernatant. The mixture was heated in boiling water for 30 min, and then quickly cooled in an ice bath. After centrifugation at 10,000 × g for 10 min, the absorbance of the supernatant at 450, 532 and 600 nm was determined. The MDA concentration was estimated using the formula:

$$C\ (\mu mol\ l^{-1}) = 6.45(A_{532} - A_{600}) - 0.56A_{450}.$$

Statistical analysis

Statistical analyses were carried out by two-way classification of ANOVA to evaluate whether the means were significantly different, taking $P < 0.05$ as significance level.

RESULTS

Isolation of *StNOA1*

The cDNA of *StNOA1* has a full length of 1929 bp and contains an open reading frame (ORF) of 1683 bp. The deduced protein of *StNOA1* contains 561 amino acid residues with the crucial GTPase-specific motifs (Figure 1). The GenBank accession number for *StNOA1* is GU205181. A *StNOA1* cDNA was used in the construct of plasmid pWM-101- *StNOA1* for transformation. Abbreviated physical map of the plasmid is shown in Figure 2.

Identification of transgenic mutant *Arabidopsis* plants

Four hygromycin resistance transgenic lines showed 1 and 1.68 kb amplification products when amplified with hygromycin phosphotransferase (*Hpt*) gene-specific primer pair (Hpt-F and Hpt-R) and *StNOA1* gene specific primer pair (stnoa5 and stnoa6), respectively, while the wild type and *AtnOA1* mutant *Arabidopsis* were negative (Figure 3A). Expression of *StNOA1* gene was analyzed by RT-PCR using total RNA extracted from leaves in transgenic *AtnOA1* plants as well as wild type and *AtnOA1* plants (Figure 3B). The *StNOA1* transcripts were detected in four transgenic lines: TL1, TL3, TL6 and TL9. The expression levels differed among the four lines. TL9 had 3 folds than that of TL1 and 2 folds than that of TL3 and TL6. No expression was detected in the wild type and *AtnOA1* controls (Figure 3B).

Phenotype comparison of TL9 with *Atnoa1* and wild-type *Arabidopsis* plants

Transgenic plants expressing *StNOA1* showed a clear phenotype difference in aspects of growth and develop-ment compared to *AtnOA1* mutant plants (Figure 4). TL9 showed more shoots and leaves (Figures 4B and C). The leaves of TL9 were greener (Figures 4A, B and C).

Effect of *StNOA1* expression on seedling survival rate under NaCl stress

Four-week-old TL9, *AtnOA1* and wild type *Arabidopsis* plants growing in potting soil were irrigated with 300 mM NaCl. On the 10th day after irrigation, TL9 displayed a higher survival rate than *AtnOA1* as well as wild type plants (Figure 5). The finding reveals that transgenic TL9 was more tolerant to salt stress than *AtnOA1* as well as wild type.

Effect of *StNOA1* expression on seedling root elongation under NaCl stress

As shown in Figure 6, root elongation of TL9 was signifi-cantly higher than *Atnoa1* after growing on MS medium with different concentrations of NaCl for 7 days (Figure 6). Nevertheless, the root elongations were reduced by 27.8, 40.5 and 51%, respectively, in wild type, TL9, and *AtnOA1 Arabidopsis* seedlings when they were grown on MS medium with 50 mM NaCl for 7 days ($P < 0.05$)

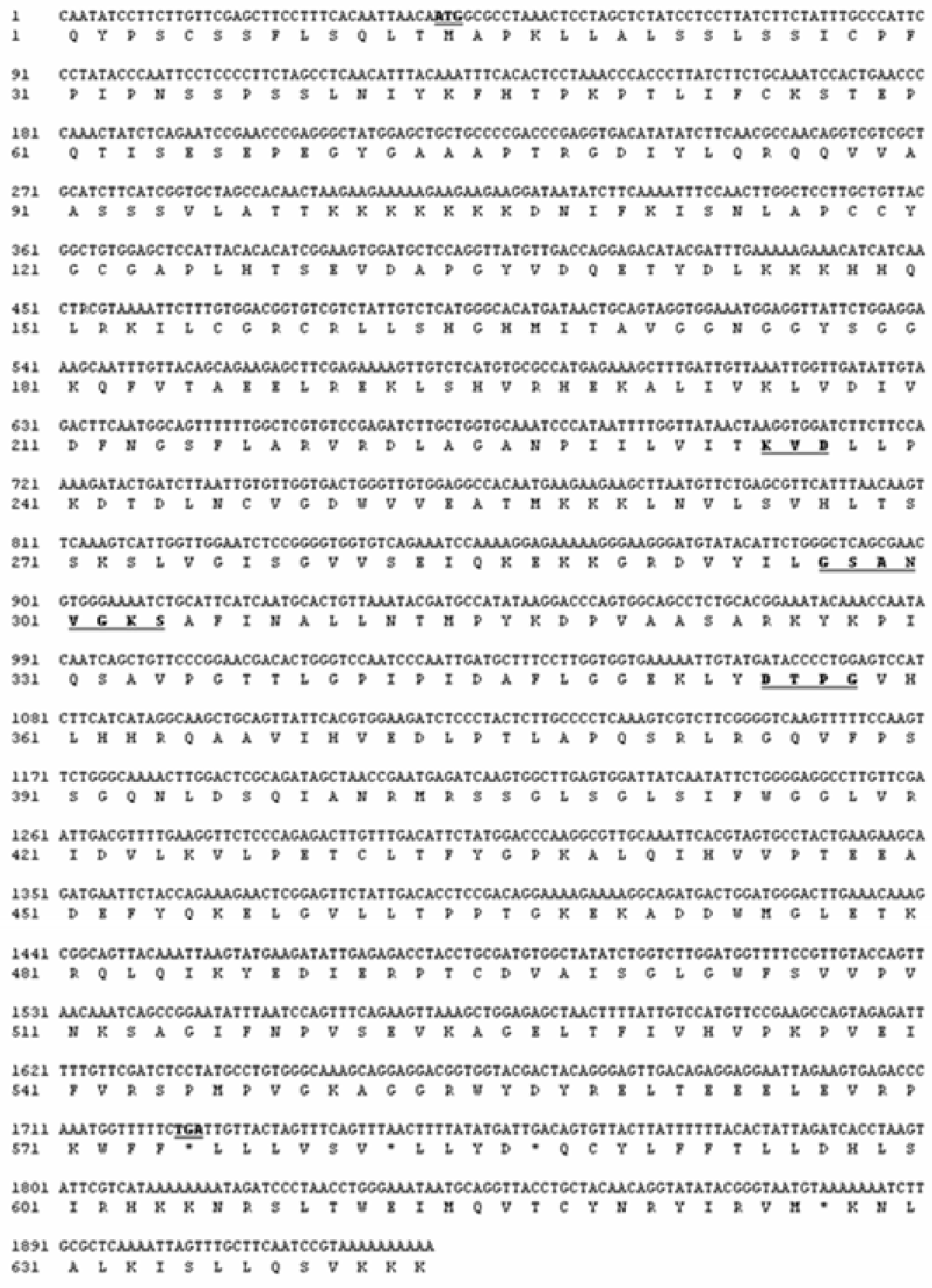

Figure 1. *StNOA1* gene sequence DNA and deduced amino acid sequence. The start and stop codes are underlined in boldface type. The crucial GTPase-specific motifs are also underlined in boldface type. [NT] KxD (the GTP-specificity motif), GxxxxGKS (Walker A), DxxG (Walker B).

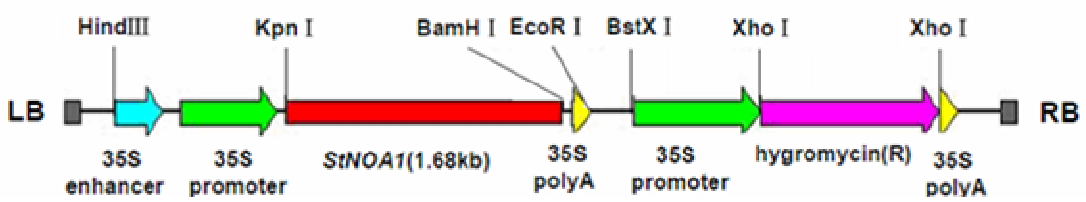

Figure 2. The *StNOA1* gene construct.

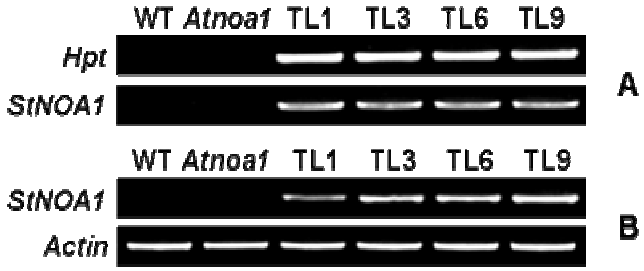

Figure 3. Molecular analysis of transgenic *AtnOA1* plants. (A) PCR analysis in 4 putatively hygromycin resistant plants of *AtnOA1* for presence of *hpt* and *StNOA1* gene, respectively. (B) The *StNOA1* gene expression was analyzed by RT-PCR. The *Actin* gene was used as internal control. WT, wild type; *AtnOA1*, *AtnOA1* mutant; TL1, TL3, TL6 and TL9, the transgenic lines.

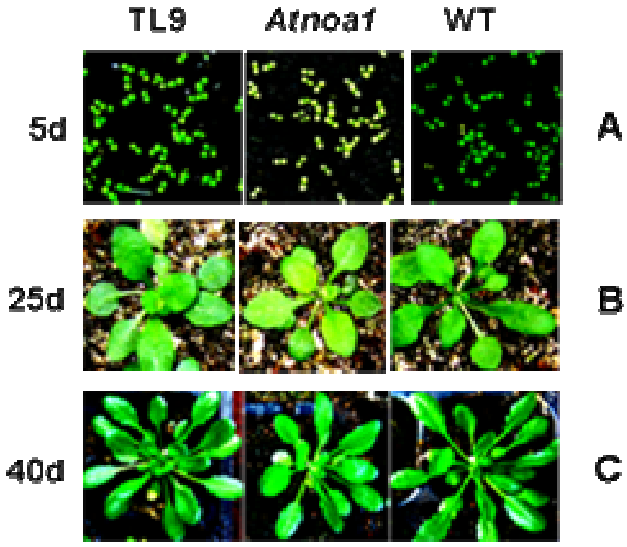

Figure 4. Phenotypic differences among transgenic *Arabidoosis* TL9, *AtnOA1*, and wild-type *Arabidopsis* in different growth periods. (A) 5 days old seedlings. (B) 25 days old plants. (C) 40 days old plants.

(Figure 6). Similar tendency was observed in other NaCl concentrations (Figure 6).

Effect of *StNOA1* expression on leaf chlorophyll and soluble protein contents under NaCl stress

Transgenic plant TL9 expressing *StNOA1* could maintain significantly higher chlorophyll and total soluble protein contents than *Atanoa1* plants under salt stress (Figures 7 and 8). Chlorophyll content in TL9 was 34.4 and 51.4% higher than in *AtnOA1* plant leaves under unstressed and NaCl stressed conditions, respectively ($P < 0.05$). Salt stress induced reduction of chlorophyll contents were 10.7, 31.4, and 22.7% in wild-type, *AtnOA1* and TL9 plant leaves, respectively (Figure 7). The soluble protein content in TL9 was 6.9 and 25.2% ($P < 0.05$) higher than *AtnOA1* in unstressed and stressed conditions, respectively (Figure 8). Salt stress induced reduction of soluble contents were 18.0, 29.6 and 17.6% in wild-type, *AtnOA1*, and TL9 plant leaves, respectively (Figure 8).

Effect of *StNOA1* expression on leaf proline accumulation under NaCl stress

The proline content of transgenic TL9 was 13.7% higher than *AtnOA1*, while 14.2% lower than wild type plants when subjected to 200 mM NaCl for 72 h ($P < 0.05$) (Figure 9). Transgenic TL9 exhibited a higher proline accumulation in unstressed condition than *AtnOA1* and wild type plants (Figure 9).

Effect of *StNOA1* expression on leaf lipid peroxidation under NaCl stress

To determine whether transgenic plants differ from *AtnOA1* and wild type plants in lipid peroxidation under salt stress, MDA contents in three types of plants were investigated. As shown in Figure 9, the MDA content was lower in TL9 than *AtnOA1* under salt stress. MDA in *AtnOA1* plants showed a marked increase in response to NaCl treatments while a marginal accumulation of MDA in TL9 and wild-type plants was observed when treated with NaCl. The MDA contents were 40.7 and 28.6% higher in wild type and TL9, than *AtnOA1* plants, respectively ($P < 0.05$) (Figure 10).

DISCUSSION

Compounds or genes that are able to reduce damaging

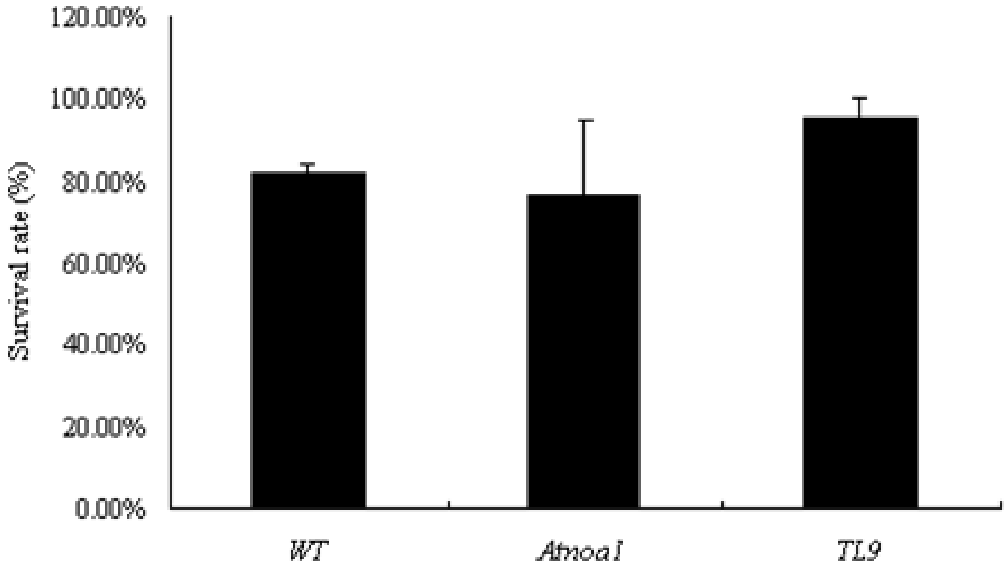

Figure 5. Survival rates of TL9, wild-type and *AtnOA1* plants in soil on the 10th day after irrigating 300 mM NaCl.

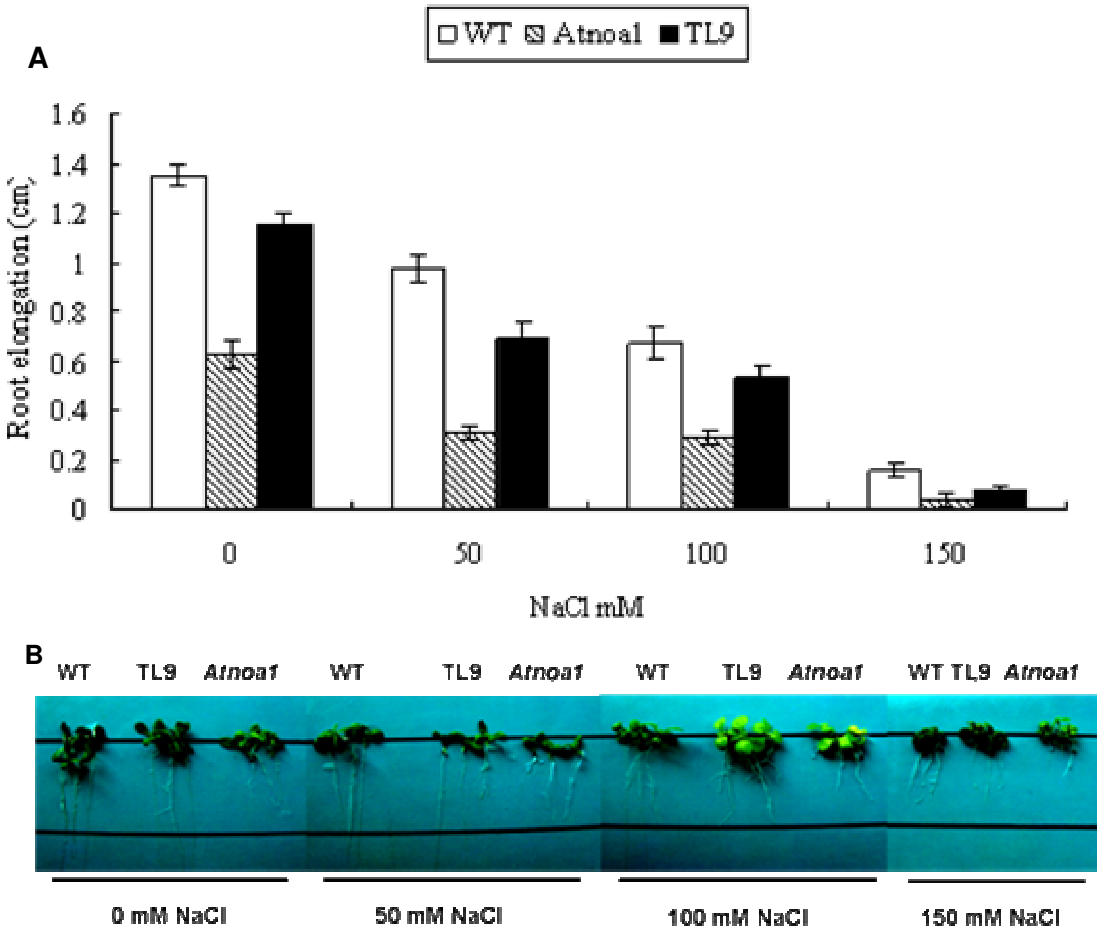

Figure 6. Effect of NaCl stress on root elongation of TL9, wild-type and *AtnOA1* seedlings. (A) Root elongation of TL9, wild-type and *AtnOA1* seedlings (7 days old) grown on MS medium with different concentrations of NaCl for 7 days. (B) Photographs of TL9, wild-type and *AtnOA1* after growing on MS with different concentrations of NaCl.

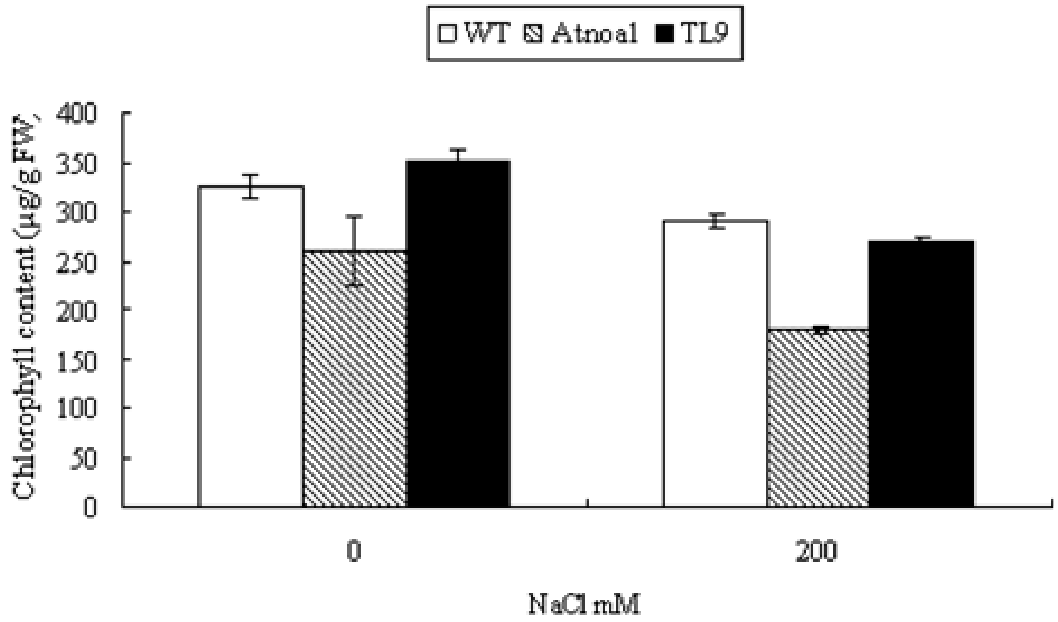

Figure 7. Effect of NaCl stress on chlorophyll contents in TL9, wild-type and *AtnOA1* plants. TL9, wild-type and *AtnOA1* plants were treated with 200 mM NaCl for 72 h and the chlorophyll contents were measured. Data are mean ± SE from three repetitions.

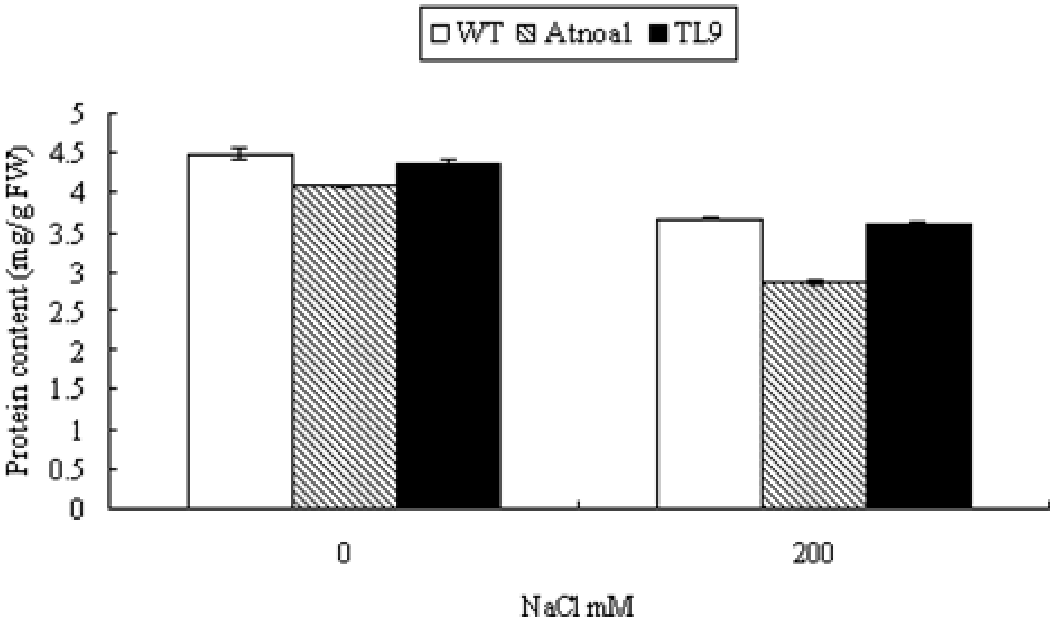

Figure 8. Effect of NaCl stress on soluble protein contents in TL9, wild-type and *AtnOA1* plants. TL9, wild-type and *AtnOA1* plants were treated with 200 mM NaCl for 72 h and the soluble protein contents were measured. Data are mean ± SE from three repetitions.

effects of various stresses should be of great importance from both the theoretical and application points of view. In *Arabidopsis*, *AtnOA1* was identified to be a regulator protein of a putative NOS-like enzyme complex (Guo et al., 2003). *AtnOA1* has low homology of DNA sequences with any types of NOS. The mutant *AtnOA1* plants had an increased susceptibility to biotic and abiotic stresses (Zeidler et al., 2004, Zhao et al., 2007b). In the present study, we showed that transgenic *AtnOA1* expressing potato *StNOA1* gene exhibited a higher stress tolerance to NaCl than *AtnOA1* mutant plants (Figures 5-9), indicating the involvement of *StNOA1* in regulating *Arabidopsis*

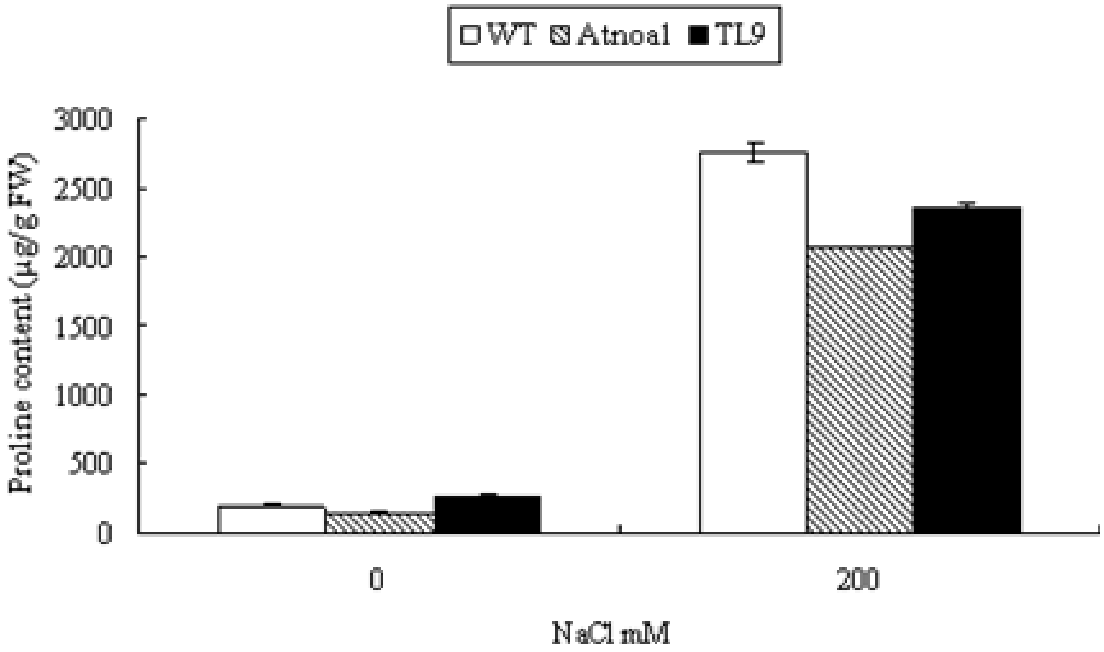

Figure 9. Effect of NaCl stress on proline contents in TL9, wild-type and *AtnOA1* plants. TL9, wild-type and *AtnOA1* plants were treated with 200 mM NaCl for 72 h and the proline contents were measured. Data are mean ± SE from three repetitions.

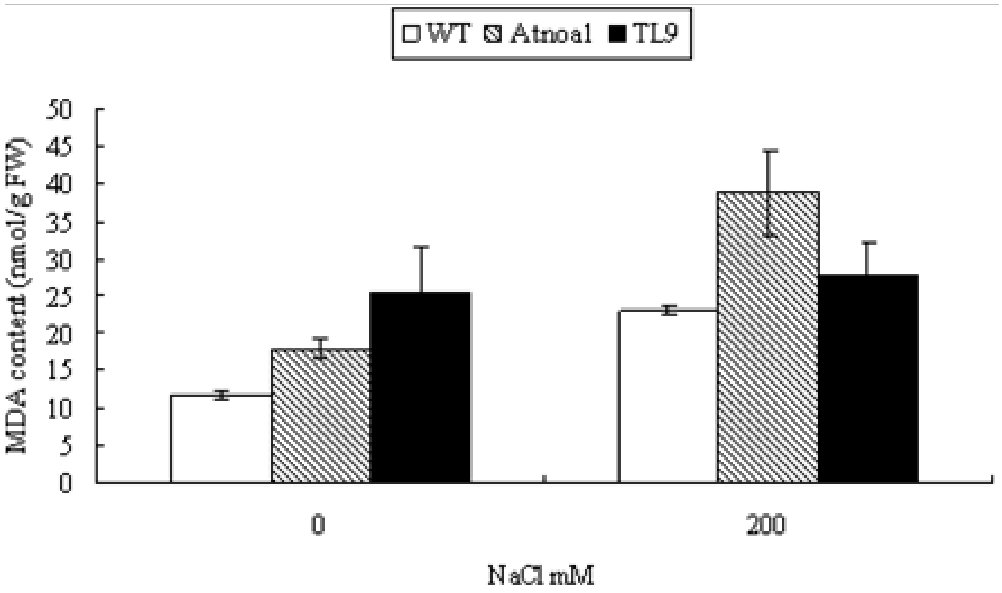

Figure 10. Effect of NaCl stress on MDA contents in TL9, wild-type and *AtnOA1* plants. TL9, wild-type and *AtnOA1* plants were treated with 200 mM NaCl for 72 h and the MDA contents were measured. Data are mean ± SE from three repetitions.

plant growth, development, and metabolisms during NaCl-mediated defense responses.

The transgenic TL9 plants expressing potato *StNOA1* gene changed original phenotype of the mutant plants in aspects of leaf yellowish and plant dwarf in different growth stages (Figure 4). The content of chlorophyll in TL9 was higher than *AtnOA1* plants either in normal or NaCl stressed conditions (Figure 7). Chlorophyll is the main component of photosynthesis. Photosynthesis has been suggested to be accompanied by S-nitrosylation of some proteins, an important protein modification involved in NO signaling (Lindermayr et al., 2005).

Proline accumulation is the common characteristic in many plants under saline conditions (Storey et al., 1977; Wyn Jones and Storey, 1978). Proline concentration in many salt tolerant plants has been found to be higher than that in salt sensitive ones. Petrusa and Winicov (1997) found that salt tolerance alfalfa plants rapidly doubled their proline content in the roots, whereas, in the salt sensitive plants the increase was low. The present result showed that TL9 exhibited a higher content of proline in stressed condition than *AtnOA1*. This indicates that transgenic plants were more tolerant to NaCl stress than *AtnOA1*.

Salt stress has been widely shown to cause an induction of lipid peroxidation (Xiong and Zhu, 2002; Xiong et al., 2002). *AtnOA1* plants have been proved to have higher MDA contents than wild-type plants under stresses (Guo and Crawford, 2005; Zhao et al., 2007b). In the present investigation, we found that TL9 and wild type plants suffered from an alleviated lipid peroxidation than *AtnOA1* plants, as revealed by lower MDA contents in TL9 and wild type plants in the absence and presence of NaCl (Figure 10), indicating that *StNOA1* plays a role in the reduction of lipid peroxidation in *Arabidopsis* leaves under salinity stress.

Although *AtnOA1* has been reported to be a cGTPase, it bears some different characteristics from small GTPase like Ras, Rho and Cdc42. For example, the guanine-binding motif of its central domain has different arrange-ment; furthermore, it possesses a C-terminal domain which is suggested to play crucial roles in plants (Moreau et al., 2008). Bacterial cGTPases are essential for cell growth (Krishna et al., 2003; Arigoni et al., 1998; Morimoto et al., 2002). Transgenic *AtnOA1* expressing *StNOA1* showed more shoots and leaves than *AtnOA1* (Figures 4B and C), which might shed light on the possible role of *StNOA1* in *Arabidopsis* growth and development.

StNOA1 as well as *AtnOA1* is predicted to be targeted to mitochondria or chloroplasts, which are both sites of electron transfer that leads to reactive oxygen species production (Gechev et al., 2006). The elevated level of ROS and oxidized lipids as well as lower NO accumulation has been reported in *AtnOA1* mutant plants (Guo et al., 2003; Zeidler et al., 2004; Guo and Crawford, 2005; Zhao et al., 2007a, 2007b). While there are also reports showing *AtnOA1* is not always impaired in NO accumu-lation (Arnaud et al., 2006; Bright et al., 2006; Shi and Li, 2006; Kolbert et al., 2008; Tun et al., 2008). Exogenous NO application can partially rescue the yellowish and pale *AtnOA1* but cannot rescue the other physiological traits associated with *AtnOA1* (Flores-perez et al., 2008). The above literatures indicates that NO is not sufficient for the loss of function of *AtnOA1*.

Our work demonstrated that *StNOA1* played a significant role in NaCl stress responses, including enhancing root elongation, elevating seedling survival rate, increasing the accumulation of leaf proline, decreasing leaf MDA content, increasing leaf chlorophyll and soluble protein content under NaCl stress.

Regardless of the nature of *StNOA1* as well as other NOAs in plants, the present study indicated that *StNOA1* functioned in *Arabidopsis* salt stress responses. We hypothesize that the enhanced salinity tolerance by *StNOA1* transformation may come from the synergisms of its C-terminal domain unknown function and its regulation of NO accumulation.

ACKNOWLEDGEMENTS

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