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EXPRESSION OF *AtNHX1* GENE UNDER DIFFERENT SALINITY LEVELS

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Abstract

Antiporter activity in the plant cell is one of the most important salt tolerance mechanisms. Studies proved that introduction of the *Arabidopsis* vacuolar membrane Na^+/H^+ antiporter (*AtNHX1)* conferred salt stress tolerance in many plants. *Arabidopsis* was treated with four different salinity levels (0, 50, 100 and 150 mM) in MS medium. Expression of *AtNHX1* was measured by quantitative real time PCR. It was found that *AtNHX1* gene was up regulated under different salinity levels. Due to the imposition of 50, 100 and 150 mM NaCl, expression level was higher 25, 90 and 110%, respectively compared to control.

Introduction

Soil salinity is a global environmental challenge, affecting crop production on over 800 million hectares, or a quarter to a third of all agricultural land on earth (Rengasamy 2010). The problem is particularly severe in irrigated areas where as much as one-third of global food production takes place. While saline soils contain numerous salts at elevated concentrations, NaCl typically dominates and it is believed that the harmful effects of saline conditions on most species are principally brought about by a combination of osmotic and ionic stresses exerted by the sodium component of NaCl (Munns and Tester 2008, Zhang *et al.* 2010). When plants are exposed to salt stress, cellular ion homeostasis may be disturbed. Na^+ entering the roots and transported to the leaves must be compartmentalized in the vacuoles in order to avoid accumulation of levels of Na^+ that are toxic to proteins in the cytoplasm. The strategy of accumulation of Na^+ inside vacuoles is used by many plants to survive under salt stress, an active vacuolar antiporter utilizes the proton motive force generated by vacuolar ATPases and pyrophosphatases to sequester excess Na^+ into the vacuole, thereby reduce the toxic effects of Na^+ inside the cytosol (Munns and Tester 2008, Plett and Moller 2010). Vacuolar *NHX*-type Na^+/H^+ antiporters play a key role in mediating the transport of Na^+ into the vacuoles, lowering the cytosolic Na^+ concentrations, and ameliorating the toxic effects of Na^+ on metabolism (Apse and Blumwald 2007). It has been reported that different *NHX1*; *OsNHX1* (Fukuda *et al.* 2004), *AgNHX1* (Hamada *et al*. 2001), *SsNHX1* (Ma *et al*. 2004), *GhNHX1* (Wu *et al.* 2004), *RhNHX1* (Kagami and Suzuki 2005), *TaNHX1* (Brini *et al.* 2005), *AlNHX1* (Zhang *et al.* 2008), *PeNHX1* (Wu and Liu 2009), *HcNHX1* (Guan *et al.* 2011), *SaNHX1* (Lan *et al*. 2011), *SbNHX1* (Jha *et al*. 2011), transcripts were increased due to NaCl treatment. *Arabidopsis thaliana* is used as model plant worldwide. In the present study, the expression pattern of *AtNHX1* (vacuolar membrane Na^+/H^+ antiporter) under different salt stress conditions was investigated.

Materials and Methods

Seeds of *Arabidopsis thaliana*, ecotype (Col-0) were collected from Key Laboratory of the Ministry of Education for Coastal and Wetland Ecosystems, College of the Environment and Ecology, Xiamen University, Xiamen, Fujian, China and the experiment was carried out in that

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laboratory. After sterilizing the seeds with 75% ethanol and 10% NaOCl, the seeds were sown on Petri dish with half strength of MS medium containing 0.8% (w/v) agar and 3% sucrose and then they were sealed with surgical tape. The seeds were kept under 8 hrs/16 hrs day/night cycle at 22°C. One week old seedlings of *Arabidopsis* were then transferred to half strength MS medium supplemented with 0, 50, 100 and 150 mM NaCl. Weight of plant biomass was measured after one week.

Total RNA was isolated from *Arabidopsis* fleshy leaves. About 0.5 g plant materials were ground in liquid nitrogen with 2% polyvinylpyrrolidone and extracted by vortexing with 700 µl RNA purification reagent (Invitrogen, California, USA) in 200 µl RNase free Eppendorf tube and kept in ice for 10 min. The homogenate was centrifuge for 10 min at 12000 rpm. The supernatant was mixed with 200 µl 5M NaCl and 300 µl trischloromethene followed by centrifugation and this procedure was repeated. The supernatant was then mixed with equal volume of 8M lithium chloride and kept in –20°C for 2 hrs. The RNA pellet was harvested by centrifugation at 12000 rpm for 10 min. The RNA pellet was washed twice with 75% ethanol, briefly dried and dissolved in 50 µl RNase-free water. After adding 1 µl RNase inhibitor, the samples were stored at –80°C for further use. One µg of total RNA was reverse transcribed to produce cDNA using cloned AMV first-strand cDNA synthesis kit (Invitrogen, Inc.). Reverse transcription proceeded for 60 min at 42°C followed by 10 min for 99°C and 10 min for 4°C and the resulting cDNA mixture was used as templates for subsequent PCRs. Extracted DNA was stored at –20°C for further use.

Quantitative real-time PCR was performed in the Rotor-Gene™ 6000 real-time analyzer (Corbett Research, Mortlake, Australia) using the FastStart Universal SYBR Green Master kit (ROX, Roche Ltd., Mannheim, Germany) according to the manufacturer's instructions. Reaction conditions (10 µl volumes) were optimized by changing the RNA concentration and annealing temperature to minimize primer-dimer formation and to increase PCR efficiency. Specific primers (forward 5′-CACCAGAAC GCCACCACGAGCAT-3′and reverse 5′-CCAGTAGTAATGCACG GTTCGAGTG-3′) were designed from mRNA sequence of *Arabidopsis* to quantify the expression levels of the examined genes *AtNHX1*. Software Primer Premier 5.0 was used to verify the quality of the designed primers for melting temperature, GC content, to avoid primer-dimer, formation of false priming sites and formation of hairpin etc. The primers were tested by running the PCR product in 1% agarose gel electrophoresis. The Actin 2 primers (forward 5′-AACTCTCTGGGTTTTTACTTACGTCTGCG-3′ and reverse 5′-AGGGAACAAAAGGAAT AAAGAGGCATCAA-3′) were used as a housekeeping gene control measured in parallel for each sample. The following PCR profile was used: 95°C for 5 min, 40 cycles at 95°C for 30 sec, the appropriate annealing temperature 55°C for 30 sec and 72°C for 30 sec, followed by recording of a melting curve. The lack of primer dimmer or non-specific product accumulation was checked by melt-curve analysis. Each run included standard dilutions and a negative reaction controls. The mRNA expression levels of the genes were expressed as the normalized ratio using the ΔΔCt method according to Livak and Schmittgen (2001). The Ct values of each target gene was calculated by Rotor-Gene 6000 Application Software (Version 1.7), and the ΔCt value of the Actin 2 gene was treated as an arbitrary constant for analyzing the ΔΔCt value of samples. Three independent pools for each target gene were averaged, and the standard error of the mean was recorded.

Results and Discussion

Morphological variations of the plants were observed due to the application of NaCl treatments (Fig. 1). Plants displayed progressive chlorosis and a general growth inhibition, and the inhibitory effect became more pronounced with increasing NaCl concentrations. Increased salinity levels decreased the total biomass of *Arabidopsis*. Due to the increase of salinity by 50, 100 and

150 mM NaCl, biomass decreased as 13, 48 and 63%, respectively (Fig. 2). Significant variation of biomass was not found with the application of 50 mM NaCl but it was prominent at 100 mM NaCl and upward level of salinity.

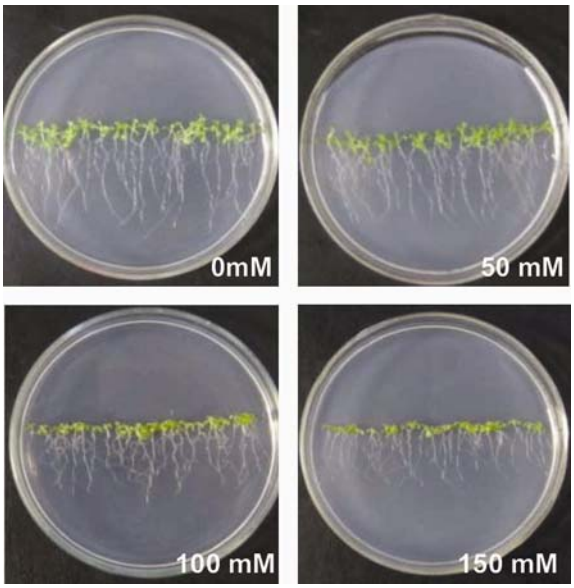

Fig. 1. *Arabidopsis thaliana* treated with different NaCl concentration.

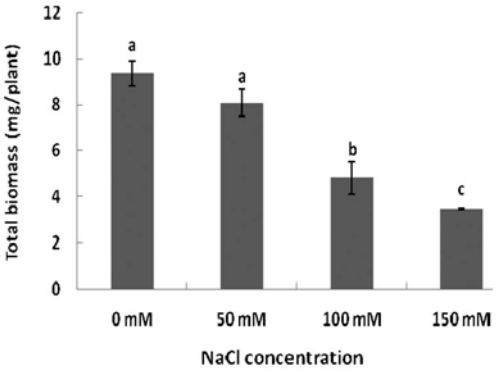

Fig. 2. Effect of NaCl on the leaf biomass of *Arabidopsis.* Columns labeled with the same letter indicate that they are not significantly different at the level of p = 0.05 and error bar represents the standard error.

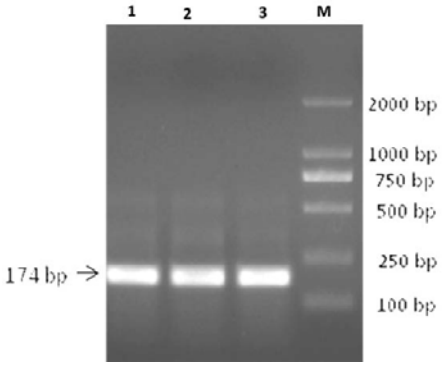

Fig. 3. PCR amplification with the primers for *AtNHX1* M: DNA ladder, Lane 1 - 3 : target bands).

Higher concentration of RNA was found at the 50 mM NaCl salinity (Table 1). The RNA concentration at ratio of 260/280 and 260/230 under different treatments were near to 2.0 and more than 2.0, respectively which indicated its purity. Extracted RNA was checked by 1% agarose gel electrophoresis and showed two bands at 28 S and 18 S indicated its good RNA integrity.

Table 1. RNA concentration of leaves of *Arabidopsis* under different salinity stresses.

Treatments (mM NaCl)	Concentration (ng/µl)	Absorbance (260/280 nm)	Absorbance (260/230 nm)
0	2477	2.11	2.39
50	3386	2.03	2.27
100	2065	2.13	2.43
150	2506	2.13	2.40

The PCR amplification products for designed primers for quantitative real time PCR were visualized at 1% agarose gel. The target band at 174 bp indicated the validation of primers (Fig. 3). The shape of amplification and melting curve of real time PCR for *AtNHX1* gene indicated its specificity (Fig. 4).

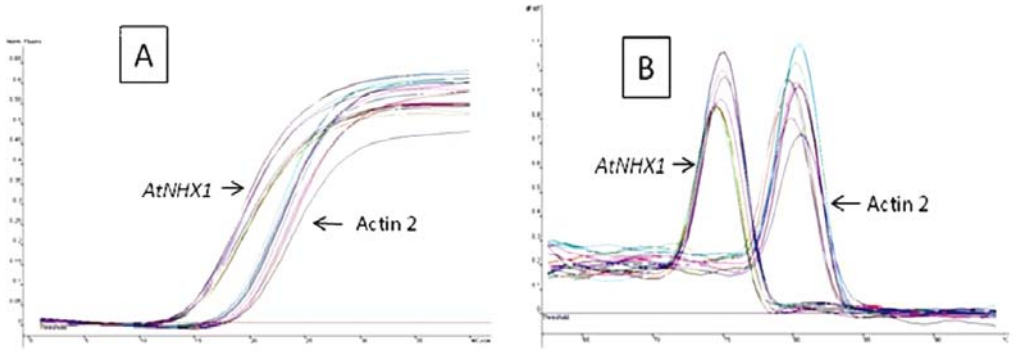

Fig. 4. The amplification curve (A) and melting curve (B) by real time PCR of *AtNHX1* gene.

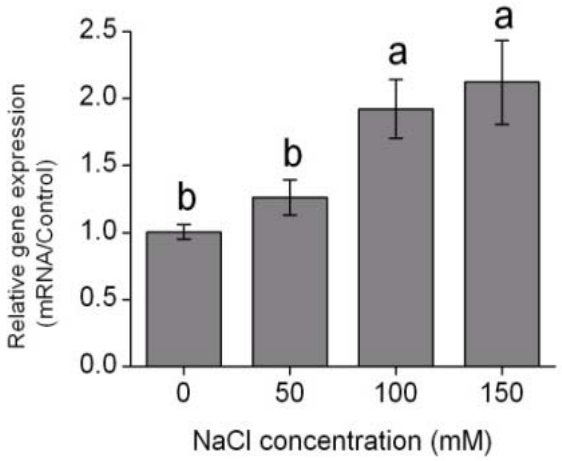

Fig. 5. Expression analysis of the *AtNHX1* under treatment of different NaCl concentrations.

Among different salinity stresses, 50 mM NaCl concentration did not show significant difference of expression from the control (Fig. 5). Significant variation of *AtNHX1* expression was found at the 100 mM NaCl salinity level. With the increasing of the salinity level from 100 to 150 mM NaCl, expression was increased but it was not significant. Compared to control, 25, 90, and 110% higher expression were observed due to the imposition of 50, 100 and 150 mM NaCl, respectively. Higher expression of genes linked to salt acclimation has been positively correlated with tolerance, which presumably is linked to a signaling cascade (Dassanayake *et al*. 2011). Apse *et al.* (1999) found the *AtNHX1* transcripts in root, shoot, leaf, and flower tissues of *Arabidopsis* under salt stress and it has been shown to increase in the presence of higher salinity levels (Gaxiola *et al*. 1999). *NHX* overexpression (endogenous or transgenic) has been shown to confer salt tolerance in a wide range of plant species (Zhang and Blumwald 2001, Ohta *et al.* 2002, Yin *et al.* 2004, Xue *et al.* 2004, He *et al.* 2005, Lu *et al.* 2005, Liu *et al.* 2008, Leidi *et al*. 2010, Li *et al*. 2010). In this study, absence of NaCl condition, expression of *AtNHX1* was low and it increased under salinity stress conditions indicating that the expressing of *AtNHX1* was induced by salt stress.

This finding indicates that there is a great potentiality of this gene in improving the mechanism of salt tolerance of crop plants. After cloning, it may be transformed into crop to develop salt tolerant varieties.

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