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ORIGINAL ARTICLE

Quantitative gene expression analysis of some sodium ion transporters under salinity stress in *Aeluropus littoralis*

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Abstract Plant sodium transporters activity is one of the most important salt tolerance mechanisms to keep normal status of cytosolic sodium content. In the present study, expression pattern of genes encoding Na^+/H^+ antiporters in the plasma membrane (*SOS1* gene), vacuolar membrane (*NHX1* gene) and H^+-ATPase pump (*VHA* gene) in *Aeluropus littoralis* under different treatments of NaCl was measured by the semi-quantitative RT-PCR method. Our results indicated that root and shoot sodium contents were increased along with increasing salinity pressure. In response to 200 and 400 mM NaCl, mRNA level of *SOS1* and *NHX1* was increased in the shoot and root tissues, while *VHA* transcripts were increased only under 400 mM of NaCl. Transcripts of *VHA-c* and *NHX1* in root were higher than shoot in all treatments. In general, our results indicated that transcriptional level of *SOS1*, and *NHX1* genes induced in parallel with *VHA* expression may be involved in controlling cytosolic Na^+ concentration in *A. littoralis*.

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1. Introduction

High concentration of Na^+ ions adversely affected the growth of plants due to deleterious effects on enzyme activities, photosynthesis and metabolism (Niu et al., 1995). Plants employ different mechanisms such as restriction of Na^+ entry to cell, increasing the extrusion of Na^+ from the cell and compartmentalization of Na^+ in the vacuoles to reduce Na^+ toxic effects (Aharon et al., 2003; Blumwald et al., 2000). Na^+ efflux from the cell and vacuolar Na^+ compartmentalization can be

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accomplished by the action of Na^+/H^+ antiporters in the plasma and vacuolar membranes. The driving force for Na^+ transport using the antiporters provided by membrane H^+-ATPase and H^+-PPiase pumps (Blumwald et al., 2000; Barkla and Blumwald, 1991; Hasegawa et al., 2000; Horie and Schroeder, 2004). The vacuolar H^+-ATPase (V-ATPase) is the dominant H^+-pump at cellular membranes of most plant cells. Under different stress conditions such as salinity and drought survival of the cells depends strongly on maintaining or adjusting the activity of the V-ATPase (Dietz et al., 2001). There are different catalytic and non-catalytic subunits in the dissociable head groups of V-ATPases, among them C subunit has a critical role in salt tolerance (Haro et al., 1993). Several Na^+/H^+ antiporter genes have been reported in glycophytes such as *Oryza sativa* (Fukuda et al., 1999), *Arabidopsis thaliana* (Yokoi et al., 2002), *Gossypium hirsutum* (Wu et al., 2004), *Rosa hybrida* (Kagami and Suzuki, 2005), *Trifolium repens* (Tang et al., 2010) and halophyte plants such as *Mesembryanthemum crystallinum* (Chauhan et al., 2000), *Atriplex gmelini* (Hamada et al., 2001), *Beta vulgaris* (Xia et al., 2002), *Hordeum brevisubulatum* (Lu et al., 2005) and *Aeluropus littoralis* (Zhang et al., 2008). Under salt stress conditions, the transcript levels of these genes or their protein activity increased and thereby the activity of Na^+/H^+ antiporters up-regulated (Blumwald et al., 2000; Shi et al., 2003). The overexpression effectiveness of Na^+/H^+ antiporter genes in improving plant salt tolerance has been investigated in several studies. Shi et al. (2003) overexpressed *SOS1* gene in Arabidopsis and achieved high levels of salt tolerance in transgenic plants (Zhang and Blumwald, 2001). Similarly, salt tolerance ability of different *NHX1* gene overexpressed plants has been shown.

A. littoralis Parl. is a C4 perennial halophyte plant that grows as weed in dry salty areas. This plant grows normally in high saline conditions without toxicity symptoms. Thus, it has the potential to become an important genetic resource to understand the molecular basis of membrane transporters and their role in salt tolerance (Zouari et al., 2007). In the present study, the expression patterns of *NHX1*, *SOS1* genes and also subunit C of V-ATPase pump in *A. littoralis* under different salt stress conditions were investigated.

2. Materials and methods

2.1. Plant material preparation

Seeds of *A. littoralis* were collected from natural habitats and cultured in acid washed and nutrient free sand in plastic pots. Pots were kept in greenhouse under controlled conditions of 45–65% relative humidity at a day/night temperature of 25 °C/16 °C and a 14/10 h (light/dark) photoperiod. Pots were irrigated with full strength Hoagland's solution for five weeks. To induce salt stress, irrigation solution was supplemented with NaCl to final concentrations of 100, 200, 300 and 400 mM. The plants were irrigated daily with solutions and the salt concentrations of irrigation solutions were gradually increased by 100 mM NaCl to reach the favorite level of treatment. Roots and shoots of treated plants were harvested after two weeks of saline conditions. A portion of each sample was oven dried for measurement of physiological parameters and another part was immediately frozen in liquid nitrogen and stored at −80 °C for RNA isolation and DNA synthesis.

2.2. Measurement of Na^+ content

After two weeks of salt treatment, twenty plants within each pot were selected randomly and used for assessing shoot salt secretion. Shoots of each treatment were rinsed thoroughly with cold double-distilled water to remove all secreted salts from shoot surfaces. Electrical conductivity of each solution was considered as secreted salt amount. Dried shoot and root samples were ashed at 540 °C for 18 h and the resulting ash was weighed and digested by hydrochloric acid. Na^+ content of all samples was determined using a flame Photometer (Corning-EEL Model 430).

2.3. Semi-quantitative RT-PCR analysis

2.3.1. RNA isolation and cDNA synthesis

Total RNA was isolated from the shoot and root of treated samples with TRIZOL reagent (Invitrogen Inc., CA, USA) according to manufacturer's manual, and then treated with DNaseI (Fermentase, Germany) to remove DNA contamination. First-strand cDNA was synthesized from 2 μg of total RNA using the RevertAid™ Reverse Transcriptase kit (Fermentase, Germany) according to manufacturer's protocol. Semi qRT-PCR of *NHX1*, *SOS1* and *VHA-c* genes was carried out using specific primers for amplification of PCR products around 200–600 bp length. The Oligo software (V. 7) was used to design specific PCR primers from conserved sequences of these genes (Table 1). *A. littoralis actin* gene was used for designing a pair of primers that amplify a 330 bp PCR product which served as an internal reference in qPCR reactions. PCR condition for all genes was as follows: 95 °C for 3 min; 25 cycles of 94 °C for 45s, 55 °C for 45s and 72 °C for 1 min; and a final extension at 72 °C for 2 min. The PCR products were separated by electrophoresing on a 1.2% agarose gel in TBE buffer. Gel images were quantified using TotalLab TL120 software (Nonlinear Dynamics Ltd).

2.4. Statistical analysis

All data statistically analyzed and mean comparison was performed by Duncan's multiple range test with critical value of $P < 0.05$ using SPSS 16.0 software.

Table 1 Gene-specific primers for the amplification of *NHX1*, *SOS1*, *VHA-c* and actin genes.

Gene	Primer sequences
NHX1	5′-CTCATCATTGGGCTGTGC-3′
	5′-TGTAAGAGAGGTAAGCCATG-3′
SOS1	5′-GCTGCRTTTCTYCGYGCWCATA-3′
	5′- CCAGTTGGCYTTGARCCYTC-3′
VHA-c	5′-ATGTCGTCGGTGTTCAGC-3′
	5′- CGTAGATACCGAGCACAC-3′
Actin	5′-GTGCCCATTTACGAAGGATA-3′
	5′-GAAGACTCCATGCCGATCAT-3′

3. Results

3.1. The effect of salt stress on salt secretion and Na^+ content

After 14 days, total salt secretion and Na^+ content of *A. littoralis* plants were measured under different NaCl treatments. As shown in Table 2, total secreted salt in the all treatments was significantly different compared with control plants. The secreted salt (13.289 ds m^{-1}g DW^{-1}) and Na^+ content (3491.440 μmol g DW^{-1}) at 400 mM salt concentration was 15.5- and 32-fold greater than control plants, respectively. The Na^+ content of roots and shoots of treated plants was increased under salt treatment. The amount of increase in roots was slightly higher than shoots, but it was not statistically significant (Fig. 1).

3.2. Expression pattern of Al-NHX1 gene under salt stress

To assess the effect of salt on the expression pattern of *Al-NHX1* gene, total RNA from shoots and roots of NaCl treated plants was isolated. The expression levels of *Al-NHX1* gene in these tissues were evaluated by semi-quantitative RT-PCR. The results indicated that *Al-NHX1* was expressed both in roots and shoots and the transcript levels of *Al-NHX1* were induced by NaCl treatment. The expression levels of *Al-NHX1* in shoots increased 1.8- and 2.7-fold relative to the control at 200 and 400 mM NaCl concentrations, respectively, while the level of transcripts in roots was 1.4- and 1.8-fold higher than control (Fig. 2). Generally, higher transcript levels of *Al-NHX*1 were detected in roots compared with shoots in all treatments.

3.3. Expression pattern of Al-SOS1 gene under salt stress

Significant increase in *Al-SOS1* transcript levels was observed in shoots and roots of salt treated plants. Under 200 and 400 mM of NaCl concentration, the expression levels in shoots were 2.9- and 3.5-fold higher than control and in the roots were 1.7- and 2.5-fold higher than control plants, respectively. Mean comparison analysis showed that *Al-SOS1* mRNA abundance in shoots was higher than roots in all salt treatments (Fig. 3).

3.4. Expression pattern of V-ATPase subunit C gene under salt stress

The mRNA abundance of V-ATPase subunit C in root tissues at 0, 200 and 400 mM NaCl was 2.3, 2.2 and 3 times higher than shoot tissues. The expression pattern of this gene in 200 mM salt treated plants was not significantly different from control plants. In plants treated with 400 mM NaCl, the transcript level of V-ATPase subunit C was increased markedly. The increment in shoot and root tissues was 1.3- and 1.7-fold higher relative to control plants, respectively (Fig. 4).

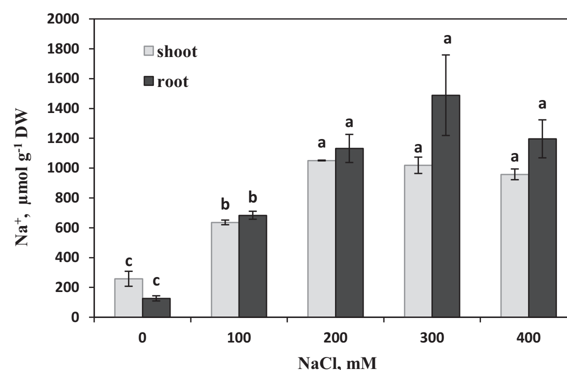

Figure 1 Effect of NaCl concentrations on Na^+ contents in shoots and roots of *Aeluropus littoralis*. Plants were kept under saline conditions supplied with different NaCl concentrations for 2 weeks. Values are means ± SE of three replications and bars indicate SE. Columns with different letters indicate significant differences at $P = 0.05$ by Duncan's test.

4. Discussion

The increment of salt secretion rate of shoot in response to salinity stress has been reported in a number of halophyte plants such as *Avicennia germinans* (Suarez and Medina, 2008), *Avicennia marina* (Downton, 1982) and *Virginicus sporobolus* (Marcum and Murdoch, 1992). As indicated by our results, along with increasing sodium content in shoot tissues, salt secretion from them dramatically increased at high salinity treatments to get rid of excessive Na^+ ions (Tester and Davenport, 2003). This phenomenon indicated the important role of salt glands at salt secretion and protection of plant tissues against toxic ions, without losing indispensable nutrients (Barhoumi et al., 2007).

Significant up-regulation of *Al-NHX1* gene under salt stress was shown in our experiments. The response to stress condition in the root was stronger than shoot tissues and higher transcript levels of *Al-NHX1* were observed in this tissue. Similar results have been reported by other researchers in *A. littoralis* (Zhang et al., 2008), *At-NHX1* of *A. Thaliana* (Yokoi et al., 2002), *Hv-NHX1* of *Hordeum vulgare* (Fukuda et al., 2004) and *Cm-NHX1* of *Cucumis melo* (Wang et al., 2011).

Transcript abundance of NHX1 gene in response to salinity can trigger high activities of tonoplast Na^+/H^+ antiporter (Blumwald et al., 2000; Shi et al., 2002). Induction of expression of different isoforms of NHX gene under NaCl treatment including *At-NHX1*, 2 and 5 in *A. thaliana* (Yokoi et al., 2002)

Table 2 Effect of different NaCl concentrations on salt secretion and secreted Na^+ contents of shoots of *Aeluropus littoralis* plants.

NaCl (mM)	0	100	200	300	400
EC (ds m^{-1}g DW^{-1})	0.856 c	8.882 b	8.685 b	10.684 b	13.289 a
Na^+ (μmol g DW^{-1})	108.581 c	2204.441 b	2443.295 b	3261.784 ab	3491.440 a

Plants were kept under salinity treatments (0, 100, 200, 300, and 400 mM NaCl) during two weeks. Values followed by the same letter are not significantly different ($P = 0.05$) as described by Duncan's test. Data are means of three replications.

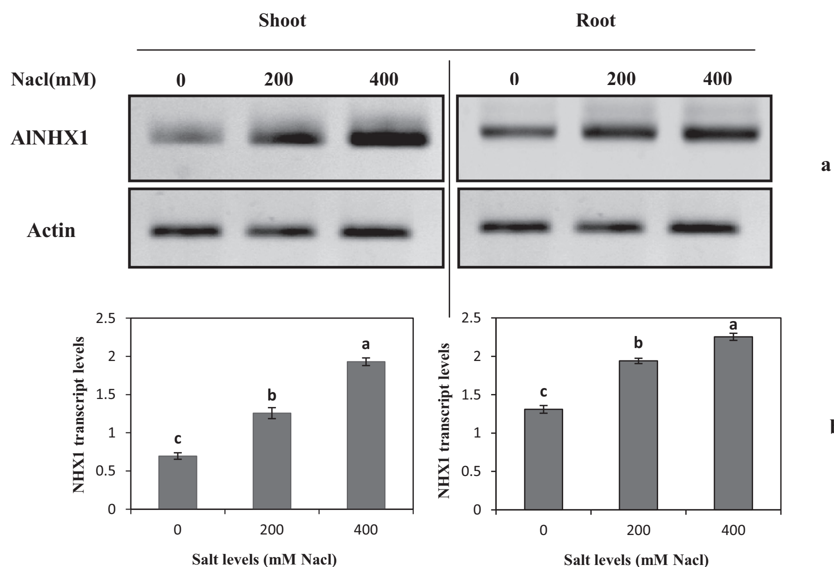

Figure 2 Expression of *Al-NHX1* in the shoot and root of *A. littoralis* under different concentrations of NaCl. (a) Semi-quantitative RT-PCR analysis of *Al-NHX1* mRNA in plants treated with 0, 200 and 400 mM NaCl for two weeks. ACTIN was used as an internal control. The final value was the average of at least three independent experiments. Only the optimal pictures are shown. (b) The relative expression level of *Al-NHX1* (related to ACTIN) under different concentrations of NaCl. Values are means ± SD and bars indicate SD. Columns with different letters indicate significant differences at P = 0.05 (Duncan's test).

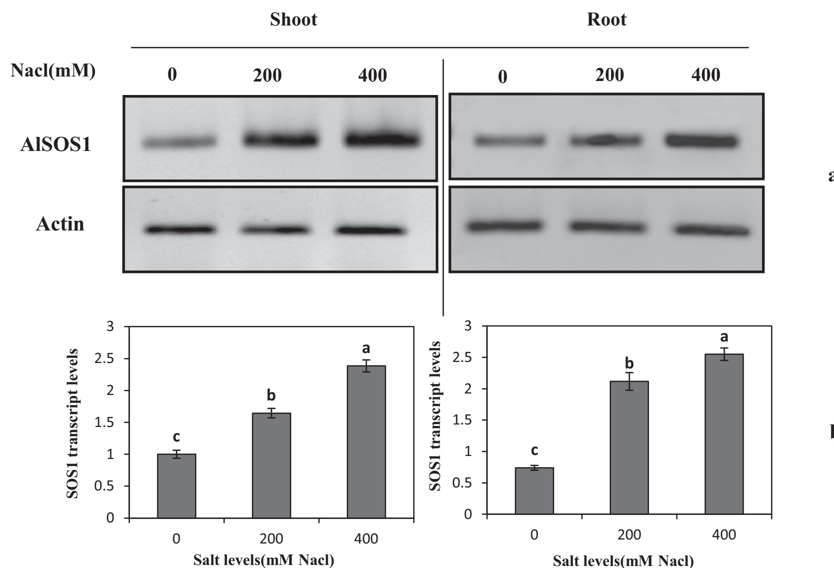

Figure 3 Expression of *Al-SOS1* in the shoot and root of *A. littoralis* under different concentrations of NaCl. (a) Semi-quantitative RT-PCR analysis of *Al-SOS1* mRNA in plants treated with 0, 200 and 400 mM NaCl for two weeks. ACTIN was used as an internal control. The final value was the average of at least three independent experiments. (b) The relative expression level of AlSOS1 (related to ACTIN) under different concentrations of NaCl. Values are means ± SD and bars indicate SD. Columns with different letters indicate significant differences at P = 0.05 (Duncan's test).

and *Pe-NHX1*, 2, 3, 5 and 6 in *Populus euphratica* (Ye et al., 2009) was shown. Although the transcript level of some NHX isoforms such as *In-NHX1* in *Ipomoea nil* (Ohnishi et al., 2005) and *Vv-NHX1* in *Vitis vinifera* (Hanana et al., 2007) was not influenced by salt stress.

Accumulation of sodium ions and expression level of *Al-NHX1* in root tissues might be correlated with each other. The up-regulation of *Al-NHX1* gene expression might diminish Na^+ translocation from root to shoot via Na^+ accumulation in the root vacuoles (Zhang et al., 2008; Wang et al., 2011; Fukuda et al., 2004).

Up-regulation of *Al-VHA-C* in root tissues parallel with *Al-NHX*1 and *Al-SOS*1 genes might result from induction of Na^+/H^+ antiporter activity and essential regulation of electrochemical proton gradient across tonoplast. As explained by Fukuda et al. (2004) alkalization of vacuolar lumen

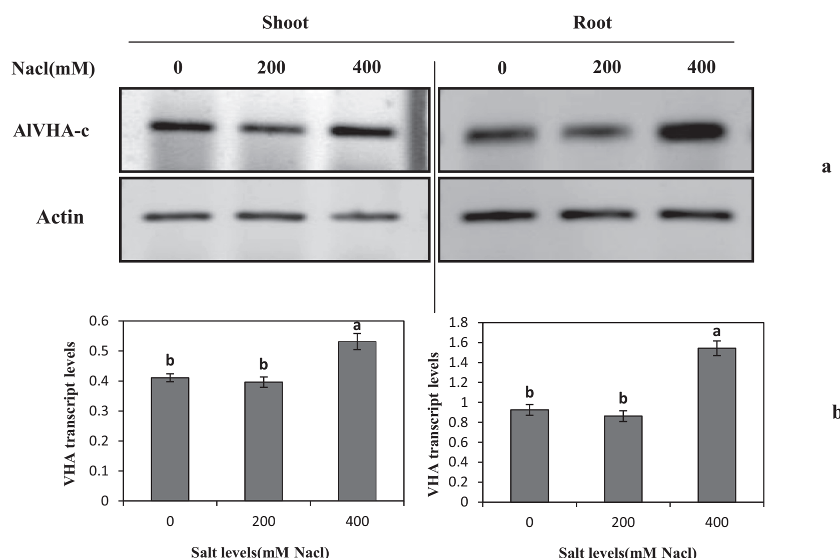

Figure 4 Expression of AlVHA-c in the shoot and root of *A. littoralis* under different concentrations of NaCl. (a) Semi-quantitative RT-PCR analysis of AlVHA-c mRNA in plants (see Section 2 for details of sand culture) treated with 0, 200 and 400 mM NaCl for two weeks. ACTIN was used as an internal control. Experiments were repeated at least three times to obtain similar results. Only the optimal pictures are shown. (b) The relative expression level of AlVHA-c (related to ACTIN) under different concentrations of NaCl. Values are means ± SD and bars indicate SD. Columns with different letters indicate significant differences at $P = 0.05$ (Duncan's test).

might regulate the H^+-pump gene expression and its acidification induces Na^+/H^+ antiporters. Overexpression of H^+-pumps in coordination with Na^+/H^+ antiporter may govern salt tolerance mechanisms in plants. Several reports revealed salt stress based induction of *VHA* in mRNA and protein levels in various plants (Fukuda et al., 2004; Rockel et al., 1998).

Transcriptional levels of *Al-SOS1* in the shoot and root of *A. littoralis* plants significantly increased in response to salinity. This expression pattern was similar to *Th-SOS1* expression of *Thellungiella halophila* (Kant et al., 2006; Oh et al., 2009). However, Taji et al. (2004) and Gong et al. (2005) did not show expression induction of *Th-SOS1* by salt treatment. High abundance of *Al-SOS1* transcript level in the shoot was accompanied with a lower sodium accumulation in comparison with those of the root. Parallel activity of *Al-NHX1* and *Al-SOS1* that were induced by salinity may result in compartmentalization of sodium ions in shoot vacuoles. On the other hand, remarkable induction of *Al-NHX1* expression in roots might alleviate Na^+ translocation from the root to shoot via sodium accumulation in the root vacuoles (Tester and Davenport, 2003). It appears that simultaneous induction of *Al-SOS1* and *Al-NHX1* in shoot and root tissues of *A. littoralis* is determinant and effective factors to control Na^+ translocation and accumulation in shoot tissues as indicated by Shi et al. (2002).

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