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Full Length Research Paper

Over-expressing *Salicornia europaea* (*SeNHX1*) gene in tobacco improves tolerance to salt

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Vacuolar Na^+/H^+ antiporters provide an efficient mechanism to avert the deleterious effects of Na^+ by its compartmentalization into vacuoles. In this study, *SeNHX1*, a vacuolar Na^+/H^+ antiporter from *Salicornia europaea*.L, was introduced into tobacco to investigate its response to salt tolerance, and to study how over-expression of this gene can affect other physiological responses. An increase of salt tolerance in transgenics was observed both *in vitro* and in pot culture. When exposed to either 138 mM or 172 mM NaCl *in vitro*, the transgenic apical meristem and stem segments with buds exhibited stronger salt-tolerance than their wild-type counterparts. In pot soil with 10.2 mg g^{-1} DW Na^+ stress, transgenic plants over-expressing *SeNHX*1 accumulated 1.2 mg g^{-1} FW Na^+ greater than wild type in old leaves. Meanwhile, no difference was observed in young leaves. Dry weight and height of transgenic plants were reduced less compared to that of wild type. Moreover, under high salt stress, the malondialdehyde (MDA) content in transgenic plants was significantly lower, but proline content and activity of antioxidant enzymes were obviously higher than that of wild-types. These results confirm and support the potential application of *SeNHX1*-transgenic plants for being cultivated in saline soil.

Key words: Antioxidant enzyme, MDA, Na^+/H^+ antiporter, Na^+, proline.

INTRODUCTION

Salinity stress is one of the most serious factors limiting the growth of plants. Salt-resistant plants have developed a variety of adaptive mechanisms, such as ion homeostasis, osmotic adjustment, detoxification and growth regulation (Zhu, 2001). It is important to maintain a low cytosolic Na^+ concentration, which was attained by the operation of Na^+/H^+ antiporters located in both the plasma membrane (Shi et al., 2002) and the tonoplast(Apse et al., 2003). In plant, the vacuolar Na^+/H^+ antiporter provides an efficient mechanism to avoid the deleterious effects of Na^+ in the cytosol and maintains osmotic balance by exchanging Na^+ for H^+ across vacuolar membranes and compartmentalization of Na^+ in the vacuoles (Zhu, 2003). To date, many genes encoding vacuolar Na^+/H^+ antiporters have been identified, for instance, *AgNHX*1(Ag*, Atriples gmelini*) in *Atriples*, *SsNHX*1 (Ss, *Suaeda salsa*) in *Suaeda*, *TaNHX*1 (Ta, *Triticum aestivum*) in wheat, *LeNHX*l (Le, *Lycopersicon esculentum*) in tomato and *AtNHXI* (At, *Arabidopsis thaliana*) in *Arabidopsis*. They share similar molecular characters with 10 to 12 transmembrane domains, hydrophobic C-terminus in vacuole and highly conserved FFIYLLPPI sequence for amiloride binding (Hamada et al., 2001).

Over-expression of the vacuolar Na^+/H^+ antiporter genes can lead to enhancing plant tolerance to salinity. The *Arabidopsis* vacuolar Na^+/H^+ antiporter mutant *nhx1* is more sensitive to salinity stress than its wild-type counterpart, and over-expression of *AtNHX1*

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Abbreviations: MDA, Malondialdehyde; **MS medium,** murashige and skoog medium; **PCR,** polymerase chain reaction; **FW,** fresh weight; **DW,** dry weight; **SOD,** superoxide dismutase; **POD,** peroxidase; **CAT,** catalase.

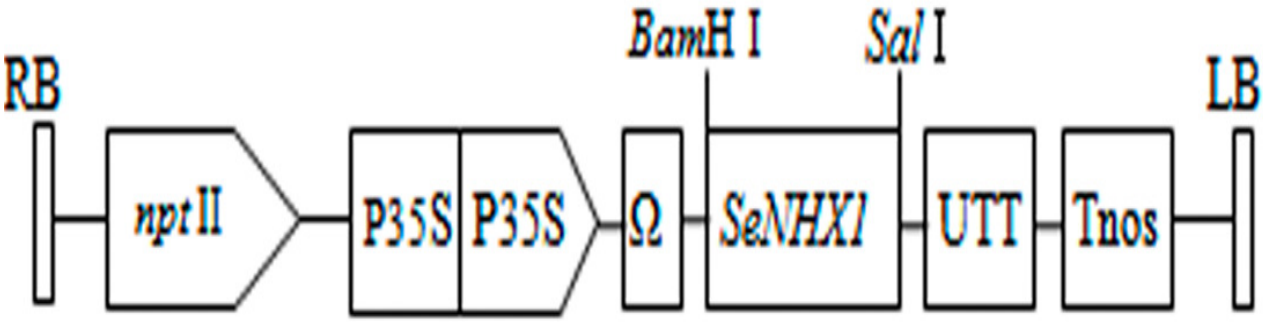

Figure 1. Schematic diagram of plant expression binary vector *T-DNA* region of *PBin438-SeNHX1*. *npt*II, neomycin phosphotransferase; P35S, cauliflower mosaic virus 35S promoter; Ω, TMV translation enhancer; UTT, termination sequence of transcription; Tnos, nopaline synthase terminator; RB and LB, right and left border.

complements the phenotype (Apse et al., 2003). *AtNHX1* transgenic tomato (Zhang and Blumwald, 2001) and *Brassica* plants (Zhang et al., 2001) are able to grow blossom and produce fruit in the presence of 200 mM NaCl. Similar results were previously obtained with transgenic rice, maize, wheat, and other crop plants (Ohta et al., 2002; Chen et al., 2007; Yin et al., 2004; Xue et al., 2004). These results suggested that the importance of vacuolar Na^+/H^+ antiporters to improve plants salinity tolerance is regulating ion homeostasis. This capacity of vacuolar compartmentalization can be taken as an adaptation mechanism to high salt environment of halophytes and glycophytes (Blumwald et al., 2000).

The *SeNHX1* gene (Se: *Salicornia europaea*) (GenBank Accession number AY131235) was previously cloned from typical halophyte, *S europaea* L. which is one of the most important leaf succulent euhalophytes without salt glands or salt bladders and can tolerate coastal seawater salinity and tidal inundation. The gene shares more than 70% sequence and structural similarity with *AgNHX*l and *SsNHX1* genes of halophytes, and more than 55% sequence and structural similarity with *OsNHX*l, *AtNHX*l and *TaNHX*l genes of glycophytes. Additionally, *SeNHX1* gene was confirmed as vacuolar Na^+/H^+ antiporters by amino acid sequence analysis and hydrophobicity analysis (Lv et al., 2003). Jha et al. (2010) investigated the function of *SbNHX*1 gene from *Salicornia brachiata*, which was 99% amino acid similar to *SeNHX1* gene. It was also found that *SbNHX*1-transgenic tobaccos exhibit higher fresh weight and chlorophyll content than wild type tobaccos when exposed to 200 mM NaCl in MS basal medium. However, there have been only few reports elucidating salt tolerance and the physiological responses of *SeNHX1* in the transgenic plants. In this study, we introduced the *S. europaea* L. vaculolar Na^+/H^+ antiporter *SeNHX1* into tobacco plants to investigate whether the salt tolerance of the transgenic tobaccos is increased. Furthermore, the effects of over-expressing of this gene on other physiological responses were studied.

MATERIALS AND METHODS

Binary plant expression vector construction and regeneration of tobacco plants

Agrobacterium tumefaciens (C58) carrying a binary vector *pBin438-SeNHX1* was used for transformation. *SeNHX1* was inserted between the *Bam*HI and *Sal*I sites of the plasmid *pBin438*, and the recombinant plasmid was named as *pBin438-SeNHX1* (Figure 1). The plasmid was introduced into *A. tumefaciens* C58 by electroporation. Then, *SeNHX1* was introduced into tobacco (*Nicotiana tabacum*) using the leaf disc transformation protocol (Horsch et al., 1985). Transformants were screened on Murashige and Skoog medium (MS medium), including 100 mg/l kanamycin and 500 mg/l cefotaxime, 30 g/l sucrose and 8 g/l agar, and were assayed by genomic DNA analysis and semi-quantifying RT-PCR.

Genomic DNA analysis and semi-quantifying RT-PCR

Genomic DNA was isolated from the leaf tissues of transgenic and non-transgenic tobacco (Murray and Thompson, 1980). For PCR analyses, primers used were 5'-GGATCCATGTTGTCACAA TTGAGCT-3' (forward) and 5'-GGGGTCGACCTATGTTCTGT CTAGC-3' (reverse), which amplified an about 1700 bp full-length fragment *SeNHX*1. PCR condition were 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 56°C for 30 s,72°C for 2 min and 72°C for 10 min.

Total RNA from leaf tissue of the putative transformants was extracted (RNeasy mini kit, QIAGEN) and used as templates for the cDNA synthesis (iScript cDNA Synthesis kit, BioRad). Semi-quantifying RT-PCR was carried out by specific primers: 5'-CACTGGTATCATTCTCCGTC-3' (forward) and 5'-GCGACAT-GACAATCCCACAGAAG-3' (reverse), which amplified about 500 bp linear range. PCR condition were 94°C for 3 min, followed by 27 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 1 min and 72°C for 10 min. 18S rRNA was used as control.

Salt treatment *in vitro*

The transformants with the relatively high, medium and low level of SeNHX1-expression were selected, transplanted to soil and grown in a greenhouse until harvest (greenhouse condition: 30 ± 2°C day/22 ± 2°C night, about 90% humidity for rooting and 60% humidity for growth, 14/10 h light/dark photoperiod, 800 μmol/m^2s photosynthetically active radiation). The T1 seeds were collected

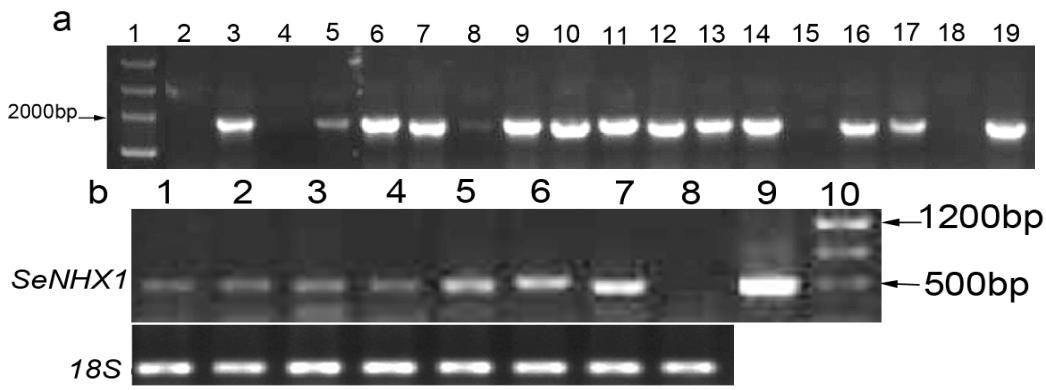

Figure 2. Electrophoresis of genomic DNA PCR and semi-quantifying RT-PCR products. (a) Genomic DNA PCR (about 1700 bp). lane 1, marker; lane 2, wild-type; lane 3, vector *pBin438-SeNHX1* as a positive control and lane 4 to 19, transformed plants. (b) RT-PCR (about 500 bp and 18S RNA as control). Lane 1, Se-1 transgenic plants; lane 2, Se-2 transgenic plants; lane 3, Se-3 transgenic plant; lane 4, Se-4 transgenic plants ; lane 5, Se-5 transgenic plants ; lane 6, Se-6 transgenic plants ; lane 7, Se-7 transgenic plants; lane 8, wild-type; lane 9, vector *pBin438-SeNHX1 as* a positive control and lane 10, marker.

and germinated on MS medium containing 100 mg/l kanamycin.

The survived T1 generation transgenic seedlings were used for salt treatment *in vitro*. Shoot tips and stem segments with 1 to 2 auxiliary buds were cut down from *SeNHX1*-transgenic and wild-type plants, and cultured in MS medium with 138 mM (8 g/l) or 172 mM (10 g/l) NaCl under light/dark cycle conditions of 16/8 h at 25°C. After 1 month, some growth characteristics including survival rate, rooting rate, leaf size and leaf color were observed and recorded. Eleven replicates of shoot tip and twenty-nine replicates of stem segment were performed for each treatment.

Salt treatment in pot soil

Uniformly developed T1 transgenic seedlings, which survived and rooted on MS medium containing 100 mg/l kanamycin, and non-transgenic seedlings were transplanted into 10 × 11 cm plastic pots with 150 g dry weight mixture soil of turf/vermiculite/perlite (50:25:25, by vol.), acclimated for 2 weeks in greenhouse. Fifteen days after the transplantation, the plants were watered weekly with 250 ml nutrient salt solution (1/10 strength MS medium salts) containing 100 mM NaCl (5.8 g/l). The 1/10 strength MS medium salts without NaCl was used as a control group. At the end, the Na^+ contents in pot soil were determined according to Volkov and Amtmann (2006) using a flame photometer (Corning Ltd, Essex, England).

Physiological responses under salt stress

The top young leaves were used for measuring contents of proline, soluble sugar and MDA after irrigation NaCl for 2, 5, 8 and 11 times (once every 6 days), respectively. Proline, MDA and soluble sugar content were determined (Monreal et al., 2007; Li et al., 2007; Dorion et al., 1996).

The top young leaves were also used for measuring contents of enzyme activities after watering NaCl for 0, 2 and 11 times. 0.5 g tobacco leaves were homogenized on ice and ground in 5 ml of 0.1M Tris-HCl buffer (pH 7.8) containing 1 mM DTT, 1 mM EDTA and 8% glycerol, and then centrifuged at 12,000 ×g for 10 min (4°C). The supernatants were collected and the activities of the superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) were measured (Donahue et al., 1997).

After 0, 2 and 11 times of watering with 1/10 MS medium containing 100 mM NaCl, about 1 g old and young leaves from each treatment were collected and digested with HNO_3-H_2O_2, respectively. The Na^+ content in leaves was measured using a flame photometer (Corning Ltd, Essex, England).

Statistical analysis

The data were subjected to analysis of variance using SPSS, mean comparisons were made using Fisher's LSD test ($P<0.05$ or $P<0.01$).

RESULTS

SeNHX1 gene expression in transgenic tobacco

The *SeNHX1* was introduced into tobacco (*N. tabacum*) by *A. tumefaciens*-mediated transformation under the control of double CaMV 35S promoter (35S to 35S). A series of transformations with kanamycin-resistance were identified by polymerase chain reaction (PCR) and SeNHX1 expression level was confirmed by semi-quantifying RT-PCR (Figure 2). As shown in Figure 2b, transcript level differed in different transgenic plants. Three transgenic plants with different expression levels (Se-2, Se-5 and Se-7) were selected and transplanted to grow in greenhouse for harvesting T1 seeds.

Over-expression of *SeNHX1* improves salt tolerance *in vitro*

The resistance of the transgenic plants to salt was confirmed by an *in vitro* culture of shoot tips and stem

Table 1. Effect of NaCl on "*in vitro*" explants development and growth of transgenic and wild type tobacco plants.

NaCl (mM)	Tissue type	Plant type	Survival rate (%)	Rooting rate (%)	Biomass (g/plant FW)
138	Shoot tips	WT	36.4	9.1	1.003 ± 0.203^{B}
		Se-7	100	90.9	4.105 ± 0.627^{A}
	Stem segments	WT	17.2	0	0.432 ± 0.165^{b}
		Se-7	82.8	18.2	1.194 ± 0.235^{a}
172	Shoot tips	WT	9.1	0	0.661
		Se-7	54.6	9.1	1.135±0.311
	Stem segments	WT	0	0	0
		Se-7	34.5	0	0.718±0.145

Tobacco plants were cultured in MS medium with 138 mM (8 g/l) or 172 mM (10 g/l) NaCl for one month, survival rate, rooting rate and plant biomass were counted. Survival rate = survived plant number / n and rooting rate=rooted plant number/n (n=11 for shoot tips and n =29 for stem segments). Values marked with the different letter within shoot tips or stem segments are significantly different at P≤0.01 (capital letter) or P≤0.05 (miniscule) by Fisher's LSD test. Values shown are means±SE (n=survived plant number in experiment).

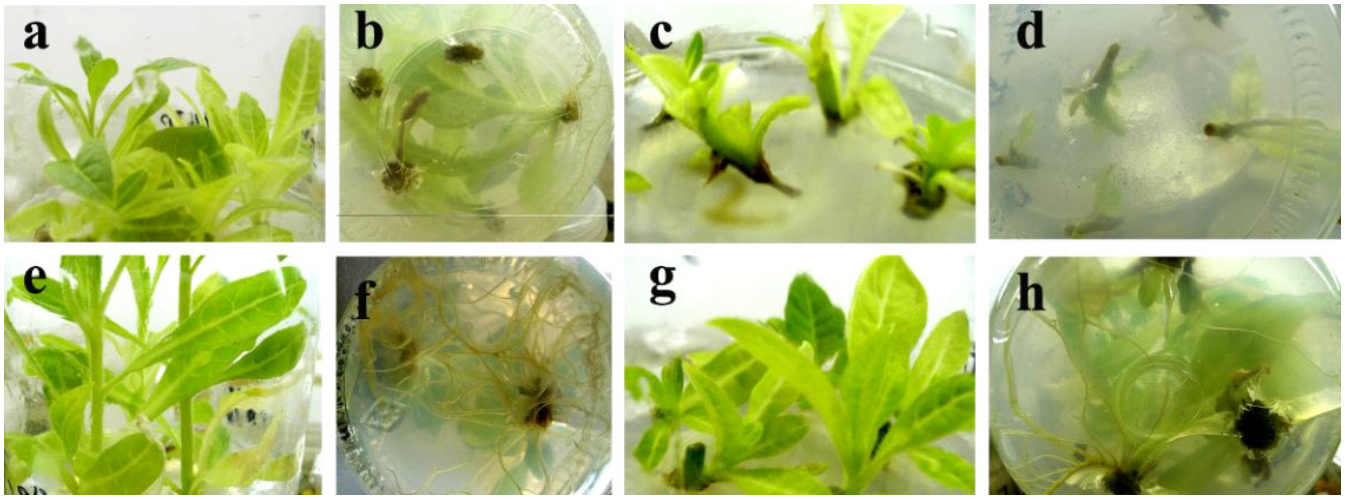

Figure 3. Effect of NaCl on *in vitro* explants shoots production and rooting of transgenic and wild type (WT) tobacco plants. Tobacco plants were cultured in MS medium with 138 mM NaCl for one month. Leaves developed from shoot tip explants of WT (a) and transgenic plants (e); rooting from shoot tip explants of WT (b) and transgenic plants (f); Leaves developed from stem segment explants of WT (c) and transgenic plants (g); rooting from stem segments explants of WT (d) and transgenic plants (h).

segments from T1 seedlings in MS medium with 138 mM or 172 mM NaCl. Wild type plants were used as control. Increased salt tolerance in transgenic plants was observed after one month (Table 1 and Figure 3). Cultured in MS medium with 138 mM NaCl, shoot tips from wild-type plants were developed into albino seedlings with low survival rate, rooting rate and biomass, while seedlings from *SeNHX1*-transgenic explants exhibited higher tolerance to NaCl with 100% survival rate, 90.9% rooting rate and normal leaf size and color. Seedlings from stem segments of *SeNHX1* transgenic plants displayed weaker resistance than shoot tips, but exhibited higher tolerance than wild-type counterpart. In MS medium with 172 mM NaCl, shoot tips and stem segments of transgenics which indicated only 54.6 and 34.5% survival rate, respectively, were still strongly resistant to salt than that from wild type plants (Table 1).

Transgenic plants reduce sensitivity to salt stress in pot culture

There were no obvious morphological differences between the wild-type and transgenic plants under non-salt treatment. When treated with 100 mM NaCl, both the wild type and transgenic plants exhibited chlorosis and growth inhibition (Figure 4). However, the growth of the former was affected more severely than that of the later under salt stress. As shown in Table 2 and Figure 4, a

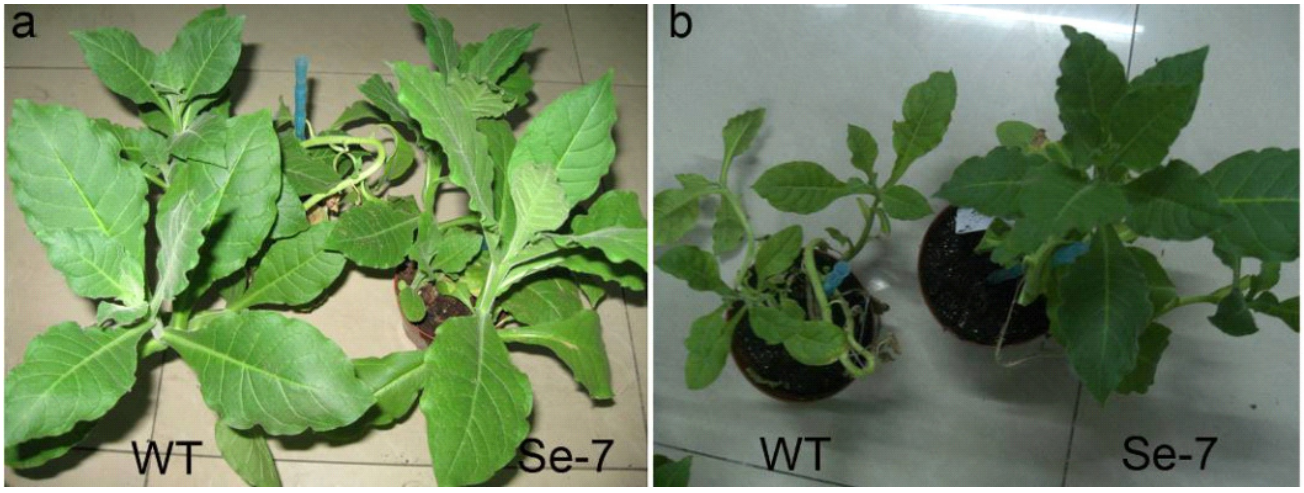

Figure 4. Effect of NaCl on growth of wild type (WT) and transgenic plants in pot culture. The age of plants was 60 days after transplanting,(a) wild type (WT) and transgenic plants (Se-7) under normal condition; (b) wild type (WT) and transgenic plants (Se-7) irrigation with 100 mM NaCl once every 6 days.

Table 2. Effect of NaCl stress on growth of wild-type and the transgenic plants in pot culture.

Treatment	Non-salt treatment		Salt treatment	
	Biomass (g/plant DW)	Plant height (cm)	Biomass (g/plant DW)	Plant height (cm)
WT	39.3 ± 3.1^{a}	98.1 ± 3 .7^{a}	10.3 ± 2.92^{b}	39.1 ± 2.8^{c}
Se-2	36.2 ± 2.6^{a}	100.4 ± 4.9^{a}	15.5 ± 1.5^{a}	45.8 ± 2.3^{b}
Se-5	40.5 ± 4.1^{a}	103.2 ± 5.3^{a}	16.8 ± 2.2^{a}	47.5 ± 3^{ab}
Se-7	37.5 ± 4.98^{a}	96.8 ± 2.2^{a}	17.6 ± 2.2^{a}	52.3 ± 2.1^{a}

Plants were treated with 100 mM NaCl for 11 times (once every 6 days), plant height and dry weight (DW) were determined after transplanting 75 days. Se-2, Se-5 and Se-7: transgenic plants in different level expression of SeNHX1. WT, wild-type plants. Values marked with the same letter within the same column are not significantly different at P≤0.05 by Fisher's LSD test. Values shown are means ±SE (n = 5).

remarkable reduction in whole-plant dry weight and height of the wild-type was observed.

Transgenic plants exhibit greater Na^+ amounts under salt stress

After irrigation with 100 mM NaCl for 11 times, there was average of 10.2 mg g^{-1} DW Na^+ in the pot soil, and no obvious difference between salt treatment groups. In contrast, only average of 1.58 mg g^{-1} DW Na^+ was found in the pot soil of the control groups.

Na^+ accumulation in leaves was assayed to test the difference between transgenic and wild-type plants. Na^+ contents in the transgenics and wild type were low and comparable under control condition (Figure 5). When treated with NaCl for 11 times, plants displayed at least 10-fold increase in Na^+ contents with the highest of 4.83 mg g^{-1} FW in old leaves of Se-7 plants and 2.87 mg g^{-1} FW in young leaves of wild type plants. Although, there was no significant difference in Na^+ content in young leaves between transgenic and wild-type plants under salt stress (Figure 5a), average Na^+ content in the old leaves of transgenic plants over-expressing *SeNHX1* was 1.2 mg g^{-1} FW higher than that of wild-type plants, which was significantly different (Figure 5b). These results imply that tobacco plants over-expressing *SeNHX1* accumulated more Na^+ in old leaves, keeping the young tissues out of toxicity.

Physiological responses in transgenic plants changed under salt stress

In order to study how over-expression of SeNHX1 in tobacco affected other physiological responses, antioxidant enzymes, MDA, proline and soluble sugar were measured. The activities of the antioxidant enzymes SOD and POD in both transgenic plants and wild-type plants are shown in Figure 6. When tobacco

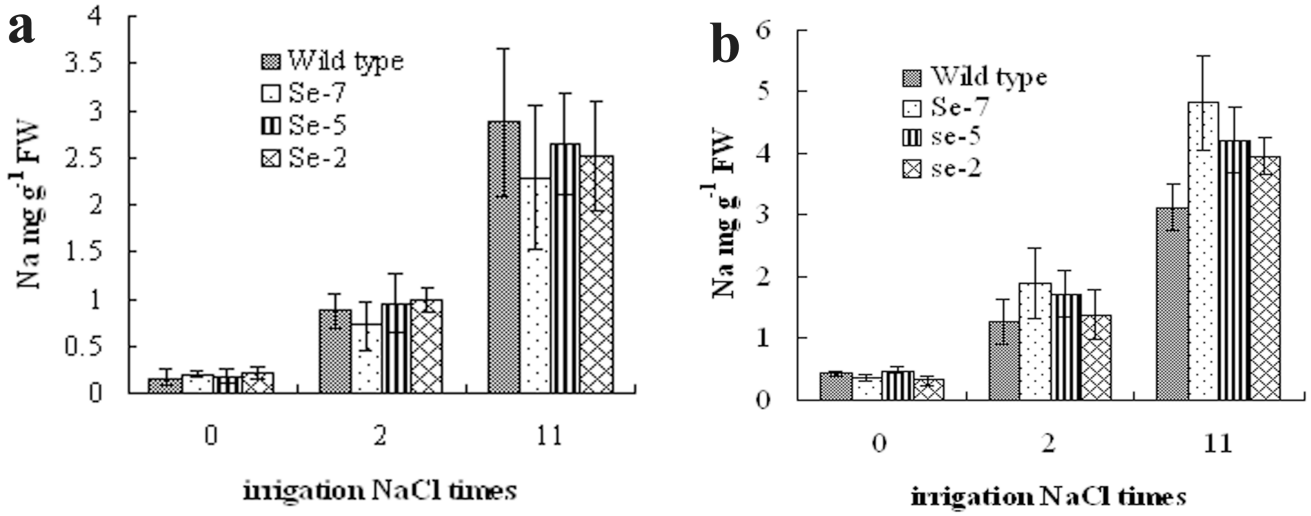

Figure 5. Effects of NaCl stress on Na^+ contents in leaves of wild-type and the transgenic tobacco plants. Tobacco plants were treated with 100 mM NaCl for 0, 2 and 11 times (once every 6 days), and Na^+ contents in young leaves (a) and old leaves (b) were determined after transplanting 80 days. Se-2, Se-5 and Se-7: transgenic plants in different expression level of SeNHX1. WT: wild-type plants. Values shown are means ± SE (n=5). We showed Na^+ concentration in leaves by FW, instead of DW, because a few samples in young leaf of wild-type were collected when wild-type plants were exposed to salt stress.

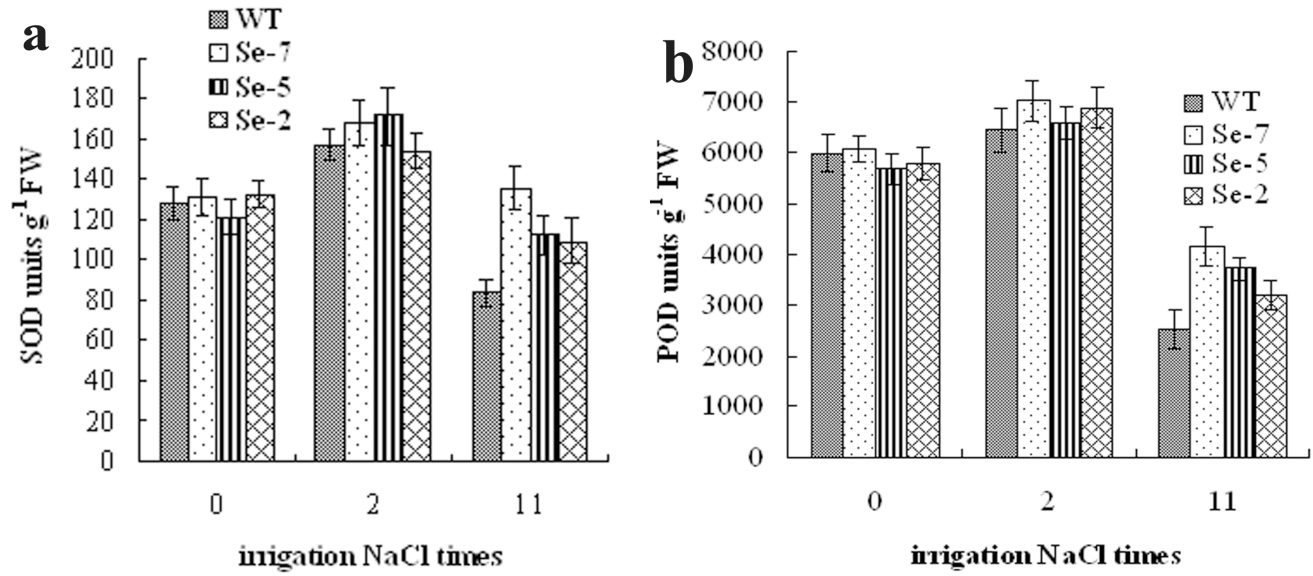

Figure 6. Effects of NaCl stress on activities of antioxidant enzymes in wild type and transgenic plants. Tobacco plants were treated with 100 mM NaCl for 0, 2 and 11 times (once every 6 days), and then activity of SOD (a) and activity POD (b) were determined after transplanting 75 days. Se-2, Se-5, Se-7: transgenic plants in different expression level of SeNHX1. WT: wild-type plants. Values shown are means ±SE (n = 5).

plants were exposed to low salt (watering NaCl for 2 times), the activities of SOD and POD increased and there was no striking difference between *SeNHX1*-transgenics and wild-type plants. Under 10.2 mg g^{-1} DW Na^+ stress (watering NaCl for 11 times), the activities of SOD and POD decreased, whereas obvious difference between *SeNHX1*-transgenics and wild-type plants was found. The activity of CAT was too low to be measured and not reported here.

The MDA contents were measured to investigate the effect of salt stress on lipid peroxidation. As shown in Table 3, the MDA content in the leaves of both transgenic plants and wild type enhanced with increase of irrigation NaCl times. When plants grew in normal condition or in

Table 3. Effect of NaCl stress on MDA, proline and soluble sugar content in leaves of *SeNHX1*-transgenics and wild-type plants.

Parameter	Treatment	Watering time				
		0	2	5	8	11
MDA (μmol g^{-1} FW)	WT	2.41 ± 0.21	2.79 ± 0.31	2.86 ± 0.38	4.25 ± 0.64^{a}	5.66 ± 0.46^{a}
	Se-2	2.37 ± 0.25	2.69 ± 0.35	2.71 ± 0.26	3.71 ± 0.19^{a}	4.79 ± 0.21^{b}
	Se-5	2.26 ± 0.37	2.53 ± 0.23	2.67 ± 0.31	3.46 ± 0.37^{ab}	4.31 ± 0.26^{bc}
	Se-7	2.52±0.26	2.74 ± 0.18	2.63±0.18	3.17 ± 0.27^{b}	4.19 ± 0.33^{c}
Proline (mg g^{-1}FW)	WT	0.83 ± 0.03	0.95 ± 0.03	1.07 ± 0.04	1.27 ± 0.08	1.63 ± 0.09^{a}
	Se-2	0.86 ± 0.06	0.92 ± 0.04	1.08 ± 0.03	1.35 ± 0.08	1.81 ± 0.07^{b}
	Se-5	0.91 ± 0.05	0.98 ± 0.04	1.11 ± 0.02	1.38 ± 0.09	1.85 ± 0.08^{b}
	Se-7	0.79 ± 0.08	0.91 ± 0.05	1.15 ± 0.05	1.47 ± 0.16	1.99 ± 0.12^{b}
Souble sugar (mg g^{-1} FW)	WT	5.9 ± 1.0	4.6 ± 0.7	5.8 ± 0.9	5.2 ± 0.9	5.3 ± 1
	Se-2	6.3 ± 0.7	5.2 ± 1.1	6.0 ± 0.8	4.9 ± 0.3	5.5 ± 0.6
	Se-5	5.1 ± 1.2	4.4 ± 0.9	5.7 ± 1	5.2 ± 0.7	5.0 ± 0.9
	Se-7	6.0 ± 0.9	4.8 ± 0.4	5.5 ± 0.4	5.1 ± 0.2	5.6 ± 0.4

Tobacco plants were treated with 100 mM NaCl for 0, 2, 5, 8, and11 times, respectively, and the plants were about 80 days after transplanting. The top young leaves were used for measuring contents of MDA, proline and soluble sugar. Se-2, Se-5 and Se-7: transgenic plants in different expression level of SeNHX1. WT, wild-type plants. Values marked with the different letter are significantly different at P≤0.05 by Fisher's LSD test. Values shown are means ± SE (n = 5).

low salt treatment, there was no significant difference in MDA content between *SeNHX1*-transgenics and wild-type plants. However, lower MDA content was observed in *SeNHX1*-transgenics under NaCl stress. This difference gradually increased and obvious difference was found when plants were treated with NaCl for 8 and 11 times. Moreover, under 10.2 mg g^{-1} DW Na^{+} stress, the MDA content in Se-7 transgenic plants expressing more SeNHX1 was significantly lower than that in Se-2 transgenic plants which expressed less SeNHX1. These results show that under sodium exposure, the transgenic plants and expressing more SeNHX1 are physiologically healthier than wild type.

Proline, one of the small osmoprotectant molecules, confers and reflects plant tolerance to various forms of abiotic stress. Our study indicates there was a rise of proline content both in *SeNHX1*-transgenic and wild-type plants under salt stress (Table 3), suggesting proline accumulated in response to salt stress. Proline content between *SeNHX1*-transgenics and wild-type plants is not significantly different before watering with NaCl for 8 times, but increment of proline in the transgenic plants was higher than that in wild-type plants. Especially, the plants were treated with NaCl for 11 times, the content of proline in transgenic plants was 0.25 mg g^{-1} higher on average than that of wild-type plants, which was significantly different.

Soluble sugar, another kind of osmotic compound, also seems to play an important role in resistance to abiotic stress. However, in this study, no significant difference was found neither between salt-stressed treatment and non-salt treatment nor between *SeNHX1*-transgenic and wild-type plants (Table 3).

DISCUSSION

When NaCl concentration around roots is approximately 40 mM, the growth of most plants was inhibited (Munns and Tester, 2008). In this study, adverse effects on wild type tobacco plants were observed (Tables 1 and 2; Figures 3 and 4) when tobacco explants of shoots and stem segments were exposed to 138 mM NaCl stress *in vitro* or tobacco seedlings were treated with 100 mM NaCl for 11 times in pot cultured, respectively. The deleterious effects of salinity on plant growth are associated with (1) low water potential of root medium which causes a water deficit within the plant; (2) toxic effects of ions-mainly Na^{+} and Cl^{-}; (3) nutritional imbalance caused by reduced nutrient uptake and transport to the shoot and (4) accumulation of reactive oxygen species, which lead to a reduction of antioxidant enzymes activities. In this study, Na^{+} content, activity of SOD and POD, proline, soluble sugar and MDA content were used as assessment for salt tolerance of transgenic plants. These parameters can reflect physiological state within plants under salt stress. For example, higher Na^{+} content in plant can promote water uptake and mitigate the deficit of water, but it is toxic to antioxidant enzymes and causes accumulation of reactive oxygen species. Reactive oxygen damages membrane lipid peroxidation which increases MDA content (Yamauchi, 2008), proline and soluble sugar are osmotic compound, which protect antioxidant enzymes and enhance their activity (Ashraf

and Foolad, 2007).

The concentration at which Na^+ becomes toxic is not well defined. *In vitro* studies showed that it started to inhibit most enzymes at concentrations approaching 100 mM Na^+. But concentrations well over 200 mM on a tissue basis are common (Arzani, 2008; Munns and Tester, 2008). In this case, vacuolar Na^+/H^+ antiporter in transgenic plants played important role in compartmentalization of Na^+ in the vacuoles to maintain low Na^+ in the cytosol (Zhu, 2003). In our studies, SeNHX1 was localized on the vacuolar membrane by SeNHX1-GFP fusion protein expressed in *Saccharomyces cerevisiae* cells and fluorescence observation (data not shown). We have also indicate that the Na^+ concentration in old leaves of Se-7 transgenics was 4.83 mg g^{-1} FW, which equaled to 210 mM if 1 g FW was taken as 1 g water, (Figure 5). We speculate *SeNHX*1-transgenic plants could get more inorganic ion for osmotic adjustment to maintain water uptake. Higher Na^+ content in plant is toxic to antioxidant enzymes. However, in comparison with wild type plants upon same salt treatment, higher activities of SOD, POD, as well as lower MDA content was found in transformants (Figure 6 and Table 3). Therefore, these results perhaps support the idea that *SeNHX1*-transgenic plants obtain the enhanced ability of salt-tolerance by efficiently sequestering excessive Na^+ into vacuole and reduce Na^+ toxicity to the cytoplasm.

The accumulation of proline and soluble sugar in response to high salinity were well documented (Liu and Zhu, 1997; Zhao et al., 2006). Zhang et al. (2001) reported that compared to wild-type tomato, 3-fold increase of proline content in *AtNHX1* transplants was found in leaves and no significant difference of soluble sugar was observed. In this study, over-expression of *SeNHX1* resulted in a similar change in proline and soluble sugar content (Table 3). So we thought antioxidant enzymes in *SeNHX1*-transgenic plants were protected by higher content of proline. Meanwhile, the result implies that proline, rather than soluble sugar, might contribute to osmotic adjustment in *SeNHX1*-transgenic plants.

Tolerance to salt was improved in transgenic plants expressing the vacuolar Na^+/H^+; *juncea* with *AgNHX1* showed higher resistance to Na^+ than the wild-type (Rajagopal et al., 2007). However, earlier reports revealed the transgenic plants with higher salt-resistance by irrigating 200 mM or 300 mM NaCl for a few times, and were unclear how much the Na^+ content was accumulated in the soil. Here, our data show *SeNHX1*-transgenic tobacco plants grew better than wild type plants in pot soil with 10.2 mg g^{-1} DW Na^+ (Table 2 and Figure 4) and we are the first to report the detailed Na^+ content in the soil.

In vitro culture, besides its use as a tool for obtaining salt tolerant plants, may offer potential for quick evaluation against salt stress (Arzani, 2008). Cano et al. (1998) evaluates salt tolerance of tomato through *in vitro* shoot apex culture. *In vitro* root growth is found to be more adversely affected than leaf growth by an increasing supply of NaCl. In this study, root and leaf growth were inhibited by salt, and both parameters were useful traits for the assessment of salt tolerance of transgenic plants. Our result also shows the *SeNHX1*-transgenic plants *in vitro* improve the salt-tolerance (Table 1 and Figure 3).

In conclusion, *SeNHX1*-transgenic plants can improve salt resistance in both *in vitro* and pot culture. The physiological mechanism for salt-tolerance includes: Accumulating Na^+ in old leaves to promote water uptake and pumping Na^+ into vacuole for detoxification.

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