



***Salicornia europaea* L. Na⁺/H⁺ antiporter gene improves salt tolerance in transgenic alfalfa (*Medicago sativa* L.)**

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Genet. Mol. Res. 13 (3): 5350-5360 (2014)

Received May 27, 2013

Accepted October 30, 2013

Published July 24, 2014

DOI <http://dx.doi.org/10.4238/2014.July.24.14>

ABSTRACT. In order to obtain a salt-tolerant perennial alfalfa (*Medicago sativa* L.), we transferred the halophyte *Salicornia europaea* L. Na⁺/H⁺ antiporter gene, *SeNHX1*, to alfalfa by using the *Agrobacterium*-mediated transformation method. The transformants were confirmed by both PCR and RT-PCR analyses. Of 197 plants that were obtained after transformation, 36 were positive by PCR analysis using 2 primer pairs for the *CaMV35S-SeNHX1* and *SeNHX1-Nos* fragments; 6 plants survived in a greenhouse. RT-PCR analysis revealed that *SeNHX1* was expressed in 5 plants. The resultant transgenic alfalfa had better salt tolerance. After stress treatment for 21 days with 0.6% NaCl, the chlorophyll and MDA contents in transgenic plants were lower, but proline content and SOD, POD, and CAT activities were higher than those in wild-type plants. These results suggest that the salt tolerance of transgenic alfalfa was improved by the overexpression of the *SeNHX1* gene.

Key words: *Medicago sativa*; *Salicornia europaea*; Transgenic alfalfa; Na⁺/H⁺ antiporter gene; Salt tolerance

INTRODUCTION

Salinity is the most widespread and adverse condition affecting approximately one-fifth of irrigated agricultural land. High salt levels increase the pH of soil, and most crop plants do not grow well at high pH (Nawaz et al., 2010). Thus, the productivity of agricultural crops and grasses is generally limited by salinity.

Salinity results in cellular ionic imbalances, which can lead to ion toxicity (Mittler, 2002). In plants, the Na^+/H^+ antiporter in the plasma and vacuolar membrane pumps excessive Na^+ from the cytosol or compartmentalizes it into tonoplasts to maintain higher K^+/Na^+ ratios in the cytoplasm, protecting cells from sodium toxicity (Fukuda et al., 1999; Xia et al., 2002). Na^+/H^+ antiporters are ubiquitous membrane proteins that mediate Na^+ homeostasis and catalyze the exchange of Na^+ for H^+ across membranes (Apse et al., 1999).

Recently, many Na^+/H^+ antiporter genes have been identified and characterized in various plant species, including *Arabidopsis thaliana* (Apse et al., 1999), *Oryza sativa* (Fukuda et al., 1999), alfalfa (An et al., 2008), *Salsola soda* (Li et al., 2011), *Chrysanthemum crassum* (Song et al., 2012), *Cucumis sativus* (Wang et al., 2013), and *Zea mays* (Pitann et al., 2013). The primary function conferring salt tolerance of these genes had been manifested by transgenic events in many plants (Asif et al., 2011; Tian et al., 2011; Uliiaie et al., 2012; Jiang et al., 2012; Yarra et al., 2012). These transgenic events have clearly shown the feasibility of breeding salt-tolerant crops by using the Na^+/H^+ antiporter genes.

Alfalfa, a perennial and outcrossing species, is one of the most valuable forage crops with high-protein content and biomass production and has been widely cultivated as an economic crop worldwide. However, it is moderately tolerant to salinity and can only be cultivated in moderate salt-alkaline soils, which limit the growth range of alfalfa (Suárez et al., 2009; Li and Brummer, 2012; Zhang et al., 2012). The expression of foreign genes, such as *GmDREB1*, *SsNHX1*, *AhBADH*, and *TaNHX2*, increases salt tolerance in transgenic alfalfa plants compared with that of wild-type plants (Jin et al., 2010; Li et al., 2011; Liu et al., 2011; Zhang et al., 2012). These results suggest the possibility of introducing salt stress-resistance genes to improve the tolerance of alfalfa to salinity stress. In this study, we introduced *SeNHX1* into a commercial alfalfa cultivar, Algonguin, by using *Agrobacterium*-mediated transformation and showed that the transgenic progenies have markedly improved tolerance to salt.

MATERIAL AND METHODS

Vector construction

A recombinant expression vector that contained *SeNHX1* was constructed under the control of the *CaMV35S* promoter (Figure 1). *SeNHX1* was amplified by polymerase chain reaction (PCR) from pBI121-Se (Zhou et al., 2008) by using primers 5'-CGTCTAGAAATGTTGTCACAATTGAGCTC-3' and 5'-GCGGTACCCTATGTTCTGTCTAGATTG-3', which contain *Xba*I and *Kpn*I sites (underlined), respectively. After digestion with *Xba*I and *Kpn*I was performed, the *SeNHX1* fragment was cloned into the binary vector pPZP221 (*CaMV35S::Nos*) to generate pPZP221 (*CaMV35S::SeNHX1::Nos*), which carries the gentamycin-resistance gene *aacC1* as a selectable marker (Hajdukiewicz et al., 1994).

Generation of alfalfa transformants

Seeds of the alfalfa cultivar Algonquin were surface-sterilized with 70% alcohol for 5 min and 0.1% HgCl₂ for 10 min and washed twice with sterile distilled water. The transformation with cotyledonary node as explants by *Agrobacterium* was performed according to Zhou et al. (2004). The *A. tumefaciens* strain AGL1, containing pPZP221 (*CaMV35S::SeNHX1::Nos*), was used for the transformation. Well-developed resistant shoots (approximately 1.5 cm) separated from explants developed roots into plantlets on SH medium (Schenk and Hildebrandt, 1972), containing 0.5 mg/L IBA and 10 mg/L gentamycin. Finally, plantlets that were higher than 5 cm were transplanted into plastic pots that contained a mixture of vermiculite, turf, and hums (1:1:1, v/v/v) and grown in a greenhouse under natural light at 25°C.

Molecular analysis

PCR analysis of transformed plants

The insertion of the *SeNHX1* gene in the genomic DNA of transformed alfalfa plants was identified by PCR analysis by using 2 primer pairs (Figure 1 and Table 1). Template DNA was isolated from transformed and untransformed plants by using a modified phenol and chloroform method.

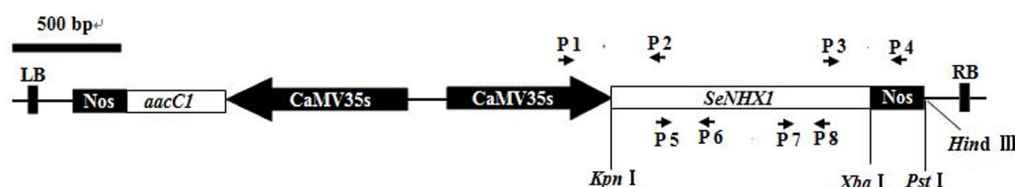


Figure 1. Schematics of the T-DNA region of the binary vector pPZP221 (*CaMV35S::SeNHX1::Nos*) and primer landing sites. The components contained the *CaMV35S* promoter, the *SeNHX1* gene, the *nos* terminator, and the *aacC1* gene, with T-DNA left and right borders (LB and RB). Primers P1-P4 were used for PCR analysis and primers P5-P8 were used for RT-PCR analysis.

Table 1. Primer pairs used to molecular analysis.

Primer	Sequence (5'-3')	Fragment length (bp)	Application
P1	ACAGAACTCGCCGTAAAGAC	670	PCR analysis
P2	CCAATTAGTAAAGCGGTGATG		
P3	ATCAGTTTACGAGGTCAGGG	629	PCR analysis
P4	AAAACCCATCTCAIAAATAACG		
P5	CGACCTCACGAATATTGATCACAG	394	RT-PCR analysis
P6	TCTCCACTTCTCAATGTCCAATGC		
P7	TTCATGAGAGGTGCTGTTCC	311	RT-PCR analysis
P8	CATAGCGGTTGTTGACTGCTTC		
Actin1	CTGCCATGTATGTGGCCATCCAGGC	434	Internal control
Actin2	AATCCAGCAGCTTCCATTCCGATC		

RT-PCR analysis of transformed plants

RT-PCR was performed using total RNA that had been pretreated with RNase-free

DNase I. The total RNA was isolated from the leaves of untransformed and transgenic plants by using the RNAiso™ plus kit (TaKaRa, Dalian, China), and first-strand cDNA was synthesized from 2 g total RNA by using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to manufacturer instructions. PCR was performed using 2 primer pairs (Figure 1 and Table 1), which were designed by comparing *SeNHX1* (GenBank: AY131235) and *MsNHX1* (GenBank: AY513732). The actin gene (GenBank: XM003602498) was amplified as an internal control.

Salt stress treatment of transgenic plants

Whether the overexpression of *SeNHX1* conferred resistance to salt stress was determined by selecting T₀ transgenic plants (lines MSeNHX24, MSeNHX43, MSeNHX72, MSeNHX75, and MSeNHX86) as test materials. When the plants grew to a height of approximately 20 cm, they were watered with NaCl solution and maintained for 21 days, resulting in 0.6% NaCl in the soil mixture. The control consisted of treatment with wild-type plants.

Determination of chlorophyll, malondialdehyde, and proline content

Chlorophyll and proline contents were measured using a spectrophotometer (DU640; BECKMAN, USA) according to Zhang and Zhai (2006) and Bates et al. (1973). The malondialdehyde (MDA) content was measured by the thiobarbituric acid reaction (Li et al., 2000).

Enzyme extraction

Enzymes were extracted by homogenizing 1.5 g fresh leaves in an ice bath (4°C) with 50 mM sodium phosphate buffer, pH 7.0, containing 1 mM ethylenediaminetetraacetate and 4% polyvinylpyrrolidone (w/v). The homogenates were centrifuged at 12,000 g for 20 min at 4°C, and the supernatants were collected and assessed for enzyme activity.

Superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) activity analysis

SOD activity was measured by monitoring the inhibition of the photochemical reduction of nitro-blue tetrazolium at 560 nm (Giannopolitis and Ries, 1977). POD activity was assayed by the guaiacol colorimetric method (Li et al., 2000). CAT activity was assayed by measuring the decrease at 240 nm for 1 min, due to H₂O₂ consumption (Zhang and Yin, 2009).

Salt treatment of transgenic progeny plants

The seeds of T₁ transgenic progenies were planted in plastic pots and grown in a greenhouse under natural light at 25°C. Three-week-old seedlings were treated with NaCl solution for 21 days, resulting in 0.6% NaCl in the soil mixture.

Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA) using SAS to

detect significant differences. Results are reported as means \pm SD for $N = 3$ plants per group (for at least 3 independent experiments). Differences between treatments were separated by least significant difference (LSD) test at a probability level of 0.05.

RESULTS

Identification of transgenic alfalfa plants

SeNHX1 was transformed into the alfalfa genome by *Agrobacterium*-mediated method (Figure 2a-d). Of the 197 surviving plants, 36 plants expressed the diagnostic band by PCR analysis (Figure 3A and B), and 6 plants grew vigorously in the greenhouse (Figure 3C). RT-PCR analysis suggested that *SeNHX1* was expressed in 5 plants (Figure 4).

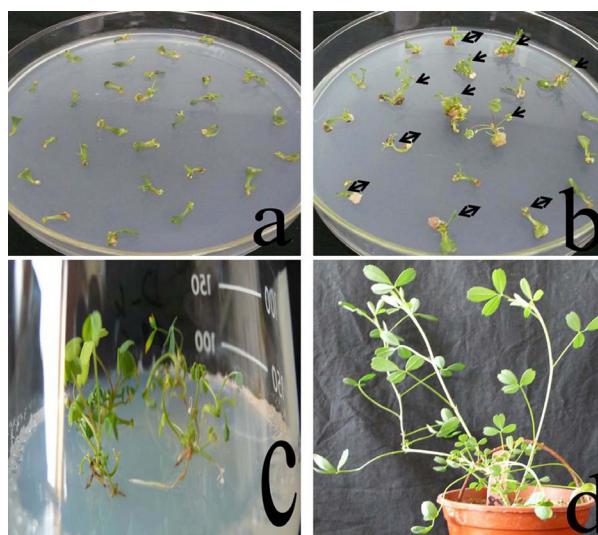


Figure 2. Regeneration of *SeNHX1*-transformed plants by *Agrobacterium*-mediated method, by using the cotyledonary node as explants. **a.** Explants incubated for 15 days on regeneration medium. **b.** Selection of regenerating plant shoots on selection medium containing 20 mg/L gentamycin. Green, resistant shoots are indicated by arrows. Brown, nontransformed shoots are indicated by double arrows. **c.** Plantlets with developing roots in flasks. **d.** Transplanted plant in the greenhouse.

Chlorophyll, MDA, and proline content

Salinity stress reduced the chlorophyll content of transgenic alfalfa plants. The amounts of chlorophyll determined are shown in Figure 5A. The chlorophyll content of transgenic plants was lower than that of the wild-type plant ($P < 0.05$), and the difference between transgenic plants, except for MSeNHX24, was also significant ($P < 0.05$). The amounts of chlorophyll decreased by 4.4, 5.1, 0.8, and 18.5% in transgenic plants MSeNHX43, MSeNHX72, MSeNHX75, and MSeNHX86, respectively, which were significantly lower than those in the wild-type plants (24.4%), indicating that the effects of salt stress on chlorophyll content in transgenic alfalfa plants were less extensive than in the wild-type plants. MDA

and proline contents in the transgenic plants increased under salt stress compared with those in the wild-type plants (Figure 5B and C). The increase in MDA content in the transgenic alfalfa plants by salt treatment, except for MSeNHX86, was more significant, increasing from 3.7 to 12.9% compared with 47.3% in the wild-type plants. Proline content also significantly increased by 2.3-, 4.1-, 0.7-, 0.7-, and 4.2-fold in transgenic plants MSeNHX24, MSeNHX43, MSeNHX72, MSeNHX75, and MSeNHX86, respectively, compared with a 0.57-fold increase in the wild-type plants.

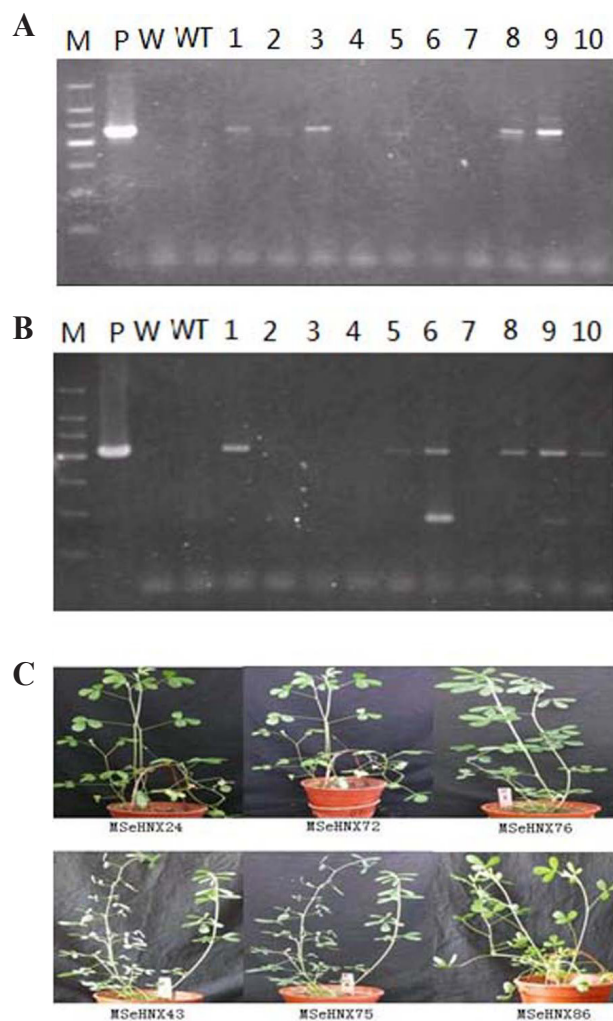


Figure 3. PCR analysis of T_0 transgenic alfalfa plants. **A.** Primers P1/P2 analysis for the *CaMV35S-SeNHX1* fragment with a 670-bp PCR product. **B.** Primers P3/P4 for the *SeNHX1-Nos* fragment with a 629-bp PCR product. The specific PCR products were detected in 36 plants among 197 surviving alfalfa plants. **C.** Six PCR-positive transgenic alfalfa plants survived in the greenhouse. Lane *M* = 150-bp ladder (TaKaRa), with fragment sizes of 12,000, 900, 750, 600, 450, 300, and 150 bp from top to bottom; lane *P* = pPZP221*CaMV35S::SeNHX1::Nos* plasmid; lane *W* = H_2O as blank control; lane *WT* = wild-type plant; lanes 1-10 = T_0 transgenic plants.

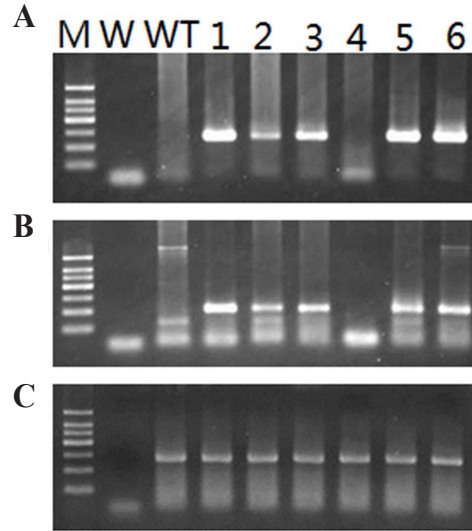


Figure 4. RT-PCR analysis of *SeNHX1* transcripts in survived T_0 transgenic alfalfa plants. **A.** Primers P5/P6 analysis with a 394-bp PCR product. **B.** Primers P7/P8 analysis with a 311-bp PCR product. The specific PCR products were detected in the 5 transgenic lines MSeNHX24, MSeNHX43, MSeNHX72, MSeNHX75, and MSeNHX86 (lanes 1-3, 5, and 6). **C.** A 434-bp actin fragment was amplified by RT-PCR as an internal control. Lane M = 150-bp ladder (TaKaRa); lane W = H_2O as blank control; lane WT = wild-type plant; lanes 1-6 = survived T_0 transgenic plants.

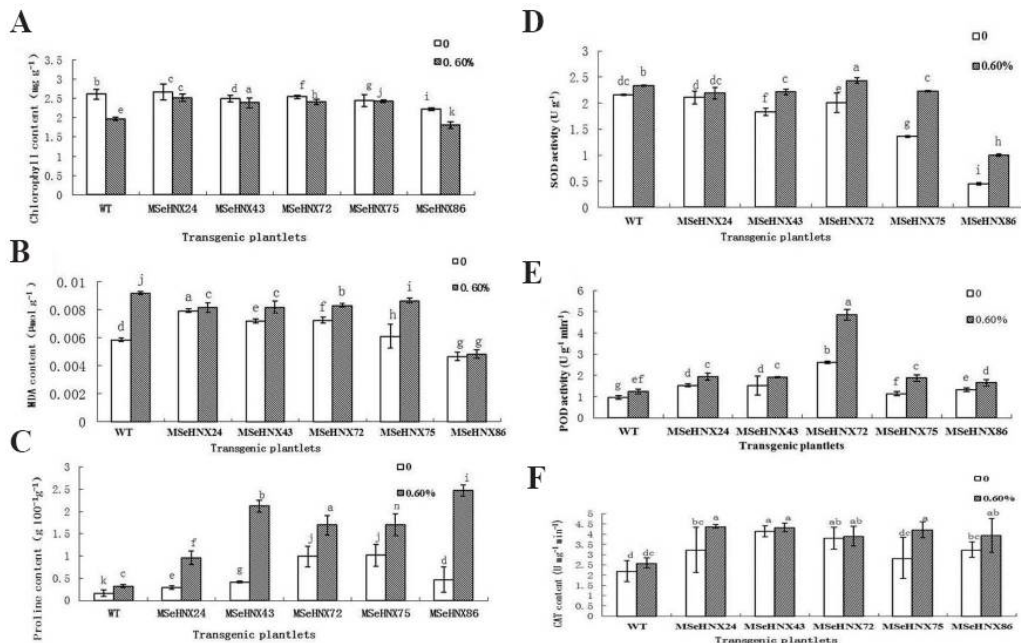


Figure 5. Change in the amounts of chlorophyll, MDA, and proline, and effects on SOD, POD, and CAT activities in wild-type and T_0 transgenic alfalfa plants under 0 and 0.60% NaCl treatment for 21 days. WT = wild-type plant. Data are reported as means \pm SD (N = 3). Letters indicate significant difference at $P < 0.05$ by the LSD test.

Antioxidant enzyme activity

After the salt stress treatment, SOD, POD, and CAT activities increased in all alfalfa plants (Figure 5D-F). The transgenic alfalfa plants had significantly higher antioxidant enzyme activities than the wild-type plants, and differences were significant, except for MSeNHX24 with regard to SOD activity. SOD activity increased by 123.1% in transgenic plant MSeNHX86 and by over 20% in the other transgenic plants, except for MSeNHX24, versus 8.2% in the wild-type plants. The POD activity of transgenic plants was enhanced without salt stress and was higher than that of the wild-type plants under salt stress. Except for MSeNHX24 and MSeNHX75, there was no difference in CAT activity before and after the salt stress. After the salt stress, CAT activity of the transgenic plants MSeNHX24 and MSeNHX75 increased by 35.2 and 48.2%, respectively, vs 18.2% in the wild-type plants.

Growth of transgenic progeny under salt stress

Three-week-old MSeNHX24 and MSeNHX75 seedlings treated with 0.6% NaCl for 21 days (Figure 6a and b) showed vigorous growth in plastic pots. However, the wild-type and other transgenic plants wilted under the same treatment. Surviving transgenic progeny seedlings were transplanted to plastic pots and grown in a greenhouse under natural light at 25°C for 1 month (Figure 6c and d).

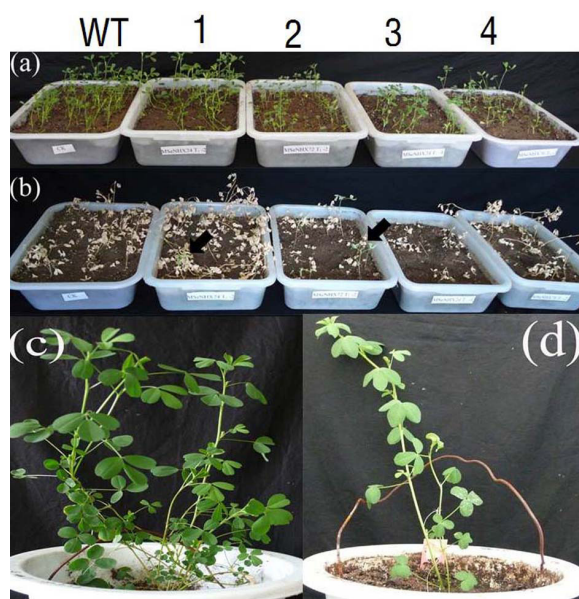


Figure 6. Salt stress test of T_1 transgenic alfalfa plants treated with 0.6% NaCl. **a.** Transgenic line and wild-type plants in plastic pots before salt stress treatment. **b.** Transgenic line and wild-type plants in plastic pots after salt stress treatment for 21 days. Transgenic MSeNHX24 and MSeNHX75 with vigorously growing seedlings are indicated by arrows. **c.** Surviving seedlings of MSeNHX24 transplanted to plastic pots and grown in a greenhouse. **d.** Surviving seedlings of MSeNHX75 transplanted to plastic pots and grown in a greenhouse. Photo obtained 1 month after transplantation. WT = wild-type plant. 1 = MSeNHX24; 2 = MSeNHX75; 3 = MSeNHX72; 4 = MSeNHX86.

DISCUSSION

Na⁺ compartmentation, driven by vacuolar Na⁺/H⁺ antiporter, is an economical means of preventing Na⁺ toxicity in the cytosol, because Na⁺ can be used as an osmolyte in the vacuoles to achieve osmotic homeostasis - a strategy on which many halophytes rely (Li et al., 2011).

Recently, many vacuolar Na⁺/H⁺ antiporter genes have been isolated and identified, and previous studies have shown that the overexpression of vacuolar Na⁺/H⁺ antiporters enhances the survival of plants under salt stress. For example, transgenic *A. thaliana*, groundnut (*Arachis hypogaea*), kiwifruit (*Actinidia deliciosa*), and poplar plants with *AtNHX1* grew normally in 200 mM NaCl (Apse et al., 1999; Asif et al., 2011; Tian et al., 2011; Jiang et al., 2012). Alfalfa and tomato that overexpressed *TaNHX2* showed improved tolerance to salt (Zhang et al., 2012; Yarra et al., 2012). *S. europaea* is an annual species of halophyte that can tolerate extreme salinity (Riehl and Ungar, 1982). *SeNHX1* was transformed into tobacco, and transgenic plants were found to be tolerant to 200 mM NaCl stress (Zhou et al., 2008). In our study, *SeNHX1* transgenic alfalfa had significant salt tolerance under 0.6% NaCl stress for 21 days (Figures 5 and 6). Two transgenic lines, MSeNHX24 and MSeNHX75, showed better growth than the wild-type and other transgenic plants under salt stress. Thus, these lines have the potential to breed salt-resistant alfalfa.

Soil salinity affects many physiological processes, including photosynthesis and membrane function. Chlorophyll content has been implicated as a parameter of salt tolerance in crop plants (Sairam and Srivastava, 2002). Our data indicate that treatment with 0.6% NaCl reduces chlorophyll content in wild-type plants to a greater extent than in transgenic alfalfa plants (Figure 5A).

MDA is a decomposition product of polyunsaturated fatty acids of membranes under salt stress; thus, MDA content reflects the tolerance of plants to salt stress (Wang and Han, 2009). MDA content was significantly increased in wild-type and transgenic alfalfa plants under salt stress, except in MSeNHX86 (Figure 5B); however, the MDA content was lower in transgenic alfalfa than in wild-type alfalfa, indicating less damage to cell membranes by salt stress.

Proline accumulates in higher plants in response to salinity stress and mediates osmotic adjustments, and proline accumulation contributing to salt tolerance in alfalfa was reported by Wang and Han (2009). We also measured proline concentration in transgenic and wild-type alfalfa plants under salt stress. Greater accumulation of proline content was observed in transgenic alfalfa plants versus wild-type plants ($P < 0.05$; Figure 5C), indicating enhanced osmotic adjustment in transgenic alfalfa plants under salt stress.

Salinity can induce the generation of reactive oxygen species in plants, which inhibits plant growth and development (Li et al., 2011). The alleviation of oxidative damage often correlates with an efficient antioxidative system under NaCl stress (Smirnov, 1998). By analyzing antioxidases with regard to SOD, POD, and CAT activities, we observed that such activity in transgenic alfalfa plants was higher than that in wild-type plants. Thus, the salt tolerance of transgenic alfalfa plants improved through the development of antioxidase activity.

In conclusion, the exogenous gene *SeNHX1* was transformed into alfalfa by *Agrobacterium*-mediated transformation. Overexpression of *SeNHX1* in transgenic alfalfa plants enhanced their tolerance to salt through improved photosynthesis and membrane protection, as revealed by chlorophyll and MDA content, osmotic adjustment, and scavenging of free

radicals by the increase in proline content and SOD, POD, and CAT activities. MSeNHX24 and MSeNHX75 T₁ transgenic alfalfa could be used in alfalfa-breeding programs for salt stress tolerance as resistant resources.

ACKNOWLEDGMENTS

We are grateful to Prof. Yinxin Li (Institute of Botany, Chinese Academy of Science) for providing the pBI121-Se plasmid (containing *SeNHX1* gene). Research supported financially by the Natural Science Foundation of Inner Mongolia, China (Grants #2011ZD03, #200607010503) and the Sciences Research Project for Colleges and Universities of Inner Mongolia Autonomous Region, China (Grant #NJZY12003).

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