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Overexpression of *HvNHX2, a* vacuolar Na^+/H^+ antiporter gene from barley, improves salt tolerance in *Arabidopsis thaliana*

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Abstract

One possible mechanism to avoid Na+ toxicity in the cytosol is compartmentalization of Na+ ions away from the cytosol. In the present work transgenic *Arabidopsis* plants overexpressing *HvNHX2*, a vacuolar Na+/H+ antiporter gene from barley, were generated using *Agrobacterium tumefaciens* transformation. We show that these plants are able to grow normally in presence of 200 mM NaCl, while wild type *Arabidopsis* plants showed necrosis. When grown on MS medium containing NaCl, transgenic plants overexpressing *HvNHX2* accumulated more Na+ in the shoots, and had longer roots in the early seedling stage. These results show that improved salt tolerance could be achieved by compartmentalization of Na+ in the vacuoles.

Keywords: *Arabidopsis thaliana*, *Agrobacterium*-mediated transformation, salt tolerance and vacuolar antiporter.
Abbreviations: wt-wild type.

Introduction

Salt tolerance is a complex trait that is controlled by multiple genes and numerous biochemical and physiological mechanisms. To improve salt tolerance of plants genetically, different genes involved in specific mechanisms must be combined to substantially increase tolerance. Intracellular K+ and Na+ homeostasis is fundamental to the physiology of living cells and maintaining this homeostasis under salt stress conditions is essential for survival. Therefore, any mechanism that allows a plant to maintain desirable K+/Na+ ratios in cytosol may contribute to improved salinity tolerance. Under salt stress, the Na+ concentration in plant cells is expected to increase to toxic levels. Three mechanisms are known that may help plant cells to prevent excess Na+ accumulation in the cytosol. Firstly, control of sodium uptake. So far, components that control Na+ influx across plant plasma membranes have not been identified. Under salinity Na+ enters the root cell cytosol either through selective/non selective channels and transporters or through the root xylem stream via an apoplastic pathway. So, improving salt tolerance at this level may be limited to improving selectivity of essential cation (K+)/channels (Chinhusamy et al., 2005). Secondly, stimulation of efflux of Na+ from the cytoplasm back to the external medium or the apoplast via plasma membrane Na+/H+ antiporters. The role of cellular efflux of Na+ is not intuitive in multicellular plants, as Na+ transported out of one cell would be a problem for neighboring cells, so the role of Na+ efflux has to be considered in specific tissues and in the context of the whole plant (Zhu, 2003). Thirdly, sequestration of accumulated Na+ from the cytosol into vacuoles through vacuolar Na+/H+ antiporters. Moreover, the compartmentalization of Na+ into the vacuole allows the plants to use Na+ as an osmoticum, maintaining an osmotic potential that drives water into the cells. V-type H+-ATPase and H+-PPase generate the necessary proton gradient required for activity of Na+/H+ antiporters (Gaxiola et al., 2002). Increased salinity tolerance of a range of plant species overexpressing *NHX* genes (Apse et al., 1999; Zhang and Blumwald, 2001; Zhang et al, 2001; Fukuda et al., 2004; Yin et al., 2004) indicates the feasibility of such a mechanism. The use of *Arabidopsis* as a molecular genetic model system in abiotic stress research has facilitated the identification of numerous salt adaption genes using loss- or gain-of-function approaches (Kasuga et al., 1999; Bressan et al., 2001; Apse and Blumwald, 2002, Zhu, 2002, 2003). In this study, effect of vacuolar antiporter gene *HvNHX2* from barley was studied in *Arabidosis* and analysis of salt stress tolerance of the transgenic lines indicates that this approach is promising for improving salt tolerance.

Materials and methods

Generation of transgenic Arabidopsis plants

The full-length *HvNHX2* open reading frame (ORF) was cloned into *Bam*HI and *Sac*I sites of the pBI121 binary vector (Clontech, USA). The resulting plasmid was introduced into *Agrobacterium* strain AGL0 and used for plant transformation. Five-week-old *Arabidopsis* plants were infected with the *A. tumefaciens* by floral dipping method (Clough and Bent, 1998) and grown in a growth room under long days (16/8 Day/Night) at 22-24˚C to induce flowering and T1 seeds were collected. Transgenic T1 plants were selected by screening seeds on MS medium containing 50 mg/l kanamycin. The T2 transgenic lines with a 3:1 (resistance:sensitive) segregation ratio were selected by screening on MS medium containing 50 mg/l kanamycin. The T3 progenies homozygous for kanamycin resistance were used for further studies.

Table 1. The root length of wt and 3 lines of T3 *Arabidopsis* plants under 0, 150 and 200 mM NaCl.

Salt level (mM)	RL (cm)			
	col	TC1	TC2	TC3
0	1.94 a*	2.02 a	2.03 a	1.94 a
150	0.91 d	1.45 b	1.23 c	1.15 c
200	0.44 f	0.96 d	0.84 de	0.77 e

* Numbers followed by different letters within columns are significantly different at the 0.05 level of confidence as tested by LSD.

Molecular analysis of transgenic Arabidopsis

RT-PCR analysis was used to confirm the presence of *HvNHX2* mRNA in T3 plants of several homozygous transgenic lines. Total RNA was extracted from transgenic lines and wt *Arabidopsis* plants and reverse-transcribed according to the manufacturer's instructions (Fermentas, Lithuania). One microliter of the reverse transcription reaction mix (first-strand cDNA) was used as a template for PCR with gene-specific primers *NHXF* (5'-CGGGATGGATGCATTGGAC-3') and the reverse primer *NHXR* (5'-CCCAACAAGCTCGCCGTAA-3') using the following conditions: 94 °C for 2 min followed by 30 amplification cycles (92 °C for 1min, 70 °C for 1min) and the final extension at 70 °C for 10 min in a thermocycler Tertsik (DNA Technology, Russia). The PCR products were resolved by electrophoresis on a 1% agarose gel using 0.5× TAE buffer.

Analysis of transgenic Arabidopsis plants for salt tolerance

The T2 seeds from each T1 individual transgenic line were tested for germination on MS plates supplemented with 0, 100, 150, 200 or 250 mM NaCl. Three independent transgenic lines which revealed higher salt tolerance were used for further investigation and their T3 progenies homozygous for kanamycin resistance were used for further analysis. For determination of salt tolerance of the transgenic plants, seeds from wt and T3 homozygous lines were surface sterilized and placed on MS medium containing 0, 150 or 200 mM NaCl. To determine whether *HvNHX2*-expressing plants have elevated salt tolerance, transgenic lines and wt *Arabidopsis* seeds were grown in pots containing 1:1 commercial horticultural soil (Seliger-Agro, Russia): perlite for 3 weeks, with a light/dark cycle of 16/8 h at 22-24 ˚C. For salt-stress treatment, the water solution was supplemented with NaCl to a final concentration of 200 mM and plants were watered in saucers underneath the pots (capillary uptake) every other day over the 16-day watering period. The control plants were watered using the same method but without NaCl. After 16 days, shoot fresh and dry weight, Na+ and K+ content were measured.

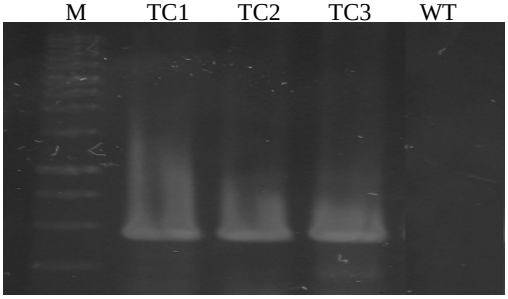

Fig1. RT-PCR of transgenic *Arabidopsis* plants. *M* 1kb DNA size marker (Sibenzyme, Russia), *TC1-3*correspond to the independent transgenic lines, *WT* wild type plant**.**

Results and discussion

Arabidopsis thaliana (var. Columbia; Col-0) plants were transformed with a construct containing the *HvNHX2* cDNA driven by the cauliflower mosaic virus (CaMV) 35S promoter. Sixteen kanamycin-resistant T1 transgenic plants harboring the 35S:*HvNHX2* were obtained. T2 seeds from each T1 individual transgenic line were tested for germination on MS plates supplemented with 0, 100, 150, 200 or 250 mM NaCl. Three independent transgenic lines revealed higher salt tolerance and their T3 progenies homozygous for kanamycin resistance were used for further studies. RT-PCR results showed expression of the *HvNHX2* gene in T3 lines (Fig. 1). To determine whether the transgenic plants have elevated salt tolerance during early seedling development, seeds from these transgenic lines and wt plants were germinated on MS medium containing different levels of NaCl. After one week, there was no difference between transgenic and wt in terms of germination and seedling size in the absence of salt. On 150 mM NaCl there was also no difference between germination of transgenic lines and wt, but the seedling size was smaller in wt compared to the transgenic lines. On 200 mM NaCl there was a dramatic difference between wt and transgenic lines (Fig. 2a); after 10 days growth on MS, root length of the transgenic lines and wt plants was measured. Without NaCl treatment, there was no significant difference among root length of wt and transgenic plants but at 150 and 200 mM NaCl, transgenic plants had longer roots compared to wt plants (Table 1 and Fig. 3). Transgenic *Arabidopsis* plants had normal growth in the presence of 200 mM NaCl in pots while wt plants showed leaf necrosis. In the absence of salt, transgenic and wt *Arabidopsis* plants had a similar growth rate and flowered at the same time (Fig. 2b). Fresh and dry weight accumulation of transgenic *Arabidopsis* plants was not significantly different from the wt (Fig. 4). K+ content in presence of 200 mM NaCl was higher only in one transgenic line compared to the wt, but two other transgenic lines did not differ from the wt (Fig. 5a). In the presence of 200 mM NaCl all transgenic lines accumulated higher levels of Na+ in shoots compared to the wt (Fig. 5b). In order to avert Na+ toxicity in the cytosol, the sequestration of Na+ into the vacuole and pre-vacuolar compartment needed to be enhanced. For a more accurate analysis of the tolerance mechanism of the transgenic lines obtained in this study, vacuolar and cytosolic ion contents needed to be determined. However, the increased Na+ content of transgenic shoots compared to wt, together with less injury of transgenic plants under Na+ stress conditions (leaf necrosis), suggests that vacuolar Na+ compartmentalization does improve salt tolerance, which is similar to results of Islam et al., 2009 on transgenic rice overexpressing *OsNHX1*. Physiological evidences suggest that halophytes and salt-resistant glycophytes actively transport Na+ from the root to the shoot, whereas salt-sensitive glycophytes appear to limit Na+ entry into the transpirational stream to prevent Na+ accumulation in the

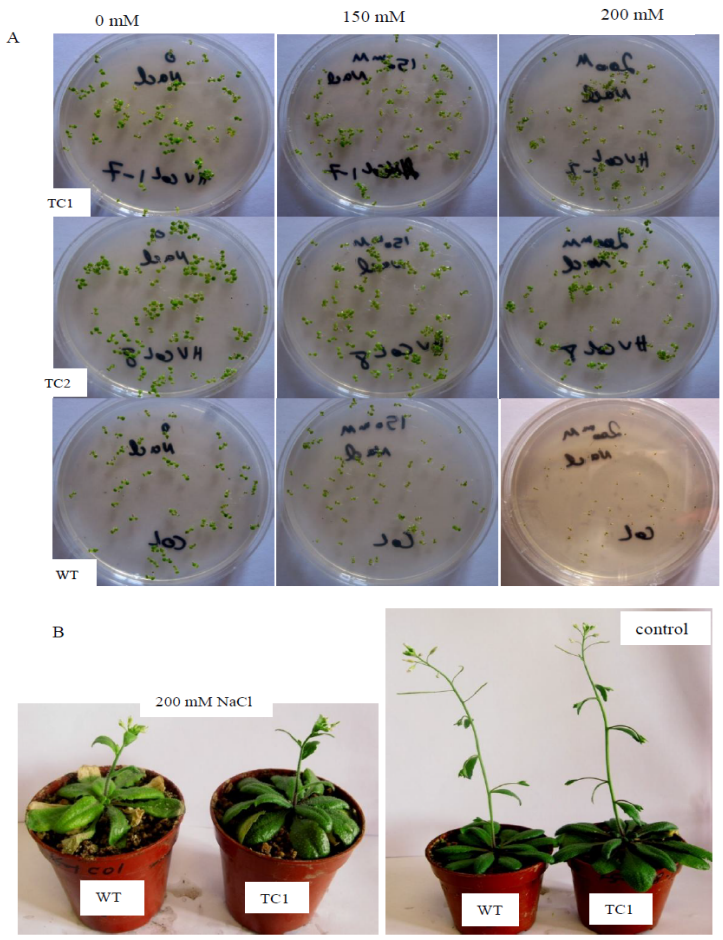

Fig 2. Analysis of salt tolerance of transgenic and wt *Arabidopsis* plants. (A) Seed germination of T3 transgenic and wt *Arabidopsis* after one week on MS medium containing 0, 150 or 200 mM NaCl. (B) Transgenic and wt plants in pots were treated with 200 mM NaCl for 16 days every other day.

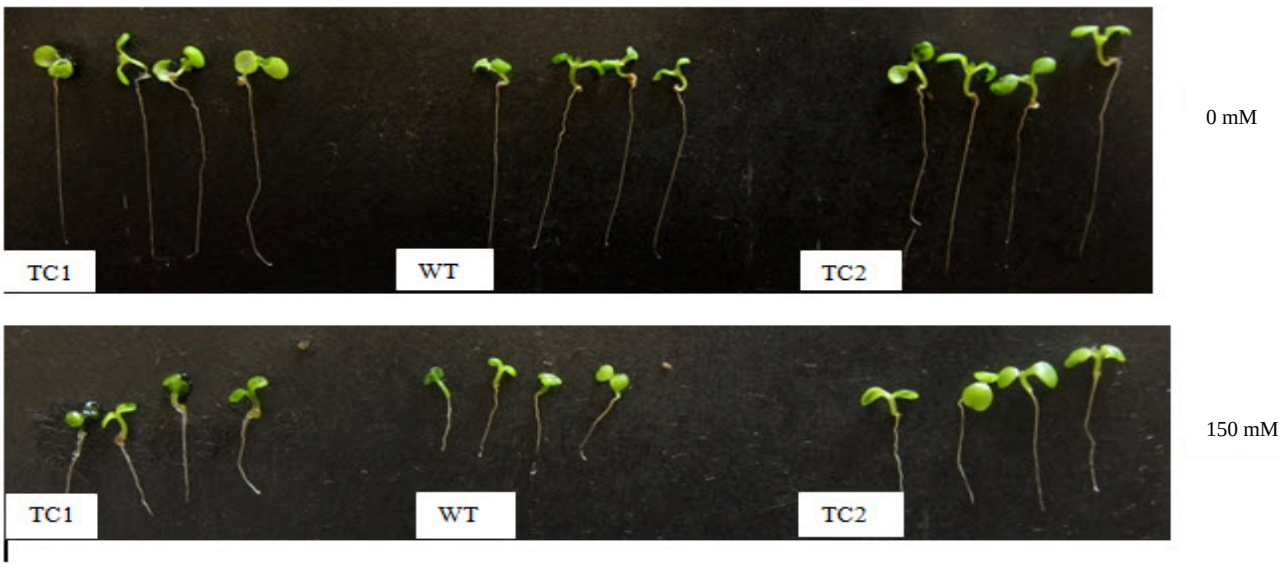

Fig 3. A representative picture of root lengths of two transgenic lines and wt *Arabidopsis* grown in the presence of 150 mM NaCl.

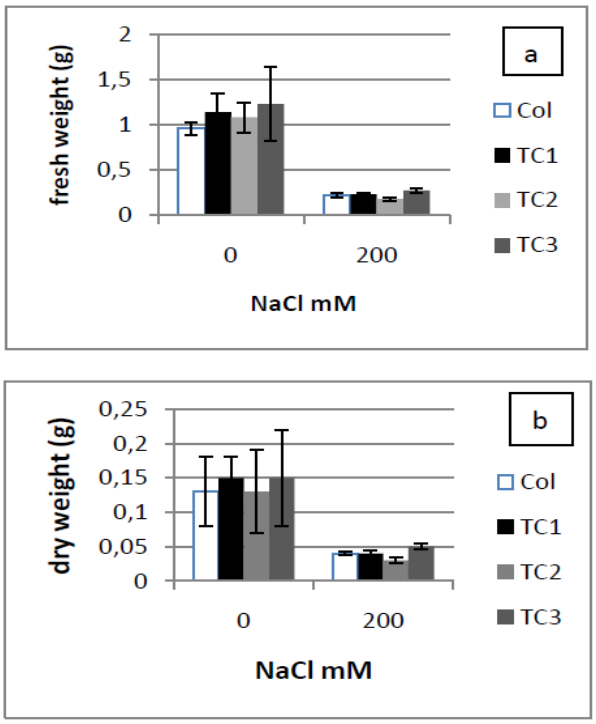

Fig 4. Effect of NaCl on Shoot Fresh Weight (a) and Shoot Dry Weight (b) of transgenic and wt plants

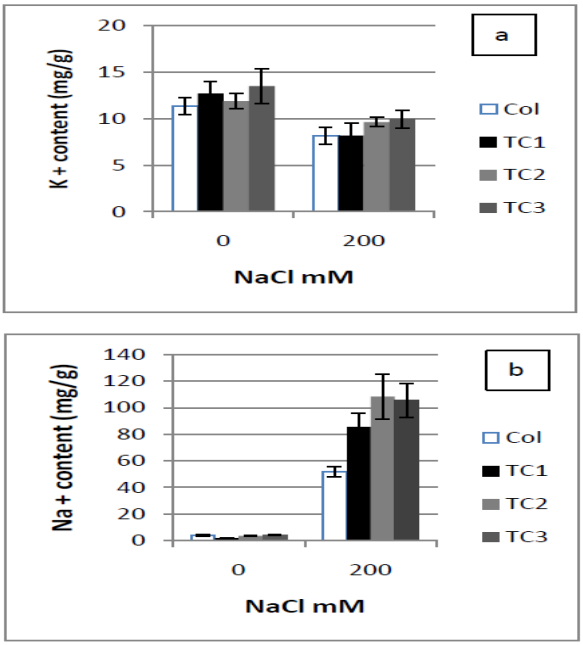

Fig 5. Effect of NaCl on K^+ and Na^+ content (mg/g of dry weight) in shoots of *Arabidopsis* plant. Shoot K^+ (a), shoot Na^+ (b).

shoot (Flower et al 1977; Lauchli 1984). SOS1 is a plasma membrane Na+ transporter essential for controlling long-distance movement in *Arabidopsis*, suggesting that SOS1 functions in Na+ retrieval from the xylem sap under severe salt stress conditions, whereas SOS1 may load Na+ into the root xylem when mild salt stress is applied (Shi et al 2002). Our results from experiments with potato (Bayat et al., 2010) showed that, in contrast to Arabidopsis, higher K+ accumulation occurred in transgenic potato transformed with the *HvNHX2* gene, suggesting that in potato the K+/Na+ selectivity of the antiporter is enhanced similar to Venema et al (2003). It can be concluded that apparently in potato K+ transport is important in salt tolerance, while in *Arabidopsis* higher salt tolerance is accompanied with higher shoot Na+ content. A possible explanation for the accumulation of different cation species in the *HvNHX2*-expressing potato and *Arabidopsis* plants may be the effect of the cellular environment on the specificity of the foreign antiporter. In *Arabidopsis* the SOS pathway appears to regulate the activity of the vacuolar Na+/H+ antiporter. The SOS pathway plays a central role in coordinating the activities of several of the transporter systems (Shi et al., 2000). The location of the protein in different membrane systems may also affect its specificity and function, e.g. ion accumulation. Ion specificity of the antiporter is dictated by specific residues within the membrane domain. *AtNHX1* removes Na+ out of the cytosol by compartmentalizing it into the vacuolar lumen (Yamaguchi et al., 2003), whereas *AtNHX4* transports Na+ out of vacuolar lumen to the cytosol (Li et al., 2009). Topological analysis revealed that whereas the N-terminus of *AtNHX1* is facing the cytosol, almost the entire C-terminal hydrophilic region of the protein resides in the vacuolar lumen. Moreover, the deletion of the C-terminus of *AtNHX1* doubled the Na+/K+ selectivity ratio of the antiporter, suggesting a regulatory role of the C-terminus of the antiporter (Yamaguchi et al., 2003). *AtNHX4* holds a C-terminus which dissociates in the cytosol out of the vacuole. Furthermore, similar to *AtNHX1*, deletion of the hydrophilic C-terminus of *AtNHX4* dramatically increased the hypersensivity (Li et al., 2009). Antiporters are regulated by phosphorylation by various kinases and by interactions with other cellular proteins, and in different plants the difference between these binding factors could modify the activity and specifity of the antiporter. For example, in *Arabidopsis* the binding of AtCaM15 (calmodulin like protein 15) to the C-terminal domain of *AtNHX1*, modified the Na+/K+ selectivity of the antiporter (Yamaguchi et al., 2005), presumably through conformational changes that could conceivably be brought by mutation in other critical amino acid residues. Also antiporters are regulated at the transcriptional level, allowing both mRNA levels and the amount of antiporter produced to be controlled, which may be the reason for the different activity of the *HvNHX2* antiporter in *Arabidopsis* and potato. Another explanation may be that potato and *Arabidopsis* imports K+ and Na+ into their cytoplasms differently, so different concentrations of these ions are available for transport across the tonoplast. Also, Na+ toxicity differs between *Arabidopsis* and potato. For example, *Arabidopsis* grows and builds up Na+, while Na+-accumulation in potato causes the plant to die. Correlations between Na+ accumulation and Na+ toxicity in potato and *Arabidopsis* remain elusive (Velasquez 2005).

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