BIOLOGIA PLANTARUM 64: 50-57, 2020 DOI: 10.32615/bp.2019.126

Overexpression of *Iris lactea* tonoplast Na^+/H^+ antiporter gene *IlNHX* confers improved salt tolerance in tobacco

Q. GUO, X.X. TIAN, P.C. MAO, and L. MENG*

Beijing Research and Development Center for Grass and Environment,
Beijing Academy of Agriculture and Forestry Sciences, Beijing 100097, P.R. China

Abstract

Sodium cation compartmentalization into vacuoles is one of the effective strategies for adaptation of halophytes to saline environments. Tonoplast Na^+/H^+ antiporter (NHX) is involved in Na^+ sequestration into vacuoles under salt stress. However, the function of NHX in halophyte *Iris lactea* is still unclear. In this study, a significant positive correlation was observed between Na^+ accumulations and *IlNHX* expression in tissues under 0 - 200 mM NaCl, indicating IlNHX might be responsible for Na^+ accumulation of *I. lactea* under salt stress. More important, IlNHX was specifically localized to the tonoplast. Transgenic tobacco expressing *IlNHX* grew better and showed higher tolerance to 200 mM NaCl than respective wild type (WT). Compared to WT, transgenic tobacco accumulated more Na^+ and K^+ and maintained higher K^+/Na^+ ratios in tissues, accompanied by the reduction of chlorophyll loss and lipid peroxidation in the presence of NaCl. Moreover, transgenic tobacco exhibited markedly higher vacuolar H^+-ATPase (V-ATPase) activity relative to WT when subjected to salt stress. The findings suggest that transgenic plants overexpressing *IlNHX* could compartmentalize more Na^+ into vacuoles in tobacco *via* enhanced V-ATPase activity, which further contributes to maintaining K^+ and Na^+ homeostasis, to improved photosynthesis, and to protection of cell membrane integrity under salt stress.

Additional key words: K^+/Na^+ ratios, Na^+ compartmentalization, *Nicotiana tabacum*, tonoplast Na^+/H^+ antiporter, vacuolar H^+-ATPase.

Introduction

Soil salinity is a major factor that limits growth, development, and productivity of plants (Bao *et al.* 2016). Generally, most plant species mainly suffered from the osmotic stress and Na^+ specific toxicity under salt stress (Blumwald *et al.* 2000, Munns and Tester 2008). This is attributed to the fact that over-accumulation of Na^+ in the cytosol leads to the inhibitions of protein synthesis, many enzymatic reactions, and photosynthetic processes (Murguía *et al.* 1995, Tsunekawa *et al.* 2009). However, halophytes have evolved various mechanisms to withstand salt stress *via* long term natural selection (Flowers and Colmer 2008). Therefore, by understanding the mechanisms leading to successful adaptation to salinity in halophytes, it might be possible to identify key genes able to alter metabolism and increase plant tolerance to salt stress (Ma *et al.* 2014).

One of adaptation strategies in halophytes is that they preferentially transport Na^+ into the shoots, and further compartmentalized it into vacuoles maintaining a low osmotic potential and avoiding Na^+ toxicity in cytosol under salt stress (Yeo 1981, Shabala 2013). A tonoplast Na^+/H^+ antiporter (NHX) mediated Na^+ compartmentalization is driven by proton motive force across tonoplast generated by H^+-ATPase (Bassil and Blumwald 2014). The first plant NHX family was isolated from *Arabidopsis thaliana* (Gaxiola *et al.* 1999). Some evidences have demonstrated that overexpression of *NHX* can improve the salt tolerance of *Arabidopsis* (Apse *et al.* 1999), *Brassica napus* (Zhang *et al.* 2001), and sugar beet (Wu *et al.* 2015). These suggested that increased Na^+ sequestration into vacuoles can remarkably improve salt tolerance in plants subjected to salt stress. Besides, several documents indicate a series of other functions of NHX in terms of pH regulation (Yamaguchi *et al.* 2001), cellular K^+ homeostasis (Leidi *et al.* 2010), and cell expansion (Bassil *et al.* 2011, Barragán *et al.* 2012). Taken together, NHX is not only involved in intracellular Na^+ compartmentalization, but also in cell expansion, intracellular ions and pH homoeostasis, and so

Submitted 16 June 2019, last revision 7 October 2019, accepted 15 October 2019.
Abbreviations: Chl - chlorophyll; CHLA - chloroplasts autofluorescence; GFP - green fluorescent protein; GLA - glufosinate ammonium; MDA - malondialdehyde; NHX - tonoplast Na^+/H^+ antiporter; V-ATPase - vacuolar H^+-ATPase.
Acknowledgements: This work was supported by the National Natural Science Foundation of China (Grant Nos 31601991 and 31971752), the Beijing Natural Science Foundation (Grant No. 6152008), the Scientific Innovation Ability Construction Project of BAAFS (Grant No. KJCX20170110), and the Institute for Science and Technology Innovation Team Project of BAAFS (Grant No. JNKST201604).
* Corresponding author; fax: (+86) 10 515 03297, e-mail: menglin9599@sina.com

salt stress response (Reguera *et al.* 2014).

Iris lactea var. *chinensis* (Fisch.) Koidz. is a perennial monocotyledonous halophyte found in saline meadow and desert steppe in north China and it is used as an ornamental plant for the reclamation of salt wastelands (Bai and Li 2005). This species has attractive leaves and flowers, abundant seeds, salt and drought tolerance, pest and disease resistance, and easy cultivation (Guo *et al.* 2017a). In our previous study, sixteen genotypes of *I. lactea* from northern China were screened out to be the salt-sensitive (*e.g.* BJCY035) or salt-tolerant (*e.g.* BJCY007) by the comprehensive assessment of *I. lactea* tolerance to salt stress (Mao *et al.* 2013). Compared with the BJCY035, the specific loci ISSR841-300 was found in BJCY007 using inter-simple sequence repeat (ISSR) marker that linked with salt tolerance gene *NHX*. Further isolation and characterization of the *IlNHX* from *I. lactea*, and its expression under salt stress has been done previously (Guo *et al.* 2015a). However, mechanisms underlying the function of IlNHX in the salt tolerance of *I. lactea* have not been fully explored.

To reveal the contribution of IlNHX in salt tolerance of plants, the correlation between accumulation of Na^+ and its expression in shoots and roots of *I. lactea* exposed to salt stress was investigated in this study, and its subcellular localization was verified. Furthermore, the aim of this study was to determine growth performance, Na^+, K^+ accumulation, and V-ATPase activity of WT and transgenic tobacco expressing *IlNHX*.

Materials and methods

Plant growth conditions and treatments: Seeds of *Iris lactea* var. *chinensis* (Fisch.) Koidz. genotype BJCY007 were collected from the experimental site at Xiaotangshan in Beijing Academy of Agriculture and Forestry Sciences. The seeds were sterilized with 5 % (v/v) NaClO for 5 min and rinsed thoroughly with distilled water, soaked in water for 56 h at 40 °C, then 10 seeds were sown in plastic culture pots (8 cm in diameter, 12 cm in height) containing peat and sand (v/v, 2:1). They germinated under a 16-h photoperiod, an irradiance of 600 μmol m^{-2} s^{-1}, a temperature of 28 ± 2 °C, and a relative humidity between 50 and 60 %. Pots were watered every 3 d for 6 weeks until emergence. Seedlings with three leaves were selected for uniformity, and then transferred into plastic containers filled with modified Hoagland solution and grown for 2 weeks in the same chamber. The 8-week-old seedlings were treated with nutrient solution supplemented with 0, 25, 50, 100, and 200 mM NaCl for 48 h.

Plasmid construction and subcellular localization of IlNHX: Open reading frame of *IlNHX* was amplified using *Tks gflex* DNA polymerase according to Guo *et al.* (2015a), and then was cloned to pMD-19T vector and sequenced. The plasmid of pCAMBIA3301 binary vector was cut using *Nco* I and *Bst* EII restriction enzymes (named as pCAMBIA3301-X), and then the open reading frame fragment of *IlNHX* was sub-cloned into the binary vector pCAMBIA3301-X under the control of CAMV35S promoter.

To further construct C-terminal green fluorescent protein (GFP) fused gene expression vector, the fragment of *IlNHX* without the stop codon was firstly obtain using *IlNHX* primer pairs P3 and P4 (Table 1 Suppl.). The *IlNHX* fused with GFP was inserted via *Eco RI/Bam HI* sites into pBSHES-NL vector using the *In-Fusion* system (*Clontech*, Mountain View, CA, USA) to generate IlNHX::GFP constructs driven by CaMV 35S promoter.

The IlNHX::GFP fusion construct was transiently expressed in protoplasts isolated from *Arabidopsis thaliana* Col-0 cell suspensions based on PEG-mediated method (Guo *et al.* 2017b). The protoplasts containing the plasmids mentioned above was transferred into 5 cm^3 Petri dish and incubated in the dark at 23 °C for 2 - 3 d. Fluorescent signals from GFP and chloroplast autofluorescence (CHLA) in the protoplasts were then observed using an inverted *FV10-ASW* confocal laser scanning microscope (*Olympus*, Tokyo, Japan).

Genetic transformation and molecular characterization: The above-mentioned constructs were introduced into *Agrobacterium tumefaciens* L. strain EHA105 by the chemical method and then it was used for tobacco (*Nicotiana tabacum* L. cv. Wisconsin 38) transformation using the leaf disc method as described by Horsch *et al.* (1985) with minor modifications. The infected leaf sections were cultivated on Murashige and Skoog (MS) medium containing 2 mg dm^{-3} 6-benzylaminopurine (6-BA) and 0.2 mg dm^{-3} 1-naphthaleneacetic acid (NAA) and pH 5.8 for 3 d. Subsequently, the explants were placed into the selection MS medium containing, in addition to 6-BA and NAA, 8 mg dm^{-3} glufosinate-ammonium (GLA) and 500 mg dm^{-3} cefotaxime and grown in a greenhouse at a 16-h photoperiod, an irradiance of 600 μmol m^{-2} s^1, a temperature of 25 °C, and a relative humidity of 60 % for 4 weeks to induce shoot development. We further aseptically excised 3 - 5 cm long shoots and transferred them into rooting 1/2 MS medium supplemented with 0.05 mg dm^{-3} indole-3-butyric acid, 8 mg dm^{-3} GLA and 250 mg dm^{-3} cefotaxime (pH 5.8) and independent transformed lines were obtained. Finally, the fifty surviving resistant plants (T_0 generation) were transferred into pots containing a mixture of *Vermiculite*, *Perlite*, and peat moss (v/v, 1:1:1) and cultured in the greenhouse and irrigated with modified Hoagland nutrient solution as described above. T_1 seeds were harvested after four months, and grown on MS selection medium with 8 mg dm^{-3} GLA, and T_2 seeds were produced in the same way. Genomic DNA was extracted from young leaves of WT and putative transgenic tobacco plants of T_2 generation using the *MiniBEST* universal genomic DNA extraction kit (*TaKaRa*, Dalian, China). The transgenic plants were confirmed using forward and reverse primer pairs P5 and P6 for *IlNHX* (Table 1 Suppl.). Total RNA was extracted with a *Trizol* kit following the manufacturer's instructions (*TaKaRa*). First strand cDNA was synthesized from 2 μg of total RNA using an *Oligo dT* primer and *PrimeScriptRTase* (*TaKaRa*). The expressions were validated by reverse transcription reverse

transcription PCR using *IlNHX* specific primers pairs P7 and P8. *IlACTIN* was regarded as the normalizer in the semi-quantitative reverse transcription PCR. The fragment of *IlACTIN* was amplified by primers pairs A1 and A2.

Assessment of salt tolerance in transgenic tobacco: Tobacco WT or transgenic T_2 generation seeds were disinfested with 5 % (v/v) NaClO for 8 min, and then were grown on MS agar medium with 8 mg dm^{-3} GLA or without it for 2 weeks. The seedlings were selected for uniformity once the plumule emerged, and were subsequently transferred into pots (18 cm in diameter, 25 cm in height) containing *Vermiculite*, *Perlite*, and peat moss (v/v, 1:1:1) irrigated with modified nutrient solution and grown eight weeks in the above mentioned greenhouse. Then, these plants were treated with the nutrient solution containing 0 mM NaCl (control) or 200 mM NaCl for 10 d.

Isolation of tonoplast vesicles, determination of vacuolar H^+-ATPase, and Western blot analysis: According to the method of Wang *et al.* (2000) with minor modifications, tonoplast enriched membrane vesicles were isolated. We select the top fully expanded tobacco leaves treated by 200 mM NaCl for 10 d and homogenized them in extraction medium containing 250 mM mannitol, 1 mM dithiothreitol (DTT), 3 mM ethylene glycol tetraacetic acid (EGTA), 1 % (m/v) polyvinylpyrrolidone (PVP), 0.25 mM phenylmethylsulfonyl fluoride (PMSF), 100 mM tricine, 3 mM $MgSO_4$, and adjusted to pH 7.8 with Hepes-Tris. The homogenate was filtered through four layers of cheese cloth and then centrifuged at 12 000 *g* and 4 °C for 15 min. The resulting supernatant was centrifuged at 300 000 *g* for 45 min, and the resulting pellet was suspended in suspension buffer containing 250 mM mannitol, 2 mM DTT, 3 mM EGTA, 10 mM Hepes and adjusted to pH 7.5 with Hepes-Tris. The microsomal membrane vesicle suspension was loaded on a 1/18 % (m/m) dextran T_{70} gradient in suspension buffer and centrifuged at 100 000 *g* for 2 h. The tonoplast-enriched membrane vesicle fraction located at the 1/8 % dextran T_{70} interface was carefully collected, diluted 4- to 5-fold with dilution buffer containing 1 mM DTT, 3 mM $MgSO_4$, 50 mM Hepes, 0.2 mM PMSF and adjusted to pH 7.0 with KOH, and then centrifuged at 300 000 *g* for 45 min. Finally, the pellets were suspended in storage buffer containing 1 mM DTT, 40 % (v/v) glycerol, 10 mM Hepes, adjusted to pH 7.0 with KOH. The tonoplast enriched membrane vesicles were frozen in liquid nitrogen and stored at -80 °C until use. The activity of H^+-ATPase was further determined (Wang *et al.* 2000).

For Western blot, 20 μg tonoplast proteins were separated using 12 % (m/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotted with antibody against polyclonal Na^+/H^+ antiporter (*AS09484*, *Agrisera*, Vännäs, Sweden). Blots were performed according to methods of Xia *et al.* (2002) and Kumari *et al.* (2017).

Determination of chlorophyll content and lipid peroxidation: Chlorophyll (Chl) content was estimated using a modified method of Guo *et al.* (2014). Briefly, 200 mg fresh leaf samples were ground using quartz sand and extracted with 80 % (v/v) acetone. The absorbance of solution was recorded at 645 nm and 663 nm using an UV spectrophotometer (*UV-T6*, *Persee*, Beijing, China).

Lipid peroxidation was evaluated by quantifying malondialdehyde (MDA) content based on the method of Dhindsa *et al.* (1981) with slight modifications. Fresh leaf samples (300 mg) were well ground with quartz sand containing 0.05 mM phosphate buffer, and then supernatant was mixed with 20 % (m/v) trichloroacetic acid containing 0.5 % (m/v) thiobarbituric acid. The absorbance was measured at 450 nm, 532 nm, and 600 nm by a *UV-T6* spectrophotometer.

Determination of Na^+ and K^+ content: At the end of each treatment, harvested plants were washed thoroughly with running distilled water, separated into shoots and roots. Roots were washed twice for 8 min in ice-cold 20 mM $CaCl_2$ to exchange cell wall-bound Na^+, and then shoots were rinsed in deionized water to remove surface salts (Guo *et al.* 2015b). Both plant parts were oven dried at 80 °C for 3 d to obtain dry mass. Na^+ and K^+ were extracted from dried plant tissue in 100 mM acetic acid at 90 °C for 2 h and ions analysis were performed using an atomic absorption spectrophotometer (*AA-6300C*, Shimadzu, Kyoto, Japan).

Statistical analysis: Each treatment was repeated six times independently with three seedlings in each replicate. All data are presented as means with standard deviation (SD). One-way analysis of variance (*ANOVA*) and the Duncan's multiple range tests were performed by statistical software (*v.13.0*, *SPSS Inc*, Chicago, IL, USA).

Results

The content of Na^+ after 48 h increased progressively in shoots and roots of *I. lactea* with increase of external NaCl concentration (25 - 200 mM) and Na^+ content in roots was a 1.3- to 2.2-fold higher than that in shoots (Fig. 1*A*). Our previous study showed that the expression of *IlNHX* was up-regulated in both shoot and roots of *I. lactea* with increasing concentration of NaCl, and the expression was always higher in shoots than in roots. Further we found a significant positive correlation between Na^+ accumulation and expression of *IlNHX* in shoots and roots of *I. lactea* under 0 - 200 mM NaCl treatment for 48 h (Fig. 1*BC*). These suggested that IlNHX might be one of major component for Na^+ accumulation in *I. lactea* under salt stress.

As concerns the subcellular localization of IlNHX in *Arabidopsis thaliana* mesophyll cells, the control (35S::GFP) construct without IlNHX showed GFP fluorescence and CHLA throughout the nucleus and chloroplasts of mesophyll cells, respectively (Fig. 2*A*). However, once IlNHX::GFP was introduced into the protoplasts of mesophyll cells, the GFP signals were clearly distinguished from CHLA signals in the area of the

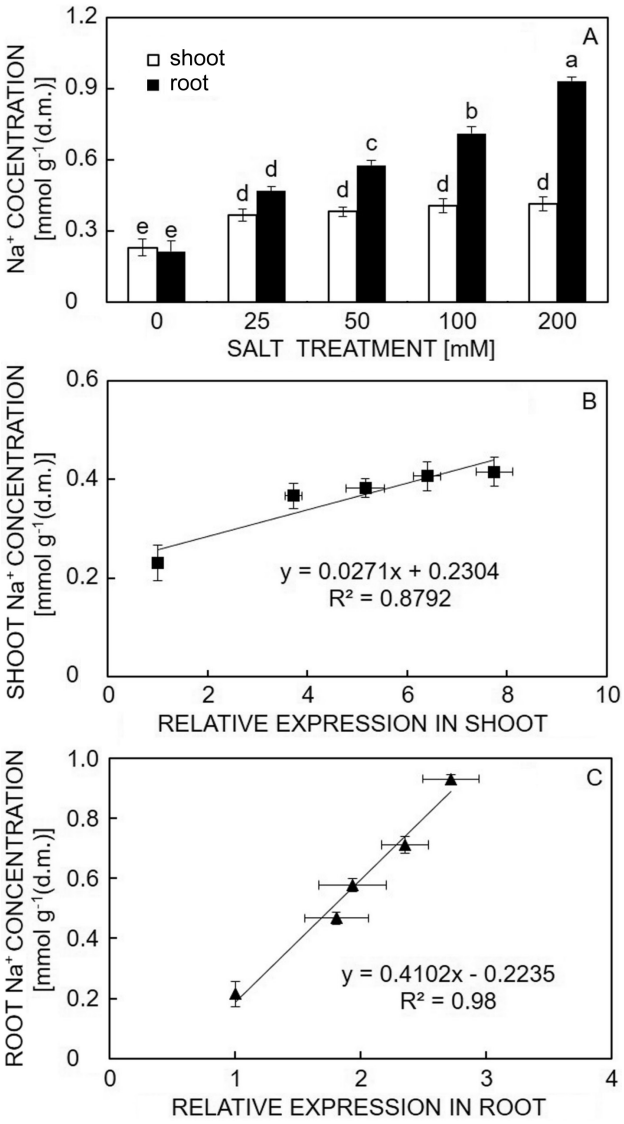

Fig. 1. Sodium cation content in shoots and roots of *Iris lactea* grown under different concentrations of NaCl (0, 25, 50, 100, and 200 mM) for 48 h (*A*). A relationship between Na^+ content and relative *IlNHX* expression in shoots (*B*) and roots (*C*) under different NaCl concentrations. Means ± SDs, $n = 3 - 6$, different letters indicate significant difference at $P < 0.05$ (the Duncan's test).

tonoplast and chloroplasts (Fig. 2*B*), implying that IlNHX is specifically localized to tonoplast.

We introduced the *IlNHX* into tobacco by *Agrobacterium* containing a binary vector pCAMBIA3301. We obtain eighteen plantlets survived after selection on GLA and nine of them were positive transgenic tobacco confirmed by PCR amplification (data not shown). The expression of *IlNHX* was further quantified by reverse transcription PCR performed on young leaves of all positive plants (L1 - L9) and WT plants (Fig. 1 Suppl.). The *IlNHX* showed the highest expression in L1 and L2 and the lowest expression in L7, L8, and L9, but it was not detected in WT. Therefore, L1 and L7 were selected to carry out the further assays. In addition, compared with WT plants, tonoplast IlNHX protein increased significantly in the L1 and L7 using Western blot analysis, and the content of this protein in L1 was higher than in L7 (Fig. 1 Suppl.).

As shown in Fig. 3*A*, both WT and transgenic tobacco plants grew well under control conditions, however, transgenic tobacco plants exhibited better growth in the presence of 200 mM NaCl relative to WT plants. For example, dry masses of shoots and roots was 1.84- and 2.27-fold higher in L1, and they were 1.74- or 2.23-fold higher in L7 than those of WT plants under 200 mM NaCl treatment (Fig. 3*BC*). These results suggested that overexpression of *IlNHX* could enhance salt tolerance of the transgenic plants compared to WT plants.

To examine whether tobacco plants overexpressing *IlNHX* could accumulate more Na^+ and K^+, their content was analyzed. At control conditions, no significant differences were observed in Na^+ and K^+ content in both shoots and roots between WT and transgenic plants (Fig. 4). In the presence of 200 mM NaCl, shoot and root Na^+ content was remarkably increased in both WT and transgenic plants relative to the control: Na^+ content in shoots or roots of L1 was 55 and 26 % higher, and of L7 it was 47 or 21 % higher compared to Na^+ content in WT (Fig. 4*AB*). In contrast, the addition of 200 mM NaCl result in a significant reduction in K^+ content in WT and transgenic plants compared to the control. However, shoot and root K^+ content was 154 and 115 % higher in L1 and 122 and 96 % higher in L7 compared with WT plants under salt stress, respectively (Fig. 4*CD*). These caused a K^+/Na^+ ratio in shoots and roots 1.64- and 3.93-fold higher in L1, or 1.52- and 3.68-fold higher in L7 than in WT (Fig. 4*EF*), indicating that transgenic plants overexpressing *IlNHX* could accumulate more Na^+ and K^+, and enhance the K^+/Na^+ ratio in tobacco exposed to salt stress.

To test whether the proton pumps activity at tonoplast was enhanced in transgenic plants, activities of V-ATPase in leaves were investigated. There was no significant difference in V-ATPase activity between WT and transgenic plants in the absence of NaCl, but the addition of 200 mM NaCl increased activity of V-ATPase by 91 or 65 % more in L1 or L7 compared to WT (Fig. 5). The results demonstrated that V-ATPase was one of important energy source for ion transport in *IlNHX* overexpressing tobacco plants under salt stress.

To investigate the effect of NaCl on photosynthetic apparatus in WT and transgenic plants, their Chl content was determined. The total Chl content was not significantly different between WT and transgenic plants at the control conditions, but total Chl content remarkably declined in both WT and transgenic plants under salt stress (Fig. 6*A*). It was further observed that Chl content of L1 and L7 was 67 and 57 % higher than that of WT under salt stress, respectively. Moreover, no marked differences were found in MDA content between WT and transgenic plants in the absence of NaCl, but the presence of 200 mM NaCl enhanced the MDA content in both WT and transgenic plants significantly. The MDA content was 43 or 38 % lower in L1 and L7 than in WT plants (Fig. 6*B*). These results suggested that transgenic plants overexpressing

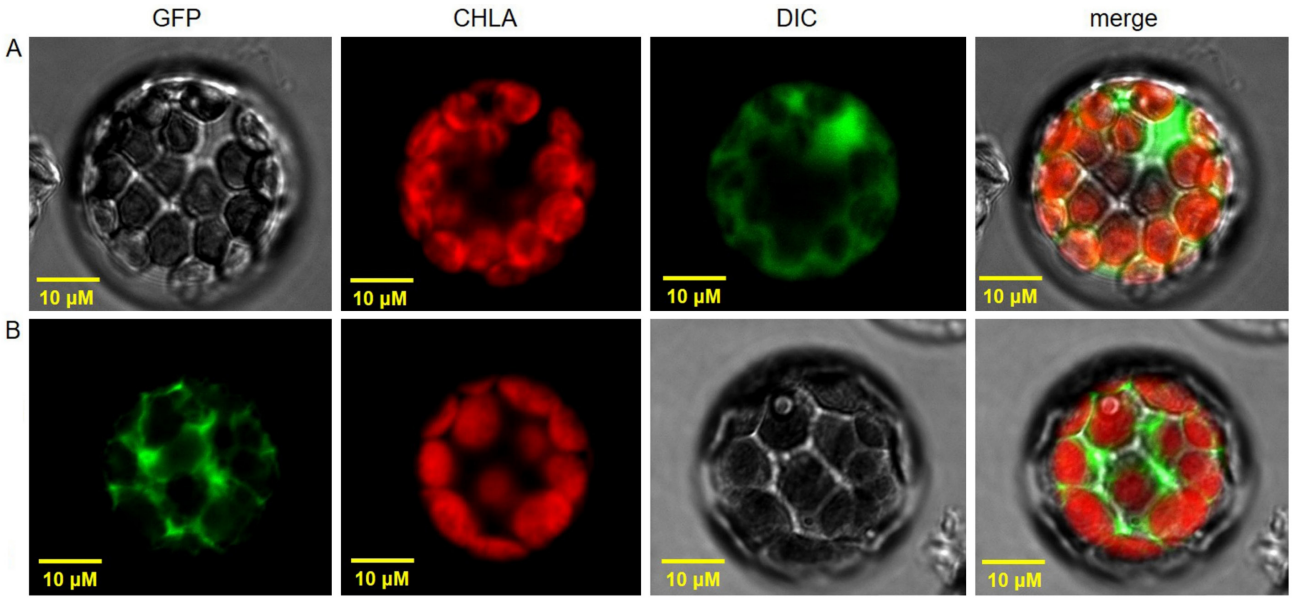

Fig. 2. Subcellular localization of *IlNHX::GFP* fusions during transient expression in *Arabidopsis* mesophyll cells. *A* - Images obtained using green fluorescent protein (GFP) alone as a control. *B* - Images obtained when GFP was fused to the C terminus of *IlNHX*. Four layers show GFP signals, chloroplast autofluorescence (CHLA), merged images of GFP and CHLA signals, and bright-field differential interference contrast (DIC). The *bar* is 10 μm.

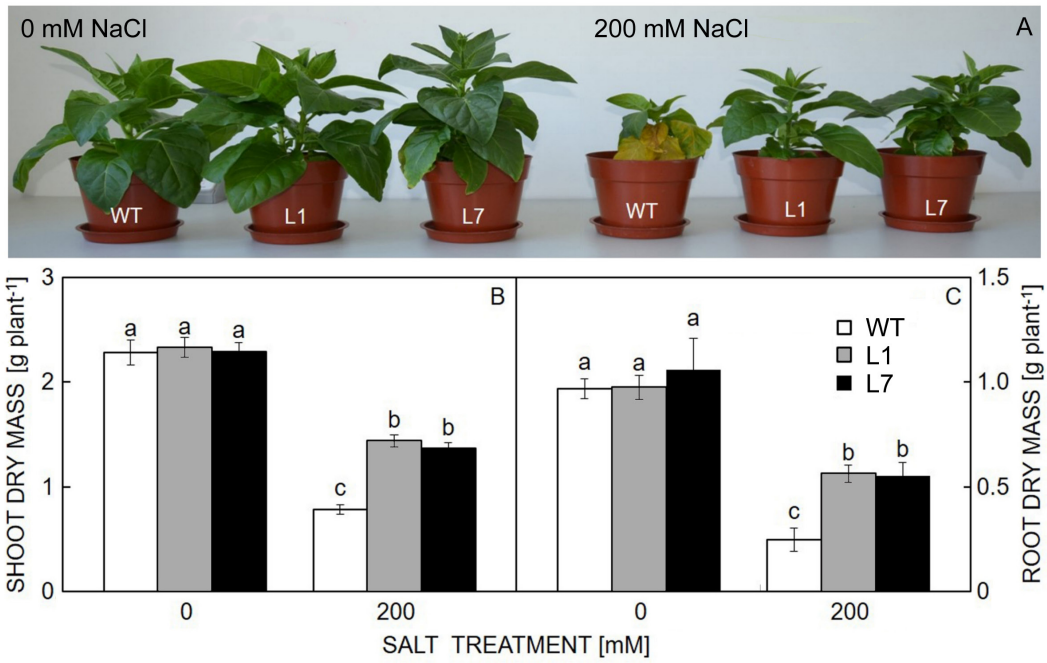

Fig. 3. Morphological characteristics (*A*), and dry mass of shoots (*B*) and roots (*C*) of 8-week-old wild type (WT) and transgenic (L1 and L7) tobacco plants overexpressing *IlNHX*, grown under control conditions or 200 mM NaCl for 10 d. Means ± SDs, $n = 6$, different letters indicate significant difference at $P < 0.05$ (the Duncan's test).

IlNHX could reduce chlorophyll content less markedly, and alleviate the Na^+ induced oxidative damage to cell membranes.

Discussion

Na^+ sequestration into vacuoles is thought to be an important mechanism to alleviate cytosolic Na^+ toxicity and it would be beneficial for supplying the additional osmoticum for pressure potential maintenance and water uptake (Apse

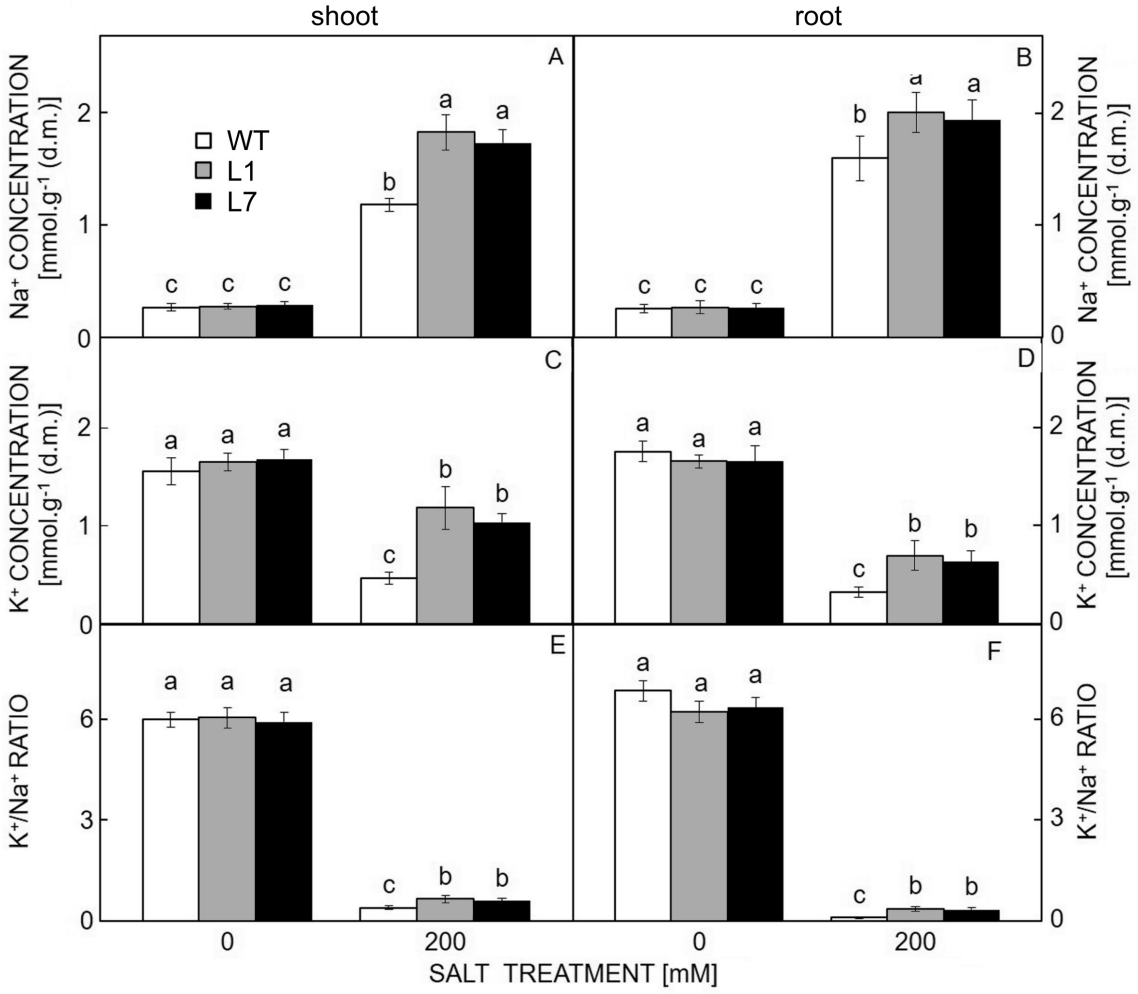

Fig. 4. Sodium cation (*A*,*B*), potassium cation (*C*,*D*) content in shoots and roots, and K^+/Na^+ ratio in shoots (*E*) and roots (*F*) of 8-week-old wild type (WT) and transgenic (L1 and L7) tobacco plants overexpressing *IlNHX*, grown under control conditions or 200 mM NaCl for 10 d. Means ± SDs, $n = 6$, different letters indicate significant difference at $P < 0.05$ (the Duncan's test).

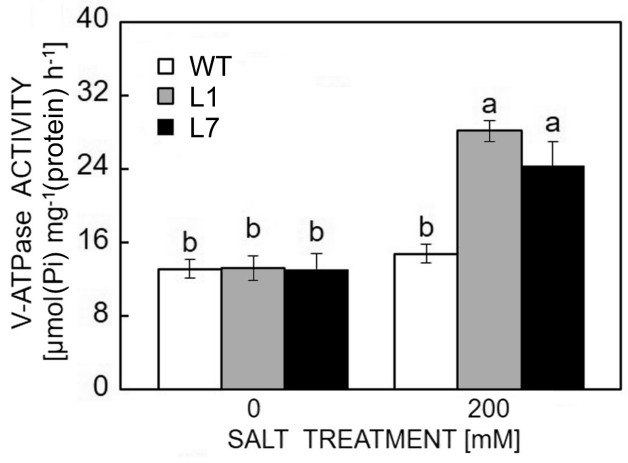

Fig. 5. Activity of V-ATPase in tonoplast vesicles from leaves of 8-week-old wild type (WT) and transgenic (L1 and L7) tobacco plants grown under control conditions or 200 mM NaCl for 10 d. Means ± SDs, $n = 6$, different letters indicate significant difference at $P < 0.05$ (the Duncan's test).

and Blumwald 2007, Yamaguchi *et al.* 2013). The NHX is mainly responsible for compartmentalization of Na^+ into vacuoles to mitigate Na^+ toxicity in the cytosol (Apse *et al.* 1999). Our previous study showed that the expression of *IlNHX* is up-regulated in both shoots and roots under salt stress (Guo *et al.* 2015a). This up-regulation might be responsible for increased NHX activity and so enhanced Na^+ compartmentalization into vacuoles (Shi and Zhu 2002, Pardo *et al.* 2006). Further investigation showed a positive correlation between Na^+ accumulations and up-regulation of *IlNHX* in both shoots and roots of *I. lactea* subjected to different NaCl concentrations (Fig. 1*BC*). Meanwhile, it was confirmed that IlNHX was located at the tonoplast specifically (Fig. 2), implying that it plays an important role in Na^+ compartmentalization into the vacuoles (Gaxiola *et al.* 1999, Ye *et al.* 2009).

To further prove the role of NHX in salt tolerance, the gene *IlNHX* was transferred into tobacco genome. Our findings showed that transgenic tobacco plants displayed better growth as well as higher biomass under salt stress, compared with WT (Fig. 3). It is no doubtfully

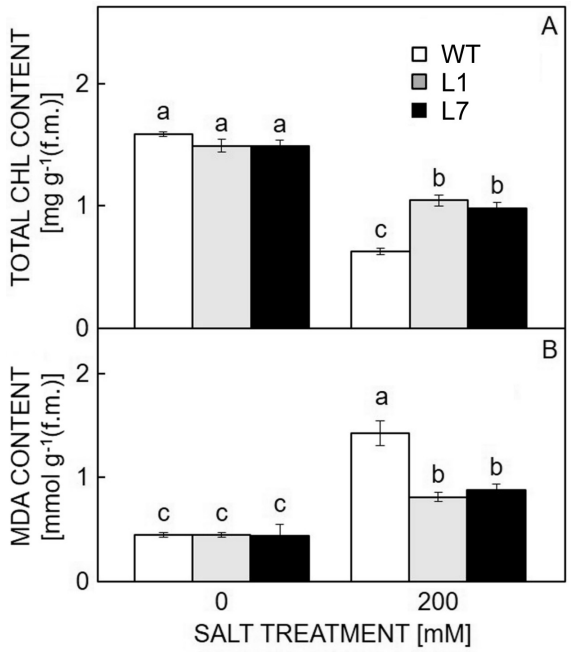

Fig. 6. Total chlorophyll (CHL, *A*) and malondialdehyde (MDA, *B*) content in leaves of 8-week-old wild-type (WT) and transgenic (L1 and L7) tobacco plants overexpressing *IlNHX* under control (0 mM NaCl) and salt (200 mM NaCl) treatments for 10 d. Means ± SDs, $n = 6$, different letters indicate significant difference at $P < 0.05$ (the Duncan's test).

that overexpressing *IlNHX* is not only an optimal way to improve tobacco salt tolerance as shown by higher biomass of transgenic plants, but also it can regulate plant development or cell expansion (Reguera *et al.* 2014, Zhang *et al.* 2015)

In addition, ion homeostasis with low Na^+ and high K^+ content in the cytoplasm is crucial for maintaining normal metabolic and physiological processes (Zhu 2003). NHX is able to keep low Na^+ and high K^+ cytosolic content *via* sequestering Na^+ into vacuoles, thus maintaining cellar ion homoeostasis in plants subjected to salt stress. Our result demonstrated that transgenic tobacco plants accumulated more Na^+ in tissues (Fig. 4*AB*), but enhanced V-ATPase activity might ensure more Na^+ sequestered into vacuoles to maintain cytosol ion homeostasis (Gaxiola *et al.* 2007, Zhou *et al.* 2011). Coincidentally, previous study reported that transgenic tomato overexpressing *NHX* accumulate more K^+ in vacuoles due to higher K^+ uptake relative to WT (Leidi *et al.* 2010). This suggests that NHX is helpful to improve cellular K^+ uptake in transgenic plants *via* enhanced vacuolar K^+ compartmentalization (Bassil *et al.* 2011). These evidences were also supported by our data: we observed that transgenic tobacco retained more K^+ in tissues than WT (Fig. 4*CD*). Therefore, we found that transgenic plants maintained higher K^+/Na^+ ratios in tissues in comparison with WT plants under salt stress (Fig. 4*EF*), which was very important for transgenic plants to cope with salt stress.

Salt stress caused the inhibition of photosynthesis including decreased content of photosynthetic pigments (Akbari *et al.* 2018). Our data showed that the Chl content in transgenic tobacco plants was always higher than that in WT plants subjected to salt stress (Fig. 6*A*). The similar results are also observed in transgenic *A. thaliana* expressing *VrNHX1* (Mishra *et al.* 2014), and sweet potato expressing *AtNHX1* (Fan *et al.* 2015) and tomato expressing *TNHXS1* (Gouiaa and Khoudi 2015). In addition to this, MDA content as indicators of lipid peroxidation assessed the oxidative stress induced by salt stress (Wu *et al.* 2013). Transgenic tobacco plants exhibited a lower MDA content in leaves than WT plants (Fig. 6*B*). These findings implied that overexpressing *NHX* can protect the cell membrane and photosynthetic machinery from injuries induced by salt stress through sequestering more Na^+ into the vacuoles to reduce cytoplasmic Na^+ toxicity.

In conclusion, our results indicated that overexpression of *IlNHX* in transgenic tobacco increased the compartmentalization of Na^+ into vacuoles and higher K^+/Na^+ *via* enhanced tonoplast proton pumps activity. This contributed to maintaining K^+ and Na^+ homeostasis, higher Chl content and less membrane damage under salt stress.

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