



# Ectopic expression of *Arabidopsis thaliana* Na<sup>+</sup>(K<sup>+</sup>)/H<sup>+</sup> antiporter gene, *AtNHX5*, enhances soybean salt tolerance

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**ABSTRACT.** Drought and salt stresses are the two major factors influencing the yield and quality of crops worldwide. Na<sup>+</sup>(K<sup>+</sup>)/H<sup>+</sup> antiporters (*NHXs*) are ubiquitous membrane proteins that play important roles in maintaining the cellular pH and Na<sup>+</sup>(K<sup>+</sup>) homeostasis. The model plant *Arabidopsis* potentially encodes six *NHX* genes, namely *AtNHX1* to *6*. In the present study, *AtNHX5*, a comparatively less well-studied *NHX*, was cloned and transferred into a soybean variety, Dongnong-50, via *Agrobacterium*-mediated cotyledonary node transformation to assess its role in improving salt tolerance of

the transgenic plants. The transgenic soybean plants were tolerant to the presence of 300 mM NaCl whereas the non-transgenic plants were not. Furthermore, after NaCl treatment, the transgenic plants had a higher content of free proline but lower content of malondialdehyde compared to the non-transgenic plants. Our results revealed that that *AtNHX5* possibly functioned by efficiently transporting Na<sup>+</sup> and K<sup>+</sup> ions from the roots to the leaves. Overall, the results obtained in this study suggest that soybean salt tolerance could be improved through the over expression of *Arabidopsis AtNHX5*.

**Key words:** *AtNHX5*; Soybean; Na<sup>+</sup>(K<sup>+</sup>)/H<sup>+</sup> antiporters; Salt tolerance

## INTRODUCTION

Drought and salinization cause tremendous losses to agricultural production and economy worldwide. One third of the irrigated land in the world is estimated to be affected by drought and salinization. China is one of the countries that has been facing severe scarcity of water and has struggled with drought and salinization for many years; this severe problem has not only been caused by the long-term excessive use of fertilizer, improper irrigation, and deterioration of vegetation, but also by the global change in the environment. Enhancing the salt tolerance of important crops adapted to the changing environment is, therefore, an urgent need.

Drought and salinization may influence the plant growth in various ways. However, the major cause of damage is the toxic effect of excessive Na<sup>+</sup> and K<sup>+</sup> ions in the cytoplasm (Gollmack et al., 2014). Research has shown that a protein called Na<sup>+</sup>(K<sup>+</sup>)/H<sup>+</sup> antiporter (NHX) plays important roles in maintaining the proper ion homeostasis in the cytoplasm (Mahajan and Tuteja, 2005; Bassil et al., 2012). Na<sup>+</sup>(K<sup>+</sup>)/H<sup>+</sup> antiporter works by expelling cytoplasmic Na<sup>+</sup> and K<sup>+</sup> into the vacuole or the extracellular space. The first Na<sup>+</sup>(K<sup>+</sup>)/H<sup>+</sup> antiporter gene (*NHX*) was found in shore crab (*Carcinus maenas*; Skou, 1957), while the first plant homolog was found in barley in 1976 (Ratner and Jacoby, 1976). Since then, Na<sup>+</sup>(K<sup>+</sup>)/H<sup>+</sup> antiporters have been found in many plant species, such as *Arabidopsis thaliana*, rice, maize, and wheat (Barkla et al., 1994; Brini et al., 2005; Zörb et al., 2005; Chen et al., 2008). The genome of the model plant, *A. thaliana*, encodes 6 typical NHXs, which are classified into two subgroups. Based on phylogenetic relationship, AtNHX1 to 4 are clustered into one group, whereas AtNHX5 and 6 are clustered in a separate group (Yokoi et al., 2002; Aharon et al., 2003). These NHXs play diverse roles in *Arabidopsis* development and response to biotic stress, pH and ion homeostasis, cell expansion, vesicular trafficking, and salt tolerance (Apse et al., 1999; Apse et al., 2003; Bassil et al., 2011a,b). Among these eight NHXs, AtNHX1 is the most widely studied NHX; the roles of other NHXs remain largely unknown. Studies have revealed that AtNHX1 and 2 localize in the vacuole and tonoplast and have an important role in maintaining the intracellular pH and K<sup>+</sup> homeostasis (Yokoi et al., 2002; Bassil et al., 2011b; Barragán et al., 2012). Furthermore, *atnhx1* and *atnhx2* double knockout mutants displayed high sensitivity to salt stress, whereas overexpression of these genes increased the salt tolerance of the plants (Apse et al., 1999; Yokoi et al., 2002; Bassil et al., 2011b; Barragán et al., 2012). Interestingly, ectopic expression of AtNHX1 can enhance the salt stress tolerance of other plants, such as tomato, kiwifruit, wheat, tobacco, buckwheat, and peanut (Chen et al., 2008; Leidi et al., 2010; Zhou et al., 2011; Banjara et al., 2012; Moghaieb et al.,

2014). These studies suggested that salt tolerance of crops could be improved through ectopic overexpression of NHXs by genetic transformation.

In the present study, we cloned the *AtNHX5* and heterogeneously expressed it in soybean via *Agrobacterium*-mediated cotyledonary node transformation. We chose soybean, instead of *Arabidopsis* to study the function of *AtNHX5* because it is one of the most important crops in the world, with yields next to those of rice, wheat, and corn. In addition to being used as a food by humans and animals, soybean also provides raw material for industry and is used in medical research. Our results demonstrate that like *AtNHX1*, overexpression of *AtNHX5* could greatly enhance the salt tolerance of soybean, suggesting the role of NHX5 in maintaining the cellular ion homeostasis. However, a comparison of the  $K^+$  and  $Na^+$  concentrations between the transgenic and non-transgenic plants in the leaves and roots suggested that *AtNHX5* enhanced the salt tolerance in soybean through a mechanism different from that employed by *AtNHX1*.

## MATERIAL AND METHODS

### Growth conditions for soybean

Soybean variety Dongnong-50 plants were grown at  $24 \pm 1^\circ\text{C}$  under a 16:8-h (light: dark) photoperiod in a growth chamber.

### Plasmid construction

The *A. thaliana*  $Na^+(K^+)/H^+$  antiporter gene (*AtNHX5*; GenBank accession No. AT1G54370) was amplified using the primers NHX5-F (5'-CCCGAGTCTATGGAGGAAGTG ATGATTTC-3') and NHX5-R (5'-TTTGGATCCCCCATCTCCATCACCATCTC-3'; the underlined bases denote the restriction sites for *SacI* and *BamHI* in NHX5-F and NHX5-R, respectively), ligated into pGEM-T easy vector (Promega, Beijing, China) and the cloning was verified by sequencing. The *AtNHX5* gene fragment was released by *SacI* and *BamHI* digestion and ligated into a modified pCAMBIA 3300 vector, in which the sequence between *EcoRI* and *HindIII* in the multiple-cloning site (MCS) was replaced by a cassette of  $\beta$ -glucuronidase (GUS) reporter gene from pBI121 vector. The modified plasmid was named pCAMBIA 3300-121-NHX5 and was confirmed by DNA sequencing.

### Soybean transformation

Soybean transformation was performed as reported earlier (Liu et al., 2012), with a few modifications. The optimal time for infection with *Agrobacterium* and the concentration of cefotaxime and DL-phosphinothricin (PPT) were estimated to be 4 days, 250 and 6 mg/L, respectively.

### Detection of transgenic soybean plants

Total DNA from the transgenic and non-transgenic soybean plants was extracted by the Cetyltrimethyl ammonium bromide (CTAB) method, as described earlier (Keim et al., 1988). The transgenic plants were identified by polymerase chain reaction (PCR) and Southern blotting. PCR was performed in a 25- $\mu\text{L}$  volume using the primers NHX5-F and

NHX5-R, under the following conditions: pre-heating at 94°C for 5 min followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 58°C, and elongation for 1.5 min at 72°C. After the thermocycling, a final extension was performed for 10 min followed by cooling of the reaction mixture to 4°C. The PCR product was separated by electrophoresis and visualized under UV light using a BioSpectrum® Imaging System (UVP LLC, Upland, CA, USA). The amplified fragment was recovered using the AxyPrep™ DNA Gel Extraction Kit (Axygen Biosciences, Hangzhou, China) and its identity was further confirmed by DNA sequencing.

### Salt stress treatment

Seedlings of T<sub>2</sub> transgenic and non-transgenic soybean plants at three-compound leaf-stage were watered adequately before treatment with 100, 200, and 300 mM NaCl. After the treatment, the plants were kept in a growth chamber for monitoring of symptoms and analysis of physiological indicators. For gradient salt treatment, three-compound leaf-stage T<sub>2</sub> transgenic and non-transgenic soybean seedlings were treated with 50, 100, 200, and 300 mM NaCl at 7 day-intervals. All the treatments were repeated twice, using at least 10 plants for each treatment.

### Detection of free proline content

The content of free proline was determined by proline-ninhydrin reaction (Bates et al., 1973). Based on the consumption values of proline-ninhydrin reaction mixtures in a series concentration of standard L-proline solutions, at 520 nm, a calibration curve was constructed. To determine the proline content in soybean leaves, free proline extracted from 500 mg fresh leaves was mixed with proline-ninhydrin reaction mixture and incubated at 100°C for 40 min. After the incubation, 5 mL toluene was added to the reaction mixture and the consumption value at 520 nm was determined with a BioMate spectrophotometer (Thermo Scientific, Waltham, MA, USA). The proline concentration was determined using the standard concentration curve and calculated on a fresh weight basis.

### Determination of malondialdehyde (MDA) content in leaf

MDA levels were calculated according to the corrected thiobarbituric acid (TBA) method (Hodges et al., 1999). MDA was extracted from 1 g fresh soybean leaves with 2 mL extraction buffer. After adding 3 mL 0.5% TBA [including 5% Trichloroacetic acid (TCA)], the mixture was heated for 30 min at 95°C and then cooled on ice. After centrifugation at 5000 g for 15 min, the consumption of the supernatant at 450, 532, and 600 nm was determined. The concentration of MDA was calculated using the formula:

$$C_{MDA} (\mu M) = 6.45 \times (D_{532} - D_{600}) - 0.56 \times D_{450}$$

where  $D_{450}$ ,  $D_{532}$ , and  $D_{600}$  were the absorbance of the supernatant at 450, 532, and 600 nm, respectively.

### Calculation of Na<sup>+</sup> and K<sup>+</sup> content

After salt treatment, the transgenic and non-transgenic soybean plants were dried and

ground into powder. Dried soybean powder (0.1 g) was mixed with 5 mL H<sub>2</sub>SO<sub>4</sub> and boiled for 3 h at 360°C in the presence of 30% H<sub>2</sub>O<sub>2</sub>, used as a catalyst. After the treatment, the reaction mixture was cooled to room temperature and was gradient diluted. The Na<sup>+</sup> and K<sup>+</sup> concentration in the reaction mixture was determined by flame atomic absorption spectroscopy.

## Gene expression analysis

Total RNA was extracted from soybean leaf tissue samples using an AxyPrep Multisource Total RNA Miniprep Kit (Axygen Biosciences, Hangzhou, China) and digested with RNase-free DNase I (Sangon, Shanghai, China) prior to cDNA synthesis using oligo-dT<sub>18</sub>. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed on a CFX96™ Real-Time PCR detection system (Bio-Rad, Shanghai, China) with an iQTM SYBR Green Supermix kit (Bio-Rad, Shanghai, China). Each PCR was performed in triplicate. The amplification condition was set as follows: denaturation at 95°C for 20 s, 40 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 15 s, extension at 72°C for 15 s, and amplification at 65-95°C for 5 s. The primer set, NHX5 (5'-GCTACGGACCCTGTCACTGT-3') and QTR1R (5'-TTGAGCCAGCAAAGTCTCA-3') was designed to amplify a 208 bp *AtNHX5* fragment. A 202 bp fragment of soybean actin gene (GenBank accession No. GQ339774) was amplified as an internal control for normalization of *AtNHX5* mRNA levels using the primer set, actinF (5'-CACAATTGGGGCAGAGAGAT-3') and actinR (5'-CTTGCTCATACGGTCTGCAA-3').

## RESULTS

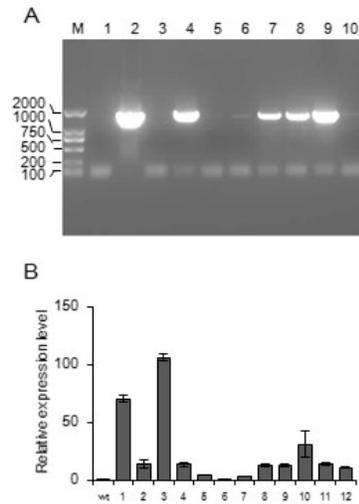
### *AtNHX5* transformation and phenotype analysis

A total of eight DL-phosphinothricin (PPT) resistant plants were generated by *Agrobacterium*-mediated cotyledonary node transformation. PCR analyses showed that, in six of the eight plants, a fragment with the predicted size (~1500 bp) was amplified (Figure 1A). Seeds from these eight plants (T1 generation) were collected separately for further analysis. Positive T1 seedlings that contained *AtNHX5* gene were pollinated and the seeds (T2 generation) were collected separately. The expression of *AtNHX5* in T2 transgenic soybean seedlings (12 individual lines) was analyzed by qRT-PCR (Figure 1B). The results demonstrated that *AtNHX5* was overexpressed in most of the transgenic lines with the line 3 (*AtNHX5oe-3*) showing the highest expression (Figure 1B). Therefore, the line *AtNHX5oe-3* was used in all the subsequent experiments. Compared to the wild-type soybean, *AtNHX5oe-3* showed no obvious differences in seed germination, plant development, and flowering (data not shown). These data suggest that overexpression of *AtNHX5* had no obvious influence on the development of soybean plants under normal conditions.

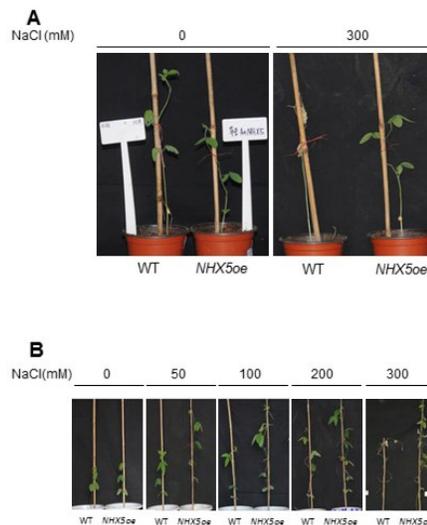
### Overexpression of *AtNHX5* enhanced salt tolerance in transgenic soybean plants

In order to verify the salt tolerance of transgenic soybean plants, T<sub>2</sub> transgenic (*AtNHX5oe-3*) and non-transgenic soybean seedlings were treated with 300 mM NaCl. After the treatment, the growth of the plants was monitored for 15 days. Results showed that the transgenic plants were still alive after 15 days of treatment, although the height of the treated plants was less than that of non-treated transgenic plants. Compared to the transgenic soybean

plants, the non-transgenic plants wilted as early as 1 day after the treatment and died after 7 days of the treatment (Figure 2A). These results indicated that the salt tolerance was enhanced in the transgenic plants.



**Figure 1.** Detection of *AtNHX5* in transgenic soybean plants. **A.** PCR detection of *AtNHX5* gene in transgenic soybean plants. Lane M, DL-2000 marker; lanes 1 to 10 are negative control, positive plasmid control, transgenic soybean ( $T_0$  generation) lanes 1 to 8. **B.** qRT-PCR analysis for *AtNHX5* expression in homozygous transgenic soybean ( $T_2$ ) (columns 1-12). The expression of *AtNHX5* was normalized to the expression of soybean *Actin* gene (GQ339774).



**Figure 2.** Phenotypes of transgenic soybean plants after NaCl treatment. **A.** Phenotype of *NHX5oe* transgenic soybean plant treated with 300 mM NaCl. **B.** Phenotype of *NHX5oe* transgenic or wild-type soybean plants under gradient salt treatment. Pictures were taken 7 days after the NaCl treatment. All the experiments were repeated at least three times.

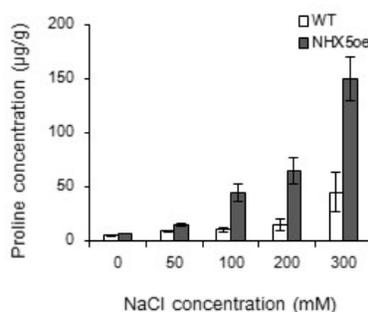
We further confirmed the salt tolerance with gradient salt treatment. In this experiment, the T<sub>2</sub> transgenic and non-transgenic soybean seedlings at the same growth stage were treated with 50, 100, 200, and 300 mM NaCl at seven-day intervals, respectively. Seven days after the treatment with 50 mM NaCl, the non-transgenic plants showed stunted growth and yellowing symptoms, whereas the growth of the transgenic plants was similar to that of the control plants (Figure 2B). In the presence of 300 mM NaCl, the non-transgenic plants died in 7 days, whereas the transgenic plants showed a growth similar to that of the untreated plants (Figure 2B). Taken together, these results clearly showed that the over-expression of *AtNHX5* significantly enhanced the salt tolerance of soybean.

### Higher proline concentration in transgenic soybean plants

The capacity to accumulate proline has been correlated with stress tolerance for many years (Barnett and Naylor, 1966; Singh et al., 1972; Stewart and Lee, 1974). The concentration of free proline could serve as an important indicator of plant resistance against abiotic stress. Therefore, the concentrations of free proline in the transgenic and non-transgenic soybean plants, treated with gradient NaCl (50, 100, 200, and 300 mM), were determined. As showed in Table 1 and Figure 2, under normal conditions (no salt stress), the concentration of free proline in the transgenic soybean plants was similar to that in the non-transgenic soybean plants (about 5 mg/g). However, upon treatment with 50 mM NaCl, the concentration of free proline in the transgenic soybean plants increased to a level much higher than that present in the non-transgenic soybean plants (14.3 to 8.6 mg/g). Furthermore, the free proline in the transgenic soybean plants was 149.8 mg/g three days post-treatment with 300 mM NaCl, which was 44.6 mg/g higher than the concentration of free proline in the non-transgenic soybean plants under the same conditions (Table 1 and Figure 3). These results indicated that under salt stress, free proline accumulated much quickly in the transgenic soybean plants than in the non-transgenic plants.

**Table 1.** Free proline concentration in wild-type and transgenic soybean plants under different NaCl treatments.

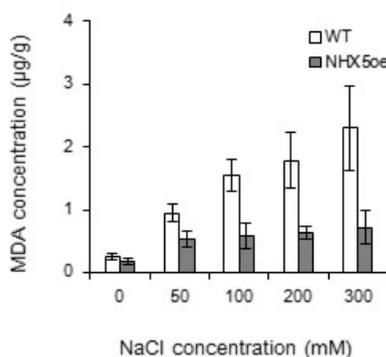
NaCl concentration (mM)	Free proline concentration ( $\mu\text{g/g}$ )	
	Wild-type	<i>NHX5oe</i>
0	4.80 $\pm$ 0.82	6.5 $\pm$ 0.64
50	8.62 $\pm$ 1.28	14.3 $\pm$ 1.21
100	10.2 $\pm$ 1.82	44.3 $\pm$ 7.63
200	14.7 $\pm$ 5.86	64.2 $\pm$ 12.53
300	44.6 $\pm$ 18.43	149.8 $\pm$ 20.47



**Figure 3.** Contents of free proline in *NHX5oe* transgenic and wild-type soybean plants under different NaCl treatments. The columns and error bars represent the average values and errors calculated from three replicate.

### Lower content of MDA in transgenic soybean plants

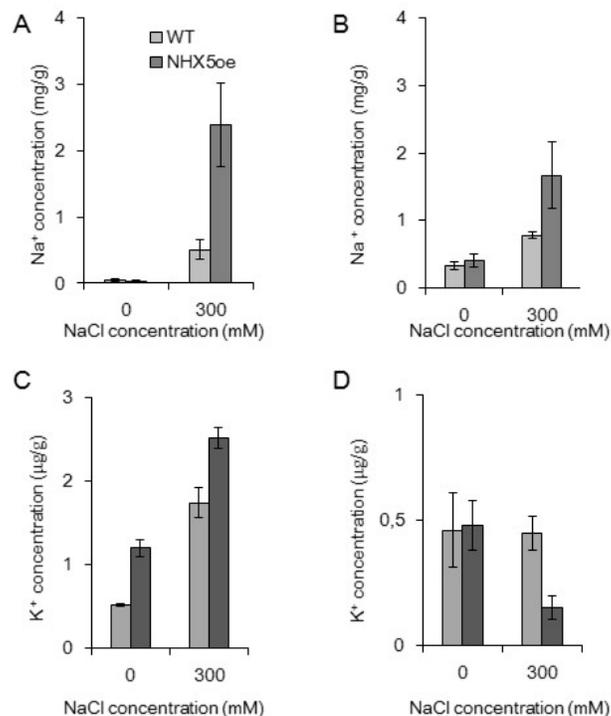
Under stress, plant cell membrane lipid has been reported to get damaged by hydroperoxidation caused by oxidative stress, induced by molecules such as superoxide ( $O_2^-$ ) and nitric oxide (NO; Mittler, 2002). Lipid hydroperoxidation is an effective indicator of oxidative damage in cells that can be measured by determining the MDA content in the leaves (Roxas et al., 1997). Therefore, we determined the MDA concentration in the transgenic and non-transgenic plants treated with NaCl. The results showed that under the normal condition, the MDA concentration was similar in both the transgenic and non-transgenic plants (Figure 4). Upon comparing the MDA concentration in the non-transgenic and transgenic plants treated with different concentrations of NaCl, it was clear that the lipid hydroperoxidation increased with the increasing NaCl concentration as indicated by the MDA concentration. However, the MDA concentration in the non-transgenic plants was significantly higher than that in the transgenic plants treated with the same NaCl concentration. For example, MDA in the non-transgenic plants was more than 3 times higher than in the transgenic soybean plants treated with 300 mM NaCl (Figure 4). These results suggested that the cell membrane of the non-transgenic plants was damaged much more severely than the transgenic plants under the same NaCl stress.



**Figure 4.** Contents of MDA in *NHX5oe* transgenic and wild-type soybean plants under different NaCl treatments. The columns and error bars represent the average values and errors calculated from three replicates.

### Na<sup>+</sup> and K<sup>+</sup> ion concentration

We further compared the Na<sup>+</sup> and K<sup>+</sup> concentration in the root and leaves of the transgenic and non-transgenic soybean plants before and after the treatment with 300 mM NaCl. Under normal conditions (no NaCl stress), the concentration of Na<sup>+</sup> in the roots and leaves was similar between the transgenic and non-transgenic soybean plants. However, the Na<sup>+</sup> concentration in the roots was higher than in the leaves in both the non-transgenic and transgenic soybean plants (Figure 5A and B). After NaCl treatment, the concentration of Na<sup>+</sup> increased in both the roots and leaves of the transgenic as well as the non-transgenic plants. However, the increase in Na<sup>+</sup> in the transgenic plants was significantly higher than that in the non-transgenic plants. Interestingly, transgenic soybean plants accumulated higher Na<sup>+</sup> in the leaves than in the roots, whereas Na<sup>+</sup> in the roots of the non-transgenic plants was higher than in the leaves, under the same treatment (Figure 5A and B).



**Figure 5.** Ion concentration in *NHX5oe* transgenic and wild-type soybean plants under 300 mM NaCl treatment. **A.** Concentration of Na<sup>+</sup> in the leaf tissue. **B.** Concentration of Na<sup>+</sup> in roots. **C.** Concentration of K<sup>+</sup> in the leaf tissue. **D.** Concentration of K<sup>+</sup> in the roots.

Under normal conditions, the K<sup>+</sup> concentration in the leaves was similar between the non-transgenic and transgenic plants, whereas the transgenic plants had a higher concentration of K<sup>+</sup> in the root (Figure 5C and D). After NaCl treatment, K<sup>+</sup> increased in the leaves of both the transgenic and non-transgenic plants. However, the increase in the transgenic plants was higher than in the non-transgenic plants. Interestingly, the concentration of K<sup>+</sup> significantly decreased in the root of the transgenic plants, whereas it was unaffected in the non-transgenic plants (Figure 5C and D). This suggested that the K<sup>+</sup> in the roots of the transgenic plants was efficiently transported to the leaves, which might help in keeping the low K<sup>+</sup> concentration in the root cell cytoplasm to promote the absorption of water from the soil.

## DISCUSSION

In the present study, the *Arabidopsis AtNHX5* gene was transformed into soybean and its role in increasing the salt stress tolerance of soybean was evaluated. The results demonstrated that the transgenic soybean plants expressing *AtNHX5* could still grow under 300 mM NaCl, whereas the non-transgenic soybean died under the same concentration (Figure 2). Furthermore, the analysis of intracellular free proline and MDA concentration revealed that the transgenic soybean accumulated higher free proline but lower MDA than the non-transgenic plants administered the same salt treatment (Figures 3 and 4). These results clearly

showed that the overexpression of *AtNHX5* could enhance the salt stress tolerance in soybean.

In order to maintain suitable cytoplasmic concentration of ions for normal water absorption under salt stress, plant cells can expel cytoplasmic ions, mainly  $\text{Na}^+$  and  $\text{K}^+$ , into the vacuole or extracellular space. This process is usually accomplished by the  $\text{Na}^+(\text{K}^+)/\text{H}^+$  antiporter proteins.  $\text{K}^+$  and  $\text{Na}^+$  concentration analysis showed that the transgenic plants expressing *AtNHX1* gene could efficiently promote the transfer of  $\text{K}^+$  and  $\text{Na}^+$  into the leaves (most possibly inside the vacuoles of the leaf cells) to maintain a low  $\text{K}^+$  and  $\text{Na}^+$  concentration in the root cytoplasm (Figure 5). These results were largely consistent with the results obtained by expressing other *Arabidopsis NHX* genes, such as *AtNHX1* (Chen et al., 2008; Leidi et al., 2010; Zhou et al., 2011; Banjara et al., 2012; Moghaieb et al., 2014).

Although the overexpression of either *AtNHX1* or *AtNHX5* could enhance the salt tolerance in the transgenic plants, the difference between *AtNHX5* and *AtNHX1* was because of the difference in their phylogeny and subcellular localization. In the phylogenetic tree, *AtNHX5* and 6 constituted a separate phylogenetic branch different from that formed by *AtNHX1* to 4 (Yokoi et al., 2002; Aharon et al., 2003). Furthermore, *AtNHX5* and 6 localize in the Golgi and *trans*-Golgi network (Bassil et al., 2011a), whereas *AtNHX1* to 4 localize in the vacuole and tonoplast (Yokoi et al., 2002; Bassil et al., 2011b; Barragán et al., 2012). These data suggest existence of possibly different mechanisms by which *AtNHX1* and 5 maintain the intracellular ion homeostasis and enhance the plant salt stress tolerance. Therefore, it would be interesting to measure the salt resistance in transgenic plants expressing both the *AtNHX1* and *AtNHX5*.

### Conflicts of interest

The authors declare no conflict of interest.

### ACKNOWLEDGMENTS

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