J. Plant Biol. (2009) 52:453–461
DOI 10.1007/s12374-009-9058-2

ORIGINAL RESEARCH

Overexpression of *AtNHX1*, a Vacuolar Na^+/H^+ Antiporter from *Arabidopsis thalina*, in *Petunia hybrida* Enhances Salt and Drought Tolerance

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Received: 12 April 2009 / Accepted: 12 July 2009 / Published online: 4 August 2009

Abstract Plant Na^+/H^+ antiporter plays a critical role in plant salt tolerance. In this study, *AtNHX1*, a vacuolar Na^+/H^+ antiporter from *Arabidopsis thalina*, was introduced into *Petunia hybrida* using *Agrobacterium*-mediated methods. Polymerase chain reaction (PCR) and DNA gel blot analysis confirmed that *AtNHX1* was successfully integrated into the *P. hybrida* genomes. Reverse transcription (RT)-PCR analysis indicated that *AtNHX1* expressed highly in transgenic plants. It was found that the 35S::*AtNHX1* transgenic *P. hybrida* plants showed much more resistant to high concentrations of NaCl and to water deprivation than the isogenic wild-type (WT) *Petunia* plants. The transgenic plants accumulated more Na^+, K^+, and proline in their leaf tissue than that of the WT *Petunia* plants and maintained high water contents and high ratio of K^+/Na^+. These results demonstrated that overexpression of the vacuolar Na^+/H^+ antiporter conferred both salt tolerance and drought tolerance to the transgenic *Petunia* plants.

Keywords Drought tolerance · *Petunia hybrida* · Salt tolerance · Transgenic plants · Vacuolar Na^+/H^+ antiporter

Abbreviations

AtNHX1	A vacuolar Na^+/H^+ antiporter of *Arabidopsis*
CaMV	Cauliflower mosaicvirus
HYG	Hygrimycin phosphotransferase
MDA	Malonyldialdehyde
MS	Murashige and Skoog
RT	Reverse transcription
RWC	Relative water content
WT	Wild type

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Introduction

Abiotic stresses, especially salinity and drought, are the primary causes of crop loss worldwide. High concentration of salts and deficiency of water cause ion imbalance, hyperosmotic stress, and subsequent secondary stresses in plants. To cope with salt and drought stress, plants have developed a variety of adaptive mechanisms, including ion homeostasis, osmotic adjustment, detoxification, and growth regulation (Bohnert et al. 1995; Zhu 2001). Recently, more attention has been paid on the mechanism of ion homeostasis in plant cells (Yamaguchi and Blumwald 2005). A large number of studies have shown that salt-tolerant plants are able to adopt efficient strategies to prevent excessive accumulation of Na^+ in cytosol, thus sustain the ion homeostasis in plant cells. These strategies include restricting the uptake of environmental Na^+, increasing the efflux of Na^+ from the cell and compartmentation of Na^+ within the vacuole. As the development of yeast and plant molecular biology, Na^+/H^+ antiporters were confirmed to be the major of Na^+ extrusion and compartmentation (Blumwald et al. 2000).

Na^+/H^+ antiporters that are localized in plasma membrane and vacuolar membrane catalyze the exchange of Na^+ for H^+ across membranes. Plasma membrane Na^+/H^+ antiporters mediate the Na^+ extrusion from plant cells,

providing an effective salt tolerance mechanism by limiting Na^+ accumulation in plant cells. Overexpression plasma membrane Na^+/H^+ antiporters *SOS1* from *Arabidopsis thaliana* (Shi et al. 2003), *SOD2* from *Schizosaccharomyces pombe* (Gao et al. 2003), and *nhaA* from *Escherichia coli* (Wu et al. 2005) improved salt tolerance in *Arabidopsis* and rice, respectively. Driven by the proton-motive force generated by the H^+-adenosine triphosphatase and H^+-inorganic pyrophosphatase, plant vacuolar Na^+/H^+ antiporters transport Na^+ into the vacuole from the cytoplasm (Blumwald et al. 2000). It contributes to ion homeostasis, and it is important for salt tolerance and development (Apse et al. 2003). Under salt stress, Na^+ compartmentation mediated by plant vacuolar Na^+/H^+ antiporters is an economical means of preventing Na^+ toxicity in the cytosol because Na^+ can be an osmolyte to help in achieving low osmotic potential, consequently sustaining water absorption from saline soil solution (Zhu 2001). Since *AtNHX1*, a vacuolar Na^+/H^+ antiporter gene of *Arabidopsis*, was firstly isolated (Gaxiola et al. 1999), a series of Na^+/H^+ antiporter coding genes have been cloned and identified from *Oryza sativa* (Fukuda et al. 1999), *Atriplex gmelini* (Hamada et al. 2001), *Beta vulgaris* (Xia et al. 2002), and some other glycophytes and halophytes. Overexpression *AtNHX1* improved salt tolerance in *Arabidopsis* (Apse et al. 1999), tomato (Zhang and Blumwald 2001), and *Brassica* (Zhang et al. 2001). Similar results were also obtained with transgenic rice (Ohta et al. 2002; Chen et al. 2007), maize (Yin et al. 2004), wheat (Xue et al. 2004), and other crop plants, further indicating the paramount role of vacuolar Na^+ compartmentation in plant salt tolerance.

Previous works have been focused on the evaluation of salt tolerance of Na^+/H^+ antiporter in transgenic plants (Zhang and Blumwald 2001; Zhang et al. 2001; Fukuda et al. 2004; Wu et al. 2004), whereas the function of drought tolerance of plant Na^+/H^+ antiporters has not been widely investigated. In principle, the salt-responsive genes may be able to activate the expression of the genes that are related with drought tolerance pathways. Meanwhile, the ions sequestered into vacuoles by plant vacuolar Na^+/H^+ antiporters may act as the osmoticum, which promotes driving water into the cell. Therefore, overexpression of vacuolar Na^+/H^+ antiporter may also enhance the drought tolerance of transgenic plants. Overexpression of the vacuolar H^+-pyrophosphatase AVP1 in *Arabidopsis* (Gaxiola et al. 1999) and tomato (Park et al. 2005) showed the improvement of drought and salt tolerance. These results provide additional evidences that supported the potential feasibility of improving plant drought tolerance conferred by overexpressing vacuolar Na^+/H^+ antiporters gene. The properties are ascribed to the increased accumulation of ion, which is likely to be a consequence of the activity of plant vacuolar Na^+/H^+ antiporter owing to the enhanced vacuolar H^+-pumping.

Petunia hybrida is a kind of important virescence and ornamental plant. Few studies have been done on genetic engineering for improving salt and drought tolerance of *P. hybrida*. Furthermore, transferring *AtNHX1* into *P. hybrida* and evaluating its effect on salt and drought tolerance have not been examined. In this study, we report the introduction of *AtNHX1* gene into *P. hybrida* using *Agrobacterium*-mediated methods and evaluate its effects on the physiological and morphological responses under salt and drought stress. This research suggests that overexpressing *AtNHX1* not only plays an important role in plant salt tolerance but also may have a great effect on plant drought resistance.

Materials and Methods

Cloning of *AtNHX1*

The total RNA was isolated from the 2-week-old leaves of *A. thaliana* using Trizol reagent method (Invitrogen). The full-length cDNA of *AtNHX1* was obtained by reverse transcription-polymerase chain reaction (RT-PCR) with the primers: 5′-GGGGTACCATGTTGGATTCTCTAGTG-3′ and 5′-CGGGATCCTCAAGCCTTACTAAGATC-3′, which contain *Bam*HI and *Kpn*I adaptor sequence (underline), respectively. PCR conditions were as follow: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 60°C for 45 s, 72°C for 90 s, and 72°C for 10 min. The *AtNHX1* PCR product was cloned into pGM-T vector (Tiangen) and confirmed by sequencing the full open reading frame.

Construction of the Plant Expression Vector

The binary Ti vector pCAMBIA1301-35SN was used for transformation. Produced by adding the cauliflower mosaic-virus (CaMV) 35S promoter and nopaline synthase terminator region into the vector pCAMBIA1301, the binary Ti vector pCAMBIA1301-35SN contains hygromycin phosphotransferase (HYG) gene for resistance to hygromycin B as a selectable marker. After being digested with *Bam*HI and *Kpn*I restriction enzymes, the cDNA of *AtNHX1* was inserted between the CaMV35S promoter and nopaline synthase terminator region of the Ti vector pCAMBIA1301-35SN, which has been digested with the same enzymes. The new construct, named pCAMBIA1301-35SN-*AtNHX1*, was electroporated into *Agrobacterium tumefaciens* strain LBA4404. The positive clones were selected on LB plates containing 50 mg L^{-1} kanamycin and identified by PCR amplification using *AtNHX1* specific primers.

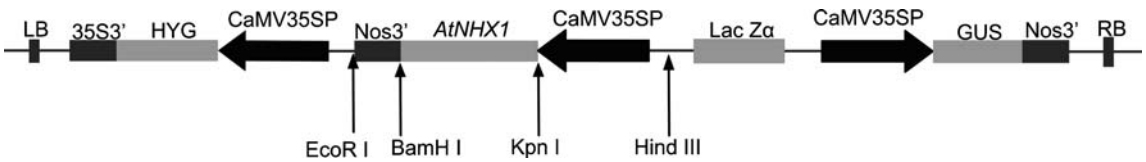

Fig. 1 Schematic of the expression vectors pCAMBIA1301-35SN-*AtNHX1*. The PCR-amplified *AtNHX1* ORF was inserted between the CaMV 35S promoter (CaMV 35SP) and nopaline synthase terminator (Nos) region. HYG gene was used as the selection marker. *LB* left T-DNA border, *RB* right T-DNA border

Production of Transgenic *Petunia* Plant

P. hybrida seeds were germinated on Murashige and Skoog (MS) medium. Eight-week-old *P. hybrida* leaves were prepared for transformation using leaf disc transformation method. For cocultivation, *Agrobacterium* LBA4404 containing pCAMBIA1301-35SN-*AtNHX1* was suspended in MS liquid medium, and the OD_{600} was adjusted to 0.5. The *P. hybrida* leaves were dipped into the *Agrobacterium* solution for 10 min and dried on sterilized filter paper before they were placed on the cocultivational medium (MS plus 1.0 mgL^{-1} 6-benzylaminopurine and 0.1 mg L^{-1} 1-naphthaleneacetic acid) for 3 days at 25°C in the dark. Then, the leaves were transferred onto the selective regeneration medium (MS plus 1.0 mg L^{-1} 6-benzylaminopurine, 0.1 mg L^{-1} 1-naphthaleneacetic acid, 200 mg L^{-1} cefalotin, and 60 mg L^{-1} hygromycin B). Regenerated shoots were transferred to fresh medium biweekly. When the shoots were 1–2 cm tall, they were separated from the calli and transferred onto rooting medium (0.5 MS medium). Rooted shoots were transplanted to soil and grown until harvest. The T1 seeds were collected and were geminated on MS medium containing 60 mg L^{-1} hygromycin B; the survived T1 generation transgenic *Petunia* plants were transferred to the 19-cm pots filled with a mixture 2:1:1(*v*/*v*/*v*) of soil/perlite/vermiculite and grown for the next experiments.

Molecular Analysis of Transgenic *Petunia* Plant

Genomic DNA was isolated from the transgenic and wild-type (WT) *Petunia* plants. For PCR analysis, the primers used were 5′-AGCTGCGCCGATGGTTTCTACAA-3′ and 5′-ATCGCCTCGCTCCAGTCAATG-3′, which amplified a 492-bp fragment of the HYG gene. PCR conditions were as follow: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 59°C for 45 s, 72°C for 90 s, and 72°C for 10 min.

Each 10 μg genomic DNA used for DNA gel blot analysis was digested with *Bam*HI before being separated by electrophoresis on a 0.8% agarose gel and then transferred to a nylon membrane. The membrane was hybridized with DIG-labeled a 300-bp fragment of the *AtNHX1* cDNA probe as the instruction of DIG High Prime

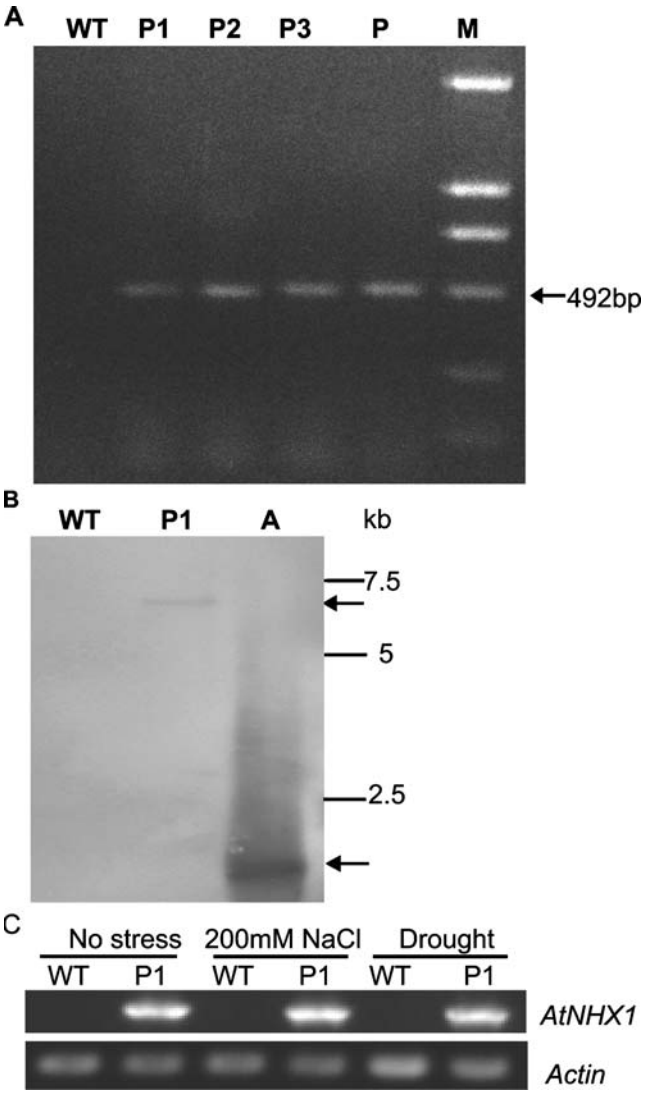

Fig. 2 Molecular identification of transgenic *petunia* plants. **a** PCR analysis of transgenic *petunia* plants. *P1–P3* independent transgenic *P. hybrida* line, *P* plasmid control, *M* marker (DL2000). *Top to the bottom*: 2,000, 1,000, 750, 500, 250, and 100 bp. **b** DNA gel blot analysis of transgenic *P. hybrida* lines (*P1*). **a** *AtNHX1* as a positive control. The genomic DNA was digested with *Bam*HI, separated by electrophoresis on a 0.8% agarose gel and then transferred to a nylon membrane. The membrane was hybridized with DIG-labeled a fragment of the *AtNHX1* cDNA probe. The positions of the molecular size standards are indicated. **c** Expression analysis of *AtNHX1* transgenic line (*P1*) and WT line under salt and drought treatments. Total RNA of transgenic plants and WT plants treated with 200 mM NaCl or water deprivation for 24 h were used for RT-PCR

DNA Labeling and Detection Starter Kit I (Roche). The cDNA of *AtNHX1* was used for positive control.

For RT-PCR analysis, total RNA was extracted using Trizol reagent method (Invitrogen) from the leaves of WT *P. hybrida* and transgenic lines treated with 200 mM NaCl or water deprivation for 24 h. One microgram total RNA from each sample was used to synthesis the first-strand DNA using Quant Reverse Transcriptase (Tiangen); the *AtNHX1* gene and the control Actin gene were amplified by PCR. The primer specific for Actin gene were 5′-CCTGATGAA GATCCTCACCGA-3′ and 5′-CAAGAGCCACATAGGCA AGCT-3′, The following PCR program was used: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55°C (for Actin gene fragment) or 60°C (for *AtNHX1* gene) for 45 s, 72°C for 90 s, and 72°C for 10 min.

NaCl and Drought Treatment

Plants were grown on soil for 8 weeks under growth chamber (≈500 μmol of photons per square meter per second, 16 h

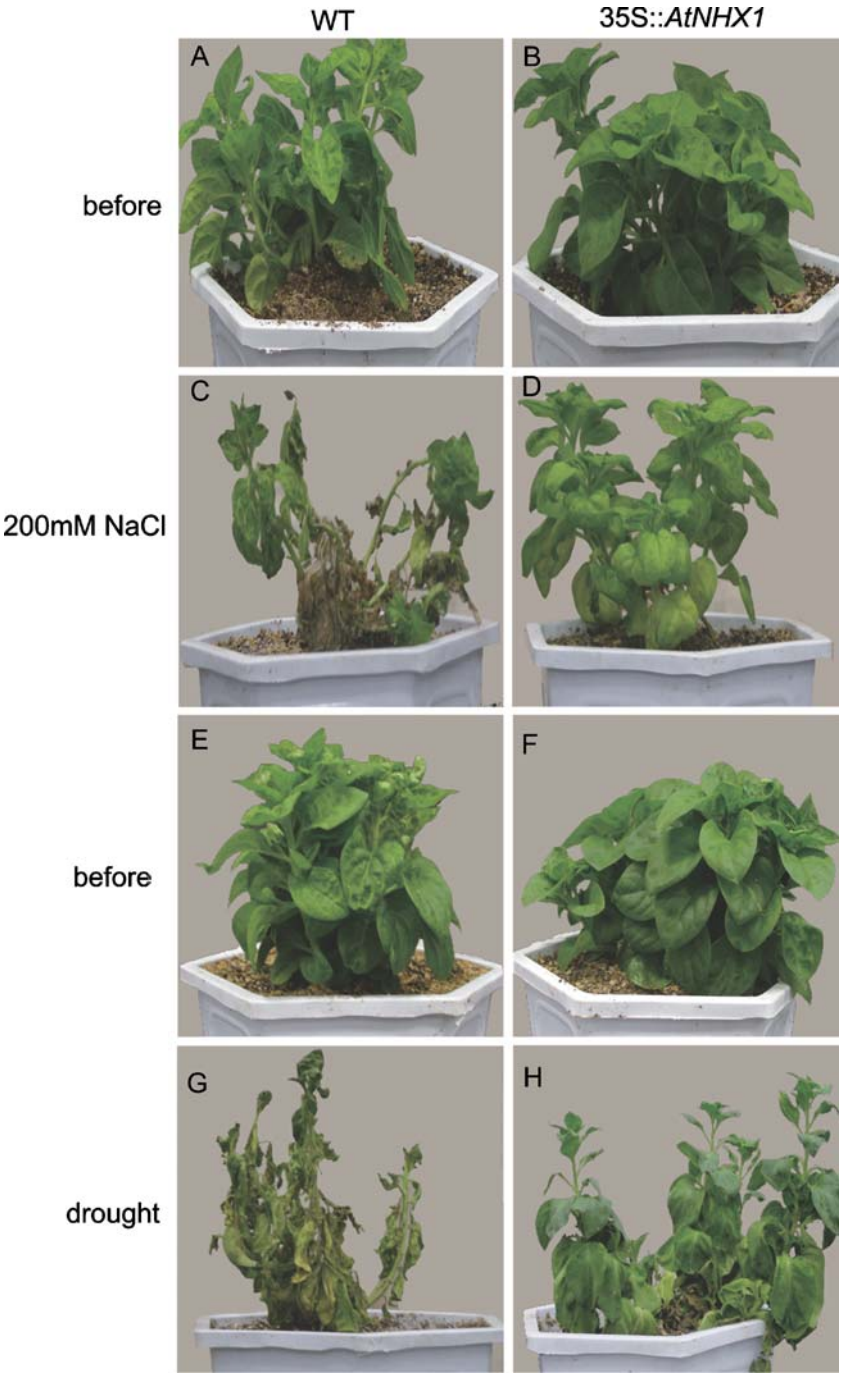

Fig. 3 Phenotype of the over-expressing *AtNHX1* transgenic (*P1*) plant and WT plant under salt and drought treatments. **a**, **b** Eight-week-old plants grown before salt treatment, **c**, **d** plants grown after 200 mM NaCl treatment for 21 days, **e**, **f** 8-week-old plants grown before drought treatment, and **g**, **h** plants grown after drought treatment for 21 days

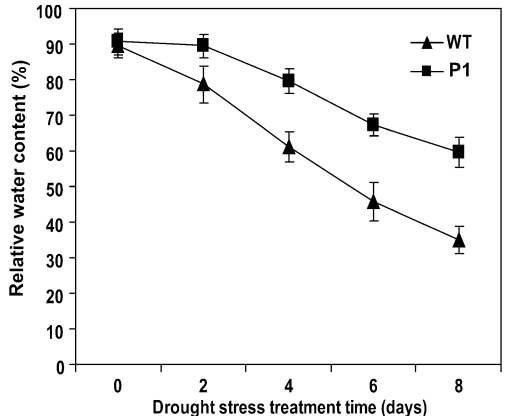

Fig. 4 RWC of WT and transgenic (*P1*) plant under drought stress. Values are means±SE (n=3)

photoperiod, 25°C) and then were placed in a container with capillarity uptake of a nutrient solution 0.125× MS salt in water supplemented with different concentrations of NaCl. The concentrations of NaCl supplementation were increased stepwise by 50 mM every 4 days for each group, to the final concentration of 50, 100, 150, and 200 mM, respectively. For drought treatment, watering was withheld for 21 days. Later, all the plants were rewatered, and the growth performance of the treated plants was checked.

Biochemical and Physiological Analysis of Transgenic *Petunia*

For malonyldialdehyde (MDA) content measurement, 2 g of fresh leaves was ground in 10 mL of 10% (*v*/*v*) acetic acid, and the homogenate was centrifuged at 13,000 rpm for 10 min. MDA concentration was determined as described previously (Quan et al. 2004). The samples were obtained from the plants treated with 0, 50, 100, 150, and 200 mM NaCl for 10 days and withhold water for 5, 10, 15, and 18 days, respectively.

During the period of drought stress, the treated leaves were excised, and their fresh weights were scored immediately. After floating them in deionized water at 4°C overnight, their turgid weights were determined. Finally, they were dried in an oven at 70°C overnight and weighted. The relative water content (RWC) was calculated as indicated: RWC=(fresh weight−dry weight)/(turgid weight−dry weight).

The roots and leaves were harvested at the end of the salt treatment, and their dry weight was measured after 48 h at 70°C. These samples were digested with HNO_3. Na^+ and K^+ contents were determined using a Z-5000 polarized Zeeman atomic absorption spectrophotometer.

For proline content measurement, 2 g of fresh leaves was ground in 10 mL of 5% (*v*/*v*) acetic acid, and the homogenate was diluted to 50 mL with distilled water. Proline concentration was determined as described previously (Troll 1955). The samples were obtained as described in that of the MDA measurement.

Results

Genetic Transformation

The leaves of 2-month-old *P. hybrida* plants were infected with *Agrobacterium* LBA4404 containing pCAMBIA1301-35SN-*AtNHX1* (Fig. 1) by leaf disc method as described, and 15 initial hygromycin B-resistant plants were obtained. The offspring of these lines were used for molecular and physiological analysis and further experiments. There were no obvious morphological or developmental differences between the transgenic and WT plants.

Molecular Characterization of the Transgenic Plants

The hygromycin B-tolerant plants were checked by PCR. The amplification was carried out in the presence of 5′ and

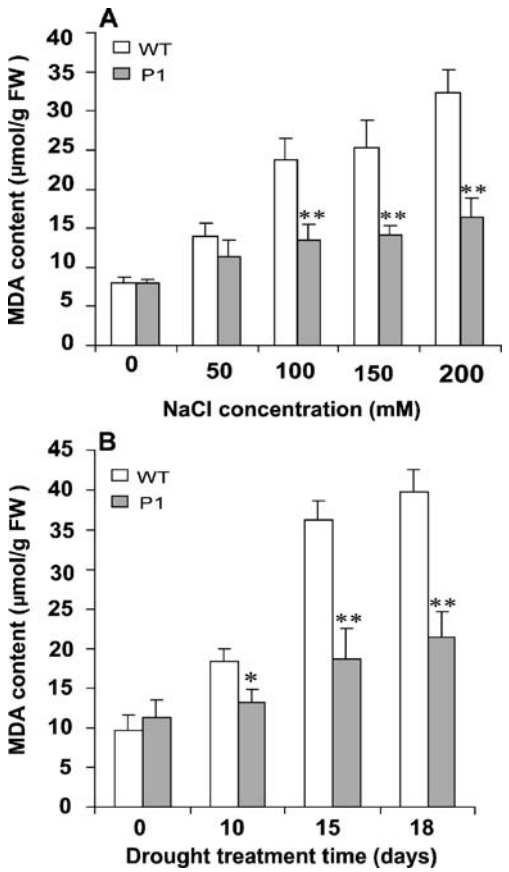

Fig. 5 MDA content of WT and transgenic (*P1*) plant under salt and drought stress. **a** MDA content of plant after salt treatment; **b** MDA content of plant after drought treatment. Values are means±SE (n=3). *P<0.05 and P<0.01, significant difference from the WT. *FW* fresh weight

3′ primers of HYG gene. An intense 492-bp band corresponding in size to the HYG gene was obtained from the hygromycin B-tolerant plants, whereas nothing was obtained from non-transgenic plants (Fig. 2a).

To further check the transgenic plants and characterize the copy number of integrated foreign *AtNHX1* gene, the genomic DNA of transgenic plants digested by *Bam*HI was hybridized with the *AtNHX1* probe. DNA gel blot analysis showed that the transgenic line P1 had hybridization signals, whereas no signal was present in wild plants. The result also showed that the line had been integrated a single copy of the *AtNHX1* gene (Fig. 2b).

In order to determine the expression of CaMV 35S promoter, which drives the expression of *AtNHX1* gene in transgenic lines and WT lines, RT-PCR was carried out using *AtNHX1* cDNA specific primers. An *AtNHX1* band with the expected size (1,617 bp) was amplified from transgenic plants but not from WT plants (Fig. 2c).

Overexpression of *AtNHX1* in *P. hybrida* Enhanced Tolerance to the Salt Stress as well as to the Drought Stress

To assess whether enhanced expression of *AtNHX1* allow plants to grow in different concentration of NaCl, WT and transgenic plants were grown in the presence of 50, 100, 150, 200 mM NaCl. There were no obvious morphological differences between the WT and transgenic plants before salt treatment (Fig. 3a, b). However, during the treatment with increasing concentration of NaCl, the growth of WT plants was severely affected, and the whole plants exhibited more and more serious chlorosis and growth inhibition. After treating with 200 mM NaCl for 21 days, the WT plants almost died (Fig. 3c), whereas the transgenic plants grew well and showed normal phenotype, and only a few of old leaves displayed marginal chlorosis in the presence of 200 mM NaCl (Fig. 3d).

WT and *AtNHX1* transgenic plants were tested for drought tolerance by growing the plants under conditions of water deprivation. Plants were grown for 8 weeks under a fully watered regimen and grown without water supplement for 21 days afterwards. As shown in Fig. 3e and f, although there were no significant morphological differences between WT and transgenic plants before drought treatment, the WT plants wilted, senesced, and seared progressively during the process of drought stress, whereas the transgenic plants wilted partially (Fig. 3 g and h). After the treatment of drought stress, the plants were rewatered. The WT plants did not recover from the stress and died later. However, the transgenic plants recovered and resumed their growth quite well. Four days later, after rewatering, most leaves of the transgenic plants displayed full turgor, and the plants grew vigorously, flowered and produced seeds normally.

The RWC of plant leaves were measured every 2 days during the treatment of drought stress. As Fig. 4 shows, a

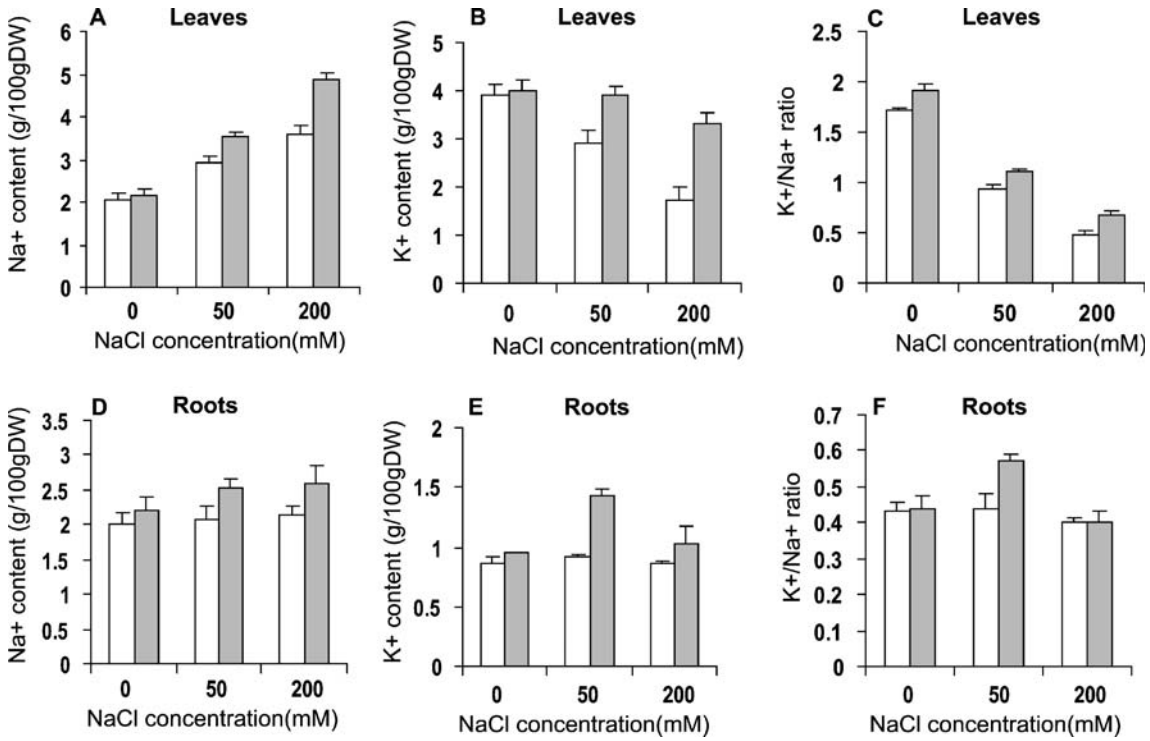

Fig. 6 Na^+ and K^+ content of WT (*white bar*) and transgenic plant (*cross-hatched line bars*) under salt stress. **a** Na^+ content of the leaves. **b** K^+ content of the leaves. **c** K^+ to Na^+ ratio of the leaves. **d** Na^+ content of the roots. **e** K^+ content of the roots. **f** K^+/Na^+ ratio of the roots. Ion contents were determined using an atomic absorption spectrophotometer. Values are means±SE (n=3). *DW* drought weight

marked reduction of the RWC in WT plants, reaching 36.3% after withholding water for 8 days, was observed. However, the RWC of transgenic plants decreased by 30.2%, from 90.8% to 60.6%. The result implied that the transgenic plants were somewhat superior to the WT plants in the character of maintaining leaves water.

In addition, the MDA contents were measured to investigate the effect of salt and drought stress on lipid peroxidation. After 18 days of 200 mM NaCl treatment, the MDA content of WT plants increased more than sevenfold to a value of 32.3, whereas the MDA content of transgenic plants merely increased twofold to 16.4 (Fig. 5a). A similar change was seen in the MDA content during the process of drought stress (Fig. 5b). These results indicated that the overexpression of plant vacuolar Na^+/H^+ antiporter effectively reduced lipid peroxidation, which was caused by salt and drought stress

Overexpressing *AtNHX1* Increased Na^+ and Proline Accumulation

The Na^+, K^+, and proline contents were measured in WT and transgenic plants. It was found that there was a steady rise of Na^+ content both in the leaves and roots of transgenic plants and WT plants, while the transgenic plants accumulated more Na^+ compared with that of the WT plants (Fig. 6a, d). The leaf K^+ content of transgenic plants was distinctly higher than that of WT plants, whereas the leaf K^+ content of WT plants was decreased as the NaCl concentration increased (Fig. 6b). Differently, few changes of root K^+ content were observed both in WT and transgenic plants, except that the root K^+ content in transgenic plants showed a rapid increase under the treatment of 50 mM NaCl (Fig. 6e). In addition, it was found that the leaf ratio of K^+/Na^+ was higher in transgenic lines than that in WT plants under different salt stress treatment (Fig. 6c).

The proline content was similar in the WT and transgenic plants before salt and drought treatment. During the period of salt and drought stress, the concentrations of proline dramatically increased in all plants. However, the transgenic plants accumulated more proline than that of WT plants under the same treatment (Fig. 7), except in the 18-day drought stress condition.

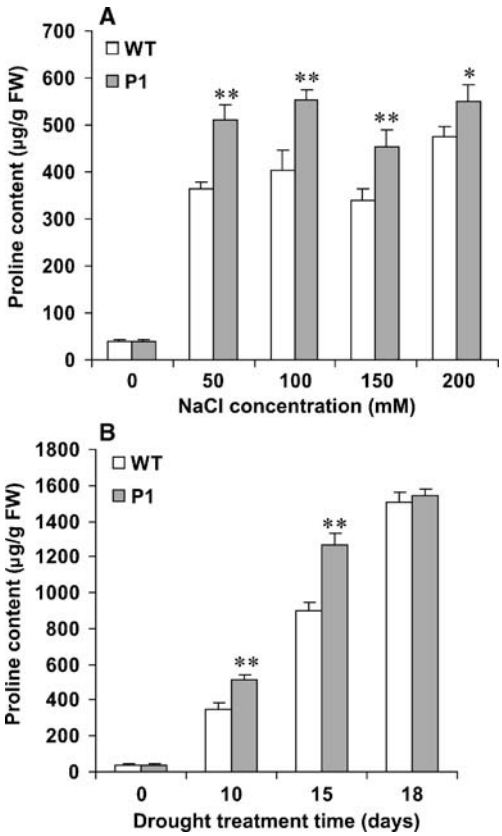

Fig. 7 Proline content of WT and transgenic (*P1*) plant under salt and drought stress. **a** Proline content of plants after salt treatment. **b** Proline content of plants after drought treatment. Values are means± SE (n=3). *P<0.05 and P<0.01, significant difference from the WT. *FW* fresh weight

Discussion

Drought and soil salinity, the main abiotic stresses that cause adverse effects on the growth of plants and the productivity of crops, are already widespread in many regions and are predicted to cause serious salinization of more than 50% of all arable lands by the year 2050 (Vinocur and Altman 2005). The study of plant salt and drought tolerance, with a view to identify and eventually manipulate the genes involved in plant salt and drought perception and responses, seems to be a promising approach for overcoming severe environmental stress (Zhu 2000). Recently, great progress of improving plant salt tolerance has been made through adopting the strategies of reestablishing ion homeostasis, such as manipulating plant vacuolar Na^+/H^+ antiporter to produce transgenic plants. Overexpression of plant vacuolar Na^+/H^+ antiporter gene in *Arabidopsis*, tomato, *Brassica*, rice, wheat, and other crops enable transgenic plants to grow in high concentration of salt, demonstrating the feasibility of producing salt-tolerant crop plants via introducing plant vacuolar Na^+/H^+ antiporter into aimed plants (Yamaguchi and Blumwald 2005).

In this study, *AtNHX1*, a vacuolar Na^+/H^+ antiporter from *Arabidopsis thalina*, was introduced into *P. hybrida*. It was found that the 35S::*AtNHX1* transgenic *P. hybrida* plants were much more resistant to high concentrations of NaCl and to water deprivation than the isogenic WT strains. Under salt and drought stress, the transgenic plants showed much better growth than the control plants (Fig. 3). Furthermore, increased salt and drought tolerance of

transgenic plants were correlated with more accumulation of Na^+, K^+ (Fig. 6), and proline (Fig. 7) in their leaf tissues, which result in abating lipid peroxidation and promoting plant cells to maintain water corroborated by low MDA content (Fig. 5) and relatively high RWC (Fig. 4), respectively. These results not only further support the important role of *AtNHX1* in plant salt tolerance but also indicate that the overexpression of *AtNHX1* has a great effect on plant drought tolerance.

Intracellular high concentration K^+ and low concentration Na^+ are important for the activities of many cytosolic enzymes and for maintaining membrane potential and an appropriate osmoticum for cell volume regulation. Under salt stress, the rate of transporter can be affected by excessive Na^+ in cytosol through its competition for K^+ binding sites of K^+ transporters such as AKT1 (Blumwald et al. 2000; Zhu 2003). Previous studies have shown that overexpressing Na^+/H^+ antiporter increases the Na^+ accumulation (Apse et al. 1999; Zhang and Blumwald 2001) and helps to maintain high ratio of K^+/Na^+ in the leaves of transgenic plants (Gao et al. 2003). In this study, it was also found that the leaves of transgenic *petunia* plants accumulated more Na^+ and K^+ and maintained a higher K^+/Na^+ ratio (Fig. 6). The transgenic plant cells obtained the enhanced ability to efficiently sequester excessive sodium into vacuole and decrease the sodium concentration in cytosol. It thus not only averts ion toxic effect on cytosolic enzymes and plasma membrane but also maintains higher K^+ concentration through alleviating the inhibition of K^+ uptake. These results indicated that *AtNHX1* overexpression increases the plant salt tolerance by elevating Na^+ accumulation and keeping the K^+/Na^+ balance mainly in the leaves. In addition, it is a surprise that Na^+ content in *Petunia* under non-saline condition was 2% by dry weight, which seems much higher than the other glycophytes.

Proline contributes to osmotic adjustment and protection in plants. The accumulation of proline in response to high salinity and drought is well documented (Kishor et al. 1995; Liu and Zhu 1997). Dissociated proline cannot only protect enzyme in the cytoplasm but also transfer more water into the cell. In this study, it was found that proline was dramatically increased both in WT and transgenic plants during the treatment of salt and drought stress. However, the transgenic plants accumulated much more proline than that of WT plants under the same treatment (Fig. 7). These results implied that the changes of ion concentrations led by elevating Na^+/H^+ transport activity may activate the expression of the drought and salt tolerance-related genes, which contributes to the enhancement in the accumulation of proline. However, the relationship of *AtNHX1* and tolerance-related genes has not yet been identified; further researches are required.

In this study, we conclude that increasing vacuolar Na^+/H^+ antiporter expression in plants elevates Na^+, K^+, and proline accumulation in leaves and conferred plants a significant degree of water deficit tolerance and high saline resistance. Our results indicate the potential application of these transgenic plants for being cultivated in saline and drought soil.

Acknowledgments We thank Prof. Blumwald E (Department of Plant Sciences, UC Davis) for revising the manuscript. This work was financially supported by Basic Research Project of Shanghai and National Natural Science Foundation of China.

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