Na\(^+\)/H\(^+\) and K\(^+\)/H\(^+\) antiporters AtNHX1 and AtNHX3 from Arabidopsis improve salt and drought tolerance in transgenic poplar

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Abstract

The tonoplast and plasma membrane localized sodium (potassium)/proton antiporters have been shown to play an important role in plant resistance to salt stress. In this study, AtNHX1 and AtNHX3, two tonoplast Na\(^+\)/(K\(^+\))/H\(^+\) antiporter encoding genes from Arabidopsis thaliana, were expressed in poplar to investigate their biological functions in the resistance to abiotic stresses in woody plants. Transgenic poplar plants expressing either gene exhibited increased resistance to both salt and water-deficit stresses. Compared to the wild type (WT) plants, transgenic plants accumulated more sodium and potassium ions in the presence of 100 mM NaCl and showed reduced electrolyte leakage in the leaves under water stress. Furthermore, the proton-translocating and cation-dependent H\(^+\) (Na\(^+\)/H\(^+\) or K\(^+\)/H\(^+\)) exchange activities in the tonoplast vesicles isolated from the leaves of transgenic plants were higher than in those isolated from WT plants. Therefore, constitutive expression of either AtNHX1 or AtNHX3 genetically modified the salt and water stress tolerance of transgenic poplar plants, providing a potential tool for engineering tree species with enhanced resistance to multiple abiotic stresses.

Additional key words: abiotic stress, electrolyte leakage, Populus davidiana × Populus bolleana, tonoplast vesicles, transgenic plant, water deficit.

Introduction

Salinity and drought are two major adverse environmental factors that prevent tree species from expanding their habitats. The detrimental effects of salt on plants are a consequence of both water deficit and excess of sodium and chloride ions on biochemical processes. In salt resistant plants, Na\(^+\) is compartmentalized into vacuoles through the operation of vacuolar Na\(^+\)/H\(^+\) antiporters to maintain osmotic balance by accumulating Na\(^+\) (and chloride) in the vacuole to drive water into the cells (Fan et al. 2015).

When the cytoplasmic Na\(^+\) content reaches a toxic level, excessive Na\(^+\) can be extruded by Na\(^+\)/H\(^+\) antiporters located in the plasma membrane (Qiu et al. 2003) or sequestered into vacuoles by tonoplast Na\(^+\)/H\(^+\) antiporters (Blumwald and Poole 1985). These active Na\(^+\)-transporting processes contribute to Na\(^+\) detoxification in plant cells. The Arabidopsis genome contains six intracellular and two plasma membrane Na\(^+\)/H\(^+\) exchanger (NHX) isoforms. AtNHX1 functions as a vacuolar Na\(^+\)/H\(^+\) antiporter to compartmentalize sodium ion away from the cytosol, as a K\(^+\)/H\(^+\) transporter for osmoregulation, or in Na\(^+\) detoxification upon saline


Abbreviations: BA - benzylaminopurine; MS - Murashige and Skoog; NAA - naphthalene acetic acid; NHX - Na\(^+\)/H\(^+\) exchanger; RT-PCR - reverse transcription polymerase chain reaction; TDZ - thidiazuron; WT - wild type.

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stress (Aharon 2003). Whereas AtNHX3 works as a tonoplast K+/H+ antiporter required for K+ utilization and ion homeostasis (Liu et al. 2010).

To date, a large number of studies have shown that expression of Na+/H+ antiporter encoding genes, especially AtNHX1, improves salt tolerance in transgenic plants. AtNHX1 was firstly over-expressed in Arabidopsis, leading to sustained growth and development in soil watered with up to 200 mM NaCl (Apse et al. 1999). Then, it was constitutively expressed in tomato (Zhang and Blemwald 2001), canola (Zhang et al. 2001), Petunia hybrida (Xu et al. 2009), soybean (Li et al. 2010), kiwifruit (Tian et al. 2011), peanut (Banjara et al. 2012), and cotton (Shen et al. 2015). Salt tolerant transgenic plants were also produced by ectopically expressing ZxNHX or AtNHX3 in sugar beet (Liu et al. 2008, Wu et al. 2015) or alfalfa (Bao et al. 2016).

Although expression of Na+(K+)/H+ antiporter improves salt and/or water-deficit tolerance in various plant species, studies on their possible functions in woody plants are limited (Wang et al. 2005, Qiao et al. 2011, Tian et al. 2011, Jiang et al. 2012). In this work, we generated transgenic poplar plants constitutively expressing AtNHX1 or AtNHX3 and determined their tolerance to high salinity and water deficit with the aim of possible use of both genes in the genetic engineering of trees.

Materials and methods

Plant growth and transformation: Hybrid poplar clone Shanxin Yang (Populus davidiana × Populus balsamifera) was used in our research. Poplar hybrids were micropropagated and cultured as previously (Wang et al. 2011).

The recombinant vectors 35S-AtNHX1 (Li et al. 2010) and 35S-AtNHX3 (Liu et al. 2008) were introduced into the Agrobacterium tumefaciens strain EHA105 and used to transform poplar as described previously (Wang et al. 2011) with minor modifications (Fig. 1 Suppl.). Briefly, leaf explants excised from one-month-old plantlets were inoculated with Agrobacterium cells harbouring 35S-AtNHX1 or 35S-AtNHX3 for 10 min and then plated on solid Murashige and Skoog (MS) medium supplemented with 0.4 mg dm⁻³ benzylaminopurine (BA), 0.1 mg dm⁻³ naphthalene acetic acid (NAA), and 100 μM aceto-syringone. After 2 d of co-cultivation, the explants were biweekly transferred onto MS medium containing 0.4 mg dm⁻³ BA, 0.1 mg dm⁻³ NAA, 0.02 mg dm⁻³ thidiazuron (TDZ), 400 mg dm⁻³ timentin, and 50 mg dm⁻³ kanamycin for selective regeneration. Regenerated shoots were transferred to rooting medium (MS supplemented with 0.05 mg dm⁻³ NAA and 30 mg dm⁻³ kanamycin). Rooted plantlets were transplanted into soil when they were over 5 cm tall. They were watered biweekly with 1/8 concentration of MS salt solution and grown in a chamber at a temperature 28 ± 2 °C, a relative humidity of 70 %, an irradiance of 125 μmol m⁻² s⁻¹ (cool white fluorescent tubes), and a 16-h photoperiod.

Confirmation of transgenic plants: Transgenic plants were screened for the insertion of T-DNA by polymerase chain reaction (PCR) amplification. Total genomic DNA was extracted from the leaves of wild type (WT) and kanamycin resistant plants according to the instructions of plant genomic DNA extraction kit (FCNCS, Nanjing, China). PCR reactions were performed using AtNHX1 and AtNHX3 primers (Table 1 Suppl.). For reverse transcription (RT)-PCR analyses, total RNA was isolated from leaves with TRIZOL reagent (Invitrogen, Carlsbad, USA) according to the manufacturer’s instructions. Reverse transcription of RNA was carried out using M-MLV RTase synthesis kit (Promega, Madison, USA). RT-PCR was performed using AtNHX1 and AtNHX3 primers to amplify products of 1 633 and 3 612 bp, respectively (Table 1 Suppl.). Poplar elongation factor gene EF1β was employed as an internal control (Tang et al. 2010, 2014). Expression of EF1β was also determined with its forward and reverse primers to amplify a PCR product of 265 bp (Table 1 Suppl.). All experiments were repeated three times independently.

Salt and water-deficit treatments: WT and transgenic plants were transferred to soil and grown in greenhouse. Plants with the same size (30 cm) and growth status were chosen and subjected to salt or water-deficit stress. For salt stress, plants were watered with a diluted nutrient solution (1/8 MS) supplemented with 0 or 100 mM NaCl every 3 d. For water stress, sterile seedlings of WT and transgenic plants of the same size and growth status were transferred to MS medium containing 0 or 200 mM mannitol. Plants were also transferred to soil and grown in the greenhouse. When they reached 10-leaf stage, water was withheld for 8 d before they were re-watered.

Determination of Na⁺ and K⁺ content, ion leakage, and proline content: Na+ and K+ content in the leaves of WT and transgenic plants at the end of salt stress was measured using an atomic absorption spectrophotometer (Hitachi Z-8000, Tokyo, Japan) as described previously (Wang and Zhao 1995). The degree of cellular damage was assessed by ion leakage analysis as described (Bowler et al. 1991). Proline content in the leaves of WT and transgenic plants at the end of water-deficit treatment was determined spectrophotometrically as described by Bates et al. (1973).

Membrane isolation and vacuolar transport assays: Membrane fractions were isolated from young poplar leaves and all the experiments were performed exactly as...
described previously (Liu et al. 2008). Membrane fractions were purified using the method described previously (Zhang et al. 2001) from young leaves of plants grown under control conditions. At the indicated times, the vacuolar H⁻-inorganic pyrophosphatase (H⁻-PPiase) was activated by the addition of Mg²⁺. When a steady state pH gradient (acidic inside) was formed, the PPI-dependent H⁻ transport activity was stopped by the addition of EDTA. The rates of cation/H⁻ exchange were determined in vesicles isolated from WT and transgenic plants expressing \textit{AtNHX1-3} or \textit{AtNHX3-6}. The initial rate of fluorescence quenching was used as a relative estimate of the rate of H⁻-translocation. The rates were normalized to the fluorescence change between the addition of Mg²⁺ and the addition of EDTA.

**Statistical analyses and GenBank accession numbers:** For statistical analyses, the Student’s t-test was used and the difference at $P < 0.05$ was considered as statistically significant. The data were normalized and all samples were normally distributed with homogeneity of variance.

GeneBank accession numbers used in this study: \textit{EF1β} (eugene3.00091463), \textit{AtNHX1} (At5g27150), and \textit{AtNHX3} (At5g55470).

*Fig. 1. Molecular characterization of transgenic poplar plants. A - PCR analyses of \textit{AtNHX1} and \textit{AtNHX3} in independently regenerated kanamycin resistant lines. B - Semi-quantitative RT-PCR analyses of transgene expression in different transgenic lines. The elongation factor gene \textit{EF1β} was employed as an internal control. C - Phenotypes of wild type (WT) and different transgenic lines expressing \textit{AtNHX1} or \textit{AtNHX3} after salt stress treatment. Plants were grown in greenhouse and were watered with 100 mM NaCl for 45 d.*

**Results**

To assess the functions of \textit{AtNHX1} and \textit{AtNHX3}, as well as their potential value in improving salt and water stress tolerance in woody plants, we introduced the constructs of \textit{AtNHX1} or \textit{AtNHX3} into the genome of poplar clone Shaxin Yang by \textit{Agrobacterium}-mediated transformation. Transgenic shoots grew faster and successfully
formed roots after three weeks, whereas WT failed to do so (Fig. 2A-F Suppl.). Furthermore, when leaf explants of WT and transgenic lines were re-regenerated on selection medium for 25 d, only transgenic lines reproduced adventitious shoots on medium with kanamycin (Fig. 2G-I Suppl.). In this way, at least ten independently regenerated kanamycin resistant lines were obtained for each construct.

Thidiazuron has strong cytokinin-like activity and has been shown to be an efficacious regulator of in vitro morphogenesis of many dicot plant species. We examined shoot regeneration frequency and shoot number per explant on MS medium supplemented with different concentrations of TDZ (0.02, 0.10, 0.20, 0.50 μM) (Fig. 3 Suppl.). Among various TDZ concentrations tested, 0.02 μM TDZ produced the highest regeneration frequency (99.17 %) (Fig. 3 Suppl.).

PCR analyses confirmed the integrity of *AtNHX1* or *AtNHX3* in most of randomly selected kanamycin resistant lines (Fig. 1A). The relative expressions of *AtNHX1* and *AtNHX3* in these transgenic lines were further detected by RT-PCR (Fig. 1B). Two independent transgenic lines of *AtNHX1* (*AtNHX1-3* and *AtNHX1-4*) and of *AtNHX3* (*AtNHX3-1* and *AtNHX3-6*) were selected for salt tolerance analyses.

In order to test whether expression of *AtNHX1* or *AtNHX3* would affect the salt tolerance of trees, we examined the effects of 100 mM NaCl on the growth of

![Figure 2](image_url)

Fig. 2. Increased salt tolerance in transgenic poplar plants expressing *AtNHX1* or *AtNHX3*. A, B, C, D - Wild type (WT) and transgenic plants were treated with 100 mM NaCl for 0 (A), 32 (B), 35 (C), and 38 (D) d. E - The fifth leaves detached from WT and transgenic plants after the salt stress for 35 d. F - Phenotypes of WT and transgenic plants under control conditions and 100 mM NaCl for 38 d.
transgenic plants. At least 30 plants of WT and each transgenic line were grown in a greenhouse. Ectopic expression of either \textit{AtNHX1} or \textit{AtNHX3} did not change the overall morphology of transgenic poplar under normal growth conditions (Fig. 2A,F; Fig. 3A-C). However, after the treatment with 100 mM NaCl for 45 d, all tested transgenic plants grew more vigorously than the WT plants (Fig. 1C). Therefore, we selected one representative line of each transgene for more detailed analyses. Although no significant difference was observed in the height of WT and transgenic plants in the presence of 100 mM NaCl, the leaves of WT plants became wilted after 32 d and started to fall off after 35 d, whereas transgenic plants exhibited near-to-normal leaf colour (Fig. 2B,C,E; Fig. 3A-C). After 38 d, most leaves on WT plants fell off, but transgenic plants were less impaired by the salt stress (Fig. 2D,F).

To understand how the transgene confers salt tolerance to transgenic plants, we investigated the content of Na\(^+\) and K\(^+\) in the leaves of WT and transgenic plants grown under both normal and salt stress conditions. Under normal growth conditions, both WT and transgenic plants contained approximately equal Na\(^+\) and K\(^+\) content (Fig. 4A, B). In the presence of 100 mM NaCl, Na\(^+\) content increased in all plants, accompanied by a decrease of K\(^+\) content. However, Na\(^+\) and K\(^+\) content in the leaves of transgenic plants was significantly higher than in the leaves of WT plants (Fig. 4A,B).

We also examined the water stress tolerance of transgenic plants at \textit{in vitro} and \textit{ex vitro} conditions. WT and transgenic shoots were cultured on MS medium supplemented with 200 mM mannitol. At this concentration, the growth of WT shoots was sustained and the leaves turned into yellow colour (Fig. 5). But transgenic plants expressing either transgene grew much better. In addition, all transgenic shoots produced roots successfully, but WT shoots failed to do so (Fig. 5). Further, WT and transgenic plants transplanted into soil were subjected to water withholding for 8 d and then re-watered for 1 d. After 5 d of water deficit, WT plants began to be wilted, whereas transgenic plants remained near-to-normal (Fig. 6A) until water was withheld for 8 d, when all plants showed wilted phenotype (Fig. 6B). When plants were re-watered for 1 d, WT plants became wilted permanently and died, whereas transgenic plants showed less damage and most of them recovered (Fig. 6C). After rewatering for 50 d, over 70\% of transgenic plants survived, whereas all WT plants died (Fig. 6D, Fig. 4A,B Suppl.).

The damage of plant cell membrane integrity by abiotic stress can be estimated by measuring the leakage of cytoplasmic solutes. Under normal conditions, no difference in electrolyte leakage between WT and transgenic plants was observed. After water-deficit treatment for 8 d, electrolyte leakage increased in both WT and transgenic plants. But the increase was significantly lower in transgenic plants, suggesting that the damage of membrane integrity was less severe in
transgenic plants under water stress conditions (Fig. 6E). We also compared proline accumulation in the leaves of WT and transgenic plants before and after water deficit treatment. The content of proline was about the same before the stress, and increased in all plants upon the stress treatment but more in the leaves of transgenic plants than in the leaves of WT plants (Fig. 6F).

Since \textit{AtNHX1} and \textit{AtNHX3} encode two vacuolar Na⁺(K⁺)/H⁺ antiporters, tonoplast vesicles were isolated from the leaves of four-week-old greenhouse grown wild type and transgenic plants. The cation-dependent H⁺ transport in tonoplast membranes was measured. Tonoplast vesicles isolated from transgenic plants expressing \textit{AtNHX1} (\textit{AtNHX1-3}) and \textit{AtNHX3} (\textit{AtNHX3-6}) displayed similar K⁺/H⁺ and Na⁺/H⁺ exchange rates and H⁺-translocation to those from vesicles isolated from WT, respectively (Fig. 7A,B). However, a significantly increased Na⁺/H⁺ exchange rate in \textit{AtNHX1-3} and K⁺/H⁺ exchange rate in \textit{AtNHX3-6} transgenic plants was observed, indicating that the expression of \textit{AtNHX1} and \textit{AtNHX3} increased the vacuolar Na⁺/H⁺ and K⁺/H⁺ exchange activity of transgenic plants (Fig. 7A,B).

**Fig. 4.** Na⁺ (A) and K⁺ (B) content in wild type (WT) and different transgenic lines expressing \textit{AtNHX1} or \textit{AtNHX3} genes before and after NaCl treatment. Means ± SEs, n = 3.

**Fig. 5.** Transgenic plants were more tolerant to osmotic stress. Wild type (WT) and transgenic plants expressing \textit{AtNHX1} or \textit{AtNHX3} genes were cultured on MS medium supplemented with 200 mM mannitol for 40 d.

**Discussion**

Salinity causes two major damages to plant cells; water deficit resulting from the relatively high solute concentrations in the soil solution, and ion toxicity due to altered K⁺/Na⁺ ratios as well as excessive Na⁺ and Cl⁻ content (Apse and Blumwald 2002). Correspondingly, two key processes, the cytosolic Na⁺ detoxification and cellular osmotic adjustment, are necessary for plants to tolerate salinity stress. The compartmentalization of Na⁺
into the plant vacuoles provides an efficient mechanism to avoid the toxic effects of Na\(^+\) in the cytosol (Apse and Blumwald 2007).

Poplar is a widely grown tree producing plenty of wood, fiber, and renewable biomass for energy. Since most of its cultivars are sensitive to salinity and water stress, developing cultivars with improved stress tolerance has become one of the most efficient strategies for dealing with salinity and drought conditions. Previous studies in various plant species have demonstrated that expression of tonoplast NHXs could increase the salt or water stress tolerance of transgenic plants. However, most of these studies were carried out in herbaceous plants (Zhang et al. 2001, Zhang and Blumwald 2001, Liu et al. 2008, Xu et al. 2009, Li et al. 2010, Banjara et al. 2012, Shen et al. 2015, Wu et al. 2015, Bao et al. 2016), and the dual salt and water stress tolerant trait was usually obtained by co-expression of a tonoplast NHX and an H\(^+-\)PPase (Brini et al. 2007, Bao et al. 2016). In this work, we showed that ectopic expression of a single gene,

**Fig. 6.** A, B, C - Phenotypes of wild type (WT) and transgenic plants expressing *AtNHX1* and *AtNHX3* genes after withholding water for 5 d (A) or 8 d (B), and then re-watering for 1 d (C). D - Survival rates of WT and transgenic plants after re-watering for 50 d. E, F - Relative electric conductivity and proline content before and after water-deficit treatment for 8 d. Means ± SEs, n = 4.

**Fig. 7.** Proton-translocation and cation-dependent H\(^+\) (Na\(^+\)/H\(^+\) or K\(^+\)/H\(^+\)) exchange activity in leaf tonoplast vesicles. Rates of Na\(^+\)-dependent and K\(^+\)-dependent H\(^+\) efflux in vacuoles isolated from wild type (WT) and *AtNHX1* and *AtNHX3* transgenic lines. The initial rates of fluorescence quenching (F) taken within the first 3 min after the addition of 50 mM NaCl or KCl were used for relative estimates of these parameters. Means ± SEs, n = 4.
either \textit{AtNHX1} or \textit{AtNHX3}, conferred both salt and water stress tolerance to poplar. When engineering trees with improved resistance to abiotic stresses, it is very important and practical to start with a relatively tolerant cultivar, and hybrid clone Shanxin Yang is more salt tolerant than many other poplar clones. Therefore, we constitutively expressed \textit{AtNHX1} and \textit{AtNHX3} in Shanxin Yang (Fig. 1A,B). Transgenic plants expressing either \textit{AtNHX1} or \textit{AtNHX3} showed normal growth and morphology under control conditions and increased tolerance to 100 mM NaCl, implying that \textit{AtNHX1} and \textit{AtNHX3} are mainly responsible for stress tolerance without impairing the normal growth and development of poplar (Figs. 1C, 2A-F, 3A-C). Similar results were also observed in our previous study in the same cultivar overexpressing either salt overlysensitive3 (\textit{PtSOS3}) or calcineurinB-like protein10 (\textit{PtCBL10}) (Tang \textit{et al.} 2014).

It has been well documented that both vascular Na$^+$ transport and optimal Na$^+$ distribution are critical for plant resistance to salt stress. The salt tolerant phenotype of transgenic plants expressing \textit{AtNHX3} or \textit{AtNHX1} was associated with a higher Na$^+$ and K$^+$ content in the leaves than in the WT plants (Fig. 4A,B). Data presented here are consistent with the earlier report that \textit{Populus deltoides} × \textit{P. euramericana} hybrid plants expressing \textit{AtNHX1} accumulated more Na$^+$ than WT plants (Qiao \textit{et al.} 2011) and \textit{Populus} × \textit{euramericana} cv. Neva expressing \textit{AtNHX1} accumulated more K$^+$ in the leaves than WT plants (Jiang \textit{et al.} 2011) in response to 150 mM NaCl treatment or seawater irrigation.

The increased osmotic and water stress tolerance in transgenic plants could have benefited from their improved ability in maintaining ion homeostasis. Indeed, the leaves of WT plants became chlorotic, and the growth of shoots was inhibited on MS medium containing 200 mM mannitol (Fig. 5). Under water stress conditions, WT plants showed rapid leaf desiccation and failed to survive after re-watering (Fig. 6A-D). The leakage of electrolyte increased strongly in WT plants, whereas only slightly in transgenic plants (Fig. 6E).

Proline is an important osmotic agent and protector of macromolecules during dehydration. It also functions as a hydroxyl radical scavenger. We found that proline was accumulated more in transgenic than in WT plants (Fig. 6F) under water stress. A higher increase in proline content was also observed in transgenic \textit{Petunia hybrida} (Xu \textit{et al.} 2009), kiwifruit (Tian \textit{et al.} 2011) and tomato (Zhang and Blumwald 2001) expressing \textit{AtNHX1} in comparison with respective WT plants. Therefore, the elevated accumulation of proline in transgenic poplar plants could have helped to alleviate the negative effects imposed by the salt and the water deficit on transgenic plants. All these results suggest that constitutive expression of \textit{AtNHX1} or \textit{AtNHX3} improved cell membrane integrity in transgenic plants.

As \textit{AtNHX1} and \textit{AtNHX3} function as tonoplast Na$^+$/K$^+$/H$^+$ antiporters (Apse \textit{et al.} 1999, Liu \textit{et al.} 2010), the increased Na$^+$ and K$^+$ content in the leaf tissues of transgenic plants could be due to the promoted Na$^+$/K$^+$/H$^+$ exchange activity (Fig. 4A,B). Indeed, Na$^+$/K$^+$/H$^+$ exchange across the vacuolar membrane was significantly higher in transgenic plants (Fig. 7A,B) than in WT plants. Similar results were also observed in transgenic tomato expressing \textit{AtNHX1} (Zhang and Blumwald 2001) and sugar beet expressing \textit{AtNHX3} (Liu \textit{et al.} 2008). All these observations indicate that expression of \textit{AtNHX1} and \textit{AtNHX3} promoted the vacuolar Na$^+$/K$^+$/H$^+$ exchange activity of transgenic plants, and as a result, increased Na$^+$ and K$^+$ accumulation in the vacuoles.

It has been well documented that \textit{AtNHX1} functions as a Na$^+$/K$^+$/H$^+$ antiporter. We found that transgenic plants constitutively expressing \textit{AtNHX1} also accumulated more K$^+$ (Fig. 4B). Similar results were also reported in other transgenic plants constitutively expressing \textit{AtNHX1} (Zhang \textit{et al.} 2001, Jiang \textit{et al.} 2012). In agreement with this observation, we have expected that relative to WT plants, a higher K$^+$/H$^+$ exchange activity would be detected in \textit{AtNHX1} transgenic poplar leaf tonoplast. However, only a small, but not significant, increase of K$^+$/H$^+$ exchange activity was detected (Fig 7B). One of the possible explanations could be that \textit{AtNHX1} is not the only K$^+$/H$^+$ antiporter in poplar, and ectopic expression of \textit{AtNHX1}, even under the control of the 35S promoter, was not enough to cause significant increase of K$^+$/H$^+$ antiporter activity in transgenic plants. Similarly, in our previous study on transgenic tomato plants constitutively expressing \textit{AtNHX1}, the activity of K$^+$/H$^+$ antiporter is also only slightly increased (Zhang \textit{et al.} 2001). Another possible reason could be that the K$^+$/H$^+$ antiporter activity of \textit{AtNHX1} is regulated by some other protein kinases such as PtSOS2 in poplar. In \textit{Arabidopsis}, the SOS2-SOS3 complex phosphorylates the Na$^+$/H$^+$ antiporter SOS1 (Shi \textit{et al.} 2000) to stimulate its Na$^+$/H$^+$ exchange activity at the plasma membrane (Quintero \textit{et al.} 2002, Qiu \textit{et al.} 2002). We also observed that the poplar calcineurin B-like proteins PtCBL10A and PtCBL10B regulated shoot salt tolerance through interaction with PtSOS2 in the vacuolar membrane (Tang \textit{et al.} 2014). Furthermore, transgenic plants accumulated more Na$^+$ than K$^+$. Therefore, the compartmentalized Na$^+$ in vacuoles may affect the driving force for Na$^+$/H$^+$ exchange, leading to a smaller Na$^+$/H$^+$ exchange. Therefore, the K$^+$/H$^+$ antiporter activity of \textit{AtNHX1} in transgenic poplar plants was also dependent on the content of Na$^+$ and K$^+$, the contribution of other K$^+$/H$^+$ antiporters, as well as the activity of other proteins which co-work with or regulate the expression and/or activity of \textit{AtNHX1}.

Soil salinity is one of the limiting factors that severely affect agricultural production. Therefore, maintaining low cytosol Na$^+$ content is fundamental for plant cells. Generally, the salt tolerance of plants is highly dependent on the activity of Na$^+$/H$^+$ antiporters. Na$^+$ ions can be
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removed out of the cytosol by compartmentalizing them into the vacuole or by transport out of the cell using the tonoplast and plasma membrane Na⁺/H⁺ exchangers, respectively (Apse and Blumwald 2007). In addition to the salt tolerance, transgenic poplar plants expressing AtNHX1 or AtNHX3 also showed increased resistance to water stress (Fig. 6A-D; Fig. 4A,B Suppl.). This is the first report on tree plants that showed resistance to both salt and water stresses when a single NHX family gene was introduced. Most reported transgenic plants which show resistance to both salt and water stresses are produced by co-expressing NHX and H⁺-PPase encoding genes (Brini et al. 2007, Shen et al. 2015, Bao et al. 2016), except transgenic Petunia hybrida and peanut expressing AtNHX1 (Xu et al. 2009, Asif et al. 2011). The enhanced tolerance to water deficit of transgenic plants could be benefited from the increased ion content in the vacuoles which drive increased water transport, thus promoting plant growth under the stress conditions.

Taken together, data presented in this work demonstrate that constitutive expression of either AtNHX1 or AtNHX3 in poplar enhanced the Na⁺/H⁺ or K⁺/H⁺ exchange activity, thereby increased Na⁺ and K⁺ accumulation in the vacuoles of transgenic plants, leading to improved tolerance to salt and water stresses in transgenic plants. This provides a promising strategy for the breeding of trees for improved resistance to multiple abiotic stresses.

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