Overexpression of AtNHX5 improves tolerance to both salt and drought stress in Broussonetia papyrifera (L.) Vent

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Paper mulberry (Broussonetia papyrifera L. Vent) is well known for its bark fibers, which are used for making paper, cloth, rope, etc. It was found that, in addition to its well-documented role in the enhancement of plant salt tolerance, overexpression of the Na⁺/H⁺ antiporter (AtNHX5) gene in paper mulberry plants showed high drought tolerance. After exposure to water deficiency and salt stress, the wild-type (WT) plants all died, while the AtNHX5-overexpressing plants remained alive under high salt stress, and had a higher survival rate (>66%) under drought stress. Measurements of ion levels indicated that Na⁺ and K⁺ contents were all higher in AtNHX5-overexpressing leaves than in WT leaves in high saline conditions. The AtNHX5 plants had higher leaf water content and leaf chlorophyll contents, accumulated more proline and soluble sugars, and had less membrane damage than the WT plants under water deficiency and high saline conditions. Taken together, the results indicate that the AtNHX5 gene could enhance the tolerance of paper mulberry plants to multiple environmental stresses by promoting the accumulation of more effective osmolytes (ions, soluble sugars, proline) to counter the osmotic stress caused by abiotic factors.

Keywords: AtNHX5, Broussonetia papyrifera, salt tolerance, drought tolerance.

Introduction

Plant growth and productivity are often dramatically reduced by multiple environmental stresses, such as high salt, drought, and high and low temperatures. Salinity stress is one of the most serious environmental factors reducing the yield of agricultural crops worldwide (Flowers 2004). The impact of excess salinity on forest trees, although less important economically than its impact on agricultural crops, is nevertheless widespread and costly (Allen et al. 1994, 1996, 2010). Therefore, there is an urgent need to increase the salt tolerance of plants either by genetic introgression or through the use of transgenic technology for gene transfer to enable agriculture in marginal lands (Flowers 2004).

Salinity-stress effects on plant growth are manifested by disrupting the ability of the roots to take up water efficiently and, through the perturbation of crucial metabolic reactions inside the cell by imposing osmotic stress on cell water relations, and by increasing the concentration of sodium to toxic levels in the cytosol (Hasegawa et al. 2000, Tester and Davenport 2003, Munns and Tester 2008). Plant NHX proteins, originally described as Na⁺/H⁺ antiporters located in the tonoplast, sequestrating excess Na⁺ into the vacuole, have been identified as important salt tolerance determinants (Apse et al. 1999, Gaxiola et al. 1999, Quintero et al. 2000, Blumwald 2000, Zhang and Blumwald 2001, Zhang et al. 2001).

Two major types of plant NHX have been identified (Rodriguez-Rossales et al. 2009). The SOS1-like (salt overly sensitive 1 type) NHX proteins located on the plasma membrane, which appear to have evolved separately from intracellular NHX transporters (IC-NHE/NHX), may play an important role in long-distance Na⁺ transport in plants (Shi and Zhu 2002, Olias et al. 2009). The
IC-NHE/NHX proteins can be further subdivided into Class-I and Class-II variants; Class-I NHX isoforms (AtNHX1–4) that have been studied to date are localized in the vacuolar membrane localization, while Class-II NHX isoforms (AtNHX5–6) are expressed in various endosomal compartments (Pardo et al. 2006, Rodríguez-Rosales et al. 2008, 2009). IC-NHE/NHX plays a critical role in internal pH regulation and Na\(^+\) and K\(^+\) homeostasis, regulating processes from vesicle trafficking and cell expansion to plant development (Rodríguez-Rosales et al. 2009, Leidi et al. 2010). The Arabidopsis AtNHX1 was the first NHX gene identified in plants, and has previously been suggested to catalyze Na\(^+\) accumulation in vacuoles, reduce cytosolic Na\(^+\) concentrations and ameliorate the toxic effects of Na\(^+\) on cellular metabolism (Ápse et al. 1999, Gaxiola et al. 1999). However, more recent evidence supports the hypothesis that plant NHX also plays crucial roles in pH regulation (Yamaguchi et al. 2001, Yoshida et al. 2005, 2009) and K\(^+\) homeostasis, which in turn affect plant K\(^+\) nutrition and Na\(^+\) tolerance (Munns and Tester 2008, Rodríguez-Rosales et al. 2008, 2009, Liu et al. 2008, Leidi et al. 2010). Numerous studies have shown that the AtNHX1 gene confers salt tolerance to Arabidopsis plants and various other plant species (Ápse et al. 1999, Zhang and Blumwald 2001, Zhang et al. 2001, Xue et al. 2004, He et al. 2005, Leidi et al. 2010). Furthermore, overexpression of AtNHX1 orthologs, such as AgNHX1 of Atriplex gmelini C. A. Mey, OsNHX1 of Oryza sativa L., BrNHX1 of Brassica napus L., GhNHX1 of Gossypium spp., HbNHX1 of Hordeum vulgare L., PgNHX1 of Pennisetum glaucum L., ThNHX1 of Thellungiella halophila, AtNHX3 and AtNHX4, conferred high salt tolerance to transgenic plants (Ohta et al. 2002, Fukuda et al. 2004, Wang et al. 2004, 2007, Wu et al. 2004, 2005, 2009, Lü et al. 2005, Rajagopal et al. 2007, Liu et al. 2008). Studies on the Class-II antiporters have been reported only for the Arabidopsis AtNHX5 (Shi et al. 2008) and the tomato LeNHX2 (Rodriguez-Rosales et al. 2008).

*Broussonetia papyrifera* is a widely valuable natural resource, which is a small tree or shrub belonging to the Moraceae family. It is native to eastern Asia where it is well known for its bark fibers, which were used for manufacturing high-quality papers, cloths and ropes (Oka and Ohyama 1989, Vatanawong et al. 1999, Liao et al. 2006). The tree contains rich chalcone and flavonoid derivatives that are important antioxidants (Cheng et al. 2001, Chen et al. 2002, Kwak et al. 2003), and has also been used as a traditional medicine to treat thrush and stomach pain and as a laxative (http://www.agroforestry.net/ttl/Broussonetia-papirumul.pdf). The tender leaves and twigs are widely used to feed pig and deer. Additionally, the tree is widely favored because of its fast growth, high yield and wide cultivation. However, traditional breeding methods for producing new phenotypic paper mulberry with high stress tolerance are limited by sexual compatibility, narrow adaptability, long breeding cycles (15–20 years) and difficulty of selection. More recently, an efficient genetic transformation method of paper mulberry culture established in our previous study (Li et al. 2008) opens an excellent opportunity for genetic engineering to explore the effects of AtNHX5 on tolerance to abiotic stresses in this plant.

In a previous study of AtNHX5 (Shi et al. 2008), we showed that ectopic expression of AtNHX5 in *Torenia* enhanced the ability of transgenic plants to withstand salt stress. In the current study, we investigated the effect of overexpression of AtNHX5 on the tolerance of paper mulberry to salt and drought.

**Materials and methods**

*Vector construction and genetic transformation*

The plant binary expression vector pHQSN-F was constructed as described by Shi et al. (2008). The constructed pHQSN-F carries both the selectable marker gene for hygromycin phosphotransferase (*hpt*) and the AtNHX5 gene driven by the cauliflower mosaic virus-35S (CaMV35S) promoter and the castor bean catalase intron (35S/Intron). Hybrid paper mulberry (*B. papyrifera* (L.) Vent) was transformed as previously described (Li et al. 2008). Southern and northern blot analyses were used to confirm the presence and expression of the transgenes.

*Southern blot and northern blot analyses of AtNHX5 transgenic plants*

Genomic DNA was prepared from leaves of wild type (WT) and the AtNHX5 transgenic lines (T1, T3) using the cetyltrimethyl ammonium bromide method, as described by Muhammad et al. (1994). For Southern blot analysis, 10 µg of genomic DNA per sample was digested with HindIII restriction enzyme at 37 °C overnight, separated on 0.8% (w/v) agarose gel and transferred to Hybond N\(^+\) membranes. A digoxigenin (DIG)-labeled AtNHX5 cDNA fragment was synthesized by polymerase chain reaction and used as the probe. The primers used for amplifying AtNHX5 were AtNHX5F (5’-ATG ATT TCT CCG GTG GAGCAC GAC GC-3’) and AtNHX5R (5’-CTA CTC CCC ATC TTC ATC TTC ATC TC-3’). Prehybridization, washing and chemiluminescent detection of the blots was performed according to the kit manufacturer’s instructions (Roche: Diagnostics GmbH, Mannheim, Germany). For northern blot analysis, RNA was extracted according to the Trizol method (Invitrogen, Carlsbad, CA, USA), and 10 µg of RNA from each transgenic line was used for running on a formaldehyde gel and then blotted to a Hybond N\(^+\) membrane. The same AtNHX5 probe was used to detect the transcript of AtNHX5.

*Plant growth conditions*

The hybrid paper mulberry does not produce flower, and so the first-generation transformants were used for the following experimental materials. Regenerated plantlets (WT and the AtNHX5 transgenic lines (T1, T3); ~5 cm in height) were transferred into plastic pots containing autoclaved vermiculite...
and sand (1/1, v/v), or trays containing autoclaved vermiculite in the green house, watered with 1/5-strength Murashige and Skoog (MS) medium (Murashige and Skoog 1962) (1/5 MS) salt solution (pH 7.0) once a week. When the plantlets reached ~10 cm in height, the plantlets were selected for uniformity and then subjected to the following salt treatment and drought stress. All plants were grown under cool-white fluorescent light (35 μmol m~2~ s~−1~; 12-h light/12-h dark) at 28 °C and 75% relative humidity.

**Salt stress treatment**

The plants, including control WT plants and transgenic plants (T1 and T3), were watered with 1/5-strength MS solution containing 250 mM NaCl for 15 days. To avoid salt shock, NaCl concentrations were stepped up in 50 mM day~−1~ increments until a final concentration of 250 mM was achieved. After the salt stress treatment, the phenotypes of the plants were examined, they were photographed and then the plants were rinsed five times with distilled water, transplanted to the normal irrigation condition (well watered with 1/5 MS solution) to promote recovery. Twenty days later, the phenotypes of the plants were examined and they were photographed. After recovery, plants that had green, healthy leaves were regarded as having survived, and the survival rate was calculated. Chlorophyll content, relative electrolyte leakage, the contents of Na~+~ and K~+~ of the third leaf from the top of the plants were measured on the 10th day of salt stress, while proline and soluble sugar contents of the third leaf were measured on the 6th and 10th days of salt treatment, respectively. Dry weight of shoot was also measured on the 6th, 10th and 15th days of the salt treatment.

**Drought stress treatment**

Water was withheld for 13 days from the plants, including control WT plants and transgenic plants (T1 and T3). Then, the phenotypes of the plants were examined and they were photographed. After drought stress for 11 days, the third-day leaves from the top of the plants were collected for measurement of chlorophyll content and relative electrolyte leakage, while leaf water, proline and soluble sugar contents and shoot dry weight were measured on the 9th, 11th and 13th days of the drought treatment, respectively. After recovery for 5 days, plants that had green, healthy young leaves were regarded as having survived, and the survival rate was calculated.

**Determination of K~+~, Na~+~, chlorophyll, proline and soluble sugar contents**

For K~+~ and Na~+~ content measurements, samples of leaves were collected and thoroughly rinsed in deionized water to remove possible surface contamination by ions. The samples were dried at 80 °C for 24 h, and the dry weight of each sample was measured. Nitric acid was added, the samples were ashed using a Multiwave 3000 instrument (Perkin Elmer, Shelton, CT, USA), and then dissolved in deionized water. The K~+~ and Na~+~ concentrations were measured using inductively coupled plasma atomic emission spectrometry (Optima 2000; Perkin Elmer). For the content measurement of chlorophyll, proline and soluble sugars, leaves were collected and cut with a 1-cm-diameter cork borer, and mixed. Leaf chlorophyll content measurement was measured as described by Arnon (1949). Proline of leaves was extracted in 3% sulfosalicylic acid and estimated by adding acid ninhydrin reagent and measuring the absorbance of the toluene chromophore at 520 nm. The amount of proline present in each sample was then determined from a standard curve (Bates et al. 1973) and expressed as mg g~−1~ DW. Leaf soluble sugar content was measured according to DuBois et al. (1956) and expressed as mg g~−1~ DW.

**Measurement of relative electrolyte leakage**

Briefly, leaf discs were soaked and shaken in deionized water at 28 °C for 3.5 h. The conductivity (C1) of the solutions obtained was then determined using a DDS-11A conductivity detector (Kangyi). The leaf discs were then boiled for 20 min. After being thoroughly cooled to room temperature, the conductivity (C2) of the resulting solutions was determined, using the same detector. The C1/C2 × 100% values were calculated and used to evaluate relative electrolyte leakage.

**Data collection and statistical analysis**

Each salt stress treatment consisted of nine replicate pots (three plants per pot, i.e. one WT plant and one plant of each transgenic line (T1 and T3)), and each drought stress treatment consisted of three replicate trays (nine plants per tray, i.e. three WT plants and three plants of each transgenic line (T1 and T3)). All experiments were conducted in three replicates. Statistical analyses were performed using the SPSS software package version 17.0 (SPSS Inc., Chicago, IL, USA). The significance of differences between controls and treatments was compared at the 0.05 probability levels using a one-way analysis of variance least significant difference test.

**Results**

**Generation of AtNHX5 transgenic plants and confirmation of gene expression**

In total, 20 transgenic plants were generated. Here we show the results of Southern and northern blot analysis of the transgenic lines T1 and T3, and the WT plants. As the genomic DNA was restricted with HindIII, which cuts only once inside the T-DNA region in the constructed pHQSN-F (Shi et al. 2008), the number of hybridization bands reflects the copy number of the T-DNA integrated into the paper mulberry genome. The T-DNA copies integrated into the transgenic lines T1 and T3 were two copies and one copy (Figure 1a), respectively. The results indicated that T1 and T3 plants derived from
growth, due to both the low osmotic potential of the soil
improved salt tolerance of paper mulberry plants. plants remained alive, and recovered growth quickly (Figure
in the WT plants, while chlorotic and necrotic spots appeared
chlorotic and necrotic spots on the sixth day of the salt treat-
transgenic plants. Tips and edges of the old leaves exhibited
ever, distinct differences were observed on the WT and
remained green, while leaves of the control WT plants turned from green
to yellow, and abscised (Figure 2b). The chlorophyll content
was also higher \( (P < 0.05) \) in the transgenic leaves than in the
control WT leaves (Figure 2e). In addition, salt stress caused a
significant \( (P < 0.05) \) increase in electrolyte leakage from
leaves of the WT plants, but not in the transgenic plants
(Figure 2f). These results indicate that AtNHX5 overexpression
reduces membrane damage caused by salt stress.

To assess the function of AtNHX5 in maintaining ion homeo-
thesis, one of the key factors that enables plants to grow nor-
ally under salt stress conditions (Blumwald et al. 2000,
Tester and Davenport 2003, Apse and Blumwald 2007, Munns
and Tester 2008, Rodríguez-Rosales et al. 2009), Na\(^+\) and K\(^+\)
contents in leaves of the transgenic and WT plants were mea-
sure under normal and salt stress conditions. The Na\(^+\) con-
centration of the leaves in all of the experimental plants was
increased under the salt treatment, with the Na\(^+\) concentra-
tion in the transgenic plants being higher than that in the control
WT plants (Figure 2g). The level of K\(^+\) decreased markedly in
WT leaves, but the K\(^+\) concentration remained higher \( (P < 0.05) \)
in the AtNHX5-overexpressing leaves, as well as that in the
normal growth condition (Figure 2g). These results indicate
that overexpression of the AtNHX5 gene promotes Na\(^+\) and K\(^+\)
accumulation in leaves.

Plant responses to salt stress are often related to metabolic
regulation, including the accumulation of proline and soluble
sugars, which may act to counter the osmotic stress caused by
salt stress (Hasegawa et al. 2000, Ashraf and Harris 2004,
Munns et al. 2006). In order to elucidate the physiological
basis of salt stress tolerance of AtNHX5 transgenic paper mul-
berry, proline and soluble sugar contents in AtNHX5-
overexpressing plants and the control WT plants responding to
salt stress were measured. Salt stress resulted in an increase
in the proline content in leaves; however, the transgenic plants
accumulated higher \( (P < 0.05) \) levels of proline than the WT
plants (Figure 2h). Extremely high salt stress caused a reduc-
tion in soluble sugar content of WT leaves, while moderate salt
stress caused an increase in soluble sugar content. However,
the soluble sugar content of the transgenic leaves was
enhanced by the salt stress (Figure 2h). These results indicate
that AtNHX5 overexpression accumulates osmolytes more
effectively to counter osmotic stress caused by salt stress.

Overexpression of the AtNHX5 gene in paper mulberry
increased drought tolerance

When water was withheld for 11 days from the AtNHX5-
overexpressing plants (T1 and T3) and the WT plants with

Figure 1. Southern blot (a) and northern blot (b) analysis of wild-type
plants (WT) and AtNHX5 transformed lines (T1 and T3). (a) Southern
blot analysis. DNA (10 \( \mu \)g) from each transgenic line was digested
with HindIII and loaded. The DIG-labeled AtNHX5 coding region was
used as a probe. (b) Northern blot analysis. RNA (10 \( \mu \)g) from each
transgenic line was used for the analysis. The DIG-labeled AtNHX5
coding region was used as a probe.

Overexpression of the AtNHX5 gene in paper mulberry
increased salt tolerance

Overexpression of AtNHX5 did not affect the overall growth of
the transgenic plants (Figures 2a, d and Figure 3d). Salt treat-
ment inhibited the growth of the plants (Figure 2b and d); how-
ever, distinct differences were observed on the WT and AtNHX5
transgenic plants. Tips and edges of the old leaves exhibited
chlorotic and necrotic spots on the sixth day of the salt treat-
ment in WT plants, while chlorotic and necrotic spots appeared
in the AtNHX5 transgenic plants on the 10th day of the salt
treatment. Eighty percent of the third leaves from the top of
the WT plants had abscised by the end of the 10th day of the salt
treatment, with 100% abscission having occurred by the 12th
day. By the end of the 15th day of the salt treatment, all the
other mature leaves had abscised. However, the third leaves of
the AtNHX5 plants had not abscised by the end of the 15th day
of the salt treatment. At the end of the recovery period, all of
the WT plants were dead, while all the AtNHX5 transgenic
plants remained alive, and recovered growth quickly (Figure
2c). These results indicate that AtNHX5 overexpression
improved salt tolerance of paper mulberry plants.

A saline environment has many adverse effects on plant
growth, due to both the low osmotic potential of the soil
solution and specific ion effects (Flowers 2004). Leaf electro-
lyte leakage and chlorophyll content were compared between
the transgenic and the WT plants under salt stress. There were
clear differences in leaf color retention between the transgenic
and WT plants. Leaves of the transgenic plants still remained
green, while leaves of the control WT plants turned from green
to yellow, and abscised (Figure 2b). The chlorophyll content
was also higher \( (P < 0.05) \) in the transgenic leaves than in the
control WT leaves (Figure 2e). In addition, salt stress caused a
significant \( (P < 0.05) \) increase in electrolyte leakage from
leaves of the WT plants, but not in the transgenic plants
(Figure 2f). These results indicate that AtNHX5 overexpression
reduces membrane damage caused by salt stress.
similar vitality (Figure 3a and d) under normal growth conditions, clear phenotypic changes were observed (Figure 3b). The old leaves exhibited chlorosis and wilting on the ninth day of the drought stress treatment in WT plants, while most of the old leaves from the AtNHX5 transgenic plants remained green and fully formed. By the end of the 11th day of the drought stress treatment, all the WT leaves had wilted. However, most of the leaves of the AtNHX5 plants had not wilted by the end of the 13th day of the drought stress treatment. These results indicated that leaf rolling was delayed in AtNHX5-overexpressing plants compared with the control plants. However, drought stress treatment inhibited the growth of all the plants (Figure 3d). After drought stress, the plants were rewatered to promote recovery. After 5 days of rewatering, the WT plants withered and died, whereas most of the AtNHX5-overexpressing plants survived and grew well under unstressed conditions (Figure 3c and e). These results indicated that overexpression of AtNHX5 increases the drought tolerance of paper mulberry.

Leaf water loss, electrolyte leakage and chlorophyll content were compared between the transgenic and the WT plants during and after water-deficient stress. Drought stress caused a marked leaf water loss in WT plants, but not in transgenic plants ($P < 0.05$) (Figure 3b and f). After drought stress, there were clear differences in leaf color retention between the transgenic and WT plants. Leaves of the transgenic plants still remained green, while leaves of the control WT plants changed

Figure 2. Performance of AtNHX5 transgenic (T1 and T3) and wild-type (WT) plants under salt stress. (a, b, c) Phenotypic comparison of the paper mulberry plants before (a), and after 250 mM NaCl treatment for 15 days (b) and after recovery for 20 days (c). Dry weight of shoot (d), chlorophyll content (e), relative electrolyte leakage (f), Na$^+$ and K$^+$ contents (g), and proline and soluble sugar content (h) of the third leaves from the top of the plants on the 10th day of salt treatment. CK0, CK6, CK10 and CK15 plants grown under the normal growth condition as controls, S6, S10 and S15 plants on the 6th, 10th and 15th days of the salt treatment. Asterisk indicates a significant difference in comparison with the WT at $P < 0.05$. 

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from green to faded green (Figure 3b). The chlorophyll content was also higher ($P < 0.05$) in the transgenic leaves than in the control WT leaves (Figure 3g). Drought stress also caused a significant ($P < 0.05$) increase in electrolyte leakage from leaves of the WT plants, but not in the transgenic plants (Figure 3h). These results indicate that AtNHX5 overexpression reduces membrane damage caused by drought stress.

Drought stress resulted in an increase in the proline content of leaves; however, the transgenic plants accumulated higher ($P < 0.05$) levels of proline than the WT plants (Figure 3i). Compared with the soluble sugar content in leaves of WT plants, the transgenic leaves had a high level of soluble sugar content during drought stress (Figure 3i). The results indicate that AtNHX5 overexpression accumulates osmolytes more effectively to counter osmotic stress caused by drought stress.

**Discussion**

Plant growth is often affected by salt stress, which imposes an osmotic stress on cell water relations and increases the concentration of sodium in the cytosol to toxic levels (Hasegawa et al. 2000, Tester and Davenport 2003). Thus, the compartmentation of Na\(^+\) into vacuoles provides an efficient mechanism to avoid the toxic effects of the Na\(^+\) ion in the cytosol (Tester and Davenport 2003; Apse and Blumwald 2007). Plant NHX proteins, which catalyze the exchange of Na\(^+\) and K\(^+\) for...
H+ across membranes, have been identified as important salt tolerance determinants. The overexpression of NHX-like antiporters has been previously shown to improve the salt tolerance of transgenic plants (Apse et al. 1999, Zhang and Blumwald 2001, Zhang et al. 2001, Xue et al. 2004, He et al. 2005, Wang et al. 2007, Liu et al. 2008, Wu et al. 2009). We have previously shown that the salt tolerance of *Torenia* is increased by the overexpression of *AtNHX5* (Shi et al. 2008), and here we show that not only the salt tolerance but also the drought tolerance of paper mulberry is increased by its overexpression.

Although overexpression of the plant NHX genes has been widely and successfully used to improve the salt tolerance of various plant species, the mechanism underlying the enhancement of salinity tolerance by these genes is not yet clear. *AtNHX1* was the first plant NHX gene isolated and has been shown to be primarily a Na+/H+ antiporter, which catalyzes a large accumulation of Na+ in the vacuole of *AtNHX1*-expressing plants in highly saline conditions (Apse et al. 1999, Zhang and Blumwald 2001, Zhang et al. 2001, Lü et al. 2005, Rajagopal et al. 2007). However, more recent evidence has revealed that plant NHX proteins might be Na+/H+ and K+/H+ antiporters, which have accumulated both Na+ and K+ (Wu et al. 2005, Zhao et al. 2006, Shi et al. 2008), or even only K+ (Xue et al. 2004, Liu et al. 2008, Rodriguez-Rosales et al. 2008, Leidi et al. 2010) in the cells of NHX-expressing plants in highly saline conditions. Several studies have not found the expected correlation between increased salt tolerance and enhanced accumulation of Na+ caused by the overexpression of NHX genes (Ohta et al. 2002, Fukuda et al. 2004, Xue et al. 2004, Liu et al. 2008, Rodriguez-Rosales et al. 2008, Wu et al. 2009, Leidi et al. 2010). *AtNHX5* was originally suggested to function as a Na+/H+ antiporter, similar to both *AtNHX1* and *AtNHX2* (Yokoi et al. 2002, Aharon et al. 2003). Recent studies have shown that *AtNHX5* is one of the Class-II antiporter members, according to its sequence similarity and subcellular localization. It has been suggested that *AtNHX5* might share the same K+ preference as the tomato LeNHX2, a different function from that of the Class-I antiporters (Venema et al. 2003, Pardo et al. 2006, Rodriguez-Rosales et al. 2008). Interestingly, although both K+/H+ and Na+/H+ exchange activity is enhanced in *LeNHX2* transgenic plants, they contain high K+ but less Na+ in aerial parts under Na+ stress conditions (Rodriguez-Rosales et al. 2008). In our studies, both the *AtNHX5*-overexpressing *Torenia* (Shi et al. 2008) and paper mulberry (the present study) accumulated higher contents of both Na+ and K+ in leaves under salt stress, or higher K+ content in leaves in the absence of salt stress, than control plants (Figure 2f), and also accumulated more proline and soluble sugars (Figure 2g) to counter osmotic stress caused by salt stress. Consequently, we suggest that the ability of *AtNHX5* transgenic plants to survive exposure to a highly saline environment could be associated with the accumulation of Na+, K+, proline and soluble sugars in leaves, thereby improving overall ion homeostasis and osmotic adjustment and thus plant salt tolerance.

However, it is becoming increasingly clear that plant NHX proteins are important in multiple cellular processes that are not necessarily limited to salinity tolerance regulation (Venema et al. 2003, Pardo et al. 2006), for example cellular K+ and Na+ compartmentation (Gaxiola et al. 1999, Yokoi et al. 2002; Leidi et al. 2010), vacuolar pH regulation (Yamaguchi et al. 2001, Yoshida et al. 2005, 2009), vesicular trafficking and protein processing (Sottosanto et al. 2007), and that they can be induced by NaCl, KCl, osmotic stress, dehydration or heat shock (Gaxiola et al. 1999, Quintero et al. 2000, Hamada et al. 2001, Porat et al. 2002, Yokoi et al. 2002). The plant NHX genes also play crucial roles in leaf (Apse et al. 2003) and chloroplast (Song et al. 2004) development. Overexpression of *AtNHX3* has been shown to increase the accumulation of soluble sugars in the storage roots of transgenic plants grown under high salt stress condition (Liu et al. 2008), while *AtNHX1* transgenic plants show significantly greater accumulation of free sugars, even if they are not under salt stress (Leidi et al. 2010). Accumulation of osmoregulatory molecules such as soluble sugars, soluble proteins, proline and ions in the cell is an important feature that is often linked to salt tolerance (Hasegawa et al. 2000). In support of the above suggestions, our previous study showed that overexpression of *AtNHX5* in *Torenia* not only improved the salt tolerance of this plant, but also enhanced shoot regeneration from leaf explants, stimulated vegetative growth of the transgenic plants, and increased the ability of *in vitro* transgenic plantlets to acclimate *ex vitro* conditions by increasing the waterholding capacity of the cells (Shi et al. 2008). The present study further showed that overexpression of the gene in paper mulberry not only improved the salt tolerance but also increased the drought tolerance of the plant. However, these phenomena have not been described in *AtNHX1*, *AtNHX3*, *AtNHX4* or *LeNHX2*-overexpressing plants (Apse et al. 1999, Zhang and Blumwald 2001, Zhang et al. 2001, Xue et al. 2004; He et al. 2005, Wang et al. 2007, Liu et al. 2008). To better understand the functions of plant NHXs, it would be worth further investigating whether other NHX orthologs improve plant drought tolerance.

Drought and salinity in the root environment of plants are frequently considered to impose similar effects on plants, due to the accompanying reductions in external water potentials (Plaut and Federman 1991). The ability to undergo osmotic adjustment is related to the accumulation of organic solutes such as N-compounds, especially proline and glycine-betaine, soluble sugars and salt stress proteins (Ashraf and Harris 2004, Bogeat-Triboulot et al. 2007). Thus, the accumulation of these compounds improves water uptake under abiotic stress and possibly protects the cell structure against molecular disturbances, resulting in plant acclimation (Munns et al. 2006).
As overexpression of \emph{AtNHX5} increases proline and soluble sugar levels in response to both salt (Figure 2h) and drought stress (Figure 3i), we suggest that the ability of \emph{AtNHX5}-transgenic plants to survive exposure to high salinity, water deficiency or other osmotic stress environment could be associated with the more effective accumulation of osmolytes (ions or proline) to counter the osmotic stress caused by abiotic factors.

Multiple abiotic stresses are present in the environments of plants, and plants are often affected by more than one stress. Thus, it is very important to improve the ability of plants to survive multiple environmental stresses \cite{Mittler and Blumwald 2010}. Although the underlying molecular mechanisms are not yet clear, our finding that \emph{AtNHX5} transgenic paper mulberry plants have enhanced tolerance to both salt and drought stress strongly suggests that use of the gene in genetically engineering this economically important plant’s tolerance to major abiotic stresses could be very valuable.

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