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Expression of a novel antiporter gene from *Brassica napus* resulted in enhanced salt tolerance in transgenic tobacco plants

J. WANG*,****, K. ZUO*,**, W. WU*, J. SONG*, X. SUN*, J. LIN*, X. LI***
and K. TANG*,**,[1]

*School of Life Sciences, Morgan-Tan International Center for Life Sciences, Fudan-SJTU-Nottingham Plant Biotechnology R&D Center, Fudan University, Shanghai 200433, China**
*School of Agriculture and Biology, Shanghai Jiaotong University, Shanghai 200030, China***
*College of Life Science, Sichuan University, Chengdu 610064, China****
*Mianyang Teacher's College, Mianyang 621000, China*****

Abstract

Tobacco leaf discs were transformed with a plasmid pBIBnNHX1, containing the selectable marker neomycin phosphotransferase gene (*npt*II) and Na^+/H^+ vacuolar antiporter gene from *Brassica napus* (*BnNHX1*), *via Agrobacterium tumefaciens*-mediated transformation. Thirty-two independent transgenic plants were regenerated. Polymerase chain reaction (PCR) and Southern blot analyses confirmed that the *BnNHX1* gene had integrated into plant genome and Northern blot analysis revealed the transgene expression at various levels in transgenic plants. Transgenic plants expressing *BnNHX1* had enhanced salt tolerance and could grow and produce seeds normally in the presence of 200 mM NaCl. Analysis for the T_1 progenies derived from seven independent transgenic primary transformants expressing *BnNHX1* showed that the transgenes in most tested independent T_1 lines were inherited at Mendelian 3:1 segregation ratios. Transgenic T_1 progenies could express *BnNHX1* and had salt tolerance at levels comparable to their T_0 parental lines. This study implicates that the *BnNHX1* gene represents a promising candidate in the development of crops for enhanced salt tolerance by genetic engineering.

Additional key words: *BnNHX1*, Na^+/H^+ antiporter, NaCl tolerance, *Nicotiana tabacum*, transformation.

Introduction

Soil salinity was a prevalent abiotic stress for plants (Qasim *et al.* 2003). Plant growth under salt stress depended on the re-establishment of proper cellular ion homeostasis with other concomitant processes. Low cytosolic Na^+ content was preserved by the concerted interplay of regulated ion uptake, vacuolar compartmentation and active extrusion to the extracellular milieu (Blumwald *et al.* 2000). Sodium transport out of the cell could be accomplished by the operation of plasma membrane-bound Na^+/H^+ antiporter (Shi *et al.* 2000).

For plants, the presence of large vacuoles allowed the efficient compartmentation of sodium into the vacuole through the operation of vacuolar Na^+/H^+ antiporters (Apse *et al.* 1999). It can actively move ions across the tonoplast into the vacuole, removing the potentially harmful ions from the cytosol. These ions, in turn, acted as an osmoticum within the vacuole, which maintained water flowing into the cell.

Recently, the cloning of *AtNHX1* gene coding for a vacuolar Na^+/H^+ antiporter was reported in *Arabidopsis thaliana* (Apse *et al.* 1999). Transgenic plants over-expressing *AtNHX1* were able to grow normally in the presence of 200 mM NaCl, supporting the role of the vacuolar Na^+/H^+ antiporter on salt tolerance (Apse *et al.* 1999).

In earlier screening of *Brassica* species for salt

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Abbreviations: BA - 6-benzyladenine; MS - medium of Murashige and Skoog (1962); NAA - α-naphthaleneacetic acid; PCR - polymerase chain reaction.
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[1] Corresponding author: fax: (+86) 21 65643552, e-mail: kxtang1@yahoo.com

tolerance, *B. napus* and *B. carinata* were found to be more salt-tolerant than *B. campestris*, and *B. napus* was also more salt-tolerant than *A. thaliana* (Ashraf and McNeilly 1990, Malik 1990, He and Cramer 1992). Recently, a new Na^+/H^+ vacuolar antiporter gene from *B. napus* was cloned in this laboratory. The full-length cDNA of *B. napus* antiporter gene *(BnNHX1*, GenBank Acc. No. AY189676) was 1819 bp and contained a 1545 bp open reading frame encoding a protein of 515 amino acids. Homology analysis and molecular modeling revealed that *BnNHX1* resembled other Na^+/H^+ antiporter genes such as those from *A. thaliana* (*AtNHX1*) (Apse *et al.* 1999) and *Oryza sativa* (Fukuda *et al.* 1999). Northern blot analysis revealed that *BnNHX1* was salt-inducible and its transcript level was most abundant after 24-h treatment with 200 mM sodium chloride. The cloning of the *BnNHX1* enables us to test its potential salt tolerance function by transferring the gene into tobacco, which has not been reported previously. In the present paper, we report on the generation of transgenic tobacco plants containing and expressing *BnNHX1*. The salt tolerance assay on transgenic tobacco is also presented.

Materials and methods

Cloning the *BnNHX1* gene in transformation vector: The *BnNHX1* gene was cloned from leaves of seed-germinated one-month-old *B. napus* cv. Huyou plant after treatment with 200 mM NaCl for 24 h using the RACE-PCR protocol (RACE System for Rapid Amplification of cDNA ends, *GIBCO BRL*, Carlsbad, USA) and inserted into the pGEM T-Easy Vector (*Promega*, Madison, USA), resulting in pGEMTBnNHX1. An *Xba*I/blunt-end of *BnNHX1* coding region fragment was excised from pGEMTBnNHX1 and inserted into the region between the CaMV35S promoter and nopaline synthase terminator (Nos-ter) on the *Agrobacterium* binary vector pBI121 (Rogers *et al.* 1986) pre-digested with *Xba*I/*Sac*I through the replacement of the glucuronidase gene (*gusA*). The resulting vector, pBIBnNHX1, containing the selectable marker neomycin phosphotransferase gene (*npt*II) conferring kanamycin resistance and the *BnNHX1*, both driven by the CaMV35S promoter (Fig. 1*a*), was introduced into *Agrobacterium tumefaciens* strain EHA 105 (Hood *et al.* 1993) by triparental mating using the protocol described before (Horsch *et al.* 1988) and was used to transform tobacco.

Transformation and regeneration of transgenic tobacco plants: The tobacco (*Nicotiana tabacum* L.) cultivar 'Petit Havana SR1' was used for transformation. The transformation was performed essentially as described by Horsch *et al.* (1988). *A. tumefaciens* EHA105 (pBIBnNHX1) was grown for two days at 28 °C in Luria Broth medium (10 g dm^{-3} trypton, 5 g dm^{-3} yeast extract, 10 g dm^{-3} NaCl, pH 7.0) supplemented with 50 mg dm^{-3} kanamycin and 50 mg dm^{-3} rifampicin. The bacteria were collected and suspended at a density of 1×10^9 in hormone-free half-strength MS (Murashige and Skoog 1962) liquid medium before use. The leaves from tobacco plants germinated and grown in hormone-free half-strength MS solid medium were cut into discs of approximately 0.5 - 1.0 cm^2. The discs were immersed in the bacterial suspension for 5 min, transferred onto hormone-free MS medium solidified with 2.6 g dm^{-3} *Phytagel* (*Sigma*, St. Louis, USA) and incubated at 26 °C in the dark for 2 d. After co-cultivation, the discs were placed on selection medium (MS basal medium supplemented with 1.0 mg dm^{-3} 6-benzyladenine (BA), 0.1 mg dm^{-3} α-naphthaleneacetic acid (NAA), 50 mg dm^{-3} kanamycin and 250 mg dm^{-3} carbenicillin) and cultured at 26 °C under a 12-h photoperiod (irradiance of 50 μmol m^{-2} s^{-1} provided by white fluorescent tubes). Following two rounds of selection (2 weeks/round), kanamycin-resistant shoots were regenerated, separated from the explants and transferred to hormone-free MS medium containing 50 mg dm^{-3} kanamycin for rooting. The rooted healthy plants were subjected to PCR, Southern blot and Northern blot analyses for the presence and expression of the *BnNHX1* gene in the plants.

Molecular analysis of transgenic plants: Plant DNA used for PCR and Southern blot analyses was extracted from young leaves by cetyl trimethyl ammonium bromide (CTAB) method as described before (Doyle and Doyle 1990). PCR analysis was carried out for the detection of the *BnNHX1* gene in transgenic plants using the forward primer
NHXF1 (5'-GCTCTAGAATGTTGGATTCTCTAGTGTC-3')
and the reverse primer
NHXR1 (5'-CTAGTGATTAGAGTCATCAA-3').
The expected product size was 1.55 kb. The PCR reactions were carried out in a total volume of 0.025 cm^3 comprising 50 ng tobacco genomic DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 μM dNTPs, 1.25 units of *Taq* DNA polymerase and 25 pmol of each primer. For PCR analysis, DNA was denatured at 94 °C for 3 min followed by 30 amplification cycles (94 °C for 50 s, 62 °C for 50 s, 72 °C for 50 s) and finally at 72 °C for 7 min.

Southern blot analysis was further used to confirm the transgenic status of plants positive for the *BnNHX1* PCR analysis. In total, over 20 independently derived transgenic tobacco lines including plant nos. 3, 5, 6, 10, 11, 15 and 23 were analyzed by Southern blot hybridization for the presence of *BnNHX1* gene in the plant genome. Aliquots of genomic DNA (8 μg) were digested overnight at 37 °C with *Xba*I (which cuts only once in

pBIBnNHX1), fractionated by 0.8 % agarose gel electrophoresis, and transferred to a Hybond-N^+ membrane (*Amersham Bioscience*, Piscataway, USA). The *BnNHX1* probe was generated by PCR using the pair of primers NHXF1 and NHXR1 as described above. The probe was labeled with $dAT^{32}P$ using a random-priming kit (*Ready-to-Go*, *Pharmacia*, Freiburg, Germany). Hybridization was carried out according to the method of Thomas (1980) and signals were visualized by exposure to *Fuji* X-ray film (Japan) at -70 °C for 2 d.

Northern blot analysis was carried out to investigate the expression of the *BnNHX1* in transgenic plants. In total, 20 independently derived transgenic tobacco lines confirmed by Southern blot analysis including plant nos. 5, 6, 10, 11, 15 and 23 were analyzed by Northern blot hybridization. Total RNA was isolated from 1 g leaf tissue using TRIzol reagent (GIBCO-BRL, USA). Aliquots of RNA (30 μg) were separated on a formaldehyde gel and transferred onto a Hybond-N^+ membrane (*Amersham Bioscience*) according to the method of Thomas (1980). The resulting blots were subjected to hybridization with the ^{32}P-labeled *BnNHX1* probe generated by PCR using the pair of primers NHXF1 and NHXR1 and signals were visualized by exposure to *Fuji* X-ray film at -70 °C for 2 d.

Salt tolerance assay of T_0 transgenic plants: Twelve independent transgenic tobacco T_0 lines expressing *BnNHX1* including plant Nos. 3, 5, 6, 10, 11, 15 and 23 were used for salt tolerance assay using the protocol as described before (Aspe *et al.* 1999, Zhang *et al.* 2001). Each of the independent transgenic lines and wild-type (untransformed) control tobacco plants was micropropagated into four copy plants and grown under a 14-h photoperiod (irradiance of 50 μmol m^{-2} s^{-1} provided by white fluorescent tubes). Each of the control and independent transgenic plants was subjected to four different salt treatments labeled from A to D. A dosage of 25 cm^3 of diluted nutrient solution (1/8 MS salts) every 2 d over a 16-d watering period was used. The control group received no NaCl supplementation. The remaining groups were watered with nutrient solution supplemented with NaCl. The concentrations of NaCl supplementation were increased stepwise by 50 mM every 4 d for each group, to the indicated maximum: (A) 0 mM NaCl, (B) 100 mM NaCl, (C) 200 mM NaCl and (D) 250 mM NaCl until the untransformed control plants died.

Genetic analysis of segregation of the BnNHX1 gene in T_1 progenies: Seven independent T_0 primary transgenic plants expressing *BnNHX1* (plant Nos. 3, 5, 6, 10, 11, 15 and 23) were grown to maturity. T_1 seeds were harvested and sown in soil in the greenhouse. The germinated T_1 plants (two-week-old) were analyzed for the presence of the *BnNHX1* gene by PCR using the method mentioned before for the segregation patterns. In addition, Northern blot analysis was carried out to investigate the expression of the *BnNHX1* in T_1 progenies from the seven lines (nos. 3, 5, 6, 10, 11, 15 and 23) using the method mentioned before.

Salt tolerance analysis of T_1 transgenic lines: T_1 seeds were germinated and grown in Petri dishes containing MS medium. For salt tolerance assay, the independent transgenic lines and wild-type (untransformed) control tobacco plants was micropropagated into three copy plants and grown under a 14-h photoperiod (50 μmol m^{-2} s^{-1}). Each of the control and independent transgenic plants was subjected to three different salt treatments labeled from A to C. A dosage of 25 cm^3 of diluted nutrient solution (1/8 MS salts) every 2 d over a 16-d watering period was used. The control group received no NaCl supplementation. The remaining groups were watered with nutrient solution supplemented with NaCl. The concentrations of NaCl supplementation were increased stepwise by 50 mM every 4 d for each group, to the indicated maximum: (A) 0 mM NaCl, (B) 100 mM NaCl, (C) 200 mM NaCl until the untransformed control plants died.

Results

Selection, plant regeneration and molecular analysis of T_0 transgenic plants: Tobacco leaf discs were transformed with *Agrobacterium tumefaciens* strain EHA 105 containing pBIBnNHX1 (Fig. 1) using the method described by Horsch *et al.* (1988). Following two rounds of kanamycin (50 mg dm^{-3}) selection, 130 kanamycin-resistant plants were regenerated, and 32 plants showed amplification of predicted fragment of *BnNHX1* while no amplification was observed in the control (data not shown).

The result of southern hybridization showed that each plant contained one or more *BnNHX1*-specific hybridizing bands, and the unique hybridization patterns observed indicated that each plant was derived from an independent transformation event (representative sample shown in Fig. 1). The copy number of *BnNHX1* in the independent transgenic plants ranged from one to over ten.

The expression of *BnNHX1* in independent transgenic lines was also detected by Northern blot analysis. The expression of *BnNHX1* varied among different lines, with the highest expression level being over three times as the least one (Fig. 1).

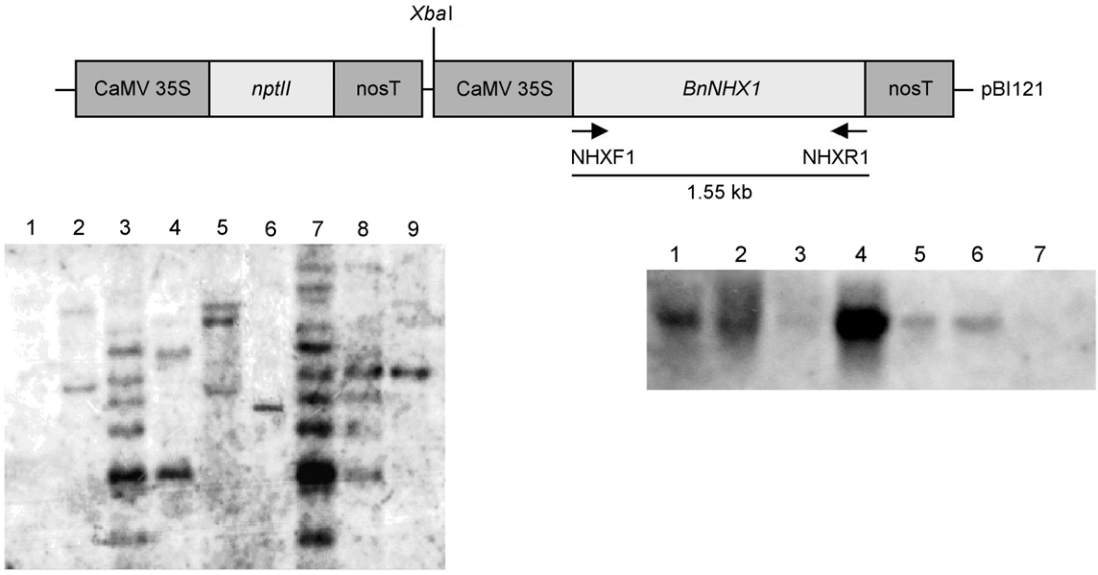

Fig. 1. The plasmid used in transformation study and molecular analyses of transgenic plants. Schematic representation of transformation plasmid (pBIBnNHX1). CaMV35S: cauliflower mosaic virus 35S promoter. *nptII*: neomycin phosphotransferase gene. nosT: 3' termination region from *Agrobacterium* nopaline synthase gene. *BnNHX1*: *Brassica napus* Na^+/H^+ antiporter gene. Primer positions, indicated by arrows, PCR product size and restriction enzyme (*Xba*I) site used for Southern analysis are also shown. (*above*). Representative Southern blot analysis for the presence of the *BnNHX1* gene in transgenic tobacco plants. Plant genomic DNA (8 μg) and pBIBnNHX1 were digested with *Xba*I, separated by 1.0 % agarose gel electrophoresis and hybridized to the ^{32}P-labeled *BnNHX1* probe. *Lane 1*: untransformed plant (negative control). *Lanes 2 - 8*: independent transgenic plants corresponding to plant nos. 3, 5, 6, 10, 11, 15 and 23. *Lane 9*: pBIBnNHX1 (positive control) (*left*). Representative Northern blot analysis for the expression of *BnNHX1* in transgenic tobacco plants. Total RNA was isolated from leaves of transgenic plants and aliquots of RNA (30 μg per sample) were subjected to electrophoresis, blotted onto a nylon membrane and hybridized to the ^{32}P-labeled *BnNHX1* probe. *Lanes 1 - 6*: independent transgenic plants corresponding to plant nos. 5, 10, 6, 15, 23 and 11 respectively. *Lane 7*: an untransformed plant (*right*).

Transgenic plants expressing BnNHX1 are salt-tolerant: Without salt treatment, there was no significant difference in the growth between the wild type control and transgenic plants. Under the treatment of 50 mM NaCl, the control plants could still maintain the growth at the same growth rate as transgenic ones. Under the treatment with NaCl at 100 mM, the leaves of the control plants became yellow and wilted quickly and no control plants could survive for more than 10 d. Under the treatment with NaCl at 200 mM, the leaves of the control plants became yellow and wilted quickly and died after 3 d (Fig. 2*a*). On the other hand, transgenic plants expressing *BnNHX1*, no matter what the expression levels of *BnNHX1* are, showed enhanced salt tolerance, and could flower and produce seeds normally under the various salt treatments compared to the control plants without salt treatment (Fig. 2*b-c* and Table 1). The endured highest NaCl concentration of transgenic plants expressing *BnNHX1* was 250 mM in which although transgenic plants could grow, but their growth was inhibited, severely stunted and no seed could be obtained.

Analysis of transgenic T_1 progenies: T_1 progenies derived from seven independently primary transformants (plant Nos. 3, 5, 6, 10, 11, 15 and 23) expressing *BnNHX1* were analyzed by PCR for the segregation patterns of the introduced *BnNHX1* gene. The transgenes in five (Nos. 3, 5, 6, 11 and 15) out of seven lines tested were inherited at a segregation ratio of 3:1 (Table 2), indicating the integration of the transgenes as Mendelian manner into tobacco genomes of the five lines.

Northern blot analysis was carried out to investigate the expression of the *BnNHX1* in the seven transgenic T_1

Table 1. Characterization of flower and seed-setting of wild-type (WT) control tobacco plants grown in 0 mM NaCl and transgenic tobacco T_0 lines expressing *BnNHX1* grown in the 200 mM NaCl.

Line	Number of flowers	Seeds [flower^{-1}]	Mass of a seed [mg]
No.3	39	883	0.063
No.5	54	920	0.075
No.6	65	955	0.068
No.10	52	985	0.071
No.11	55	860	0.069
No.15	49	862	0.059
No.23	56	895	0.068
WT.21	48	897	0.073
WT.28	36	947	0.061
WT.34	40	881	0.066

progenies (Nos. 3, 5, 6, 10, 11, 15 and 23). The result showed that the transgenic T_1 lines containing *BnNHX1* could express *BnNHX1* at levels similar to their T_0 parental lines (data not shown).

Table 2. Segregation of *BnNHX1* gene in T_1 progenies of transgenic tobacco lines. Two-week-old T_1 progeny plants were analyzed by PCR for the presence of the *BnNHX1* gene. a - abnormal, non-Mendelian segragation, $n = 48$.

Line	Segregation ratio	*BnNHX1*+	*BnNHX1*-	χ^2	*P*
No.3	3:1	32	16	1.78	0.18
No.5	3:1	30	18	4.00	0.05
No.6	3:1	35	13	0.11	0.74
No.10	a	28	20	7.11	0.01
No.11	3:1	34	14	0.44	0.51
No.15	3:1	37	11	0.11	0.74
No.23	a	29	19	5.44	0.02

A very similar result to that for T_0 plant was obtained for T_1 plant in salt tolerance assay. Under normal condition (without salt treatment), there was no significant difference for the growth between the wild-type seed-derived control and T_1 plants. Under the treatment with NaCl at 100 mM, the leaves of the wild-type seed-derived plants became yellow and wilted after one day and no wild-type control plants could survive for more than 10 d. The same phenomenon was found for the T_1 separated non-transgenic plants. However, T_1 transgenic plants could grow normally under the 100 mM NaCl treatment like no salt-treated plants. Under the treatment with NaCl at 200 mM, a concentration that inhibits the growth of almost all crop plants, the leaves of the wild-type control and T_1 separated non-transgenic plants became yellow and wilted quickly, and all these plants died three to 6 d later. On the other hand, T_1 transgenic plants could grow normally under 200 mM NaCl treatment like those T_1 separated non-transgenic plants under no salt treatment (Fig. 2*D*).

Fig. 2. Salt tolerance assay of tobacco control and transgenic lines and their T1 progenies. *A left*: wild-type control plant under no NaCl treatment; *right*: wild-type control plant under 200 mM NaCl treatment. *B left*: representative transgenic T0 plant (No. 11) under no NaCl treatment; *right*: representative transgenic T0 plant (No. 11) under 200 mM NaCl treatment. *C left*: representative flowering transgenic T0 plant (No. 11) under 200 mM NaCl treatment; *right*: wild-type control flowering plant under no NaCl treatment. *D left*: representative transgenic T1 plant (No. 11) containing and expressing *BnNHX1* under 200 mM NaCl treatment; *right*: representative separated T1 plant containing no *BnNHX1* derived from No. 11 under 200 mM NaCl treatment.

Discussion

In the present study, we generated transgenic tobacco plants containing and expressing the Na^+/H^+ antiporter gene (*BnNHX1*) from *B. napus*. PCR and Southern blot analysis confirmed their transgenic status. Northern blot analysis revealed that most of the transgenic plants tested expressed the *BnNHX1* gene at various levels. A similar phenomenon was also observed for majority of transgenic *Arabidopsis*, *Brassica* or tomato plants containing Na^+/H^+ antiporter gene from *Arabidopsis* (*AtNHX1*) in respect to the transgene expression (Apse *et al.* 1999, Zhang and Blumwald 2001, Zhang *et al.* 2001). Segregation analysis of T_1 progenies from the seven independent transgenic lines expressing *BnNHX1* (Nos. 3, 5, 6, 10, 11, 15 and 23) demonstrated that the *BnNHX1* gene was inherited into most of T_1 progenies as a single Mendelian trait and expressed *BnNHX1* at levels comparable to their T_0 parental lines, which was also observed in previous transgene segragation studies (Bano-Maqbool and Christou 1999, Tang *et al.* 1999, 2001).

Apse *et al.* reported that transgenic *Arabidopsis* plants over-expressing AtNHX1 were unaffected by up to 200 mM NaCl treatment and plant development was not compromised (Apse *et al.* 1999). However, these transgenic plants, when grown under 300 mM NaCl treatment, displayed a reduction in leaf wilt and chlorosis. Zhang *et al.* reported that transgenic *Brassica* plants over-expressing AtNHX1 from *Arabidopsis* could grow, flower, and produce normal seeds under the treatment of 200 mM NaCl while the growth of the wild-type plants was severely affected (Zhang *et al.* 2001). Recently, transgenic rice plants containing the Na^+/H^+ antiporter gene (*AgNHX1*) from *Atriplex gmelini* were found to be able to survive under the treatment of 300 mM NaCl for only 3 d although the expression of the *AgNHX1* in the plants was not be studied (Ohta *et al.* 2002). In the present study, although expressing BnNHX1 at various levels, revealed by Northern blot analysis (Fig. 1*C*), all the transgenic plants could grow, flower and produce seeds normally under the treatment of 200 mM NaCl (Table 1), in consistent with previous reports (Apse *et al.* 1999, Zhang and Blumwald 2001, Zhang *et al.* 2001). It was also found from the present study that the treatment with NaCl did not increase the expression level of BnNHX1 in transgenic tobacco plants and the transgenic plants subjected to 200 mM NaCl treatment expressed BnNHX1 at similar levels with those without NaCl treatment, revealed by Northern blot analysis (data not shown). Although expressing BnNHX1 at various levels, the independent transgenic plants had similar salt tolerance. However, in consistent with previous results (Apse *et al.* 1999, Zhang and Blumwald 2001, Zhang *et al.* 2001, Ohta *et al.* 2002), all the transgenic plants were inhibited, stunted and could not produce normal seeds under the treatment of NaCl over 200 mM. This result and previous results (Apse *et al.* 1999, Zhang and Blumwald 2001, Zhang *et al.* 2001) suggest that pyramiding strategy by transferring more than one gene with different salt-resistant mechanisms into plants may be the effective way in order to achieve higher salt tolerance of transgenic plants, which was demonstrated to be effective in achieving enhanced insect and disease resistance in transgenic rice (Datta *et al.* 2002).

From these results, it is suggested that there are different mechanisms of salt tolerance for the plants under different salinities. When the expression reaches comparable necessary level, more expression of transcripts has no aid to improve tolerance. As far as Na^+/H^+ antiporter gene, there was no significant difference in the aspects of growth and agronomic traits among the transgenic plants expressing different levels of Na^+/H^+ antiporter protein grown under 250 mM NaCl in which antiporter protein may act as main regulator. When soil NaCl concentration was more than 250 mM, signal of the membrane proteins may stimulate other pathways, such as water channel proteins and the production of stress proteins and compatible osmolytes scavenging reactive oxygen species (ROS) (Zhu 2001). Zhu reported that tuning cell division and expansion could potentially improve productivity under salt or drought stress (Zhu 2002). Blumwald *et al.* (2000) suggested that Na^+/H^+ antiporters were not a ubiquitous characteristic of all plant cells. These results and the current study indicate that, even though Na^+/H^+ antiporter plays an important role in the salt tolerance, it is difficult for developing crops which can stand severe salinity by introducing just only one gene as the salt tolerance is complex and controlled by different pathways.

In conclusion, we reported for the first time that transgenic tobacco expressing the Na^+/H^+ antiporter gene (*BnNHX1*) from *B. napus* showed enhanced tolerance to NaCl. This study implicates that the *BnNHX1* gene represents a promising candidate in the development of crops for enhanced salt tolerance by genetic engineering.

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