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Improved salt tolerance in canola (*Brasica napus*) plants by overexpression of Arabidopsis Na^+/H^+ antiporter gene *AtNHX1*

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Abstract

A significant portion of the world's cultivated land is affected by salinity that reduces crop productivity in these areas. Breeding for salt tolerance is one of the important strategies to overcome this problem. Recently, genetic engineering is becoming a promising approach to improving salt tolerance. In order to improve the yield performance of canola in saline soils, we transformed canola with Arabidopsis vacuolar Na^+/H^+ antiporter gene *AtNHX1* which enhances the plant capacity for reducing cytosolic Na^+ by transporting Na^+ into the vacuole. Southern analysis of putative transgenic plants indicated that only one copy of the gene integrated into the plant genome. Overexpression of the *AtNHX1* gene was shown in T_1 transgenic plants. Under salinity conditions, stem and root length and overall biomass of transgenic plants were significantly higher compared to those of nontransgenic plants. Moreover, salt treated transgenic plants contained high proline and K^+, but less Na^+ compared to wild type.

Keywords: Genetic engineering, Na^+/H^+ antiporter, Salt tolerance, Canola.

Introduction

According to the data gathered by Food and Agricultural Organization (FAO), salinity affects 7% of the world's land area, which amounts to 930 million ha (Szabolcs, 1994.). In many regions of the world, soil salinity is one of the major environmental factors limiting crop yield. It is estimated that 20–30% of the world's irrigated soil is adversely affected by salinity (Flowers, et al. 1995). Therefore, breeding for salt tolerance is considered as one of the most important estrategies to meet the needs of the world's increasing population (Holmberg and Bulow, 1998). In recent years, with the rapid development of plant genetic engineering, improving salt tolerance plants through transgenic technology has become feasible. After 15 years of attempts in this field, the utility of this technology have been tested in the field (Xuea et al. 2004; He et al. 2005).

The limiting effects of salt on plant growth are the consequence of both water deficit resulting from the high solute concentrations of ions in the soil and Na+ -specific stress due to alteration in Na+/K+ ratios and Na+ toxicity (Blumwald et al. 2000). Halophyte plants enzymes are not tolerant to high salt concentrations and display the same sensitivity to salt as enzymes from glycophytes

(Flowers et al. 1977). So, plants respond to higher concentrations of salts by maintaining low cytosolic Na+ concentrations and an optimum cytosolic K+/Na+ ratio. Strategies for the maintenance of high K+/Na+ ratio in the cytosol include Na+ extrusion and/or the intracellular compartmentation of Na+ (Blumwald et al. 2000). The compartmentation of Na+ into the vacuoles provides an effective mechanism to avoid the deleterious effects of Na+ in the cytosol. Moreover, the compartmentation of Na+ into the vacuole allows the plants to use NaCl as an osmoticum, maintaining an osmotic potential that drives water into the cells. The transport of Na+ into the vacuole is mediated by a Na+/H+ antiporter that is driven by the electrochemical gradient of protons generated by the vacuolar H.-translocating enzymes: Moreover, the compartmentation of Na+ into the vacuole allows the plants to use NaCl the Na+-ATPase and the H+-PPiase. (Blumwald et al. 2000). The Arabidopsis thaliana genome-sequencing project has allowed the identification of first Na+/H+ antiporter gene (AtNHX1) (Gaxiola et al. 1999). Over expression of this gene in Arabidopsis displayed significant increase of salt tolerance in transgenic plants and highlighted the role of this protein in improving plant salt tolerance (Apse et al. 1999).

Consistently, expression of AtNHX1 in Arabidopsis, tomato, canola, cotton and wheat displayed significant increases in their salt tolerance (Apsa et al., 1999, Zhang and Blumwald 2001; Zhang, et al, 2001, He et al., 2005; Xuea et al., 2005). Similar results were obtained when antiporter genes from other species were used. For example, salt tolerance was improved by expression of the rice Na+/H+ antiporter gene OsNHX1 in rice (Ohta et al., 2002), a putative vacuolar Na+/H+ antiporter gene from cotton (GhNHX1) in tobacco, Suaeda salsa vacuolar membrane Na+/H+ antiporter gene (SsNHX1) in rice (Zhao et al., 2006), and wheat Na+/H+ antiporter gene (TaNHX1) in Arabidopsis (Brini et al., 2007).

The AtNHX1 gene was also introduced into the genome of B. napus cv. Westar (Zhang et al 2001). This model cultivar is an old spring cultivar and is no longer grown in the fields due to some agronomic deficiencies. Here, we report the constitutive expression of Na+/H+ antiporter gene (AtNHX1) in canola variety PF70 which is an important commercial cultivar in Iran which has been chosen for transformation experiment due to its good response to tissue culture among several tested cultivars.

Materials and Methods

Plant material

Canola variety PF70 was used in this experiment.

Plasmid construction and Agrobacterium-preparation

The pBX1 plasmid harboring the *AtNHX1* gene has been kindly provided by Professor Blumwald from University of California. The full length of the *AtNHX1* gene with 35S promoter and NOS terminator has been isolated by digesting the plasmid with *Eco*RI and *Hin*dIII enzymes. The purified fragment has been inserted into *Hin*dIII and *Spe*I digested pLH6000 after blunting the *Eco*RI and *Spe*I ends in fragment and plasmid respectively. Resulting pLH6000:*AtNHX1* plasmid transferred to *Agrobacterium tumefaciens* strain LBA4404 by electroporation and was used for transformation. Hypocotyl was used as explants. pLH6000:AtNHX1 contains *hpt* selectable marker gene which encodes hygromycin phosphotransferase within the T-DNA region. *A. tumefaciens* was cultured overnight in YEP medium (yeast extract 10 g/L, peptone 10 g/L, sodium chloride 5 g/L, pH 7.0) supplemented with 50 mg/L Rrifampicin and 30 mg/L Hygromycine at 28 ^{0C} with shaking (150 rpm) to mid-log phase (OD600= 0.8-1). Then, the bacterial cells were collected by centrifugation at 2500 g for 5 min and resuspended in liquid MS medium up to a final OD600 of 0.2 for further infection.

Figure 1. T-DNA region of plant transformation vectors. The AtNHXI was cloned into transformation vector pLH6000 in p35s upstream.

Agrobacterium -mediated transformation

For transformation and regeneration a published protocol (Cardoza 2003) was followed with slight modifications. Briefly, seeds were surface-sterilized for 15 min with 5% sodium hypochlorite with 0.1% Tween added as a surfactant. The sterilization was followed by a 90 sec rinse with 95% ethanol. The seeds were washed three times with sterile distilled water and placed on a 1/2MS medium with 10 g/L sucrose that was solidified with 7 g/L agar for germination. Hypocotyls were excised from 10-days-old seedlings, cut into 0.5-1-cm pieces and preconditioned for 48 h on callus induction medium (MS medium supplemented with 1 mg/L 2,4-D and 30 g/L sucrose, solidified with 7 g/L agar). The preconditioned hypocotyl segments were then inoculated with *Agrobacterium* for 20 min. Explants were co-cultivated with *Agrobacterium* for 48 h on callus induction medium. Following cocultivation, the explants were transferred to the same medium containing 150 mg/L ticarcilin and 30 mg/L hygromycin to select for transformed cells. The explants with induced calli were subcultured into new medium every 2 weeks. After 4-6 weeks, the explants were transferred to MS medium with 4 mg/L 6-benzylaminopurine (BAP), and 3 mg/L silver nitrate, supplemented with antibiotics for shoot induction . After the emergence of small shoots, the explants were transferred to MS medium containing 1 mg/L BAP for further development. The developed shoots were transferred to MS medium with 0.05 mg/L BAP, for shoot elongation. The elongated shoots were transferred to half-strength MS medium supplemented with 10 mg/L sucrose, 2mg/L IBA and 15 mg/L hygromycin for rooting. All the cultures were maintained at 25±2^{oC} under a 16/8-h (light/dark) photoperiod with light supplied by cool-white fluorescent daylight. The rooted plantlets were transferred to soil and grown under a photoperiod of 16/8 h (light/dark) in a plant growth chamber for 4 weeks and finally transferred to greenhouse and maintained until they produced seeds.

Polymerase chain reaction (PCR) analysis

Genomic DNA was extracted from leaf tissue using the cetyl trimethylammonium bromide (CTAB) method (Murray and Thompson, 1980). The presence of the *AtNHX1* transgene in putative transgenic plants was initially detected with polymerase chain reaction (PCR) using Sigma's REDExtract-N-Amp Tissue PCR Kit following the manufacturers' instructions. Ten pico M of each primer (forward primer, 5'-CGCCACCACGAGCATGTTAT; reverse primer, 5'-CAAAGACGGGTCGCATGAAG) were added to each reaction. The PCR reactions were preheated to 95$^{°C}$ for 2 min, followed by 35 cycles with denaturation at 95$^{°C}$ for 30 sec, annealing at 59$^{°C}$ for 30 sec, extension at 72$^{°C}$ for 60 sec, and a final extension step at 72$^{°C}$ for 10 min.

RT-PCR analysis of AtNHX1 expression in transgenic canola

Total RNA was extracted using RNA-plus solution (SinaGene IRAN). Reverse transcription RT-PCR was performed using total RNA that was pre-treated with RNase-free DNase I. One microgram of total RNA from each sample was used in a 10 µl RT reaction using oligo dT as a primer. PCR amplifications were performed with 50 ng of cDNAs and *AtNHX1* specific primers in a reaction mixture of 50 µl, as described above.

Southern blot analysis

Ten micrograms of genomic DNA of each putative transgenic line and wild type was digested with 30 units of *Eco*RI and separated by electrophoresis in a 0.8 % agarose gel at 40 V for 18 h.Separated DNA was transferred to nylon Hybond N+ membrane. A 1.5 kb of the *AtNHX1* fragment was amplified, labeled with [α-32P] dCTP and used as probe. Hybridization was conducted in the hybridization buffer (6· SSC, 0.5% SDS, 0.2 mg/ml salmon sperm DNA) at 65_^{o}C for 24 h. The membrane was washed once at room temperature for 15 min in 0.2 SSC, 0.5% SDS and twice at 55^{oC} for 20 min in 0.1 SSC, 0.5% SDS. Membranes were then subjected to autoradiography.

Tolerance analysis of transgenic and wild-type plants under salt treatment

The seed of transgenic plants and wild type plants (coming from transformation processing) were cultured in silver sand at 2 Liter plastic pot and were grown to the 4-leaf stage. Seedlings were irrigated with Hogland solution supplemented with 200 mM NaCl for 30 days. The concentrations of NaCl addition increased stepwise (50mM per day for 4 days) to the final concentration of 200mM. At the end of the experiment, proline content, fresh weight, dry weight and length of shoot and roots of transgenic and wild types were measured.

Measurement of Na+ and K+ contents

At the end of the experiment 0.5 g of leaves and roots were collected and dried at 70°C, then the sampels were digested in nitric acid and finally, Na^+ and K^+ content were measured using a flame photometer.

Determination of proline content

Proline content was measured as described by Bates et al. (1973). One hundred mg of frozen plant material was homogenized in 10 mL of 3% sulphosalicylic acid and the residue was removed by centrifugation. 200 µl of supernatant was reacted with 200 µl glacial acetic acid and 200 µl acid ninhydrin (1.25 g ninhydrin warmed in 30 mL glacial acetic acid and 20 mL 6M phosphoric acid until dissolved) for 30 min at 100°C. The reaction was then terminated in an ice bath. The reaction mixture was extracted with 600 µl toluene. The chromophore containing toluene was warmed to room temperature and its optical density was measured at 520 nm.

Results

Generation of transgenic canola plants

We used the hypocotyledons and cotyldonary explants for transformation. All cotyledonary explants became necrotic in a few days after selection. However, transgenic calli were induced from hypocotyle explants in the presence of 30 mg L^{-1} of hygromycin. Transgenic plants were regenerated in the presence of 20 mg L^{-1} of hygromycin B (Fig.2).

Molecular analysis of transgenic plants

Initial molecular screening of putative transgenic plans have been carried out by using the tow primers to amplify a 440 bp fragment. The expected fragment of *AtNHX1* gene have been amplified in all transgenic plants (Fig.3-A). To determine gene copy numbers of *AtNHX1* in the genome of transgenic plants, Southern blot analysis of transgenic plans was conducted using a 1.5 Kbp PCR amplified fragment of the gene as probe. Detection of only a single band in three tested lines showed that one copy number of the At*NHX1* gene is inserted into the genome of each transgenic line (Fig. 3-B).

RT-PCR

Among these transgenic lines, lines L4 and L10 that exhibited apparently normal growth and morphology were chosen for functional analysis at T1 generation. But only the offspring of Line L4 expressed the *AtNHX1*. In-spite of its expression in T0 plants of line L10, no expression was observed at the progenies of this line.(data not shown). An *AtNHX1*-specific band with an expected size (440 bp) was amplified from DNase-treated RNA prepared from 10-day-old seedlings of transgenic lines L4 (Fig. 3-C), but not from L10 and wild types.

Salt tolerance of transgenic pants

To analyze the salt tolerance of *AtNHX1* transgenic plants the young T_1 seedlings of transgenic lines were grown in washed sand and irrigated with Hoagland solution supplemented with 200mM NaCl solution for 30 days. Phenotypically, the transgenic plants did not differ from non-transformed plants under non-saline conditions. Transgenic L4 lines showed survival rates than the control plants. The shoot and root fresh weight and dry weight, length of shoot and root of L4 at the end of 30-day salt treatment were higher than in nontransgenic plants (Table 1). The

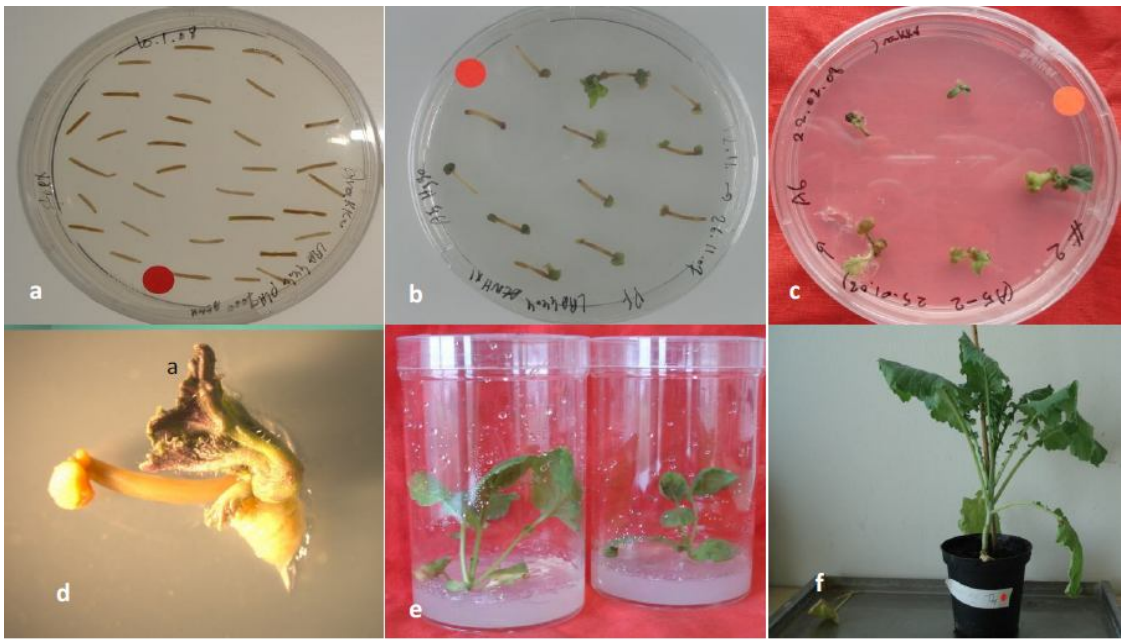

Figure 2. Regeneration of transgenic plants from hypocotyledonary explants. (a) hapocotyl preparation and cocultivation wit *Agrobacterium*; (b) callus induction in transformed tissue; (c and d) transgenic shoot regeneration in presence of 20 mg/l hygromycine; (e) shoot elongation and rooting of transgenic plants; and (f) Putative transgenic plantlets transplanted to pots.

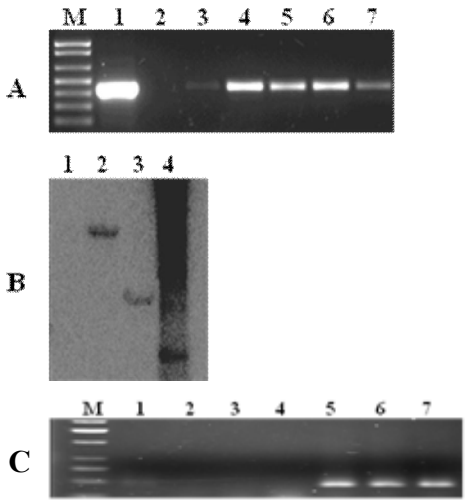

Figure 3. Analyses of *AtNHX1* gene in transgenic canola lines. (A) PCR analysis. Lane 1, Marker (1KB plus ladder); lane 2, plasmid pLH600/AtNHX1; lane 3 wild type, lanes 4–8, T0 transgenic lines. (B) Southern blot analysis of DNA isolated from leaves of T1 transgenic canola. Lanes 1, wild type; lane 2-4 transgenic lines. C; Expression analysis of *AtNHX1* transgenic lines presented in top. Lane 1, wild type; lanes 2-4, transgenic plants of L10 offspring ; lanes 5-7, transgenic plants of L4 offspring.

respective shoot and root weight of L4 was 3.19 and 3.32 fold more than nontransgenic lines. Salt treatment markedly reduced the biomass production of wild type, while line L4 showed much less growth retardation and longer root length (Fig. 4).

Na^+, K^+ and proline content in transgenic plants

The Na^+ and K^+ contents from leaves of wild-type and transgenic *AtNHX1* plants grown in the presence of 200 mM NaCl were analyzed. The Na^+ content was significantly lower in the transgenic lines than in wild-type plants, but the K^+ content of transgenic plants was higher than the wild-type plants (Fig.5,A). Prolin is one of the important osmoprotectants that response to salinity. The result indicated that the proline content in transgenic leaves was higher than in wild-type leaves. Transgenic plants were stressed with 200 mM NaCl displayed 3 fold increase in proline content compared with wild-type plant (Fig.5,B).

Figure 4. Effects of salt on the growth of *AtNHX1* transgenic canola's (Right) lines and wild type (Left). Plants with three young leaves were grown in pots containing silver sands and watered with a nutrient solution in the absence (non-saline) or presence of 200mM NaCl for 30 days. (A), Plants in pots after 30-day salt treatment; (B) whole plants after 30-day salt treatment.

Discussion

To avoid salt damage, plants have developed different mechanisms to limit Na+ uptake or its compartmentalization into vacuoles. Research efforts in plant biology have led to the identification of genes that might be useful in crop improvement (Chinnusamy et al. 2004), and among the genes identified those encoding vacuolar Na^+/H^+ antiporters appear to hold great promise in improving agricultural productivity under salt and drought conditions. The improvement of plant salt tolerance by the overexpression of a vacuolar Na^+/H^+ antiporter gene *AtNHX1* from Arabidopsis has already been demonstrated in plants such as Arabidopsis, (Apse, et al, 1999), tomato (Zang and Blomwald, 2001), wheat (Xuea, 2004) and cotton (He et al. 2005).

In the present study, we showed that the the salt tolerance of canola was improved by the introduction of the *AtNHX1* gene. The transgenic canola plants expressing AtNHX1 exhibited improved biomass production at the vegetative stage under the salt condition of 200 mM NaCl in greenhouse. A significant difference in biomass production at the vegetative growth stage was observed between the *AtNHX1* transgenic plants and non-transgenic plants.

A comparative analysis on Na^+ accumulation revealed that transgenic plants accumulated significantly less Na^+ in leaves than the non-transgenic control after 30 days of salt treatment. Similar results have been reported for vacuolar antiporter overexpressing plants such as wheat (Xeu et al. 2004), fescue (Zhao, et al. 2007), Arabidopsis, (Li et al. 2007) and sugar beet (Liu et al. 2008). This may be attributed to more Na^+ transport into root vacuoles, consequently resulting in a lower Na+ level in the cytosol and intercellular fluid of roots for transport into the shoot through the xylem transpiration stream (Xeu, et al 2004). In contrast, the AtNHX1 expressing transgenic tomato (Zang and Blumwald 2001) ryegrass (Wu et al. 2005) and maize (Chen et al. 2007) accumulated more Na+ in the leaves than nontransgenic plants. To explanation of the low Na+ content of AtNHX1 expressing transgenic plants Xeu et al. (2004) suggested three possible reason; a) operation of an unknown mechanism with preference in reduction of the Na^+ level in the leaves over the roots, b) shifting of some portion of expressed antiporter to plasma membrane pathway and c) the high dilution rate of the total Na^+ content in transgenic plants because of their higher growth rate.

The K^+ and proline concentrations of transgenic lines and wild-type under salt stress were also monitored in this study. Under salt-stress treatment, the leaf K^+ and proline concentrations of transgenic canola line were higher than that of wild-type. Similar results were observed in tomato plants overexpressing Na^+/H^+ antiporter gene *AtNHX1* (Zang et al. 2001) which indicates that overexpression of Na^+/H^+ antiporter gene could have changed the metabolism of K+ and maintained higher K+ and proline content in cells. The leaf and root K + contents of the transgenic canola plants expressing AtNHX1 grown in 200

Table 1. Effect of 200mM NaCl on the vegetative growth of T1 plants of AtNHX1 expressing *transgenic* plants and non-transgenic control.

	Parametr	Transgenic L4 Line	Control	Significance level of difference between two groups*
Shoot	FW (gr)	56.5± 4.5	17.66 ± 0.96	0.006
	DW (gr)	4.46 ±1.2	1.7± 0.53	0.016
	Length (cm)	24 ± 1.2	13.1 ± 1.85	0.003
	Number of Leaves	14± 0.81	8.33 ± 0.57	0.001
Root	FW (gr)	16.35 ±0.19	1.23 ± 0.05	0.005
	DW (gr)	2.14 ± 0.24	0.35 ± 0.22	0.001
	Length (cm)	36.16±2.46	21.3 ± 3	0.003

FW: fresh weight, DW: dry weight

mM NaCl were lower than those from plants grown in low salinity (Zang, et al, 2001). Very recently it was shown that AtNHX1 expressing transgenic plants had larger K+ vacuolar contents in all growth conditions but no stabel enhancement of Na+ accumulation under salt stress, so It was sugested that in some case AtNHX1 act as a K+ transporter (Leidi, et al, 2009).

Proline contributes to osmotic adjustment, protection of macromolecules during dehydration, and acts as hydroxyl radical scavenger (Chen, et al. 2008). Although we did not analyzed more details of transgenic plants performance in salinity condition but Zang et al (2001) find transgenic canola plants expressing AtNHX1 grown at 200 mM NaCl produced numbers of seeds similar to those of wild-type plants grown at low salinity. Moreover, qualitative and quantitative analyses of the oil content showed no significant differences between seeds from wild-type plants grown at low salinity and transgenic plants grown at high salinity.

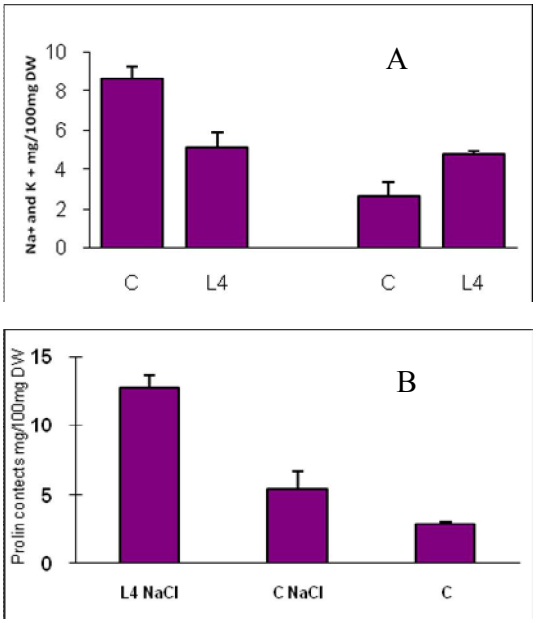

Figure 5. (A); Comparison of Na^+ and K^+ contents between AtNHX1-overexpressing line L4 and the wild-type after 30 d of 200 mM NaCl treatment: C, Wild type. (B); Proline content in leaves of transgenic (L4) and wild-type (C) plants exposed to 200 mM NaCl. L4 NaCl; transgenic L4 under NaCl treatment, C NaCl; wild type plants under NaCl treatment and C is the wild type plants under normal condition.

Improvement of salt tolerance using the transgenic approach can also be achieved by the over-expression of a plasma membrane Na^+/H^+ antiporter gene (*sos1*) to enhancing the salt exclusion capacity from the cells (Shi et al. 2003; Gao et al. 2003). This approach will leave salt in the soil. In contrast, the sequestering Na^+ into the vacuole of plant cells would be more advantageous than the salt exclusion transgenic approach in terms of the sustainability of crop production in saline soils, as the vacuolar Na^+/H^+ antiporter transgenic plants would potentially take away a significant amount of salt from the soil. Thus, it would provide a means to reduce the soil salinity problem in a long term as well.

However, careful examination of all transgenic plants expressing AtNHX1 indicted that the increased salt tolerance appeared to be limited to around 200 mM NaCl, which could not be enough to overcome the salt toxicity in many soilswhere

the salt concentrations are higher (Bartels and Sunkar, 2005, Chinnusamy and Zhu 2005). To increase salt tolerance further in plants, the simultaneous overexpression of a vacuolar H^+ pump and a vacuolar Na^+/H^+ antiporter will probably be required. We anticipate that AtNHX1/AVP1-overexpressing canola plants will be significantly more salt tolerant. This project is ongoing.

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