Expression of *OsNHX1* gene in maize confers salt tolerance and promotes plant growth in the field

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ABSTRACT

Maize yield is severely affected by soil salinity. In an effort to engineer maize for improved salt tolerance, embryogenic calli of maize were co-bombarded with plasmids containing *Oryza sativa* Na^+/H^+ antiporter gene (*OsNHX1*) and *bar* genes. For the molecular analysis of putative transgenic samples, PCR, Southern and Northern blots were carried out. The maize plants over-expressing *OsNHX1* accumulated more biomass when grown in the presence of 200mM NaCl in greenhouse conditions. Higher Na^+ and K^+ content was observed in transgenic leaves than in wild-type leaves when treated with 100~200mM NaCl, while the osmotic potential and the proline content in transgenic leaves was lower than in wild-type maize. A field trial revealed that the transgenic maize plants produced higher grain yields than the wild-type plants at the vegetative growth stage. These results demonstrate that the *OsNHX1* gene was successfully transferred into *Zea mays*, and the salt-tolerance of transgenic maize was improved by overexpression of the *OsNHX1* gene.

Keywords: Na^+/H^+ antiporter; salt-tolerance; transgenic maize

Soil salinity is a major abiotic stress factor, which severely inhibits the growth of plants and reduces the grain yield. Many crop species are glycophytes that are usually salt-sensitive. Statistics and analyses indicate that approximately 20% of cultivated lands and 50% of irrigated lands in the world have high salt content (i.e. salty soil; Yokoi et al. 2002). Therefore, it is important to understand how plants respond to salt stress at the cellular and molecular levels. Production of transgenic crops with improved stress tolerance could have important economic and social benefits.

Salt toxicity in plants mainly results from Na^+ damage. Plants utilize three mechanisms to prevent accumulation of Na^+: restriction of Na^+ influx, activation of Na^+ efflux, and compartmentalization of Na^+ in the vacuole (Blumwald et al. 2000).

The Na^+/H^+ antiporter plays an important role in resistance to salt stress by exchanging Na^+ and H^+ across the plasma or vacuolar membranes. The tonoplast Na^+/H^+ antiporter, which was found in several plant species (Barkla et al. 1990, Ballesteros et al. 1997, Fukuda et al. 1999, Gaxiola et al. 1999, Hamada et al. 2001), transports Na^+ from the cytoplasm into vacuoles, increases the cytoplasmic K^+/Na^+ ratio and thereby protects cells from sodium toxicity (Fukuda et al. 1999). Up to now, many salt-tolerant transgenic plants were generated by over-expressing a vacuolar Na^+/H^+ antiporter gene in plants. In the transgenic plants, such as *Arabidopsis*, tomato, brassica, maize, wheat and cotton, increased vacuolar uptake of Na^+ and improved salt tolerance than in wild-type plants were demonstrated (Apse et al. 1999, Zhang and

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Blumwald 2001, Zhang et al. 2001, Xue et al. 2004, Yin et al. 2004, He et al. 2005). Similar results were obtained in transgenic rice, transgenic ryegrass and poplar tree by overexpressing the halophyte (*Atriplex gmelini*) or rice vacuole-type Na^+/H^+ antiporter gene (Ohta et al. 2002, Fukuda et al. 2004, Wang et al. 2005, Wu et al. 2005).

Maize is one of the most important crops in the world. Its production and planting area are greatly affected by soil salinity. Therefore, it is of agricultural importance to improve the salt tolerance of maize. The Na^+/H^+ antiporter gene *OsNHX1* was shown to increase salt tolerance in native and heterologous systems; we hypothesized that this approach should work in maize. Therefore, we expressed the *OsNHX1* in maize to test whether it could improve its salt tolerance. As expected, *OsNHX1*-expressing maize plants were significantly more tolerant to salt treatment under greenhouse conditions, and produced more biomass yield than wild-type plants. A major result that distinguishes our study from many previous studies is that even under non-saline conditions in the field, the *OsNHX1*-expressing maize plants exhibited higher grain yield than wild-type plants. Our data suggest that the increased expression of a vacuolar Na^+/H^+ antiporter may be an effective way to improve salt tolerance for maize.

MATERIAL AND METHODS

Transgenic maize (*Zea mays*) lines and plant expression vector. Transgenic maize (*Zea mays*) lines used in this study were inbred lines Zong3 (Z3), Zong31 (Z31), P9-10, Qi31 (Q31) and hybrid line F (first filial generation of Z3 crossed with Z31). The *Oryza sativa* Na^+/H^+ antiporter gene *OsNHX1* cDNA (United States Patent 20050032112) was digested with *Bam*HI and *Bst*EII and subcloned into pCAMBIA3301 (Wu et al. 2005). The *OsNHX1* gene was under the control of the cauliflower mosaic virus (CaMV) 35S promoter, and the terminator region contained the polyadenylation signal of the nopaline synthetase gene (*Nos*).

Maize transformation. Plants of maize were grown under standard greenhouse conditions. Self-pollinations of maize plants were made. 10 to 12 days after pollination (Vain et al. 1993), the developing ear was removed and surface soaked in 70% ethanol for 5 min, then rinsed several times with sterile distilled water. Immature embryos (1.5–3.0 mm) were isolated and placed onto initiation medium consisting of N6 basal salts and vitamins with pH adjusted to 5.8. After 2–3 weeks in the dark at 28°C the callus was transferred to 'maintenance' medium.

The 'maintenance' medium differed from the 'initiation' medium in the content (700 mg/l L-proline) and lacked silver nitrate. Callus was used for transformation after approximately 16 weeks after initiation.

In preparation for tissue bombardment, 150 mg of plasmid DNA was precipitated onto 60 mg of alcohol-rinsed, spherical gold particles (1.5–3.0 mm diameter, Aldrich Chemical Co., Inc., Milwaukee, WI) by adding 74 ml of 2.5M $CaCl_2$ H_20 and 30 ml of 0.1M spermidine to 300 ml of plasmid DNA and H_2O. The solution was immediately vortexed and the DNA-coated gold particles were allowed to settle. The resulting clear supernatant was removed and the gold particles were resuspended in 1 ml of absolute ethanol. This suspension was diluted with absolute ethanol to obtain 15 mg DNA-coated gold/ml. Maize transformation was done according to Wan et al. (1995). The gene gun was PSD-1000 by Bio-Rad. After bombardment prior to blasting, the DNA-coated gold particles were further diluted 1:1 with absolute ethanol, then accelerated at the callus targets using a helium pressure of 1500 psi, with each blast delivering 15 ml of the DNA/gold suspension.

Immediately after blasting, the tissue was transferred to maintenance medium for a 16–24 h recovery period. Then calli were placed on selective medium containing 10 mg/l phosphinothricin (PPT) and subcultured at 2-week intervals.

After 8 to 12 weeks, survived shoots were transferred into root-inducing medium to induce roots.

PPT-resistance assay. PPT-resistance was measured to verify *bar* gene expression in T1 seeds. Seeds were surface-sterilized with 70% ethanol for 5 min and rinsed 3 to 4 times with sterile distilled water. Then, the seeds were placed on liquid media [1/2 MS (the basic medium) plus 10 mg/l PPT] at 28°C.

PCR, Southern blot and Northern blot analysis. PCR was used to verify the presence of *OsNHX1* and *bar* in the genomic DNA of transformed plants. The primers used in PCR were: 5'-TAG GAT CCA AGC CAT TGA TCA GGC TGC-3' and 5'-GCA AGC TTG TCT TCC ATG GCT GCT CTG-3'; 5'-GCG GTC TGC ACC ATC GTC A-3'and 5'-GTA CCG GCA GGC TGA AGT CCA-3'. In Southern and Northern blotting experiments, a 1.4 kb probe for *OsNHX1* was prepared by digesting p3301-*OsNHX1* with *Nco*I and labeled with [α-^{32}P] dCTP.

In Southern and Northern blotting experiments, 10 mg of genomic DNA of each putative transgenic line was digested with *Bam*HI and separated by electrophoresis in a 1.0% agarose gel at 45 V for 16–18 h, then transferred to nylon membrane (Biodyne A; Pall Corp., Port Washington, NY, USA) and hybridized with [α-^{32}P]-dCTP-labeled *OsNHX*1 gene probe. The hybridization was conducted as previously described (Sambrook et al. 1989).

Northern blot analysis was performed by using standard procedures (Sambrook et al. 1989). After gel electrophoresis and blotting of total RNA onto a nylon membrane (Biodyne A; Pall Corp., Port Washington, NY, USA), hybridization was performed with ^{32}P-labeled cDNA fragments prepared using a random primer labeling kit (Random Primers System; Takara, Tokyo, Japan). Equal loading of RNA blots was assessed by scanning the signals of ribosomal RNA detected with methylene blue.

Salt-tolerance assay and biomass measurements under salt treatment in the greenhouse. Transgenic seeds (T1 generation) and wild-type seeds were sterilized with 70% ethanol, and washed 3 to 4 times with sterile distilled water. Then, the seeds were placed on liquid media [1/2 MS (the basic medium) plus 10mg/l PPT] at 28°C. Seedlings with established lateral root systems were planted in plastic pots (5–7 seeds/pot) filled with silver sand and watered with MS solution. After molecular analysis, the transgenic plant with four leaves were treated with MS solution containing 200mM NaCl and irrigated thoroughly at 3-day intervals for 16 days until the death of wild-type maize. The shoot (above-ground portion) and root (under-ground portion) were harvested for fresh biomass determination after 10 days of 200mM NaCl treatment. Roots were harvested by gently flushing silver sand away with water and drying with paper (three pots replicates were used for each treatment.). Plants were photographed to document growth/survival. The temperature in the greenhouse was maintained at 28 ± 2°C, and the relative humidity was maintained at 50 ± 10%. Each treatment was repeated three times.

Determination of the Na^+, K^+ contents in leaf and root and osmotic potential in leaf. 20 Transgenic maizes and wild-type (CK) maize were cultured with MS solution containing 0–200mM NaCl (0, 50, 100 and 200mM) and watered for 1 week (three pots replicates were used for each treatment). Leaves and roots were collected and dried at 70°C. Na^+ and K^+ content were measured using a Z-5000 polarized Zeeman atomic absorption spectrophotometer (HITACHI Instrument, Japan). Leaves from the same spot were immediately frozen for 30 min in liquid nitrogen and thawed for 20 min at room temperature. The leaves were loaded into a 2.5 ml injector with filters. The injectors were inserted into 1.5 ml Eppendorf tubes and centrifuged at 4 000 rpm for 5 min. Osmomat 030 was used to determine the osmotic potential.

Determination of proline content. Proline content was measured as described by Bates et al. (1973). 500 mg of frozen plant material was homogenized in 5 ml of 3% sulphosalicylic acid and the residue was removed by centrifugation. The extract was reacted with 2 ml glacial acetic acid and 2 ml acid ninhydrin (1.25 g ninhydrin warmed in 30 ml glacial acetic acid and 20 ml 6M phosphoric acid until dissolved) for 1 h at 100°C and the reaction was then terminated in an ice bath. The reaction mixture was extracted with 1 ml toluene. The chromophore containing toluene was warmed to room temperature and its optical density was measured at 520 nm.

Field test and analysis of field-grown maize plant. Two independent lines (T1 generation Z3 and Q31) of *OsNHX1*-expressing plants, 10 seeds for each line, were field-tested, at China Agriculture University Experimental Farm in Beijing. Transgenic lines and wild-type plants were irrigated every day. The plants were harvested by hand at the end of the growing season.

Statistical analysis. Analysis of variance with a complete randomized block design and *t*-test was used for determining the existence of significant effects due to the transgene *OsNHX1*. All statistical analysis was done using Microsoft Office Excel 2000.

RESULTS AND DISCUSSION

Molecular identification of transgenic plants

Using the gene gun transformation method, 76 resistant lines were obtained. The surviving regenerated plants in the fields usually did not grow well. Most of them showed abnormal male and/or female organs. Though all transformed inbred lines could produce seeds, only a part of the plants (Q31-5, Q31-33, Z3-1 and Z3-7) produced seeds by self-pollination.

PCR and Southern blot analysis were conducted to confirm the presence of the Na^+/H^+ antiporter gene in the T1 seedlings from four transgenic lines (Q31-5, Q31-33, Z3-1 and Z3-7). Several

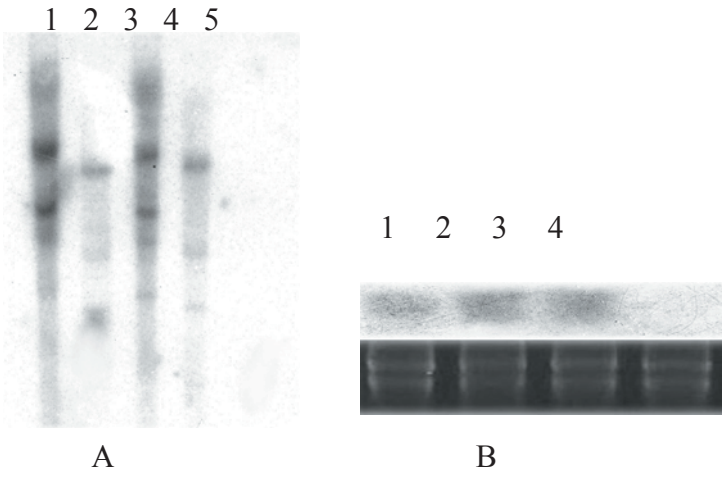

Figure 1. Southern and northern-hybridization analysis of transgenic maizes

(A) Southern blot analysis of transgenic plants: 10 mg aliquots of genomic DNA prepared from leaf tissue of T1 generation harbouring p3301-*OsNHX1* were digested with *Bam*HI and probed with a 1.4 kb [α-^{32}P] dCTP-labeled *Nco*I fragment from p3301-*OsNHX1*. Lanes 1–4: genomic DNA from transgenic plants Q31-5, Q31-33, Z3-1 and Z3-7; lane 5: genomic DNA from a non-transformed plant

(B) Northern blot analysis of T1 generation transgenic plants

Total RNA was prepared from leaves of T1 generation transgenic plants transformed with p3301-*OsNHX1*. Total RNA was separated on an agarose gel and hybridized with a 1.4 kb [α-^{32}P] dCTP-labeled *Nco*I fragment from p3301-*OsNHX1*; Lanes 1–3: T1 transgenic lines Z3-1, Z3-7 and Q31-5; lane 4: a non-transformed plant

hybridization bands were detected in all four lines (Figure 1A), indicating that the *OsNHX1* cDNA had integrated into the maize genome. It was also found that the transgenic plants had different hybridization bands, which might be caused by unequal exchange of homoeologous chromosomes in gene rearrangement, or base mutation of enzyme cutting site. The hybridization band on lane 5 of Figure 1A resembled the control one (non-transgenic plant Z3). The transgenic lines, Z3-1, Z3-7 and Q31-5, were selected for Northern blot analysis. One hybridization band was detected (Figure 1B), indicating that the integrated *OsNHX1* cDNA was transcribed in these lines.

OsNHX1-expressing maize plants were more tolerant to salt stress than wild-type plants

To test whether *OsNHX1*-expressing maize is more tolerant to salt treatment, an initial rapid screening for germination rate under selected

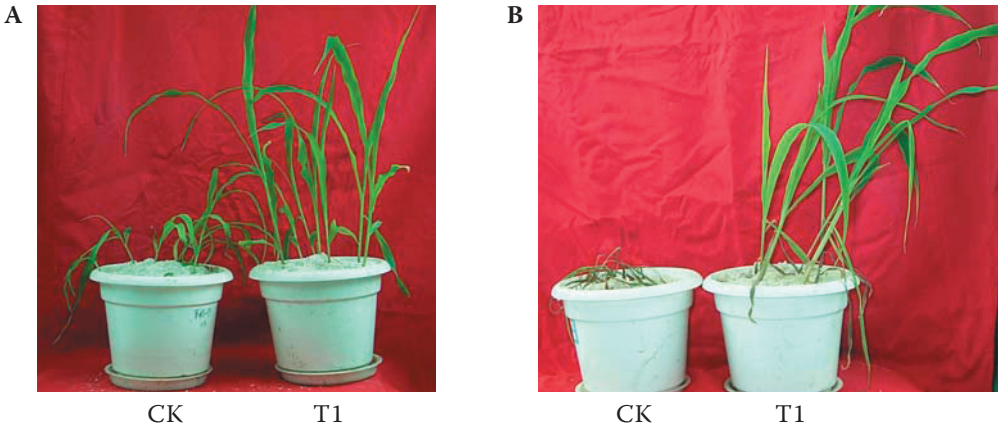

Figure 2. Salt-resistance of transgenic plants

Untransformed (CK) and Z3-1 transgenic plants (T1) treated with 200mM NaCl for 10 days (A) or 16 days (B)

herbicide treatment was performed. The germination of wild-type maize seeds was suppressed by herbicide while the germination of transgenic maize seeds was not.

Then we analyzed all transgenic lines for their salt tolerance in greenhouse conditions. All transgenic lines showed higher salt tolerance than the wild-type plants. In particular, transgenic plants Z3-1, Z3-7 exhibited relatively higher salt tolerance than other lines and therefore were used for further analysis. Young plants (4 leaves) were treated with 200mM NaCl until the wild-type plant died. Dramatic phenotypic differences appeared between wild-type plants and *OsNHX1*-expressing maize plants when treated with 200mM NaCl. Growth of wild-type plants was severely inhibited by 200mM NaCl, whereas growth of the *OsNHX1*-expressing maize plants was considerably less inhibited. After 10 days of exposure to 200mM NaCl treatment, the leaves of wild-type maize withered, while the transgenic plants grew normally and the transgenic plants were noticeably higher than wild-type plants (Figure 2). The transgenic maize accumulated more fresh shoot biomasses and fresh root biomasses than the wild-type plants did (Table 1). After 16 days of exposure to the 200mM NaCl treatment, the wild-type plants died while the *OsNHX1*-expressing maize plants survived (Figure 2). These results indicate that the expression of *OsNHX1* in maize can protect plant from damage by NaCl stress.

Na^+, K^+ content and osmotic potential in transgenic and wild-type maize

T1 plants from transgenic lines Z3-1, Z3-7, Q31-5 and Q31-33 and wild-type maize were treated with different concentrations of NaCl and the Na^+ and K^+ content in leaves and roots were measured. When plants were treated with 0~200mM NaCl, the Na^+ and K^+ content in the root of transgenic and wild-type maize was similar (Figure 3, $P > 0.05$; Figure 5, $P > 0.05$). Na^+ content in the leaves and roots of transgenic and wild-type maize increased as NaCl content increased (Figure 3), and in plants treated with 100~200mM NaCl, higher Na^+ content was observed in transgenic leaves than in wild-type leaves (Figure 3, $P < 0.01$). This result indicated that transgenic leaves absorbed more Na^+ than wild-type leaves. K^+ content in the roots of transgenic and wild-type maize decreased with increasing NaCl concentration, but to a smaller extent in transgenic than in wild-type plants (Figure 4A); however, in plants treated with 100~200mM NaCl, significantly higher K^+ content was observed in transgenic leaves than in wild-type leaves (Figure 4B, $P < 0.01$).

At 0 and 50mM NaCl, the osmotic potential was similar in roots of transgenic and wild-type maize. However, at 100 and 200mM NaCl, the osmotic potential was much lower in transgenic maize than in wild-type maize ($P < 0.01$) (Figure 5).

Determination of the proline content in leaf

Transgenic lines Q31-5 and Z3-7 and wild-type plants were irrigated with MS solution containing 200mM NaCl and the proline content in leaf was measured after 10 days. The result indicated that the proline content in transgenic leaves was lower than in wild-type leaves ($P < 0.05$) (Figure 6).

OsNHX1-expressing maize plants produced higher grain yield under irrigation in the field

To study how *OsNHX1*-expressing maize plants would perform in irrigated field conditions, we

Table 1. Effects of 200mM NaCl on biomass of T1 plants of transgenic maize plants and wild-type plant

	Shoot fresh weight (g)		Root fresh weight (g)	
	non-saline	NaCl	non-saline	NaCl
Wild-type plant	33.2 ± 0.16 aA	17.5 ± 0.12 aA	30.5 ± 0.15 aA	16.2 ± 0.11 aA
Z3-1	34.5 ± 0.15 bB	33.2 ± 0.15 bB	30.6 ± 0.14 aA	26.7 ± 0.13 bB
Z3-7	36.4 ± 0.13 cC	33.2 ± 0.12 bB	31.2 ± 0.15 bB	28.9 ± 0.14 cC

OsNHX1-positive homozygous transgenic plants (the presence of the transgene was detected by PCR analysis) and wild-type with four young leaves were treated with a nutrient solution in the absence (non-saline) or presence (NaCl) 200mM NaCl. Data were collected after 10-day treatment. Values are means ± SD ($n = 8$ individual plants). Different capital and small letters after fresh weight mean significant level at 1% and 5%, respectively

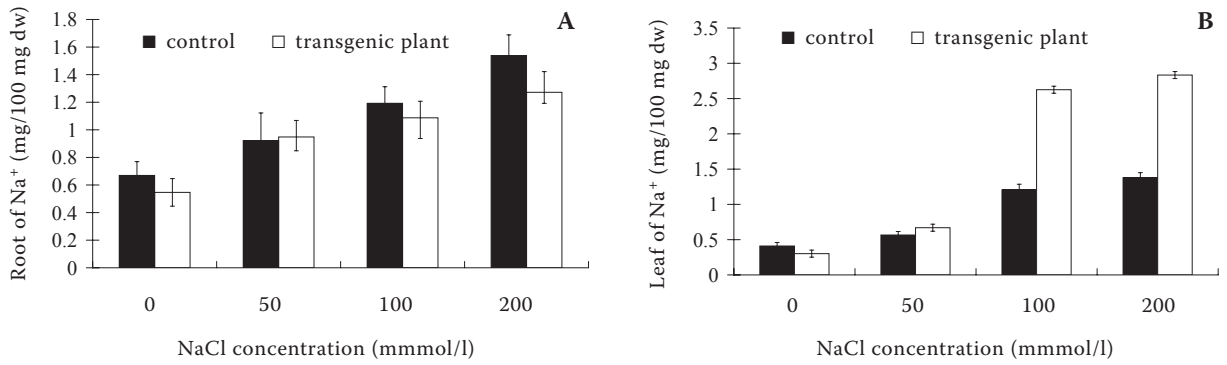

Figure 3. Na^+ content of leaves and roots of transgenic and wild-type maize exposed to salt stress

10-day-old seedlings were watered with MS solution containing NaCl (when plants had 4 leaves). The Na^+ content was measured in leaves and roots after 7-day salt treatment. Values are the mean ± SD (n = 3). Controls: non-transgenic maize; transgenic: T1 transgenic lines Z3-1, Z3-7, Q31-5 and Q31-33

tested two transgenic lines (T1 generation of Z3 and Q31) at the Experimental Farm of China Agriculture University in Beijing during the 2003 growing season. Maize seeds were planted in early April and seeds were harvested in late August. During the growing season, they were drip watered on a daily basis.

All transgenic lines exhibited higher grain yields than wild-type maize plants, with an average increase of >10% per line; the results from two of the transgenic lines are presented in Figure 7.

A major factor affecting the productivity of important world food crops is the environmental stress, such as salinity, which reduces crop yields severely. A large amount of lands are so saline that they cannot be used for crop cultivation. Therefore, it is imperative to make crops more tolerant to salt stress and more productive under such stressful conditions.

Genes encoding vacuolar Na^+/H^+ antiporters appear to hold great promise in improving agricultural productivity under salt stress. The first transgenic plant using *AtNHX1*, an *Arabidopsis* vacuolar Na^+/H^+ antiporter, was reported by the Blumwald group. Recently, Fukuda et al. (2004) used a similar approach to create salt-tolerant rice by using *OsNHX1*. In this study, we showed that the salt tolerance of a monocot species, maize, could also be improved by the introduction of the *OsNHX1* gene.

Growth conditions in the field are more complex than greenhouse conditions. Most previous transgenic studies about Na^+/H^+ antiporter transgenic plants are based on laboratory experiments. Few study pay attention to the growth and yield of transgenic plants. A major result that distinguishes our study from many previous studies is that even under non-saline conditions in the field, the

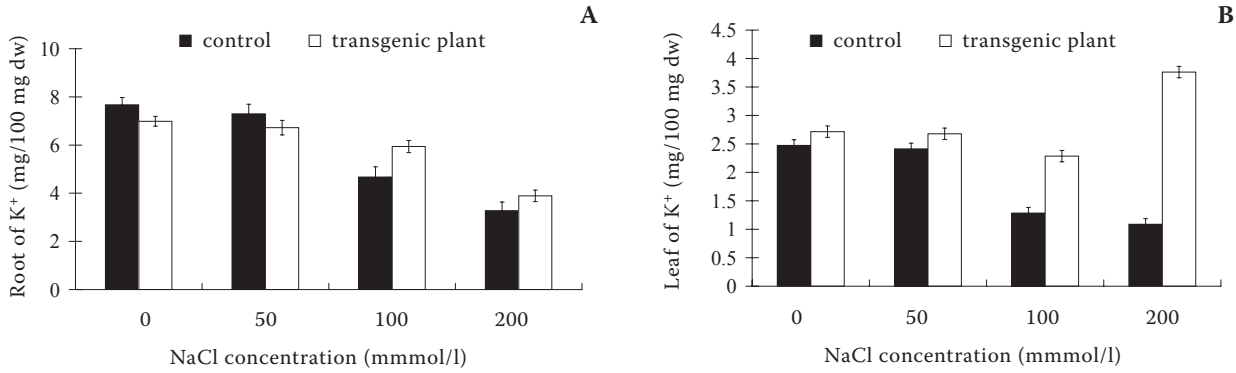

Figure 4. K^+ content in leaves and roots of transgenic and wild-type maize exposed to salt stress

10-day-old seedlings were watered with MS solution containing NaCl (when plants had 4 leaves). The K^+ content was measured in leaves and roots after 7-day salt treatment. Values are the mean ± SD (n = 3). Controls: non-transgenic maize; transgenic: T1 transgenic lines Z3-1, Z3-7, Q31-5 and Q31-33

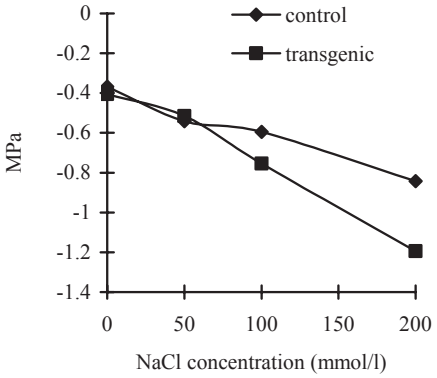

Figure 5. Osmotic potential in transgenic and wild-type maize exposed to salt stress

10- days old seedlings were watered with MS solution containing NaCl (when plants had 4 leaves). The osmotic potential was measured after 7 days salt treatment. Values are the mean ± SD (n = 3). Controls: non-transgenic maize; transgenic: T1 transgenic lines Z3-1, Z3-7, Q31-5 and Q31-33

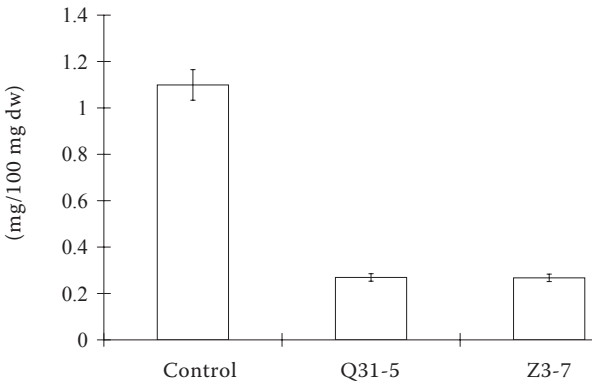

Figure 6. Proline content in leaves of transgenic and wild-type maize exposed to salt stress

10-day-old seedlings were watered with MS solution containing NaCl (when plants had 4 leaves). The proline content was measured after 10-day salt treatment. Values are the mean ± SD (n = 3). Controls: non-transgenic maize; transgenic: T1 transgenic lines Z3-7 and Q31-5

OsNHX1-expressing maize plants exhibited higher grain yield than did wild-type plants (Figure 7). Nevertheless, the underlying mechanisms of increased grain yield are not known at this time. He et al. (2005) found that expressing *AtNHX1* gene in cotton can improve photosynthetic performance. This might partly explain the improved performance of transgenic maize. In comparison with wild-type plants, *OsNHX1*-expressing maize plants may exhibit higher rates of CO_2 assimilation and a greater activity of nitrate reductase. Thus, the capacities to assimilate carbon and nitrogen appear to be maintained to a greater extent by the *OsNHX1*-expressing maize than by wild-type plants, and may contribute substantially to the improvement in biomass and grain yield.

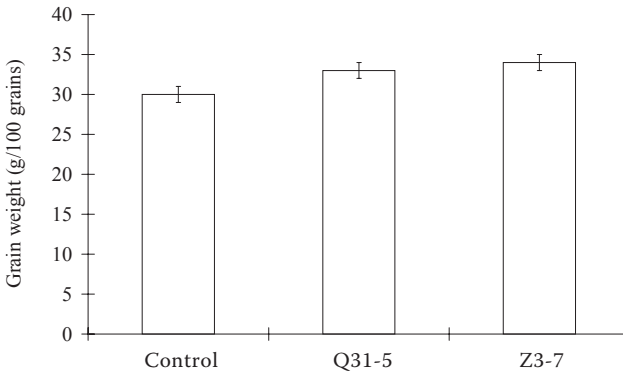

Figure 7. Grain yields of wild-type and *OsNHX1*-expressing maize plants in the field. Controls: non-transgenic maize; Z3-7 and Q31-5 two independent *OsNHX1*-expressing maize plants. Values are the mean ± SD (n = 10)

The Na^+ and K^+ contents were also higher in leaves of transgenic plants than in wild-type plants under salt stress. This is because transcript levels of *OsNHX1* in shoots were higher than those in roots irrespective of the concentration of NaCl (Fukuda et al. 2004). This result also suggested that *OsNHX1* might play an important role in the salt tolerance of shoots rather than roots.

The K^+ content in the leaves of transgenic maize increased after exposure to 100 or 200mM NaCl, and this result is somewhat different from previous studies. This discrepancy may reflect different adaptive mechanisms for handling salt stress in different plant species. Recent studies indicated that the tonoplast Na^+/H^+ antiporters have the same affinity for Na^+ and K^+ (Numata and Orlowski 2001, Zhang et al. 2001, Venema et al. 2002). Thus, the increase of K^+ content in the transgenic maize expressing *OsNHX1* may be due to increased K^+ transportation by the Na^+/H^+ antiporter, and higher K^+ content in transgenic plants could explain the decreased osmotic potential observed in these transgenic plants (Figure 6).

Previous studies indicate that proline content in maize plant leaves increases in response to salt stress. This report shows that the proline content

was lower in transgenic maize than in wild-type maize under salt stress, suggesting that expression of *OsNHX1* confers salt tolerance and reduces salt stress. The transgenic maizes described in this report are also herbicide-resistant, because they were co-transformed with the *bar* gene. Thus, the maize lines described here have a potential economic advantage, because they may reduce the need for weed removal during propagation.

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