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Over-expression of a vacuolar Na^+/H^+ antiporter gene improves salt tolerance in an upland rice

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Abstract To develop a salt-tolerant upland rice cultivar (*Oryza sativa L.*), *OsNHX1*, a vacuolar-type Na^+/H^+ antiporter gene from rice was transferred into the genome of an upland rice cultivar (IRAT109), using an *Agrobacterium*-mediated method. Seven independent transgenic calli lines were identified by polymerase chain reaction (PCR) analysis. These *35S::OsNHX1* transgenic plants displayed a little accelerated growth during seedling stage but showed delayed flowering time and a slight growth retardation phenotype during late vegetative stage, suggesting that the *OsNHX1* has a novel function in plant development. Northern and western blot analyses showed that the expression levels of *OsNHX1* mRNA and protein in the leaves of three independent transgenic plant lines were significantly higher than in the leaves of wild type (WT) plants. T_2 generation plants exhibited increased salt tolerance, showing delayed appearance and development of damage or death caused by salt stress, as well as improved recovery upon removal from this condition. Several physiological traits, such as increased Na^+ content, and decreased osmotic potential in transgenic plants grown in high saline concentrations, further indicated that the transgenic plants had enhanced salt tolerance. Our results suggest the potential use of these transgenic plants for further agricultural applications in saline soil.

Keywords Over-expression · *OsNHX1* · Vacuolar Na^+/H^+ antiporter · Salt tolerance · Transgenic upland rice plants

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Introduction

Na^+/H^+ antiporters, which catalyze the exchange of Na^+ for H^+ across membranes, are ubiquitous membrane proteins that play important roles in cellular pH and Na^+ homeostasis throughout the biological kingdom. In plants, Na^+/H^+ antiporters are located in both plasma (Shi et al. 2002) and vacuolar (Apse et al. 1999) membranes, removing Na^+ from the cytosol or compartmentalizing it in vacuoles for maintenance of a low Na^+ concentration. In particular, the vacuolar Na^+/H^+ antiporter has been investigated and proposed to play an important role in salt tolerance (Blumwald 2000). Recently, with the molecular identification and biochemical characterization of the genes encoding these vacuolar membrane Na^+/H^+

antiporters, they have been isolated from many plants, including *Arabidopsis thaliana* (Apse et al. 1999), *Oryza sativa* (Fukuda et al. 1999, 2004b), *Atriplex gmelili* (Hamada et al. 2001), *Brassica napus* (Wang et al. 2003), *Beta vulgaris* (Xia et al. 2002), *Gossypium hirsutum* (Wu et al. 2004), and *Hordeum vulgare* (Fukuda et al. 2004a). These proteins are homologous and have a similar function. However, it is still unclear whether fine variations in activity among these orthologues may contribute to differences in physiological regulation.

Manipulating the vacuolar Na^+/H^+ antiporter to improve Na^+ homeostasis is recognized as an attractive strategy in plants. Recently, *AtNHX1* has been over-expressed in several dicotyledonous plants, including *Arabidopsis* (Apse et al. 1999), tomato (*Lycopersion esculentum*) (Zhang and Blumwald 2001), and *Brassica napus* (Zhang et al. 2001). These transgenic plants displayed robust salt tolerance and could grow normally and produce fruit and seeds under highly saline conditions (200 mM NaCl). More recently, *AtNHX1* also has been introduced into crop plants such as wheat (Xue et al. 2004) and maize (Yin et al. 2004), improving salt tolerance. Moreover, over-expression of *GhNHX1* in tobacco plants also enhanced salt tolerance (Wu et al. 2004). The examples above clearly demonstrate the feasibility of engineering salt tolerance into crop plants.

Rice is an important crop and mostly grown under continuously flooded conditions in lowlands. However, an ever-increasing water shortage has become the most serious constraint to rice production and yield stability in many rice-growing areas. Therefore, the non-flooded cultivation of upland rice is an alternative to lowland rice farming that could reduce the demand for irrigation water by 50–70% (Wang et al. 2002). However, upland rice, like lowland rice, has low salt tolerance. If salt tolerance were to be conferred upon upland rice through genetic engineering, the production of upland rice would be increased in saline soil and the plants would grow in a wider area. Thus, the persistent problem of food shortage in Asia and Africa could perhaps be alleviated to some extent through these methods. Recently, *OsNHX1* has been over-expressed in rice (Fukuda et al. 2004b) and perennial ryegrass (Wu et al. 2005b). The *AgNHX1* (Ohta et al. 2002) and bacterial *nhaA* (Wu et al. 2005a) genes have been introduced into rice as well, resulting in transgenic plants that displayed enhanced salt tolerance. However, this is the first report on improving the salt tolerance of upland rice by over-expressing a vacuolar Na^+/H^+ antiporter.

In the present study we produced transgenic upland rice plants over-expressing the *OsNHX1*, using an *Agrobacterium*-mediated transformation method, and investigated whether the salt tolerance in these plants could be improved by enhancing the level of *OsNHX1* mRNA and protein. As the T_2 generation plants were more salt tolerant, the exhibition of damage, or death, caused by high saline concentration was delayed or diminished in these plants. Furthermore, they also displayed improved recovery upon their removed from salt stress. In addition to being the first report that *OsNHX1* has been over-expressed in upland rice, this is also the first description of the effects of *OsNHX1* on the plant growth phenotype.

Materials and methods

Agrobacterium-mediated transformation and growth of transgenic plants

Mature seeds of upland rice *Oryza sativa* L. (cv IRAT109) were de-hulled, surface sterilized with 70% (v/v) ethanol for 5 min and exchanged with 0.1% (w/v) mercuric chloride ($HgCl_2$) plus 0.1% (v/v) Tween-20 for 13–15 min. Following a rinse with sterile distilled water, the seeds were inoculated on callus induction medium ND_2 (N_6 medium (Zhu et al. 1975) plus 2.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 300 g l^{-1} casamino acid, 500 mg l^{-1} proline, 500 mg l^{-1} glutamine, 30 mg l^{-1} sucrose, 7 mg l^{-1} agar, pH 5.8) for callus induction. Cultures were transferred at 25°C in the dark.

Embryogenic calli were initiated from mature embryos and sub-cultured on ND_2 medium 2–3 times at 21-day intervals and kept in the dark at 25°C. The embryonic calli were pre-cultured on fresh ND_2 medium for 5 days, then infected with

Agrobacterium tumefaciens LBA4404 containing the vector p3301/*OsNHX1*(Wu et al. 2005b) for 20–30 min; thereafter, they were transferred on ND_2-AS (ND_2 plus 100 μmol l^{-1} acetosyringone and 10 g l^{-1} glucose, pH 5.2) medium for 2–3 days in the dark at 25°C. We added 5 mg l^{-1}, 10 mg l^{-1} and 20 mg l^{-1} phosphinotricine (PPT) in ND_2 medium as selective media and transferred the co-cultivation calli on them at 2-week intervals. After 6 weeks of selection culture at 25°C in the dark, PPT-resistant calli were produced and regenerated into plants by being cultivated in regenerative medium, RE1-CH [Murashige and Skoog (MS) medium plus 20 mg l^{-1} PPT, 2 mg l^{-1} 6-benzylaminopurine (6-BA), 0.25 mg l^{-1} naphthalene acetic acid (NAA), 30 mg l^{-1} sucrose, 8 mg l^{-1} agar, pH 5.8] for 15 days, then in RE2-CH (RE1-CH medium with NAA adjusted to 0.5 mg l^{-1}) for an additional 15 days. Transgenic plants of the T_0 and T_1 generation were transplanted into soil and grown in a greenhouse under natural light and controlled temperature (28 ± 2°C daytime, 23 ± 2°C night) and relative humidity (50–60%). T_2 generation plants were used in the analysis.

Polymerase chain reaction analysis

A polymerase chain reaction (PCR) strategy was used to identify the transgenic plants. The primers: P1 (5′-gcggtctgcaccatcgtaa-3′) and P2 (5′-gtaccggcaggctgaagtcca-3′), from the *bar* coding region, were used to amplify a 460 bp fragment. To avoid the interference of endogenous *OsNHX1*, we based the forward primer on the 35S promoter sequence. The primers for exogenous *OsNHX1*, P3 (5′-tcattgcgataaaggaaaggc-3′) and P4 (5′-acgaacaggttgatggacacc-3′) were used to amplify a total 453 bp DNA fragment (340 bp from the CaMV35S promoter region and 113 bp of the *OsNHX1*). The following PCR program was used: 94°C for 3 min, 40 cycles of 94°C for 30 s, 57°C (for *35S* plus *OsNHX1* fragments) or 58°C (for the *bar* gene) for 30 s, 72°C for 30 s, and 72°C for 10 min.

Northern and western blot analysis

Three-week-old seedlings of wild-type (WT) and transgenic plants cultured in vermiculite irrigated with half-strength MS solution were used for northern and western blot analyses. Before isolating tonoplast fractions, we treated seedlings of both WT and transgenic plants for 12 h with half-strength MS containing 0.25 mM NaCl. Total RNA (25 μg) isolated from leaf tissue was separated by electrophoresis on a 0.8% (w/v) agarose gel. The RNA was transferred onto Hybond N^+ membrane (Boehringer Mannheim, Germany) and hybridized with a 536 bp fragment of *OsNHX1* cDNA probe labeled with [^{32}P] dCTP (Amersham Biosciences, USA) using a random primer labeling kit (TakaRa Biotechnology, Ltd., Dalian, China). The 536bp *OsNHX1* DNA probe was amplified by PCR using the primers P5 (5′-gagcaccttccttggagtatttg-3′) and P6 (5′-gcaatcgacacagctcctctcat-3′). After hybridization for 18–20 h at 65°C, the membrane was washed once with 2× standard saline citrate (SSC) plus 0.1% sodium dodecyl sulfate (SDS) at 65°C for 30 min, then washed with 1× SSC plus 0.1% SDS at 65°C for 20 min. The membrane was exposed to X-ray film at –80°C for 7 days.

Polyclonal antibodies against the COOH-terminus of *OsNHX1* (Fukuda et al. 1999, 2004b) were generously supplied by Dr. Fukuda. For western analysis, tonoplasts were isolated from the leaves of upland rice seedlings, as previously described (Fukuda et al. 1998, 2004b). Proteins were separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Blots were then incubated with a horseradish peroxidase(HRP)-goat anti-rabbit IgG (Sigma-Alrich Biotech Co., Missouri, USA) secondary antibody. Immunoreactive bands were detected by super enhanced chemiluminescence(ECL) (Applygene Tech Co. Ltd., Beijing, China).

Seed germination in salt solution and seedling growth in water

The WT and T_2 transgenic upland rice seeds (lines L1, L3, and L5) were sterilized with 2.5% sodium hypochlorite for 15 min, washed thoroughly with water, then soaked in 9 cm Petri dishes with or without 100 mM NaCl for 5 days (2 days in the dark and 3 days in light). Each Petri dish contained two sheets of Whatman No. 1 filter paper, 36 seeds, and 20–30 ml

solution. The seeds were germinated, and the resulting seedlings were grown in a growth chamber at 25°C with a light intensity of 100 μmol m^{-2} s^{-1} and 56–60% relative humidity. After 5 days salt treatment, seedlings were transferred to distilled water for 15 days under a growth cycle (12 h light, 12 h dark); during this period the water was exchanged every day. Growth was monitored photographically.

Salt-tolerant assay in agar and measurement of ion content

Evaluation of growth performance was carried out for T_2 plants under salt-stress conditions. Seeds of these T_2 and WT plants were surface sterilized with 70% ethanol for 5 min and with 0.1% $HgCl_2$ for 12–15 min, rinsed with sterile water thoroughly, then germinated in the dark at 25°C on MS agar supplemented with (for T_2 transgenic plants) or without (for WT plants) 20 mg l^{-1} PPT, and allowed to grow for 5 days. PPT-resistant transgenic and WT plants were then transferred to 9 cm plates with 25 ml MS agar. These MS media were supplemented with different concentrations of NaCl (0 mM, 50 mM, 100 mM, 150 mM, and 200 mM). The young seedlings were grown under identical conditions in water in Petri dishes. After 18 days of culture, representative plants were chosen and photographed. For ion content measurements, the fully expanded leaves and roots were separated (growing in flasks) and rinsed quickly with distilled water to wash off possible surface Na^+ contamination. The roots were washed at least five times then oven-dried for 8 h at 80°C. We re-weighed the dried materials to obtain the dry weight, and the ion content was determined by atomic absorption spectrophotometry (Z-5000, Hitachi Instrument, Japan).

Plant growth and salt-stress treatment in soil

Seeds were germinated and grown in MS agar for 15 days then transferred into soil in trays (30 × 18 × 8 cm), with each tray containing three WT and six transgenic plants (two lines). The seedlings were grown for an additional 2 weeks before they were exposed to salt stress. Each experiment was repeated three to four times. Plants grown in different trays were watered with 900 ml solution containing different concentrations of NaCl (0 mM, 50 mM, 100 mM, and 200 mM) once and later irrigated with water. Seedlings were grown in a growth chamber at 25°C under a light intensity of 100–120 mol m^{-2} s^{-1}, 12/12 h day/night period, and 56–60% relative humidity.

Osmotic potential measurements

Four-week-old seedlings of WT and transgenic plants were treated with different concentrations of NaCl (0 mM, 50 mM, 100 mM, and 200 mM) for 24 h. The fully expanded leaves were collected and stored at –20°C for 24 h. Juice was extracted from the leaves through a pillar by centrifugation at 1,000 r.p.m. for 10 min,and the osmotic potential of leaf juice was measured with an osmometer (O30 M, Gonotec, West Germany).

Results

Production and molecular analysis of transgenic upland rice plants

Mature embryo-derived calli of the upland rice cultivar IRAT109 were transformed with *Agrobacterium* strain LBA4404 harboring the p3301/*OsNHX1* binary plasmid, and *OsNHX1* was successfully introduced into the upland rice genome following the protocol of Hiei et al. 1994 and Huang et al. 2000 with some improvements as described in the Materials and methods section. More than 150 plantlets from seven independent transgenic calli lines carrying the additional *OsNHX1* gene were identified by PCR amplification (Fig. 1A, B). Arrows indicate the target *bar* gene (460 bp) and 35S + *OsNHX1* (453 bp) PCR products. The lower bands in each lane represent primer dimers. Three independent T_2 generation lines from these transgenic plants were used in the experiments to follow. Northern blot analysis showed that these transgenic lines had higher levels of *OsNHX1* transcript than did WT plants, with the transgenic lines 1–16 and

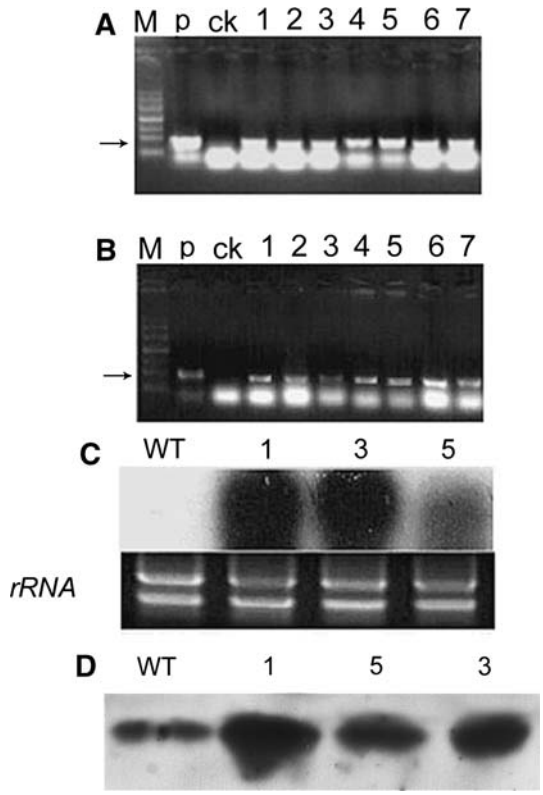

Fig. 1 PCR, northern-, and western-blot analyses of transgenic upland rice plants. **A**, **B** PCR products were amplified from the genomic DNA of independent T_0 transgenic lines (*1–7*). **A** The *bar* (460 bp) gene was identified (*arrow*); **B** a PCR product (453 bp) between the 35S promoter and *OsNHX1* was amplified (*arrow*); *the lower band* in each lane represents unincorporated PCR primers; marker III (*M*) (4,500 bp, 3,000 bp, 2,000 bp, 1,200 bp, 800 bp, 500 bp, 200 bp). *p* plasmid p3301/*OsNHX1*, *ck* wild type IRAT109. **C** Northern-blot analysis of *OsNHX1* gene expression in wild-type and three independent lines (T_2 generation). Both wild type and transgenic plants germinated and grew in vermiculite irrigated with half-strength MS solution. *WT* wild-type plants, *1* lines 1–16, *3* lines 3–12, *5* lines 5–14. **D** Western blot analysis of protein from the leaves of 3-week-old plants (seedlings of WT and transgenic plants treated with 25 mM NaCl for 12 h before isolation of tonoplast membrane proteins). Tonoplast proteins (12 μg) were probed with antibodies raised against the COOH-terminus of *OsNHX1*, revealing a reactive protein band located at 40 kDa

3–12 displaying higher levels of *OsNHX1* transcript than transgenic lines 5–14 displayed (Fig. 1C). Immunoblots of tonoplast (vacuolar membrane) fractions isolated from the leaves of WT and transgenic plants also indicated that transgenic plants had enhanced expression of the vacuolar Na^+/H^+ antiporter (Fig. 1D). The growth performance of the transgenic plants was similar but slightly higher than WT seedlings under normal conditions. However, *35S::OsNHX1* transgenic plants displayed a clear retardation phenotype and a more prolonged vegetative stage than did the WT during the late vegetative growth phase (Fig. 2). On average, WT plants flowered and ripened 10 days earlier than did the transgenic plants under normal conditions.

Growth performance of seedlings in water after salt stress treatment

To examine whether the enhanced expression of the vacuolar Na^+/H^+ antiporter conferred salt resistance to the plants, seeds of three transgenic lines and WT were germinated and grown in 100 mM NaCl solution or water for 5 days, then were transferred to water for 15 days. In water, nearly all transgenic and control seeds germinated well. Nearly no difference was seen in their seed germination rate. However, in 100 mM NaCl both transgenic and control plants germinated slowly, with a delay of about 2 or 3 days for the emergence of shoots and roots. After germination, seedlings were transferred into water. Here, both *35S::OsNHX1* and control seedlings recovered and resumed normal growth. However the

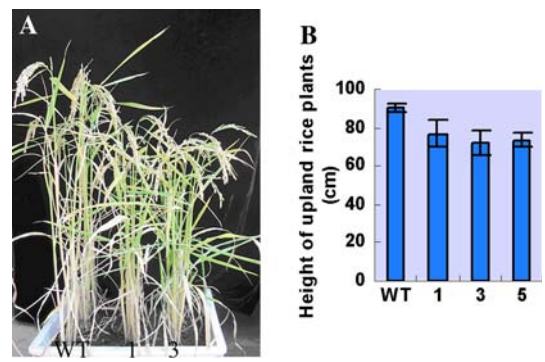

Fig. 2 Comparison of growth and plant height between wild-type and T1 transgenic plants under normal conditions. **A** Growth of WT and two transgenic plants during ripening. **B** Height of WT and transgenic plants; WT plants were taller and matured early

35S::OsNHX1 plants grew faster than the WT plants did during this recovery period. The shoots of transgenic plants were significantly taller than controls after 15 days growth, especially lines 1 and 3, which grew more vigorously than line 5 and the WT. This result correlated positively with the expression levels of *OsNHX1* mRNA and protein (Figs. 1C, D and 3; Table 1). No significant difference was observed between WT and *35S::OsNHX1* plants when the seeds were germinated and grown continuously in water.

Transgenic plants have improved salt tolerance in agar under salt-stress conditions

To further confirm the enhanced salt tolerance of transgenic plants, we tested 5-day-old seedlings germinated in MS medium from seeds of both T_2 generation transgenic and WT plants for their response to salt stress. The growth of both sets of seedlings was inhibited in agar media containing salt, and inhibition was aggravated progressively with increasing NaCl concentration, regardless of whether they were grown in plates (four–five seedlings in each plate) or flasks (ten seedlings in each flask). In the MS plus 200 mM NaCl agar plates, old leaves of WT plants started necrosis on the 11th day, and 75% had died by the 18th day. However, old leaves of transgenic plants did not undergo necrosis until the 15th day and were still alive on the 18th day (Fig. 4). Interestingly, the death of WT plants was delayed, and the old, lower leaves of these plants became yellower than those of transgenic plants grown in flasks (photograph not shown). Triplicate experiments on

Table 1 Growth of seedlings in water after germination under salt stress. Data were collected 20 days after seed-5 days in salt solution and 15 days in non-stress conditions (water). Each Petri dish contained two sheets of Whatman No. 1 filter paper with an equal volume of solution. Following 5 days of salt solution treatment, the solution was replaced with distilled water that was changed every day. Each value ± SD represents the average of 25 seedlings. For non-stressed controls, seeds were germinated and grown continuously in distilled water for 20 days. *Numbers in parentheses* denote the percentages of shoot length of transgenic seedlings compared with those of control seedlings, which was defined as 100%

Transgenic line	Length of shoot (cm)	
	H_2O	H_2O + NaCl (100 mM)
WT	7.05 ± 0.79	5.29 ± 0.59 (100)
Lines 1–16	7.43 ± 1.09	7.78 ± 1.87 (147)
Lines 3–12	8.37 ± 0.98	9.33 ± 1.90 (175)
Lines 5–14	7.20 ± 1.26	7.16 ± 1.28 (135)

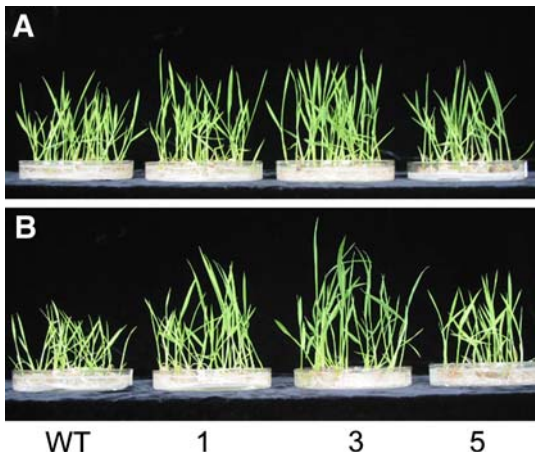

Fig. 3 Over-expression of *OsNHX1* improves the salt tolerance of upland rice by enhancing recovery upon the removal of salt stress conditions. **A** Seeds of WT and transgenic upland rice plants (lines 1–16, lines 3–12, and lines 5–14) were germinated and grown in water (without NaCl) for 20 days. **B** Seeds of WT and transgenic plants (lines 1–16, lines 3–12, and lines 5–14) were germinated in 100 mM NaCl for 5 days then transferred to water for 15 days

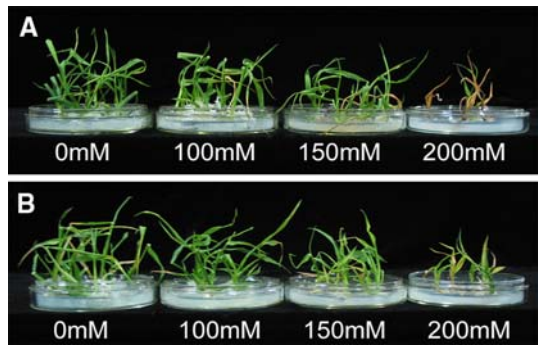

Fig. 4 Salt-stress assay of wild-type and transgenic upland rice plants in agar medium. After 5 days of germination in MS medium, the wild type and transgenic plants (lines 1–16) were transferred to MS medium plus NaCl (0 mM, 50 mM, 100 mM, 150 mM, or 200 mM) in plates. After 18 days, the plants were photographed. **A**, **B** Plants (not shown on MS plus 50 mM NaCl medium) in plates. **A**) WT, **B** transgenic plants (lines 1–16)

plants from lines 1–16, lines 3–12, and lines 5–14 produced similar results.

Growth performance of transgenic plants in soil under salt-stress conditions

In order to investigate whether tolerance for high salinity soil was improved in the transgenic plants, we planted WT and transgenic four-leaf-stage plants in soil in trays and allowed them to grow and recover for 2 weeks, then we irrigated them with different NaCl solutions (50 mM, 100 mM, and 200 mM). We found that severe salt stress (200 mM NaCl) significantly affected the growth and survival of both transgenic and control plants. After being watered with 200 mM NaCl in soil, the seedlings of the control plants gradually wilted within 4 days and died within 1 week. In contrast, the wilting of transgenic seedlings was delayed by 3–4 days and the transgenic plants continued to survive for 2 weeks (photo not shown). Under more moderate salt stress, irrigation with 50 mM or 100 mM NaCl in each tray, the transgenic plants continued to grow for more than 1 month, whereas the WT plants died within 2 weeks.

Determination of the Na^+ and K^+ content in WT and transgenic plants

We determined the Na^+ and K^+ content in leaves and roots from both WT and transgenic plants grown under control and differing concentrations of NaCl in MS agar media (Fig. 5). As anticipated, the Na^+ content in both the leaves and roots of WT and transgenic plants increased as the NaCl concentration was raised (Fig. 5A, B). However, the Na^+ content of the leaves and roots of the transgenic plants grown in MS medium containing 200 mM or 50 mM NaCl was markedly higher than in WT plants grown in the same medium. These results indicated that the transgenic plants accumulated more vacuolar Na^+ than did the WT plants under the same conditions. Conversely, the K^+ content in leaves progressively decreased in both WT and transgenic plants with increasing NaCl concentration, and no significant difference between the WT and

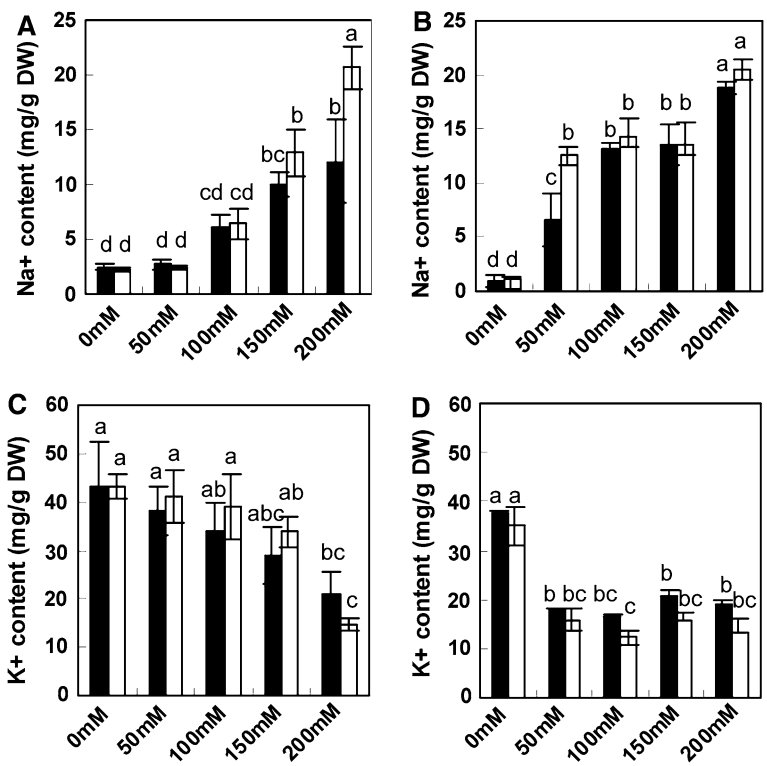

Fig. 5 Na^+ and K^+ content of leaves and roots of wild-type and transgenic plants over-expressing *OsNHX1* grown in medium with various salt concentrations for 18 days. **A**, **C** Leaves; **B**, **D** roots. *Solid black bar* wild type plants, *open bar* lines 1–16 transgenic plants. Values shown are the mean ± SD ($n = 4$). *The letters in each graph* (*a–d* in **A**, **B**, *a–c* in **C**, **D**) indicate significant differences ($P < 0.05$, analysis of variance (ANOVA) and Tukey's test). *DW* dry weight

transgenic plants was observed, except in the 200 mM NaCl condition (Fig. 5C, D). In addition, the root K^+ content of both WT and transgenic plants grown in concentrations between 50 mM and 200 mM NaCl was significantly lower than under normal conditions, and no significant difference was seen among the different NaCl concentrations. There were slightly lower K^+ levels in transgenic plant roots than in WT plants.

Lower osmotic potential in transgenic plants grown under severe salt stress

We measured the leaf osmotic potential of both WT and transgenic plants grown in soil with a series NaCl concentrations, revealing that there was no difference between WT and transgenic plants grown in the range of 0–100 mM NaCl (Fig. 6). Nevertheless, transgenic plants treated with 200 mM NaCl had lower osmotic potentials than the controls had, suggesting that transgenic plants had absorbed more Na^+ in their vacuoles. Thus, osmotic potentials decreased and the capacity of osmotic adjustment increased to absorb more water in transgenic plants.

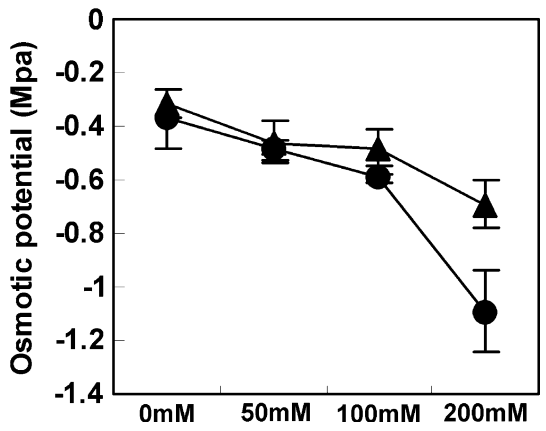

Fig. 6 Determination of the osmotic potential of leaves from wild-type and transgenic plants grown under 0–200 mM NaCl. The osmotic potential of leaves from four-leaf-stage seedlings of WT and transgenic plants that were allowed to recover in soil for 1 week and were then treated with NaCl concentrations from 0 mM to 200 mM for 24 h. Fully expanded leaves were collected as material for this assay. Each value is the mean ± SD of four independent experiments. *Triangles* WT, (*circles*) lines 3–12

Discussion

Increased expression levels of *OsNHX1* mRNA and protein, and salt tolerance in transgenic upland rice plants over-expressing *OsNHX1*

Several dicotyledonous and monocotyledonous species, including *Arabidopsis* (Apse et al. 1999), tomato (Zhang and Blumwald 2001), *Brassica napus* (Zhang et al. 2001), wheat (Xue et al. 2004), and maize (Yin et al. 2004) over-expressing *AtNHX1*, a vacuolar Na^+/H^+ antiporter gene from *Arabidopsis*, have exhibited enhanced salt tolerance. In addition, Ohta et al. (Wu et al. 2005a) also observed that transgenic rice plants over-expressing this type of gene from a halophytic plant could survive 300 mM NaCl for 3 days, while WT plants could not. Fukuda 2004b recently reported that over-expression of *OsNHX1* improved the salt tolerance of transgenic rice cells and plants. We obtained similar results in upland rice, further verifying the importance of *OsNHX1* in salt tolerance of rice.

By assessing the response to salt stress in solution, medium, and soil, we found that over-expression of the *OsNHX1* in the upland rice variety, IRAT109, improved salt tolerance. Symptoms of major damage caused by salt stress, such as wilting, necrosis, yellowing of old leaves, and death of older leaves or whole plants were moderated or delayed in transgenic plants (Fig. 4). Furthermore, enhanced salt tolerance was also exhibited when the salt stress was removed, transgenic plants displayed improved recovery in comparison with that of WT plants (Fig. 3). The three transgenic lines that we tested exhibited greatly improved performance over WT plants during salt stress. The increased salt tolerance of T_2 generation plants correlated positively with the expression level of *OsNHX1* transcript and protein (Fig. 1). Plants from lines 1–16 and lines 3–12 showed better salt tolerance than the plants from lines 5–14 did, indicating that increased expression of the rice vacuolar Na^+/H^+ antiporter confers enhanced salt tolerance in upland rice. This phenotype was reminiscent of rice over-expressing barley HVA1, a stress protein (Xu et al. 1996). These results further suggest

the potential use of transgenic plants that over-express *OsNHX1* for agricultural practice in otherwise arable saline soil.

Over-expression of *OsNHX1* in upland rice increased the Na^+ content and decreased osmotic potential under salt stress

Over-expression of *OsNHX1* in lowland rice improved the salt tolerance of transgenic rice suspension cells and whole plants and correlated with increased Na^+ content of rice suspension cells. However, it did not correlate with increased Na^+ content of young leaves when cells were grown in high NaCl medium or when plants were grown in hydroponic solutions containing 50 mM or 100 mM NaCl (Fukuda et al. 2004b). Ohta et al. 2002 observed a similar result of enhanced salt tolerance in transgenic plants, without a significant difference of leaf Na^+ content between WT and transgenic plants. These results implied that compartmentalization of Na^+ in mature leaves or tissues may improve salt tolerance in rice plants.

In this report, we also monitored the Na^+ content of transgenic upland rice treated with NaCl (0–200 mM) and found that the Na^+ content in leaves of transgenic plants was higher than in WT controls under severe salt conditions, but not in moderate saline conditions (Fig. 5). These results clearly demonstrate that transgenic plants over-expressing the Na^+/H^+ antiporter has enhanced ability to efficiently compartmentalize sodium into vacuoles of leaf cells when faced with severe salt stress, thus reducing the osmotic potential of leaf cells and increasing water uptake capacity and, consequently, maintenance of turgor and survival [corroborated by measurements of leaf osmotic potential (Fig. 6)]. When plants were grown under low or moderate NaCl conditions, the Na^+ was primarily accumulated in mature leaves. This Na^+ exclusion to mature leaves effectively protected younger leaves from damage caused by excess Na^+, thus maintaining their function (Tester and Davenport 2003). Moreover, a comparative analysis of Na^+ accumulation in the roots of upland rice was performed for the first time in this study. In roots, the transgenic plants had higher levels of Na^+ than the controls had at 50 mM NaCl. This may be due to greater Na^+ transport into the vacuoles of root hair or cortex cells (Shi and Zhu 2002), thus reducing cytoplasmic or cell wall Na^+ and consequently limiting Na^+ transport into shoots through the xylem stream.

Previous studies (Kinclova-Zimmermannova et al. 2004) have shown that *OsNHX1* has the ability to compartmentalize K^+ into vacuoles from cytoplasm. However, the K^+ content of suspension cells derived from transgenic rice (Fukuda et al. 2004b) or of roots from upland rice was less than or similar to the K^+ content of control cells and roots under nearly all conditions. When treated with 200 mM NaCl, the leaf Na^+ content of transgenic upland rice plants was higher than that of WT leaves, whereas the K^+ content of leaves from transgenic plants was lower than the K^+ content of leaves from WT plants (Fig. 5). These results suggest that Na^+ is likely to be transported into cells through K^+ carriers (Blumwald 2000; Fukuda et al. 2004b). Previous research has conjectured that Na^+ can enter cells via several high-affinity transporters (such as the HAK or HKT potassium transporters) or low-affinity K^+ transporter [i.e., LCT (Amtmann et al. 2001) and non-selective cation channels (NSCCs), including cyclic-nucleotide-gated channels and glutamate-activated channels, although the relative roles of each component seem likely to vary among species (Maser et al. 2002; Tester and Davenport 2003; Flowers 2004]. For example, parallel experiments measuring K^+ and Na^+ uptake in yeast expressing the wheat or rice HKT1 transporters verified that they were very different; TaHKT1 transported K^+ and Na^+, OsHKT1 only Na^+ (Garciadeblas et al. 2003), while OsHKT2 was found to function as a Na^+/K^+ symporter (Horie et al. 2001). We believe that the explanation for upland rice's having higher Na^+ levels and lower K^+ levels in the leaves or roots, respectively, under high salt conditions, is likely because K^+ transport can be affected by Na^+ levels through competition for the K^+ binding site of potassium transporters (Blumwald 2000; Xue et al. 2004).

Recently, Apse et al. (2003) have reported that *nhx1* plants, harboring a T-DNA insertion mutant of AtNHX1 from *Arabidopsis*, had much lower Na^+/H^+ and K^+/H^+ exchange activity in leaves,

had altered leaf development, and had reduced frequency of large epidermal cells and overall leaf area in comparison with WT plants. Those results suggest that the vacuolar Na^+/H^+ antiporter(s) contribution to ion homeostasis is important not only for salinity tolerance but also for development (Apse et al. 2003). The *35S::OsNHX1* upland rice plants showed a little accelerated growth during seedling stage (Fig. 3, Table 1), but, in the late vegetative stage, transgenic plants exhibited delayed flowering and slight retardant growth (Fig. 2), indicating that *OsNHX1* may also play an important role in rice development. However, the mechanism behind this remains to be further studied.

In summary, the physiological traits, including increased Na^+ content and decreased osmotic potential, of transgenic plants grown under severe saline stress illustrate well that these transgenic plants had enhanced salt tolerance. This, of course, implies that these plants, over-expressing the *OsNHX1*, could be cultivated in saline soil. Furthermore, these transgenic plants also had herbicide resistance, because the *bar* gene was used as a selection gene during transformation. It is known that upland fields nearly always have more weeds to compete for nutrients and light than lowland fields have. Therefore, the application of bialaphos may make possible simultaneous weed control in upland fields, reducing the labor cost of cleaning weeds. This transgenic salt- and herbicide-resistant upland rice could be widely used in upland fields, providing significant economic value.

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