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# Function, Intracellular Localization and the Importance in Salt Tolerance of a Vacuolar $Na^+/H^+$ Antiporter from Rice

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**We examined the function and intracellular localization of the product of the $Na^+/H^+$ antiporter gene (*OsNHX1*) cloned from rice (*Oryza sativa*). *OsNHX1* has the ability to suppress $Na^+$, $Li^+$ and hygromycin sensitivity of yeast *nhx1* mutants and sensitivity to a high $K^+$ concentration, a novel phenotype of the *nhx1* mutants. Analysis using rice cells expressing a fusion protein of OsNHX1 and green fluorescent protein and Western blot analysis using antibodies specific for OsNHX1 confirmed the localization of OsNHX1 on the tonoplasts. These results indicate that the *OsNHX1* gene encodes a vacuolar ($Na^+$, $K^+$)/$H^+$ antiporter. Treatment with high concentrations of NaCl and KCl increased the transcript levels of *OsNHX1* in rice roots and shoots. In addition, overexpression of OsNHX1 improved the salt tolerance of transgenic rice cells and plants. These results suggest that OsNHX1 on the tonoplasts plays important roles in the compartmentation of $Na^+$ and $K^+$ highly accumulated in the cytoplasm into the vacuoles, and the amount of the antiporter is one of the most important factors determining salt tolerance in rice.**

**Keywords**: $Na^+/H^+$ antiporter — Rice (*Oryza sativa*) — Salt tolerance — Tonoplast — Vacuole.

Abbreviations: DTT, dithiothreitol; EGTA, ethylene glycol bis(2-aminoethyl ether)tetraacetic acid; GFP, green fluorescent protein; IPTG, isopropyl-β-D-thiogalactopyranoside; MS medium, Murashige–Skoog medium; ORF, open reading frame; V-PPase, vacuolar $H^+$-inorganic pyrophosphatase.

## Introduction

$Na^+/H^+$ antiporters, which catalyze the exchange of $Na^+$ for $H^+$ across membranes, contribute to the regulation of internal pH, cell volume and the sodium level in the cytoplasm (Aronson 1985, Orlowski and Grinstein 1997). The antiporters are widespread membrane proteins found in animals, yeasts, bacteria and plants. In particular, vacuolar $Na^+/H^+$ antiporters, which compartmentalize $Na^+$ into the vacuoles for detoxification, have been investigated as the key to salt tolerance in yeasts and plants (Blumwald et al. 2000).

In *Saccharomyces cerevisiae*, the product of a gene (*ScNHX1*), which is homologous to mammalian NHE, has been shown to mediate sequestration of $Na^+$ within an intracellular compartment and to have an endosomal distribution, localizing to prevacuolar compartments (Nass et al. 1997, Nass and Rao 1998). In plants, a vacuolar $Na^+/H^+$ antiporter gene (*AtNHX1*) has been cloned in *Arabidopsis thaliana* as the first plant homolog to *ScNHX1* (Gaxiola et al. 1999). We have also cloned a vacuolar $Na^+/H^+$ antiporter gene (*OsNHX1*) from rice (*Oryza sativa*) (Fukuda et al. 1999). Recently, $Na^+/H^+$ antiporter genes have been reported from several other plants (Chauhan et al. 2000, Fukuda-Tanaka et al. 2000, Hamada et al. 2001). The increased expression of the antiporter genes by salt stress has been reported from both glycophytes, *A. thaliana* and rice, and halophytes, *Mesembryanthemum crystallium* and *Atriplex gmelini*. Salt stress also activates the antiporter in salt-tolerant plants such as *Hordeum vulgare* (Fukuda et al. 1998, Garbarino and DuPont 1988, Garbarino and DuPont 1989) and *Beta vulgaris* (Blumwald et al. 1985). These results suggest that vacuolar $Na^+/H^+$ antiporters play important roles in the salt tolerance of various plants.

Rice, one of the most important crops in the world, has low salt tolerance, and its production and planting area are greatly affected by soil salinity (Akbar and Ponnamperuma 1980). Therefore, it is of agricultural importance to analyze and improve the salt tolerance of rice. The $Na^+/H^+$ antiporter gene, *OsNHX1*, which we have cloned from rice, has been shown to play important roles in the salt tolerance of rice (Fukuda et al. 1999).

In this paper, we report a functional analysis in yeast, the intracellular localization of OsNHX1 protein in rice cells, the effects of salt and osmotic stresses on the expression of *OsNHX1*, and the salt tolerance of transgenic rice overexpressing OsNHX1. We also discuss the mechanism of salt tolerance in rice.

## Results

### *Complementation of yeast with the OsNHX1 gene*

To test the function of *OsNHX1*, we expressed the gene in yeast *nhx1* mutants. Overexpression of *OsNHX1* increased the tolerance of the mutants to high concentrations of $Na^+$ and $Li^+$

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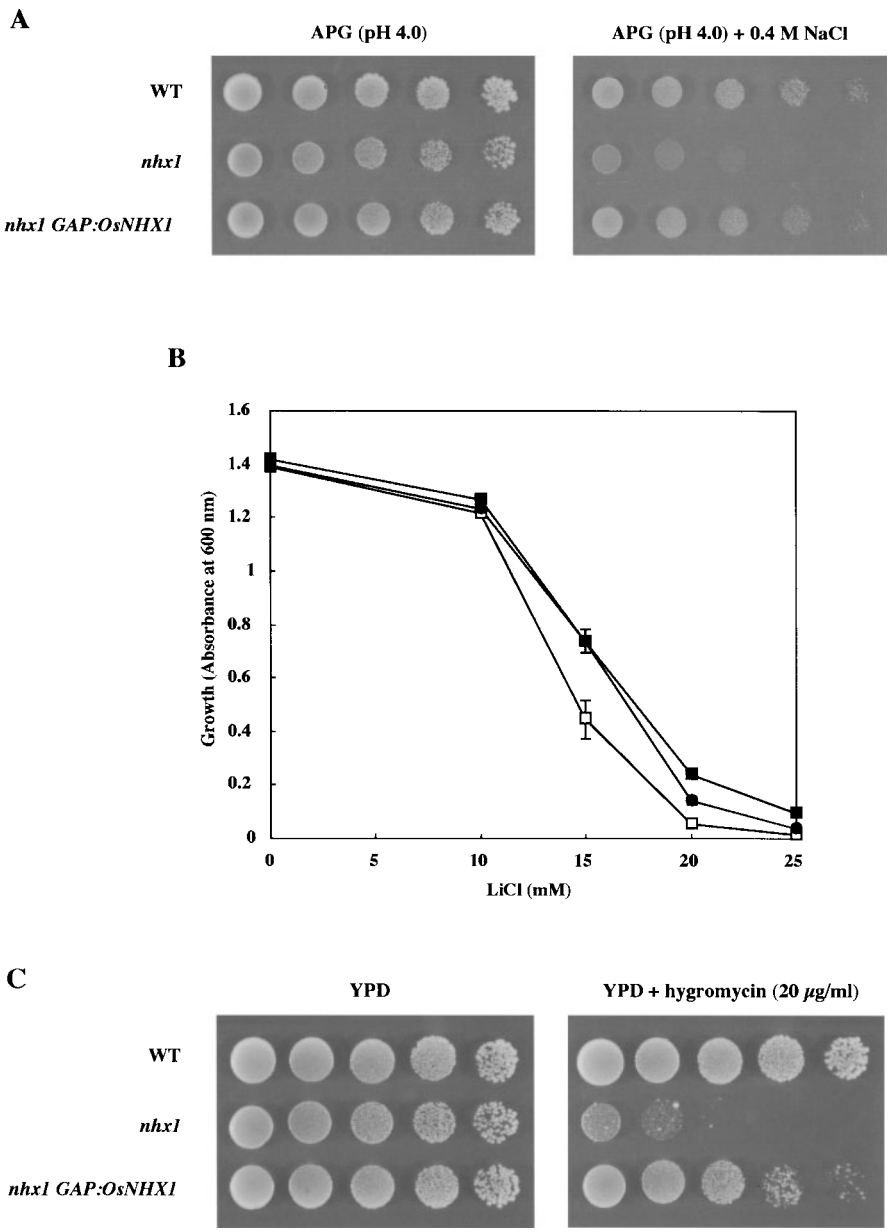



**Fig. 1** Expression of *OsNHX1* in *nhx1* yeast mutants. (A) Vector pKT10+*HIS* was introduced into wild-type (WT) (K601) and *nhx1* strains (R100). Plasmid pOsNHX1; *GAP:OsNHX1* was introduced into the *nhx1* mutant (*OsNHX1*). The strains were grown overnight in selective APG medium (pH 6.5). Aliquots (5 µl) of a saturated seed culture and 5-fold serial dilutions of the indicated strains were spotted onto APG plates (pH 4.0) supplemented with or without 0.4 M NaCl. The strains were grown at 28°C for 8 d. (B) The same strains (5 µl of a saturated seed culture) were grown in 2 ml of APG medium (pH 4.0) supplemented with or without LiCl for 48 h with shaking. Growth of the strains was detected by measuring absorbance at 600 nm. Each value is the mean ± SD of three independent experiments. Symbols and relevant genotypes are as follows: closed square, K601; open square, R100; closed circle, *OsNHX1*. (C) Serial dilutions of the same strains as in (A) were grown on YPD plates supplemented with or without 20 µg $ml^{-1}$ hygromycin for 3 d.

to an extent similar to that of wild-type yeast (Fig. 1A, B). The *OsNHX1*-complementation of $Na^+$ and $Li^+$ sensitivity of the *nhx1* mutants was obtained only when the $K^+$ concentration was reduced (APG medium containing 1 mM $K^+$) and the pH was low (pH 4.0), because the mutants showed marked $Na^+$ and $Li^+$ sensitivity compared with the wild type only under those conditions (data not shown, Nass et al. 1997). The *nhx1* mutants are also hypersensitive to hygromycin, which is a toxic cation that accumulates intracellularly in response to an electrochemical proton gradient (Darley et al. 2000). It has been suggested that ScNHX1, which affects the pH or membrane potential of the vacuolar and prevacuolar compartments, plays

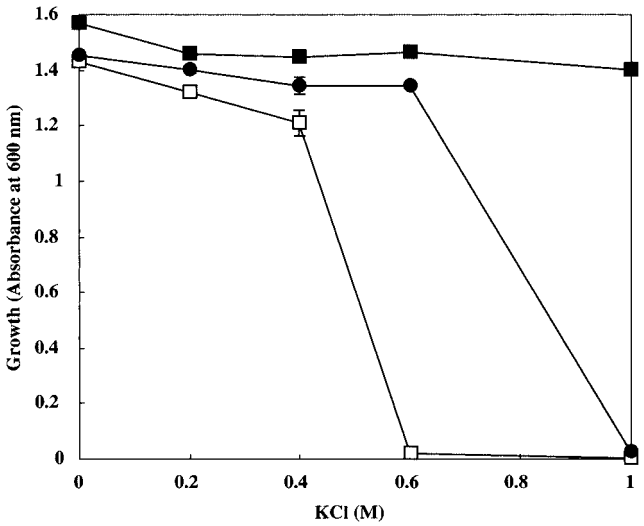



**Fig. 2** Effect of a high KCl concentration on the *nhx1* yeast mutants overexpressing *OsNHX1*. Each strain was grown in APG medium (pH 4.0) supplemented with or without KCl at 28°C for 96 h. Each value is the mean ± SD of three independent experiments. Symbols and relevant genotypes are as follows: closed square, K601; open square, R100; closed circle, *OsNHX1*.

an important role in hygromycin compartmentation into vacuoles (Gaxiola et al. 1999). *OsNHX1* also suppressed the hygromycin sensitivity of the mutants (Fig. 1C), indicating that OsNHX1 protein has the same function as ScNHX1 in yeast. In addition, we found that the *nhx1* mutants were sensitive to a high $K^+$ concentration in APG medium (Fig. 2). Introduction of *OsNHX1* into the mutants rendered the strain tolerant to a high $K^+$ concentration to an extent similar to that of the wild type. These results indicate that the *OsNHX1* gene encodes a vacuolar ($Na^+$, $K^+$)/$H^+$ antiporter that has activity similar to that of the ScNHX1 protein.

*Western blot analysis of OsNHX1 protein*

The subcellular localization of OsNHX1 protein was determined by Western blot analysis using polyclonal antibodies against the COOH-terminus of OsNHX1, as shown in Materials and Methods. Fig. 3A shows that OsNHX1 was present mainly in the tonoplast fractions from roots and shoots of rice seedlings. The apparent molecular mass of OsNHX1 of 40 kDa determined by SDS-PAGE did not correspond to the calculated molecular mass of 59 kDa. To check the apparent molecular mass found by SDS-PAGE, OsNHX1 was detected in the yeast *nhx1* mutants expressing *OsNHX1* using anti-OsNHX1 antibodies and *Escherichia coli* expressing the T7-tagged *OsNHX1* using anti-T7-Tag monoclonal antibodies (Fig. 3A, B). The apparent molecular mass of OsNHX1 in the transgenic yeast microsome was 40 kDa and that in the transgenic *E. coli* treated with isopropyl-β-D-thiogalactopyranoside (IPTG) was 42 kDa as determined by SDS-PAGE. The results indicate

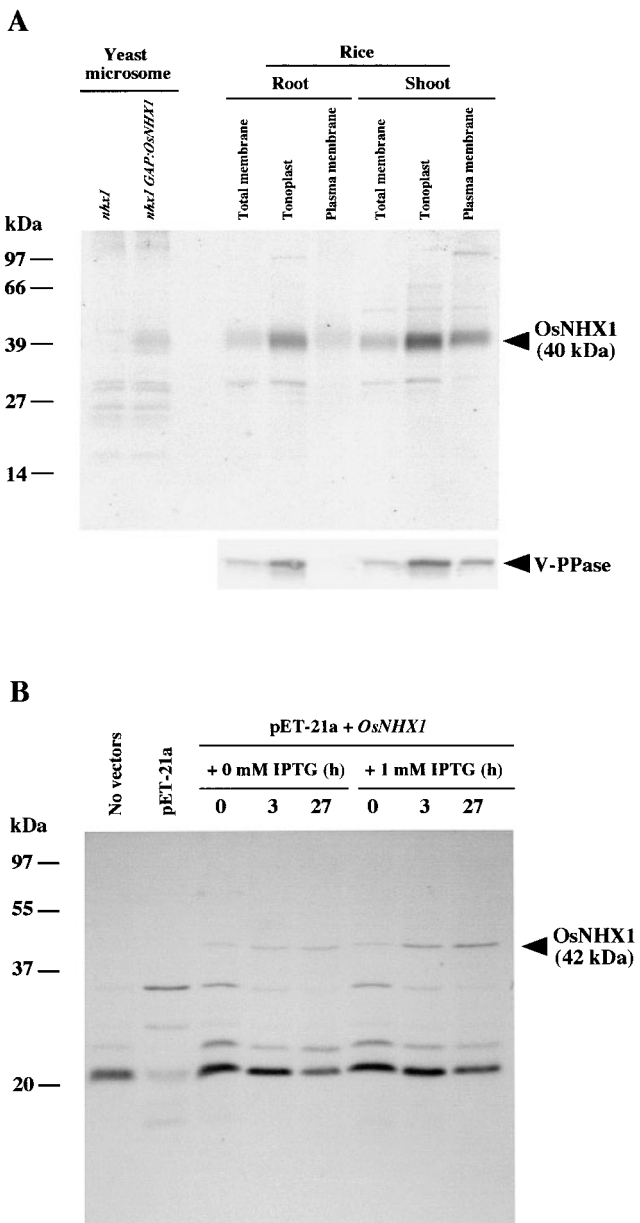
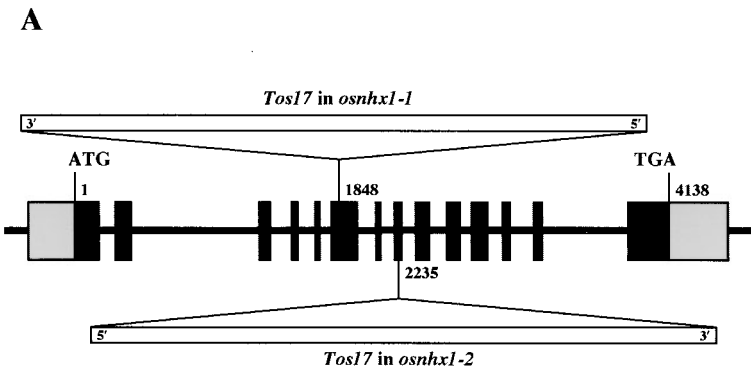



**Fig. 3** Western blot analysis of OsNHX1 protein. (A) Microsome fractions were isolated from R100 (*nhx1*) and R100 transformed with pOsNHX1. Membrane fractions were isolated from roots and shoots of 7-day-old rice seedlings. Protein [10 μg for OsNHX1 and 1 μg for vacuolar $H^+$-inorganic pyrophosphatase (V-PPase)] was separated on a 10% SDS-PAGE gel and immunoblotted. Polyclonal antibodies raised against the COOH-terminal portion of OsNHX1 were used for the immunoblot. Antibodies against *Vigna radiata* V-PPase (Maeshima and Yoshida 1989) were used as a marker for tonoplasts. (B) The fusion protein was extracted from BL21(DE3) and BL21(DE3) transformed with pET-21a or pET-21a+*OsNHX1*. The expression of the fusion protein was induced by the addition of 1 mM IPTG. BL21(DE3) containing pET-21a was grown in the medium with 0.6 mM IPTG for 3 h. Protein (14 μg) was separated on a 12% SDS-PAGE gel and immunoblotted with anti-T7-Tag monoclonal antibodies.



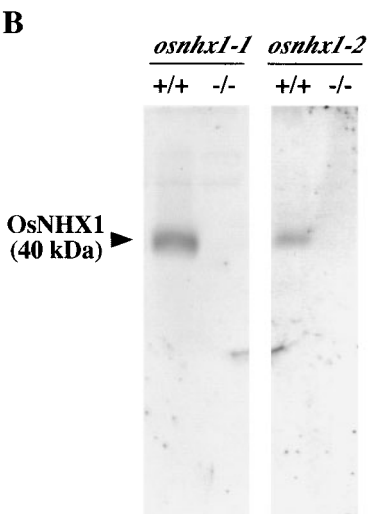




**Fig. 4** The estimation of *osnhx1* mutant lines. (A) Genomic organization of *OsNHX1* gene and insertion sites of *Tos17* gene in the *OsNHX1* gene of *osnhx1* mutant lines (*osnhx1-1* and *osnhx1-2*). The genomic organization of *OsNHX1* is derived from sequence information available in the DDBJ/EMBL/GenBank database (accession number AP004274). Gray and black boxes represent the untranslated cDNA sequence and the translated cDNA sequence, respectively. Insertion sites of *Tos17* genes (4.3 kb) (white boxes) in the *OsNHX1* gene of the mutant lines are indicated by vertical bars labeled with the range of nucleotides. (B) Western blot analysis of the tonoplast fractions isolated from segregating progeny of the *osnhx1* mutant lines. We used shoots of 1-month-old plants of lines homozygous (–/–) for the *Tos17* insertion and of progeny with wild-type *OsNHX1* alleles (+/+). Protein (14 μg) was separated on a 10% SDS-PAGE gel and immunoblotted with antibodies against the COOH-terminus of OsNHX1.

that the protein band at 40 kDa detected by immunoblotting is an actual OsNHX1 protein.

We isolated two rice *osnhx1* mutant lines, *osnhx1-1* and *osnhx1-2*, from *Tos17* insertional mutants by PCR-based screening. The amplified DNA fragments from the two mutant lines were sequenced, and the insertion sites were determined (Fig. 4A). The *Tos17* gene was inserted into the sixth exon of the *OsNHX1* gene in the *osnhx1-1* mutant and into the eighth exon in the *osnhx1-2* mutant, suggesting that the amino acid sequences were terminated incompletely. The insertion of *Tos17* into *OsNHX1* and the normal inheritance in the progeny of the mutant lines were confirmed by Southern blot analysis as described by Takano et al. (2001), and lines homozygous (–/–) for the insertion were selected (data not shown). In the Western blot analysis with antibodies against the COOH-terminus of OsNHX1, the 40 kDa protein was detected in the tonoplast fractions isolated from the progeny (+/+) with wild-type *OsNHX1* alleles but not in those from the homozygous lines (–/–) (Fig. 4B). These results confirm that the 40 kDa protein in the Western blot analysis is certainly the OsNHX1 protein and that the antibodies are specific to OsNHX1.

*Localization analysis of OsNHX1 protein using green fluorescent protein (GFP)*

The subcellular localization of OsNHX1 on the tonoplasts was confirmed by an analysis using rice cells expressing the fusion protein of OsNHX1 and sGFP. The chimeric gene for the fusion protein was constructed by the fusion of the *OsNHX1* open reading frame (ORF) to the N-terminus (OsNHX1–sGFP) or C-terminus (sGFP–OsNHX1) of sGFP. The sGFP fluorescence imaged under a laser-scanning confocal microscope showed that OsNHX1–sGFP distribution coincided with that of the FM 4–64 stain for the tonoplasts (Vida and Emr 1995) (Fig. 5A, B). However, sGFP–OsNHX1 produced a pattern similar to only sGFP (data not shown). Thus, the N-terminus of OsNHX1 has a site for specific cleavage. Several small, bright, dot-like fluorescent structures were observed in the cells by fluorescent microscopy (Fig. 5A). These dot-like fluorescent structures were also observed in cells expressing pMSH1 vector not including *sGFP* (data not shown), suggesting that the structures were due to false fluorescence, and not to fluorescence from sGFP.

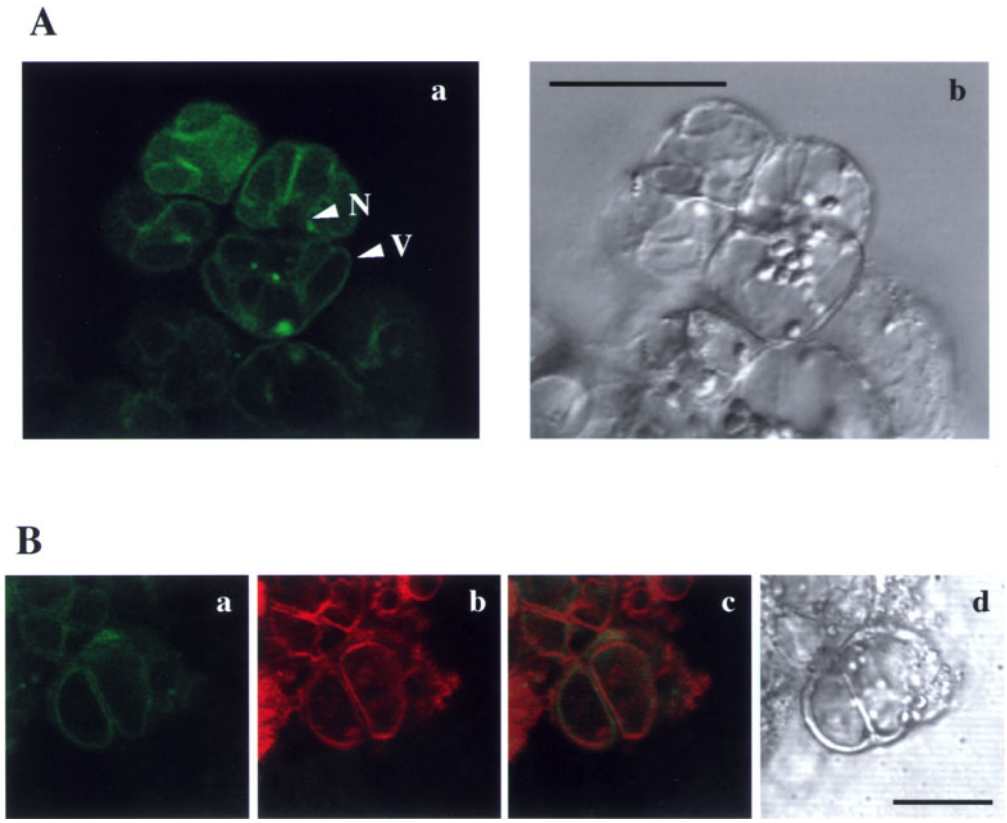



**Fig. 5** Localization of OsNHX1–sGFP fusion protein expressed in rice cells. OsNHX1–sGFP fusion protein was expressed in rice cells as described in Materials and Methods. Green fluorescence of sGFP in the cells was imaged under a laser-scanning confocal microscope (A-a and B-a). Tonoplasts are stained *red* with FM4-64 (B-b). The two images of B-a and B-b are overlaid in B-c. Contrast images of the cells are shown in A-b and B-d. N, nucleus; V, vacuole. Scale bar in A = 50 μm; scale bar in B = 10 μm.

*Expression of the OsNHX1 gene in rice seedlings subjected to various stresses*

The effects of salt and osmotic stresses on the expression of *OsNHX1* were examined by Northern blot analysis of total RNAs from roots and shoots of 7-day-old rice seedlings after 5 or 24 h of treatment with 0.2 M NaCl or 0.4 M mannitol, respectively. The salt stress increased the transcript levels of *OsNHX1* in roots and shoots (Fig. 6). As estimated by density scanning, the level of *OsNHX1* transcripts in roots increased 4- and 2-fold after treatment with NaCl for 5 and 24 h, respectively, and that in shoots increased 2-fold after both 5 and 24 h. On the other hand, the transcript levels of *OsNHX1* increased slightly only in roots after 5 h of the treatment with mannitol. The increase in the transcript level due to osmotic stress was less than that due to salt stress. These results show that mainly ionic stress increased the level of *OsNHX1* transcripts.

In addition, we performed another Northern blot analysis with 7-day-old rice seedlings after 24 h of treatment with 0 to 0.2 M NaCl or KCl. The treatments with a high concentration of each salt increased the transcript levels of *OsNHX1* in both roots and shoots (Fig. 7). Treatment with NaCl increased the

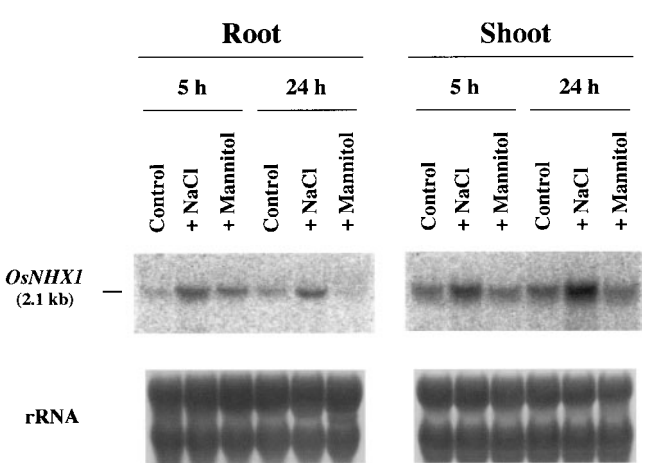



**Fig. 6** Northern blot analysis of the rice vacuolar $Na^+/H^+$ antiporter in rice seedlings treated with salt and osmotic stresses. Thirty μg of total RNAs was isolated from shoots and roots of 7-day-old rice seedlings at the indicated times after the start of treatment with 0.2 M NaCl or 0.4 M mannitol. The isolated RNA was separated, blotted onto a nylon membrane and hybridized with the $^{32}$P-labeled 3′-untranslated regions of *OsNHX1*. Methylene blue staining of the rRNA on the membrane is also shown.

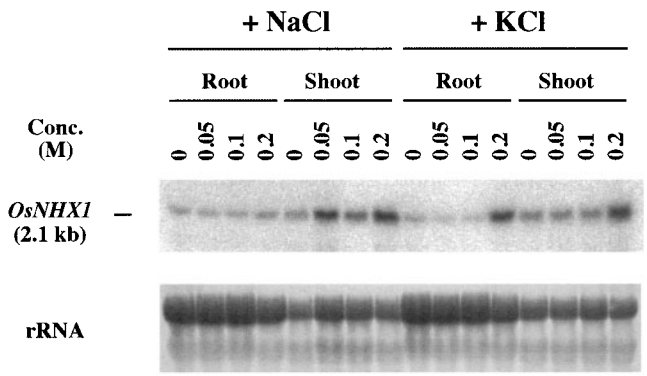



**Fig. 7** Northern blot analysis of the rice vacuolar $Na^+/H^+$ antiporter in rice seedlings treated with various concentrations of NaCl and KCl. Thirty μg of total RNAs was isolated from shoots and roots of 7-day-old rice seedlings after 24 h of treatment with NaCl or KCl. The isolated RNA was separated, blotted onto a nylon membrane and hybridized with the $^{32}$P-labeled 3′-untranslated regions of *OsNHX1*. Methylene blue staining of the rRNA on the membrane is also shown.

transcript level of *OsNHX1* in shoots at more than 50 mM. On the other hand, treatments with 0.05 to 0.1 M KCl did not increase the transcript levels of *OsNHX1* in roots or shoots. However, the treatment with 0.2 M KCl abruptly increased the levels 4-fold and 2-fold, relative to the control, in roots and shoots, respectively (signals of the Northern analysis were normalized according to the intensity of the rRNA signals), and in roots the level of increase was higher than that resulting from the treatment with 0.2 M NaCl.

*Overexpression of OsNHX1 protein in transgenic rice cells*

A construct containing *OsNHX1* was introduced into the genome of *Oryza sativa* L. cv Nipponbare. Eight independent transgenic rice calli were obtained, and cell suspension cultures were initiated from the transgenic calli. Overexpression of OsNHX1 protein was confirmed by Western blot analysis of tonoplast fractions isolated from wild-type and transgenic cells with polyclonal antibodies against the COOH-terminus of OsNHX1 (Fig. 8A). We used two of the cell lines (95-1-35 and 95-5-169) in this experiment. To examine whether the overexpression of OsNHX1 would enhance the tolerance of cells to salt stress, we cultured wild-type and transgenic cells in the presence of 0.1 or 0.2 M NaCl. Growth of the wild-type cells was inhibited by the presence of 0.1 and 0.2 M NaCl, and the wild-type cells grew poorly in the presence of 0.2 M NaCl. On the other hand, the growth of the transgenic cells in the presence of 0.1 and 0.2 M NaCl was improved relative to that of the wild-type cells in correlation with the increase in the level of OsNHX1 (Fig. 8B, C).

We determined the $Na^+$ and $K^+$ contents in wild-type and transgenic rice cells grown in the medium containing 0, 0.1 or 0.2 M NaCl (Fig. 8D, E). The $Na^+$ content of both the wild-type and transgenic cells increased, and the $K^+$ content decreased, with exposure to high concentrations of NaCl. The $Na^+$ content of the transgenic cells grown in the presence of a high NaCl concentration was higher than that of the wild-type cells (Fig. 8D), and correlated with the increase in the level of OsNHX1 (data not shown). These results indicate that the increased vacuolar compartmentation of $Na^+$ in the transgenic cells induced the higher salt tolerance of the transgenic cells.

*Overexpression of OsNHX1 in transgenic rice plants*

We assessed whether the overexpression of OsNHX1 allowed plants to grow in the presence of high NaCl. We obtained 31 transgenic plants and seven homozygous lines of the T3 generation (data not shown), and used two of the lines (95-3-2 and 95-4-12) in this experiment. We confirmed the overexpression of OsNHX1 by Western blot analysis, as shown by the experiment with the transgenic rice cells (Fig. 9A). The overexpression of OsNHX1 did not affect the growth of the transgenic plants grown under control conditions (Fig. 9B, C). We grew wild-type and transgenic rice plants in the presence of 0.05 and 0.1 M NaCl. The growth of the wild-type plants was severely inhibited, whereas that of the transgenic plants in the presence of high NaCl concentrations was improved relative to that of the wild-type plants; the transgenic plants were able to maintain growth in 0.1 M NaCl, but the wild-type plants eventually died (Fig. 9B, C).

We determined the $Na^+$ and $K^+$ contents of the leaves of wild-type and transgenic plants grown under control and high-NaCl conditions (Fig. 10) and found no significant differences (ANOVA and Tukey's test, $P > 0.05$). In contrast to the result for the transgenic cells, the $Na^+$ and $K^+$ contents in the transgenic plants were similar to those in the wild type under all growth conditions.

**Fig. 8** Growth and ion contents of wild-type (WT) and transgenic rice cells overexpressing OsNHX1 grown at various salt concentrations. Wild-type rice cells (lines 97-4-5 and 97-5-15) transformed with pMSH1 and transgenic cells (lines 95-1-35 and 95-5-169) transformed with pMSH1+*OsNHX1* were cultured in the presence of 0, 0.1 and 0.2 M NaCl for 14 d. (A) Western blot analysis of the tonoplast fractions isolated from these cells. Protein (7 μg) was separated on a 10% SDS-PAGE gel and immunoblotted with antibodies against the COOH-terminus of OsNHX1. (B and C) Growth of wild-type (white bars; 97-4-5 in B and 97-5-15 in C) and transgenic cells (black bars; 95-1-35 in B and 95-5-169 in C) is shown as % control of dry weight. Each value is the mean ± SD of three independent experiments. Asterisks represent significant differences between the wild-type and transgenic cells ($P < 0.01$, Student's $t$ test). (D and E) $Na^+$ (D) and $K^+$ (E) contents of wild-type (white bars; 97-5-15) and transgenic cells (black bars; 95-5-169). Ion contents were determined as shown in Materials and Methods. Each value is the mean ± SD of three independent experiments. Different letters in each graph (a–c in D and a–d in E) indicate significant differences ($P < 0.05$, ANOVA and Tukey's test). DW, dry weight.

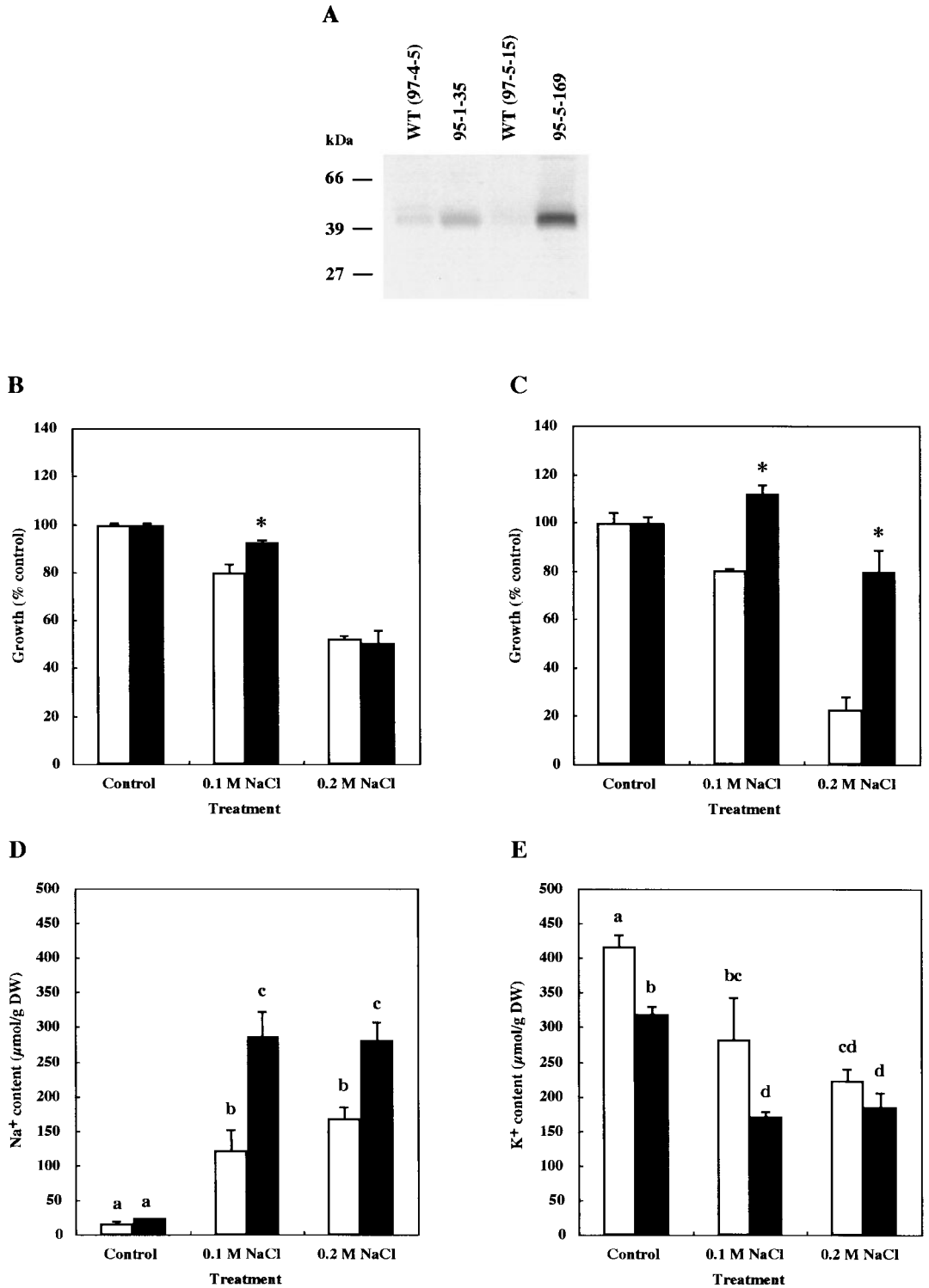



Fig. 8

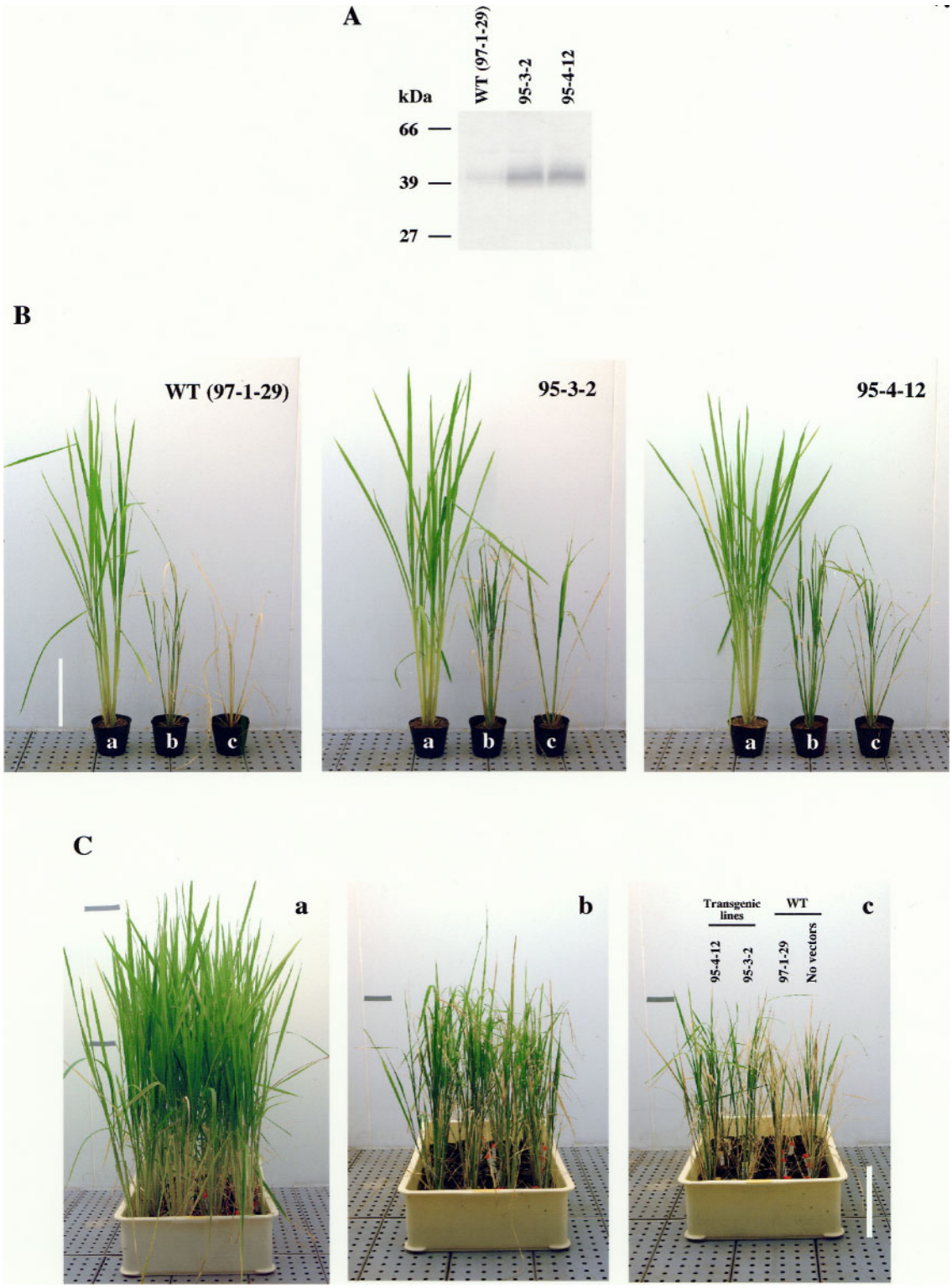



**Fig. 9** Salt tolerance of wild-type (WT) and transgenic rice plants overexpressing OsNHX1 grown at various salt concentrations. Wild-type and transgenic plants were grown under normal conditions for 18 d, and then treated with 0, 0.05 or 0.1 M NaCl for 7 weeks. (A) Western blot analysis of the tonoplast fractions isolated from the leaves of wild-type plants (line 97-1-29) transformed with pMSH1 and from those of transgenic plants (lines 95-3-2 and 95-4-12) transformed with pMSH1+*OsNHX1*. Protein (10 μg) was separated on a 10% SDS-PAGE gel and immunoblotted with antibodies against the COOH-terminus of OsNHX1. (B and C) Wild-type (no vectors and 97-1-29) and transgenic plants (95-3-2 and 95-4-12) were grown in the presence of 0 (a), 0.05 (b) or 0.1 (c) M NaCl. We obtained similar results in another experiment. Scale bar = 20 cm.

## Discussion

*The ability of OsNHX1 protein to transport several ions*

*OsNHX1* had the ability to suppress the $Na^+$, $Li^+$, $K^+$ and hygromycin sensitivity of yeast *nhx1* mutants (Fig. 1, 2). These results indicate that OsNHX1 protein has the activity of ScNHX1 protein and functions as a vacuolar ($Na^+$, $K^+$)/$H^+$ antiporter. However, the *nhx1* mutants overexpressing *OsNHX1* had lower tolerance for $Na^+$, $K^+$ and hygromycin than wild-type yeast. The lack of a signal peptide in the construction of the yeast expression vectors used in our study may cause the phenotype of the mutants overexpressing *OsNHX1*, as suggested by Darley et al. (2000). Results similar to those obtained with *OsNHX1* have been reported for *nhx1* mutants overexpressing *AtNHX1* from *A. thaliana* or *AgNHX1* from *A. gmelini* (Gaxiola et al. 1999, Quintero et al. 2000, Hamada et al. 2001). These findings suggest that NHX-type antiporters of various plants have functions similar to ScNHX1, of which they are homologs.

The present findings show that the *nhx1* mutants are sensitive to a high $K^+$ concentration, and OsNHX1 has the ability to suppress the $K^+$ sensitivity of the mutants. This is the first report that a plant vacuolar $Na^+/H^+$ antiporter has the ability to suppress the sensitivity of *nhx1* mutants to a high $K^+$ concentration. In *S. cerevisiae*, NHA1 and ENA1-4, $Na^+$ transporters in the plasma membranes, mediate $Na^+$ and $K^+$ efflux, and mutants of these genes show lower tolerance to high $K^+$ concentration than wild-type (Banuelos et al. 1998). These results suggest that $Na^+$ transporters, including ScNHX1, can function as $Na^+/K^+$ transporters and that they have an important role in the tolerance of yeast to high $K^+$ concentrations. In plants, it has been shown by the analysis of antiporter activity that AtNHX1 can catalyze $K^+$ transport (Zhang and Blumwald 2001, Venema et al. 2002). These findings suggest that plant NHX-type antiporter genes, including *OsNHX1*, encode vacuolar ($Na^+$, $K^+$)/$H^+$ antiporters, and like ScNHX1, they may play an important role in the compartmentation of $K^+$ highly accumulated in the cytoplasm into vacuoles.

*Tonoplast localization of OsNHX1 protein*

The 40 kDa protein is OsNHX1 protein (Fig. 3, 4), and anomalous migration of hydrophobic membrane proteins may be the main cause of the lower mass. Apse et al. (1999) and Venema et al. (2002) showed an apparent mass of 45–50 kDa, which is below the predicted molecular mass (58 kDa), for AtNHX1 protein in *A. thaliana* using antibodies against the COOH-terminus. The lower apparent mass of plant NHX-type antiporter proteins compared with the mass predicted by calculations based on their gene sequences may be a general character of masses determined with SDS-PAGE.

The OsNHX1 band is diffuse in the Western blot analyses of rice and transgenic yeast. However, the band is clear for the transgenic *E. coli* lacking the ability to glycosylate proteins. There are three potential glycosylation sites in the deduced sequence of amino acid residues of OsNHX1 (Fukuda et al. 1999). These results suggest that OsNHX1 is a glycoprotein. Wells and Rao (2001) have demonstrated that ScNHX1 is a glycoprotein and shows a diffuse band in Western blot analysis of the yeast. In addition, immunological analyses have shown that the bands of AtNHX1 and AgNHX1 are also diffuse (Apse et al. 1999, Hamada et al. 2001). These results support our suggestion that OsNHX1 is a glycoprotein.

The analysis using rice cells expressing the fusion protein of OsNHX1 and sGFP confirmed the localization of OsNHX1 on the tonoplasts (Fig. 5). In addition to the results from the analysis using sGFP, the Western blot analysis showed that OsNHX1 was present in the tonoplast fractions from roots and shoots of rice (Fig. 3A). Apse et al. (1999) and Hamada et al. (2001) also used Western blot analysis to show that AtNHX1 and AgNHX1 are localized in the tonoplasts. These findings suggest that plant NHX-type antiporters are vacuolar $Na^+/H^+$ antiporters that catalyze the compartmentation of $Na^+$ to the vacuoles. Homologs to plant NHX-type antiporters have been identified in animals (NHE6 and NHE7) (Numata et al. 1998, Numata and Orlowski 2001). NHE6 and NHE7 localize to the mitochondria and the *trans*-Golgi network, respectively, although these antiporters are members of the animal $Na^+/H^+$ exchanger (NHE family) localized in the plasma membranes. In addition, ScNHX1 localizes to the late endosomal/prevacuolar compartments (Nass and Rao 1998). These antiporters share a cluster in a phylogenetic analysis of various $Na^+/H^+$ antiporters from a wide variety of organisms, including animals, yeasts, bacteria and plants (Fukuda et al. 1999). Recently, Shi et al. (2002) showed that AtSOS1, a novel $Na^+/H^+$ antiporter in *A. thaliana*, is localized in the plasma membranes, and the deduced amino acid sequence of the antiporter has low similarity to those of plant NHX-type antiporters. These results indicate that the NHX-type antiporters, NHE6 and NHE7 form a family of intracellular antiporters that may have evolved independently of antiporters localized in the plasma membranes.

*The regulation of OsNHX1 gene expression under various stresses*

Treatment with a high concentration of NaCl increased the transcript levels of *OsNHX1* in rice roots and shoots (Fig. 6, 7). In addition, the increase in *OsNHX1* transcript levels by 0.4 M mannitol, iso-osmotic stress for 0.2 M NaCl, was lower than that caused by salt stress. These results indicate that the expression of *OsNHX1* is regulated by salt stress, and that this regulation is mainly due to ionic stress. Transcript levels of *OsNHX1* in shoots were higher than those in roots irrespective of the concentration of NaCl (Fig. 6, 7). OsNHX1 may play an important role in the salt tolerance of shoots rather than roots.

Treatment with 0.2 M KCl also increased the transcript levels of *OsNHX1* in roots and shoots (Fig. 7). In roots, the increase in *OsNHX1* expression by 0.2 M KCl was higher than that by 0.2 M NaCl, indicating that the increased expression of the gene was mainly due to the ionic stress of KCl. The effect of a high $K^+$ concentration on the leaf growth of rice is similar

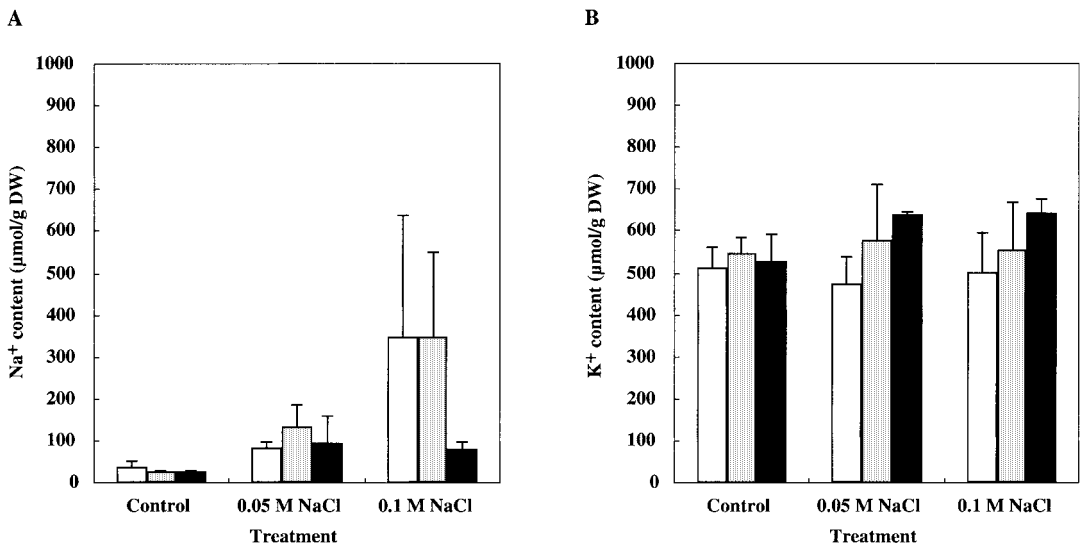



**Fig. 10** $Na^+$ (A) and $K^+$ (B) contents of wild-type and transgenic plants overexpressing OsNHX1 grown at various salt concentrations. Wild-type (no vectors; white bars) and transgenic plants (95-3-2 and 95-4-12; gray and black bars) were grown under normal conditions for 18 d, and treated with 0, 0.05 or 0.1 M NaCl for 7 weeks. $Na^+$ and $K^+$ were extracted from the leaves, and ion contents were determined as shown in Materials and Methods. Each value is the mean ± SD of three independent experiments.

to or greater than that of a high $Na^+$ concentration (Yeo et al. 1991). In *A. thaliana*, treatment with a high $K^+$ concentration has also been reported to increase the expression of *AtNHX1* (Gaxiola et al. 1999). In addition, we showed that OsNHX1 protein had the ability to suppress $K^+$ sensitivity of yeast *nhx1* mutants and, like AtNHX1, to transport $K^+$ (Fig. 2). These results support the suggestion that plant NHX-type antiporters, including OsNHX1, regulate the $K^+$ level in plant cells and play an important role in the tolerance of plants to high $K^+$ concentrations.

*The importance of OsNHX1 protein in salt tolerance of rice*

Overexpression of OsNHX1 improved the salt tolerance of transgenic rice cells, and this improvement in the salt tolerance was correlated with an increase in the $Na^+$ content of rice cells grown in a medium with high NaCl concentration (Fig. 8). These results indicate that the increased activity of OsNHX1 in the tonoplasts enhanced the compartmentation of $Na^+$ into the vacuoles and resulted in the increased tolerance of the transgenic cells to a high NaCl concentration. Similar results have been reported in some transgenic plants, *A. thaliana*, *Lycopersicon esculentum* and *Brassica napus*, overexpressing AtNHX1 (Apse et al. 1999, Zhang and Blumwald 2001, Zhang et al. 2001). These results suggest that a vacuolar $Na^+/H^+$ antiporter is effective for improving the salt tolerance of various plants. We previously reported that the activity of vacuolar $Na^+/H^+$ antiporter in rice was lower than that in barley, a salt-tolerant crop, although they had similar apparent $K_m$ values, and suggested that the amount of the antiporter is one of the most important factors in removing $Na^+$ from the cytoplasm and determining salt tolerance (Fukuda et al. 1998). The present findings confirm that an increased amount of a rice vacuolar $Na^+/H^+$ antiporter, OsNHX1, conferred higher salt tolerance in rice.

Although we showed that OsNHX1 protein had the ability to transport $K^+$ (Fig. 2), the $K^+$ content of the transgenic rice cells overexpressing OsNHX1 was less than or similar to that of the wild-type cells under all growth conditions (Fig. 8E). The $K^+/Na^+$ ratio in salt-treated cells is lower than that in control cells, because $Na^+$ can be transported into the cells through $K^+$ carriers (Blumwald et al. 2000). Although further investigations of the ion selectivity of OsNHX1 protein are needed, the larger decrease in the $K^+$ content of the salt-treated transgenic cells compared with that of the salt-treated wild-type cells might be related to the larger increase in the $Na^+$ content of the transgenic cells compared with that of the wild type. The mechanism causing the lower $K^+$ content in the control transgenic cells compared with that of the control wild-type cells is unknown. It might be difficult for the $K^+$ transport stimulated by increased OsNHX1 to affect the $K^+$ content in the whole cell. It is possible that some secondary phenomenon might have caused the result, because the $K^+$ content is much higher than the $Na^+$ content in the cells and many $K^+$ transport mechanisms exist in plant cells (Schachtman 2000).

Overexpression of OsNHX1 also improved the salt tolerance of transgenic rice plants (Fig. 9). This result indicates that OsNHX1 is effective for improving salt tolerance not only in rice cells, but also in plants. However, in contrast to the result for transgenic rice cells, no significant differences were observed in the leaf $Na^+$ content of either wild-type or transgenic plants grown in a high NaCl concentration (Fig. 10). This is the first report that shows a difference in the increase in $Na^+$ content under salt stress between transgenic cells and plants

overexpressing a vacuolar $Na^+/H^+$ antiporter. We also analyzed the $Na^+$ content in the shoots and roots of 8-day-old wild-type and transgenic seedlings grown for 5 d in the presence of 0.1 M NaCl, and found that the content in the transgenic seedlings was similar to that in the wild-type seedlings (data not shown). These results are contrary to those for three transgenic dicot plants overexpressing AtNHX1 (Apse et al. 1999, Zhang and Blumwald 2001, Zhang et al. 2001). However, Ohta et al. (2002) reported that $Na^+$ content in young leaves of transgenic rice plants overexpressing AgNHX1 was the same as that in wild-type plants after 3 d of 0.3 M NaCl treatment, although the transgenic plants had higher salt tolerance than the wild-type plants. Although only the young leaves of rice plants grown for a long time (more than 3 weeks) were analyzed in their study, Ohta et al. (2002) suggested that compartmentation of $Na^+$ into the mature leaves may improve the salt tolerance of the plants. We also observed the $Na^+$ content in surviving (younger) leaves of transgenic plants grown for a long time (more than 9 weeks) (Fig. 10). The difference in $Na^+$ content between wild-type and transgenic rice plants might be observed in the mature leaves, but not in the young leaves. Although further investigations are needed, the compartmentation of $Na^+$ into mature tissues might prevent young tissues from the impairment of salt stress and contribute to the improvement of salt tolerance in rice plants. In rice seedlings, which do not develop mature tissues, no significant differences were observed in the growth of shoots and roots between wild-type and transgenic seedlings (8-day-old) grown for 5 d in the presence of 0, 0.1 and 0.2 M NaCl (data not shown). This result supports the suggestion that compartmentation of $Na^+$ into mature tissues improves salt tolerance in rice plants.

Overexpression of a vacuolar $Na^+/H^+$ antiporter, AtNHX1, has been suggested to significantly improve the salt tolerance of the crop plants *L. esculentum* and *B. napus* (Zhang and Blumwald 2001, Zhang et al. 2001). However, for efficient improvement of salt tolerance in agricultural contexts, the mechanism of salt tolerance should be examined for each species or family, because the mechanisms may differ, as shown in this paper. Rice is a staple food of more than 50% of the world's population, and it is also a representative of monocot crops, which account for 90% of the world's staple diet. Most wild grasses are also monocots. Therefore, it is worthwhile to elucidate the mechanism of salt tolerance of rice and to improve the salt tolerance of rice plants. The present findings regarding rice and the rice vacuolar $Na^+/H^+$ antiporter OsNHX1 revealed differences in the mechanism of salt tolerance between rice, a monocot species, and three dicot species overexpressing AtNHX1 and should be useful for improving the salt tolerance of rice and other monocots significantly and efficiently for practical use in the field.

## Materials and Methods

*Yeast strains and media*

The two yeast (*S. cerevisiae*) strains, K601 (wild type) and R100 (Δ*nhx1::URA3*) (kindly provided by Dr. Rajini Rao, Johns Hopkins University, U.S.A.), used in this study are isogenic to W303 (*ade*2-1 *can*1-100 *his*3-11, 15 *leu*2-3, 112 *trp*1-1 *ura*3-1) and have been described previously (Nass et al. 1997). Yeast cells were grown in YPD [1% (w/v) yeast extract / 2% (w/v) peptone / 2% (w/v) glucose], SD [0.67% (w/v) Yeast Nitrogen Base (Difco Lab., Detroit, MI, U.S.A.) with 2% (w/v) glucose], or APG medium containing 10 mM arginine, 8 mM phosphoric acid, 2 mM $MgSO_4$, 1 mM KCl, 0.2 mM $CaCl_2$, 2% (w/v) glucose, trace vitamins and minerals, at pH 6.5 with arginine (Nass et al. 1997). Where indicated, NaCl or hygromycin-B was added, or the pH was adjusted to 4.0 with acetic acid.

*Plant materials and growth conditions*

Rice (*O. sativa* L. cv. Nipponbare) seedlings or plants were used in the experiments. For seedling culture, rice seeds were imbibed overnight and then placed on cotton mesh suspended over a nutrient solution, containing 0.5 mM $NH_4H_2PO_4$, 1 mM $KNO_3$, 0.5 mM $MgSO_4$, 12.5 μM Fe-EDTA, 1 mM $CaCl_2$ and micronutrients. Seedlings were grown by hydroponics in the nutrient solution in a growth chamber under a 14-h photoperiod with a photon flux density of 100 μmol $m^{-2}$ $s^{-1}$. In the chamber, relative humidity was 75%, and the temperature was 30°C during the day and 25°C at night. Air was continuously bubbled through the nutrient solution. Seedlings were stressed as indicated beginning 7 d after sowing.

Rice seeds were germinated and then transplanted into soil (Bonsol no.1; Sumitomo Chemical Corp., Tokyo, Japan). Plants were grown in a growth chamber under a 14-h photoperiod with a photon flux density of 200 μmol $m^{-2}$ $s^{-1}$ under a relative humidity of 70% at 28°C during the day and 24°C at night. The water in the soil was changed every 2 d. The plants were grown for 18 d and then stressed as indicated.

*Expression of OsNHX1 in yeast*

The *OsNHX1* ORF was obtained from a cDNA including the full length of the gene by PCR with the 5′-primer d(CGGAATTCATGGGGATGGAGGTGGCGGC) and the 3′-primer d(ACGCGTCGACATTCCATTCTCATTTCTTTG), which included *Eco*RI and *Sal*I restriction sites on their respective 5′ ends. The resulting fragment was subcloned into pBluescript II KS+ (Stratagene, San Diego, CA, U.S.A.) and sequenced to verify the fidelity of the PCR product. The *OsNHX1* ORF was inserted into the *Eco*RI/*Sal*I site of pKT10+*HIS3*, which was derived from the yeast-*E. coli* shuttle vector pKT10 (Tanaka et al. 1990) by insertion of *HIS3*, downstream of the *GAP* promoter, producing the plasmid pOsNHX1. K601 and R100 were transformed with lithium acetate (Gietz and Schiestl 1995).

*Expression of OsNHX1 in Escherichia coli*

For expression of T7-tagged OsNHX1 in bacteria, the *OsNHX1* ORF fragment obtained by the same method as expression of *OsNHX1* in yeast was inserted into the *Eco*RI/*Sal*I site of pET-21a(+) (Novagen, Madison, WI, U.S.A.). The plasmid was transformed into the *E. coli* strain BL21(DE3). The strain was grown in 2× YT [1% (w/v) yeast extract / 1.6% (w/v) tryptone / 0.5% (w/v) NaCl] plus ampicillin (50 mg $liter^{-1}$). Protein expression was induced at an $OD_{650}$ of 0.7 with 1 mM IPTG.

*Microsome preparation from yeast*

Yeast microsomes were prepared using a modification of the method of Jazwinski (1990). Yeast cells were grown to mid-exponential phase in APG medium at 28°C. After pelleting, cells were suspended

in 0.1 M Tris-$H_2SO_4$, pH 9.4, containing 10 mM dithiothreitol (DTT) and incubated for 30 min at 30°C with shaking. After centrifugation at 3,000×*g* for 5 min, suspended cells were digested with Zymolyase 20T (Seikagaku Kogyo Co., Tokyo, Japan) at 0.5 mg $ml^{-1}$ in 20 mM potassium phosphate buffer, pH 7.4, containing 1.2 M sorbitol for 1 h at 30°C with gentle shaking. The resulting spheroplasts were washed with ice-cold potassium phosphate buffer and suspended in 20 mM Tris-HCl, pH 7.4, 0.6 M sorbitol and 50 μM Pefabloc SC (4-(2-amino-ethyl)-benzenesulfonyl-fluoride hydrochloride) (Merck, Darmstadt, Germany). The suspension was homogenized with a Dounce homogenizer and centrifuged at 3,000×*g* for 5 min. The supernatant (crude solution) was centrifuged at 100,000×*g* for 60 min. The precipitate was resuspended in potassium phosphate buffer, and used as the microsome.

Proteins were quantified by the method of Bradford (1976).

*Transformation of rice calli and plants*

The cDNA construct of full-length *OsNHX1*, which was cloned into the *Bam*HI site of pBluescript KS+, was digested with *Kpn*I and *Not*I restriction enzymes. The resulting fragment was subcloned into the Ti-plasmid pMSH1 containing a hygromycin resistance gene (a gift from Dr. Tsutomu Kawasaki, Nara Institute of Science and Technology, Japan) downstream of the CaM 35S-promoter (Kawasaki et al. 1999). The resultant plasmid was introduced into calli derived from rice by transformation mediated by *Agrobacterium tumefaciens* (strain EHA101) (Hood et al. 1986) as described by Toki (1997). Transformed calli were selected for hygromycin resistance, and plants were regenerated from the transformed calli.

For the experiments using rice cells, we initiated cell suspension cultures from the transformed calli and maintained the cells in Murashige–Skoog (MS) medium containing 3% (w/v) sucrose, 2 μg $ml^{-1}$ 2,4-D, vitamins and 50 μg $ml^{-1}$ hygromycin in the dark at 26°C on a rotary shaker (120 rpm) and subcultured every second week.

For the experiments using transgenic rice plants, seeds of wild-type and transgenic rice were germinated on wet filter paper for 3 d, transplanted onto a 0.9% (w/v) agar plate containing 50 μg $ml^{-1}$ hygromycin for selection, and grown for 6 d. The plants grown on the plate were transplanted into soil.

*Membrane preparation from rice*

Tonoplasts were isolated from roots and shoots of rice seedlings by using a modification of a method described previously (Fukuda et al. 1998). Roots or shoots were homogenized in 50 mM MOPS-KOH, pH 7.6, containing 0.25 M sorbitol, 5 mM ethylene glycol bis(2-aminoethyl ether)tetraacetic acid (EGTA), 1.5% (w/v) polyvinylpyrrolidone K-30, 2.5 mM potassium metabisulfite and 50 μM Pefabloc SC. The resulting pellets after final centrifugation were resuspended in MOPS-KOH buffer, and used as the tonoplast fraction.

Plasma membranes were isolated from roots and shoots of rice seedlings using the aqueous two-phase system of Hodges and Mills (1986). We used a grinding medium containing 50 mM MES-Tris, pH 7.6, 0.3 M sucrose, 0.5% (w/v) BSA, 5 mM EDTA, 5 mM EGTA, 4 mM salicylhydroxamic acid, 1 mM DTT, 2.5 mM sodium metabisulfite, 20 mM NaF and 2 mM phenylmethylsulfonyl fluoride.

*Preparation of OsNHX1 antibodies and Western blot analysis*

Polyclonal antibodies were raised in rabbit against a synthetic peptide derived from the COOH-terminal region of the predicted OsNHX1 amino acid sequence (Val-Pro-Phe-Ser-Pro-Gly-Ser-Pro-Thr-Glu-Gln-Ser-His-Gly-Gly-Arg) (Fukuda et al. 1999). The antibodies were purified by affinity purification using a chromatography column linked with the synthetic peptide. SDS-PAGE was performed in 10% gels containing 20% glycerol (Schagger and von Jagow 1987) or 12% gels (Laemmli 1970). Immunoblotting was carried out with Immobilon-P membrane (polyvinylidene difluoride) (Millipore, Bedford, MA, U.S.A.) onto which proteins in the gel were transferred by an electrophoretic transfer system (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Goat anti-rabbit IgG conjugated with alkaline phosphatase (Organon Teknika Corp., West Chester, PA, U.S.A.) was used as a secondary antibody to detect the IgG on the immunoblot. Immunoreactive bands were detected by AttoPhos Substrate Set (Roche Diagnostics Corp., Indianapolis, IN, U.S.A.) using FluorImager 575 (Molecular Dynamics, Sunnyvale, CA, U.S.A.). For expression of T7-tagged OsNHX1, mouse monoclonal antibodies against the T7-tag (Novagen, Madison, WI, U.S.A.) and VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA, U.S.A.) were used.

*Isolation of osnhx1 mutants from Tos17 insertional mutants*

The Functional Genomics Laboratory at the National Institute of Agrobiological Sciences (Japan) has produced large populations of rice mutants generated by using *Tos17*-mediated mutagenesis (Hirochika 2001). We screened the populations of *Tos17* insertional mutants by PCR using *Tos17*-specific primers and *OsNHX1*-specific primers in all possible combinations as described by Takano et al. (2001). The amplified DNA fragments were analyzed by Southern blot analysis with $^{32}P$-labeled ORF of *OsNHX1* to eliminate non-specific amplifications and sequenced to confirm the insertion of *Tos17*.

*Expression of a fusion protein of GFP and OsNHX1 in rice cells*

The vector psGFP (sGFP S65T mutant) was kindly provided by Dr. Yasuo Niwa, Shizuoka Prefectural University, Japan (Niwa et al. 1999). The vector psGFPcs, which is a derivative of psGFP (Jiang et al. 2001), was kindly provided by Dr. Akiko Baba, National Institute of Agrobiological Sciences, Japan. The construct OsNHX1-sGFP was generated by PCR amplification from the cDNA including the full length of *OsNHX1* with the 5′-primer d(ACGCGTCGACCATGGGGATGGAGGTGGCGGCGG) and the 3′-primer d(CATGCCATGGATCTTCCTCCGTGGCTCTGCTCG), which included *Sal*I and *Nco*I restriction sites on their respective 5′ ends. The amplified DNA fragment was subcloned into the *Sal*I/*Nco*I sites of psGFP and sequenced to verify the fidelity of the PCR product. The *Sal*I/*Not*I fragment of the construct was subcloned into the pMSH1 vector with the *Sma*I/*Not*I sites downstream of the CaM 35S-promoter. The construct sGFP–OsNHX1 was generated by PCR amplification from the cDNA including the full length of *OsNHX1* with the 5′-primer d(ATATGGGCCCATGGGGATGGAGGTGGCGGCGG) and the 3′-primer d(ATATCCCGGGTCTTCCTCCATGGCTCTGCTCG), which included *Apa*I and *Xma*I restriction sites on their respective 5′ ends. The amplified DNA fragment was subcloned into the *Apa*I/*Xma*I sites of psGFPcs and sequenced. The *Xba*I/*Not*I fragment of the construct was subcloned into pMSH1 with the *Xba*I/*Not*I sites. The OsNHX1–sGFP and sGFP–OsNHX1 constructs were used to transform the rice calli with *A. tumefaciens*. Suspension cultures of rice cells were established from the transformed calli. The cell suspension was maintained in MS medium containing 50 μg $ml^{-1}$ hygromycin, as stated in the *Transformation of rice calli and plants* section.

*Confocal microscopy*

Rice cells were labeled with 40 μM FM 4–64 [*N*-(3-triethylammoniumpropyl)-4-(6-(4-diethylamino)phenyl)hexatrienyl]pyridinium dibromide] (Molecular Probes, Eugene, OR, U.S.A.) for 30 min, and cultured in fresh medium for 5 h as described by Nass and Rao (1998). The fluorescent images were observed using a Bio-Rad Micro Radiance 2000 confocal laser microscope. GFP and FM 4-64 were excited by wavelengths of 488 nm and 514 nm, respectively, from an Ar laser.

*Northern blot analysis*

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, U.S.A.), hybridization was performed with $^{32}P$-labeled cDNA fragments prepared using a random primer labeling kit (Random Primers System; Takara, Tokyo, Japan). The membrane was exposed to an imaging plate, and the radioimage of the plate was analyzed (BAS2000; Fuji, Tokyo, Japan). The Northern experiments were performed at least twice. Equal loading of RNA blots was assessed by scanning the signals of ribosomal RNA detected with methylene blue.

*Dry weight and ion content determination*

Rice cells were collected by filtration on preweighed Whatman Grade 113 filter paper and rinsed quickly with distilled water at least five times. Leaves were cut from rice plants and rinsed with distilled water to wash out possible surface contamination by $Na^+$. The collected cells and leaves were dried at 70°C for at least 4 h and for 3 d, respectively, and then the dried materials were reweighed to obtain the dry weight. The dried cells and leaves were extracted in boiling water for 1 h and 0.1 M acetic acid at 90°C for 2 h, respectively. The contents of $Na^+$ and $K^+$ were determined with a capillary electrophoresis system (P/ACE System 2100; Beckman Instruments, Fullerton, CA, U.S.A.) as described by the manufacturer. The running buffer used for the ion analysis contained 13 mM of α-hydroxyisobutyric acid and 5 mM UV CAT-1 (Millipore Corp., Milford, MA, U.S.A.). Statistical analyses with Student's *t* test, ANOVA and Tukey's test were performed using the TTEST procedures and the General Linear Models procedures of the Statistical Analysis System (SAS Institute Inc., Cary, NC, U.S.A.).

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