An Na^+/H^+ antiporter gene from wheat plays an important role in stress tolerance

Jia Ning Yu[1,2], Jian Huang[1], Zi Ning Wang, Jin Song Zhang[1*] and Shou Yi Chen[1,*]

[1] *National Key Lab of Plant Genomics, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China*

[2] *College of Life Science, Shaan Xi Normal University, Xi'an , Shaanxi, 710 062, China*

**Corresponding authors (Fax, 86-10-64873428; Email, sychen@genetics.ac.cn; jszhang@genetics.ac.cn)*

A vacuole Na^+/H^+ antiporter gene *TaNHX2* was obtained by screening the wheat cDNA library and by the 5′-RACE method. The expression of *TaNHX2* was induced in roots and leaves by treatment with NaCl, polyethylene glycol (PEG), cold and abscisic acid (ABA). When expressed in a yeast mutant (*Δnhx1*), TaNHX2 suppressed the salt sensitivity of the mutant, which was deficient in vacuolar Na^+/H^+ antiporter, and caused partial recovery of growth of *Δnhx1* in NaCl and LiCl media. The survival rate of yeast cells was improved by overexpressing the *TaNHX2* gene under NaCl, KCl, sorbitol and freezing stresses when compared with the control. The results imply that TaNHX2 might play an important role in salt and osmotic stress tolerance in plant cells.

[Yu J N, Huang J, Wang Z N, Zhang J S and Chen S Y 2007 An Na^+/H^+ antiporter gene from wheat plays an important role in stress tolerance; *J. Biosci.* **32** 1153–1161]

1. Introduction

Salt and osmotic stress are two of the most serious factors that limit plant growth. To cope with stress, many salt- and drought-tolerant plants have developed a variety of adaptation mechanisms (Bohnert *et al* 1995). The active transport of ion and solute molecules such as glycine, betaine, proline and polyhydric alcohols (including manitol and pinitol) in the cell is one of the main ways to maintain cell turgor at low water potentials (McNeil *et al* 1999; Gaxiola *et al* 2001; Shen *et al* 2003).

A vacuole Na^+/H^+ antiporter drives actively moving Na^+ into the vacuole by making use of the H^+-ATPase and H^+-PPiase coupled with the vacuolar H^+-translocating enzymes, the H^+-ATPase and the H^+-PPiase, which produce electrochemical H^+ gradients (Blumwald 1987). The antiporter mediates transport of Na^+ into the vacuole. The activity of Na^+/H^+ antiporter was shown to be present in tonoplast vesicles from red beet storage tissue (Blumwald *et al* 1985). A novel Na^+/H^+ antiporter (*Nhx1*) was identified from a prevacuolar compartment of yeast by analysis of genes involved in cation detoxification (Nass *et al* 1997). The first plant Na^+/H^+ antiporter, *AtNHX1*, was isolated from *Arabidopsis* by functional genetic complementation of a yeast mutant defective for endosomal Na^+/H^+ activity and was found to be similar to the mammalian *NHE* transporter (Apse *et al* 1999; Gaxiola *et al* 1999; Quintero *et al* 2000). Since then, a number of Na^+/H^+ antiporter genes have been isolated from plants such as rice (Fukuda *et al* 1999), *Atriplex gmelini* (Hamada *et al* 2001), *Brassica napus* (Wang *et al* 2003), cotton (Wu *et al* 2004) and wheat (Wang *et al* 2002; Brini *et al* 2005).

In recent years, significant advances have been made towards understanding the function of Na^+/H^+ exchanger (Schachtman and Liu 1999; Blumwald *et al* 2000). This transport protein was localized in vacuolar membranes and maintains the osmotic pressure of the plant (Blumwald *et al* 2000). Overexpression of Na^+/H^+ antiporter genes (*AtNHX1*) of *Arabidopsis* suppressed some of the salt-sensitive phenotypes of the *nhx1* yeast strain (Gaxiola *et al* 1999). In transgenic *Arabidopsis* overexpressing *AtNHX1* there was higher activity of vacuolar Na^+/H^+ antiporter and they were able to grow in the presence of 200 mM NaCl (Apse

Keywords. Na^+/H^+ antiporter; stress tolerance; transcription accumulation; yeast cells; wheat

et al 1999). Overexpression of *AtNHX1* in tomato resulted in transgenic plants that were able to grow, flower and set fruit at high salt concentrations (Zhang and Blumwald 2001). The role of sodium compartmentalization in salt tolerance has been further demonstrated in transgenic *Brassica napus* (Zhang *et al* 2001). The transcription level of *BnNHX1*, a vacuolar Na^+/H^+ antiporter from *Brassica napus*, increased upon treatment with 200 mM NaCl (Wang *et al* 2003). Overexpression of the cotton Na^+/H^+ antiporter gene *GhNHX1* in tobacco improved salt tolerance in comparison with wild-type plants (Wu *et al* 2004). All these results suggest that vacuolar Na^+/H^+ antiporter plays an important role in the salt tolerance of various plants.

Other functions of Na^+/H^+ antiporter have also been identified. In the Japanese morning glory, a shift from purple buds to blue open flowers correlated with the activity of an *NHX1* homologue in *Ipomoea nil* (Yamaguchi *et al* 2001). A T-DNA insertion mutant of *AtNHX1* showed much lower Na^+/H^+ and K^+/H^+ exchange activity. The mutant plants exhibited altered leaf development and reduced leaf area compared with wild-type plants (Apse *et al* 2003). These results suggest the Na^+/H^+ antiporter is important not only for salt tolerance but also for vacuolar pH regulation and the developmental processes of leaves.

In the present study, we cloned a new Na^+/H^+ antiporter gene, *TaNHX2*, from wheat. The expression patterns of *TaNHX2* were analysed in response to treatment with salt, drought, cold and ABA. The function of *TaNHX2* was analysed by complementation in the yeast mutant *Δnhx1*. The yeast cell with overexpressed *TaNHX2* showed higher salt, osmotic and freezing tolerance. These results provide evidence that *TaNHX2* may play an important role in salt and osmotic stresses.

2. Materials and methods

2.1 *Plant materials and RNA extraction*

Wheat cultivar (*Triticum aestivum* L.) 86211 (HD) was used in this study. The seeds of HD were germinated at 25°C for 1 day and then grown in the greenhouse at 22°C at a photoperiod of 16 h/day. For gene cloning, 7-day-old seedlings of HD were treated with 250 mM NaCl for 12 h. The plants were harvested and stored at –70°C for RNA extraction. For northern blotting, 7-day-old seedlings of HD were irrigated with a solution containing 250 mM NaCl, 26.2% PEG6000 or 20 μM ABA, or treated at 4°C. The seedlings were harvested at 0 h, 1.5 h, 3 h, 6 h, 12 h, 18 h, 24 h, 36 h and stored at –70°C for RNA extraction.

RNA was isolated according to the method of Zhang *et al* (1996). The tissues were ground to powder in liquid nitrogen. Isolation of total RNA was performed using guanidine thiocyanate, and purified with phenol–chloroform extraction.

2.2 *Cloning of the* TaNHX2

A partial 3′ sequence of *TaNHX2*, 1696 bp, was obtained by screening a salt-stressed wheat cDNA library using a rice Na^+/H^+ antiporter cDNA fragment as a probe (Wang *et al* 2002). Two specific primers were designed according to the partial cDNA sequence of *TaNHX2* (GSP, 5′-AGCAGCTCTTCCAACCAGAACCAACC-3′; NGSP, 5′-CTCTTGAGCTCTCTGTCACGTTGTGC-3′). 5′-RACE-PCR was carried out according to the SMART™ RACE cDNA amplification kit user manual (Clontech). The PCR reaction was performed for five cycles at 94°C for 5 s, 72°C for 2 min; five cycles at 94°C for 5 s, 70°C for 10 s, 72°C for 2 min; 30 cycles at 94°C for 5 s, 60°C for 10 s, 72°C for 3 min; and finally 72°C for 10 min. Subsequently, the PCR product was diluted and used in a nested PCR reaction. The nested PCR condition was at 94°C, 2 min and then 30 cycles at 94°C for 5 sec, 63°C for 10 sec, and 72°C for 2 min, plus an extension at 72°C for 10 min. The amplified DNA fragments were purified and named 5′ sequence of *TaNHX2,* cloned into pGEM®-T easy vector (Promega) and sequenced.

To get the full TaNHX2, a pair of PCR primers, 5′-GATGGGGTACCAAGTGGT G-3′ (primer 1) and 5′-CTT-CTTCGCGACGTTCATTC-3′ (primer 2), were designed according to the 5′-, 3′- sequences of TaNHX2 obtained above. RNA was isolated from 7-day-old seedlings of HD treated with 250 mM NaCl for 12 h. As a template, 1 μg cDNA reverse-transcribed from RNA with the cDNA synthesis kit (Promega,Madison, WI, USA) was used. The PCR reaction was performed at 94°C, 5 min, and followed by 30 cycles of 30 s at 94°C, 40 s at 58°C and 2.5 min at 72°C, then 10 min at 72°C. The amplified DNA fragment was purified, cloned into the pMD18-T vector (Takara) and sequenced.

The alignments of the DNA or amino acid sequences were performed with MegAlign of DNASTAR (DNASTAR Inc., Madison, WI, USA) by using the clustal method.

2.3 *RNA gel-blot analysis*

Total RNA (30 μg/lane) was denatured and fractionated on a 1.2% formaldehyde agarose gel, blotted onto a nylon membrane, and fixed by UV-crosslinking. Hybridization with the α-^{32}P-dCTP labelled full-length *TaNHX2* gene (1632 bp) was performed at 65°C for 16 h. The membranes were washed with 2×SSC, 0.1% SDS for 15 min and 1×SSC, 0.1% SDS for 5 min at 45°C. After stripping the probe, the

same blots were reprobed with the 18S rRNA gene. The filters were scanned with a phosphoimager (Molecular Dynamics, CA, USA).

2.4 *Yeast strains, media and plasmids*

Two yeast (*Saccharomyces cerevisiae*) strains, W303 (ura3-1, can-1-100, leu 2-3 112 trp 1-1, his 3-11, 15) and *nhx1*:: W303 (kindly provided by Professor Xuecheng Wang) were used as host cells. Yeast cells were grown in YPD (1% yeast extract/2% peptone/2% dextrose; Difco), YPGAL (1% yeast extract/2%peptone/2% galactose; Difco), SD (0.67% yeast nitrogen base with 2% glucose; Difco), or APG medium containing 10 mM arginine, 8 mM phosphoric acid, 2 mM $MgSO_4$, 1 mM KCl, 0.2 mM $CaCl_2$, 2% glucose, trace vitamins and minerals, at pH 4.0 with acetic acid (Nass *et al* 1997). NaCl, LiCl, KCl, sorbitol, or hygromycin-B were added as indicated.

The yeast plasmid pYES2 with GAL1 promoter was used as an expression vector. The *TaNHX2* coding region was amplified from the original plasmid with two primers 5′-CTGGAATTCATGGCCTCCAAGCAGAAC-3′ (sense primer) and 5′-ATGCTCGAGCGAGGAGTACATCAAATCG -3′ (antisense primer). The amplified product was digested with *Eco*RI plus *Xba*I and ligated to *Eco*RI/*Xba*I digested pYES2 to construct the expression vector pYES2–TaNHX2. This plasmid and the control plasmid pYES2 were used to transform *S. cerevisiae* W303 and *nhx1*::W303, respectively, using a lithium acetate method (Ito *et al* 1983).

2.5 *Expression of* TaNHX2 *in yeast cells*

The yeast transformants harbouring pYES2–TaNHX2 or pYES2 were cultured in SD-ura medium (Difco) until the OD_{600} reached 0.7. Then the culture was transferred to YPD, or YPGAL medium for further growth. When an OD_{600} of 2.0 was reached, total RNA was isolated by a hot phenol method (Schmitt *et al* 1990), fractionated on a denatured 1.2% agarose gel, and blotted onto a Hybond N^+ membrane (Amersham). The cDNA fragment of *TaNHX2* was labelled and used as the probe. Hybridization and washing was done by the method described previously (Shen *et al* 2003).

2.6 *Salt and drought tolerance assay of yeast transformants*

Yeast transformants, W303-pYES2-TaNHX2 and W303-pYES2, were grown in SD-ura medium for 1 day (OD_{600} = 0.2) and 20 μl of the culture was inoculated into 2 ml YPGAL for each treatment containing NaCl, KCl or sorbitol. Growth rates of these transformants were monitored by measuring absorbance at 600 nm after culturing them at 30°C for 48 h. Each treatment was repeated three times.

2.7 *Freezing tolerance assay of yeast transformants*

Yeast cells, W303-pYES2–TaNHX2 and W303-pYES2, were grown in SD-ura medium until they reached an OD_{600} of 0.6–0.7 at 30°C and then 1 ml of the culture was centrifuged and the yeast cells were resuspended in the same volume of sterile water. This culture was further diluted to an OD_{600} of 0.002 and four aliquots of 100 μl were spread onto YPGAL medium. Colony-forming units were counted after 2 days. Another four aliquots were frozen in an ethanol bath and kept at –20°C. After 24 h, the frozen aliquots were thawed in a water bath at 30°C and spread onto YPGAL plates. Colony-forming units were counted as above. The procedure was repeated 3 times and the data averaged (Imai *et al* 1996).

3. Results

3.1 *Cloning and structural analysis of* TaNHX2

An 873 bp fragment of *OSNHX1*, the rice Na^+/H^+ antiporter gene, was used as a probe to screen the NaCl-stressed cDNA library of wheat. Seventy-six positive clones were obtained after three cycles of screening. From these clones, a 1696 bp partial 3′-cDNA sequence of *TaNHX2*, named *TaNHX2*-1, was isolated. 5′-RACE was carried out and a product of 900 bp was obtained, which overlapped with the sequence of *TaNHX2-1*. Then the full length of *TaNHX2* (AY040246) was amplified from corresponding cDNAs with primers 1 and 2. The full-length cDNA of *TaNHX2* is 1632 bp in length including an open-reading frame of 1614 bp, and encodes a transmembrane protein of 538 amino acids with a molecular weight of 59 kD and of pI 8.21.

Structural analysis revealed that TaNHX2 possess ten membrane-spanning segment domains. A putative amiloride-binding domain LFFIYLLPPI, conserved in many vacuole Na^+/H^+ antiporters (Counillon and Pouyssegur 1993; Hamada *et al* 2001), is also found in the deduced amino acid sequence of TaNHX2 (figure 1). TaNHX2 shows high similarity with Na^+/H^+ antiporters from other plants. For example, it exhibits 66.0%, 86.7%, 69.9%, 73.3% and 95.7% identities to TaNHX1 (Wang *et al* 2002), OsNHX1 (Fukuda *et al* 1999), AtNHX1 (Gaxiola *et al* 1999), AgNHX1 (Hamada *et al* 2001) and HvNHX1 (Fukuda *et al* 2004), respectively. Therefore, it is likely that TaNHX2 belongs to the Na^+/H^+ antiporter family.

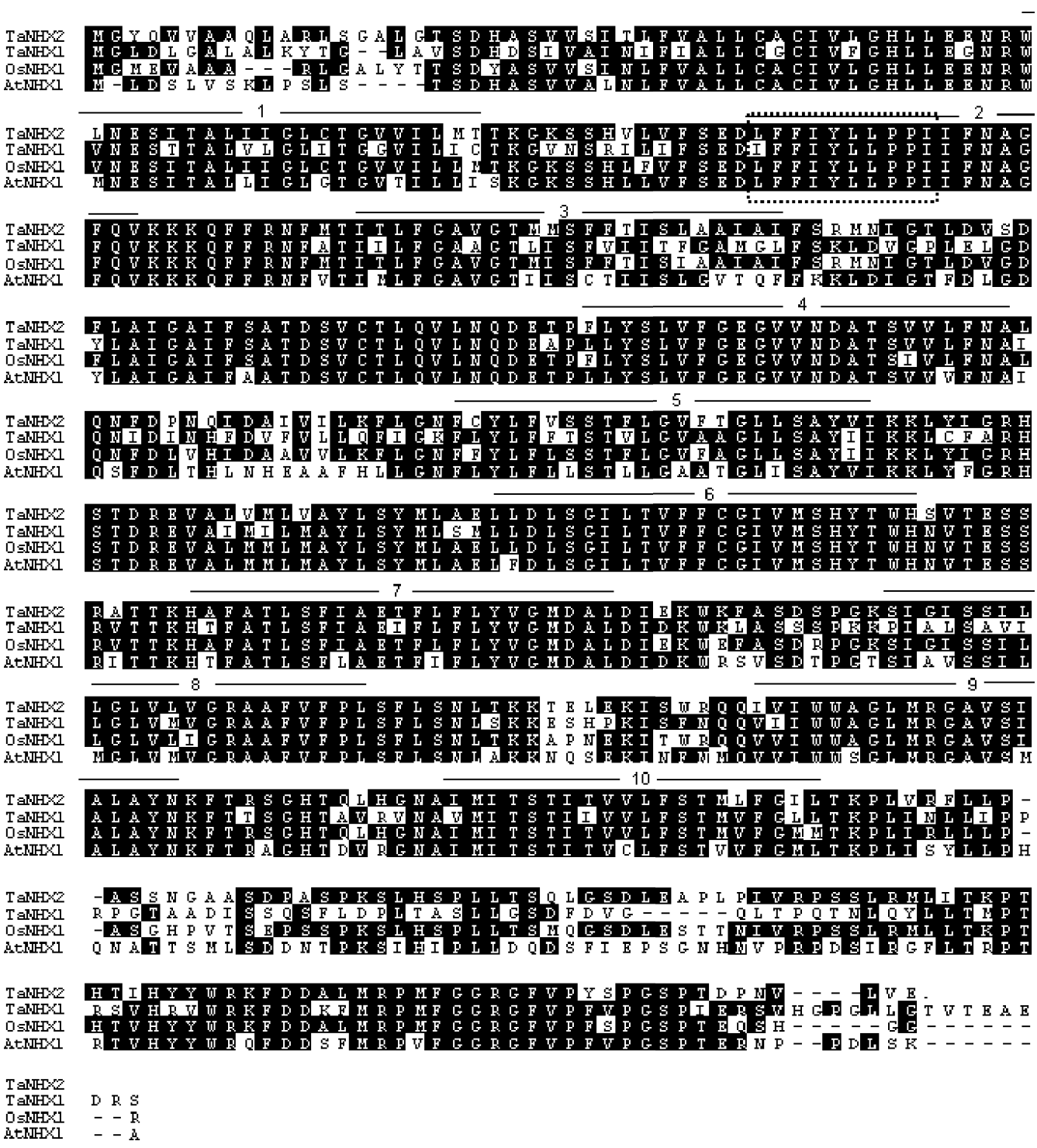

Figure 1. Comparison of the amino acid sequence of TaNHX2 with other plant NHX proteins. Identical amino acids are shaded in black. The dashes indicate gaps inserted in the alignment. A box with dotted lines indicates the putative amiloride-binding site. The putative TaNHX2 transmembrane regions are indicated by lines above the sequence. The SMART software was used for the analysis.

3.2 *Expression of* TaNHX2 *in response to different treatments*

Northern blot analysis was performed to investigate the expression pattern of *TaNHX2* under NaCl, PEG, ABA and cold treatments. The transcription level of *TaNHX2* was induced by salt treatment in both leaves and roots (figure 2A). Salt stress caused a steady accumulation of the *TaNHX2* transcript in roots, with a higher level maintained for 1.5–24 h after initiation of treatment. Thereafter, the expression decreased. In leaves, the expression patterns of *TaNHX2* were similar to those in roots, but the levels peaked at 3–6 h after initiation of treatment (figure 2A). The *TaNHX2* transcripts were also induced by ABA, PEG and cold treatments in both roots and leaves of wheat. As shown in figure 2B, the *TaNHX2* mRNA level in roots steadily increased after ABA treatment and reached a peak at 3–6 h, whereas in leaves, the expression of *TaNHX2* showed a peak at 6 h. After PEG treatment, the transcription of *TaNHX2* steadily increased in roots but showed a peak in leaves at 3–6 h (figure 2B, C). For cold treatment, the expression of *TaNHX2* was increased after 1.5 h of treatment and reached a high level thereafter in roots. It was induced to a higher level after 12 h of treatment in leaves (figure 2B, C). These results indicate that the *TaNHX2* was differentially expressed in leaves and roots following various treatments.

3.3 *Complementation of yeast* Δnhx1 *mutants with* TaNHX2

Plasmid pYES2, a high-copy-number shuttle vector, was used for expression of the recombinant plasmid in the yeast cell. The *TaNHX2* gene was inserted between the inducible yeast GAL1 promoter and the yeast CYC1 terminator, and the recombinant plasmid pYES2–TaNHX2 was obtained. The plasmid pYES2–TaNHX2 was then transferred into the yeast *Δnhx1* mutant for the functional complementation tests of *TaNHX2*. Figure 3A shows that growth of the *Δnhx1* mutant was sensitive to high concentrations of NaCl (400 mM) and LiCl (30 mM) in a low K^+ medium (APG medium, pH 4.0) (Nass *et al* 1997), conditions that did not inhibit the growth of wild-type strains. Expression of *TaNHX2* improved the tolerance of the *Δnhx1* mutants to the NaCl and LiCl stresses (figure 3A). *TaNHX2* also played an important role in suppressing the sensitivity of the *Δnhx1* mutant to hygromycin, a toxic substance that accumulates intracellularly in response to an electrochemical proton gradient (Darley *et al* 2000) (figure 3B). The result confirmed that *TaNHX2* encoded a new vacuolar Na^+/H^+ antiporter gene.

3.4 *Yeast transformants overexpressing* TaNHX2 *showed higher tolerance to Na^+, K^+ and sorbitol stress*

A yeast heterologous expression system was also used to investigate the functions of the *TaNHX2* gene *in vivo*.

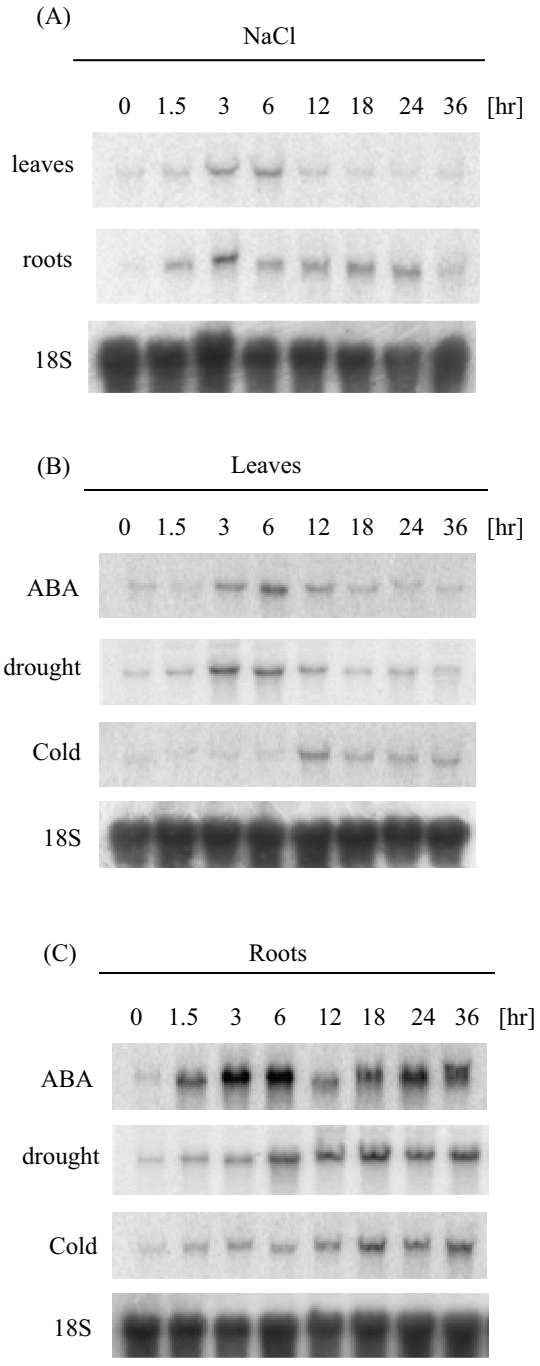

Figure 2. *TaNHX2* expression in wheat seedlings under NaCl, ABA, drought (26.2% PEG6000) and cold (4) treatments. Each lane was loaded with 30 μg of total RNA. The filter was hybridized with the full-length *TaNHX2* gene (1632 bp) labelled with α-^{32}P-dCTP. The 18S rRNA was examined as a load control. **(A)** *TaNHX2* expression in leaves and roots at different times of the 250 mM NaCl treatment. **(B)** *TaNHX2* expression in leaves upon ABA, drought and cold treatments. **(C)** *TaNHX2* expression in roots upon ABA, drought and cold treatments.

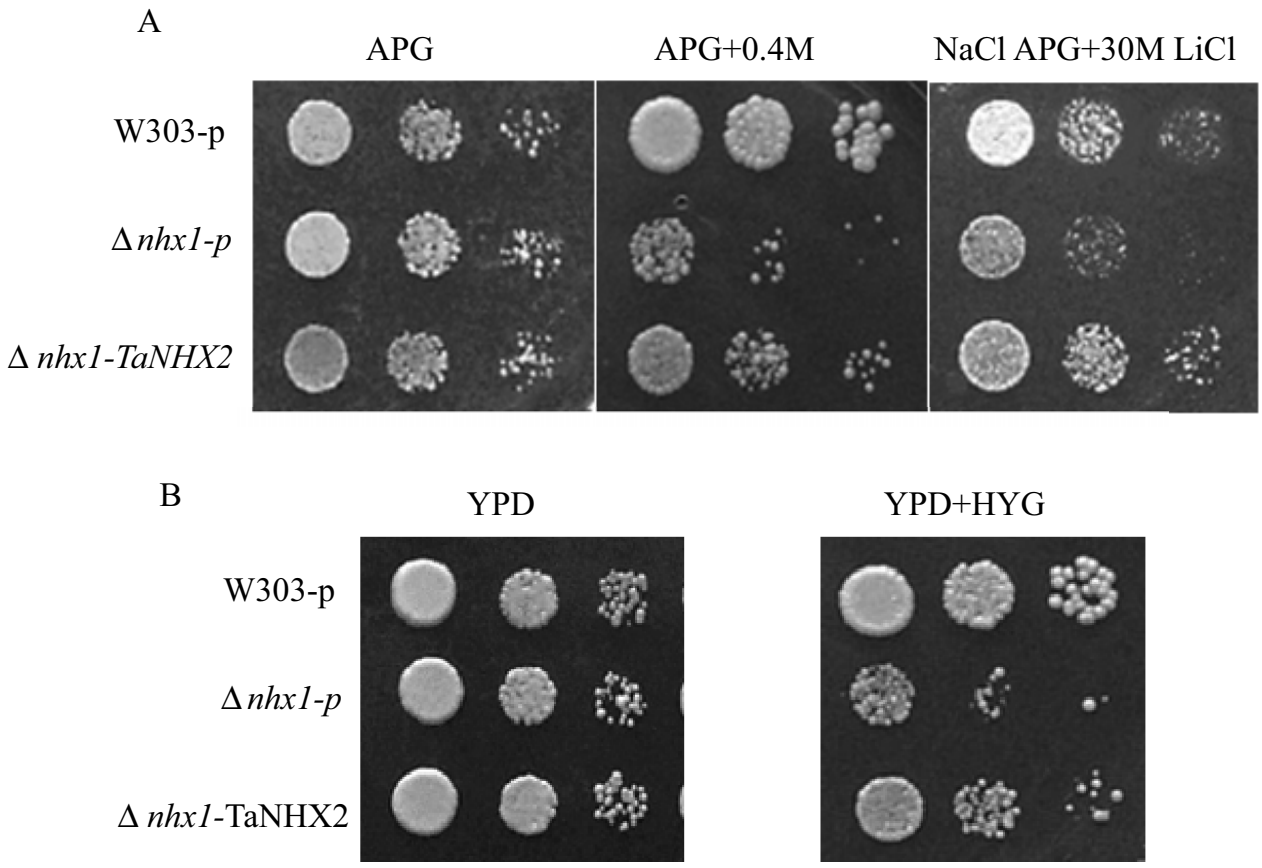

Figure 3. Comparison of growth of the yeast mutant *Δnhx1* and transformants harbouring the *TaNHX2*. The pYES2–TaNHX2 plasmid was introduced into the yeast *Δnhx1* mutants. Plasmid pYES2 was introduced into wild-type (W303) and *Δnhx1* as controls. The strains were grown until the OD_{600} reached 0.2 in YPGAL. Ten-fold serial dilution of the indicated strains was grown at 28°C for 2 days on different media. **(A)** Comparison of yeast cell growth upon hygromycin treatment. Serial dilutions of the strains were grown on YPD plates with or without 0.05 mM hygromycin supplementation. **(B)** Comparison of yeast cell growth upon NaCl and LiCl treatment. Serial dilutions of the same strains were grown on APG plates supplemented with or without 0.4 M NaCl or 30 mM LiCl.

Plasmids pYES2 and pYES2–TaNHX2 were transferred into the *S. cerevisiae* strain W303. The cells harbouring the appropriate plasmids were grown on inducing (YPGAL) or non-inducing (YPD) medium. Expressions of the transformed genes were examined by RNA gel-blot analysis (figure 4). The *TaNHX2* gene was expressed in the galactose-induced medium YPGAL in *S. cerevisiae* and the mRNA levels of *TaNHX2* were the highest in YPGAL+1.2 mol/l NaCl medium (lanes 4, 8). This implied that NaCl might induce *TaNHX2* overexpression in a galactose-induced medium. The apparent expression of the *TaNHX2* gene in the min-dextrose YPD culture was likely to be due to baseline transcription in the absence of a catabolite-repressing carbon source (figure 4, lanes 2, 6) (Swire-Clark and Marcotte 1999). No signals were detected when RNA from the transformants was harboured the vector pYES2, under either inducing or non-inducing conditions (figure 4, lanes 1, 3, 5, 7).

The yeast *TaNHX2* transformants were then examined for their growth under ionic and osmotic stresses. Yeast cells harbouring pYES2–TaNHX2 and pYES2 were grown in SD-ura medium until the OD_{600} reached 0.2. The cultures were transferred to YPGAL medium containing different concentrations of NaCl or KCl. The growth of yeast cells was measured after 48 h. The results show that the transformants had similar growth rates when the NaCl concentration was in the range of 0–0.8 M. The pYES2–TaNHX2 cells displayed better growth as compared with pYES2 cells when the concentration of NaCl increased from 0.8 M to 1.4 M. A similar result was obtained for KCl treatment (data not shown).

In a hyperosmotic medium containing sorbitol at a concentration of 1.4 M or less, the growth of transformants was barely affected. After that, an apparent difference in growth rate was observed when the concentration of sorbitol was increased to 1.6 M, and the cells with pYES2–TaNHX2 showed better growth compared with the control (data not shown).

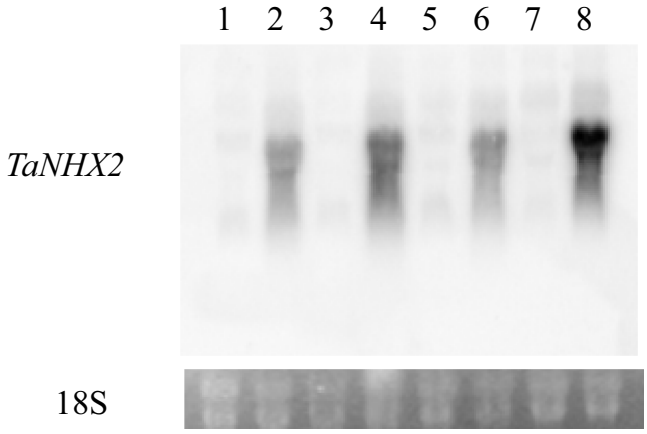

Figure 4. *TaNHX2* expression in yeast transformants upon NaCl treatment. *TaNHX2* cDNA (1632 bp) was inserted between the promoter of the *GAL1* gene (*PGAL1*) and the terminator of CYC1 of the high-copy-number shuttle vector, pYES2, resulting in pYES2–TaNHX2 expression plasmids. The plasmids were transformed into yeast W303. The transformants, with pYES2–TaNHX2 or pYES2, were grown in YPD or YPGAL with or without NaCl. When an OD_{600} of 2.0 was reached, total RNA was isolated. Northern analysis was performed using the full-length *TaNHX2* as a probe and 18S rRNA was examined as a control. Lanes 1, 3, 5, 7. pYES2 in YPD, YPGAL, YPD+1.2 M NaCl, YPGAL+1.2 M NaCl; Lanes 2, 4, 6, 8. pYES2–TaNHX2 in YPD, YPGAL, YPD+1.2 M NaCl, YPGAL+1.2 M NaCl.

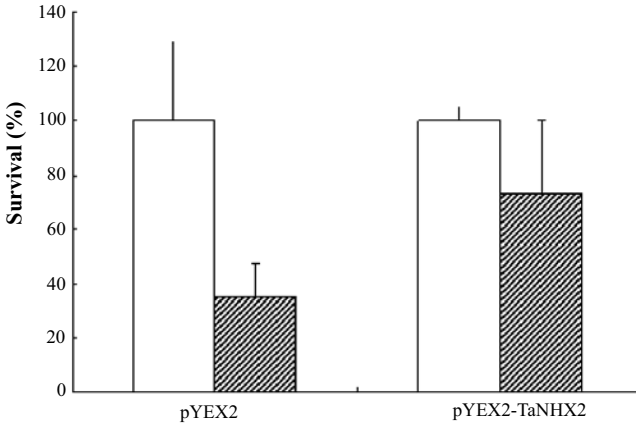

Figure 5. Freeze tolerance of yeast cells overexpressing *TaNHX2*. Transformants W303-pYES2 (pYEX2) and W303-pYES2–TaNHX2 (pYEX2–TaNHX2) were subjected to freezing at –20°C for 24 h, and then spread onto the YPGAL medium. *pYEX2* and *pYEX2–TaNHX2* colonies that appeared on YPGAL plates after –20°C treatment for 0 h and 24 h were counted. Data from three independent experiments were averaged. Survival rates of the cells are shown. Open columns indicate survival rate (100%) under normal conditions. Striped columns indicate survival rate after freezing treatment.

3.5 TaNHX2 *improved freezing tolerance of yeast*

Another abiotic stress that results in a decrease in available cellular water in the cell is freezing (Imai *et al* 1996). We were interested in testing whether overexpression of the *TaNHX2* gene could alter freeze tolerance in the yeast cell. Exponentially growing transformants were frozen rapidly and kept at –20°C for 24 h. The thawed cells were diluted and spread onto the YPGAL plates for two days and cultured at 30°C. The colonies on the plates were counted. As shown in figure 5, the transformant with pYES2 had a survival rate of 35.2±1.2% after treatment at –20°C for 24 h, while pYES2–TaNHX2 cells exhibited a survival rate of 73.3±5.0% under the same conditions compared with the non-treatment control.

4. Discussion

Plants have developed mechanisms to limit Na^+ uptake and increase Na^+ exclusion, or to sequester Na^+ into vacuoles for their survival under salt stress (Amtmann and Sanders 1999). Among them, Na^+/H^+ antiporter genes are considered to play an important role in the regulation of internal pH, cell volume and sodium level in the cytoplasm (Mahnensmith and Aronson 1985; Rausch *et al* 1996). In particular, vacuolar Na^+/H^+ antiporters, which sequester Na^+ in vacuoles to maintain low cytosolic concentrations of Na^+, have been investigated as the key to salt tolerance in the yeast and plants (Blumwald *et al* 2000). In the present study, a novel vacuole Na^+/H^+ antiporter gene *TaNHX2* was cloned from wheat and studied further.

The deduced TaNHX2 protein sequence possesses 10 membrane-spanning domains. It also has an amiloride-binding domain. The transmembrane domain is highly conserved relative to other NHX, whereas the N-terminal (1–18) and C-terminal (538–553) sequences are highly divergent. The amino acid residues 450–498 are the most variable regions (figure1A). Comparison of the amino acid sequence with other NHX proteins indicates that TaNHX2 shares a higher identity with OsNHX1 from rice (Fukuda *et al* 1999) and HvNHX1 from barley (Fukuda *et al* 2004) but lower identity with AtNHX1 from *Arabidopsis* (Gaxiola *et al* 1999), AgNHX1 from *Atriplex gmelini* (Hamada *et al* 2001) and TaNHX1 from wheat (Wang *et al* 2002). These data allowed us to classify TaNHX2 as a new member of the vacuole Na^+/H^+ antiporter family and to suggest that it might have a similar function as HvNHX1 and OsNHX1.

Most Na^+/H^+ antiporter genes are induced by salt stress (Fukuda *et al* 1999; Gaxiola *et al* 1999; Hamada *et al* 2001; Wang *et al* 2003). The expression of *TaNHX2* is also induced by NaCl treatment (figure 2A), suggesting that the function of *TaNHX2* might be related to salt tolerance in wheat. *TaNHX2* might also function in maintaining the ion balance even under normal conditions because *TaNHX2* expression is observed both in roots and leaves without treatment. However, transcript levels of *TaNHX2* in roots are induced to a higher level and maintained for a longer time compared with those in leaves by salinity stress, suggesting that TaNHX2 might play a more important role in roots than in leaves.

ABA is known to be involved in responses to various environmental stresses in plants, and many stress-responsive genes are induced by exogenous ABA treatment. Expression of the *TaNHX2* gene is also induced by ABA treatment, and the transcript levels of *TaNHX2* in roots are higher than those in shoots (figure 2B, C). It is possible that the roots absorbed more ABA than the leaves did, thus activating the expression of the *TaNHX2* gene. The transcription of *TaNHX2* is also induced by osmotic and cold treatments in both roots and shoots (figure 2B, C). This suggests that *TaNHX2* may have a role to play in the response to various abiotic stresses and ABA treatment.

The function of the *TaNHX2* gene was analysed using an yeast *Δnhx1* mutant. The *TaNHX2* gene suppresses the sensitivity of the *Δnhx1* mutant to NaCl, LiCl and hygromycin (figure 3A, 3B). Thus, *TaNHX2* has the ability to compensate partially for the function of yeast NHX1. This result is consistent with the report that *AgNHX1* from *A. gmelini* and *OsNHX1* from rice could partially recover the growth of the yeast *Δnhx1* mutant (Hamada *et al* 2001; Fukuda *et al* 2004). Therefore, *TaNHX2* has a function similar to that of *AgNHX1* and *OsNHX1*. In addition, *TaNHX2* was expressed in *S. cerevisiae* W303 and the growth rates of the transformants were examined under ionic, osmotic and cold stresses. The result of RNA gel-blot analysis showed that there was no endogenous homologue of *TaNHX2* in yeast cells (figure 4). We found that the growth rate of the pYES2–TaNHX2 cells was higher than that of transformants with pYES2 at high concentrations of NaCl and KCl, respectively (data not shown). A similar result has been reported by others – that *AtNHX1* can catalyse K^+ transport – by antiporter activity analysis in plants (Zhang and Blumwald 2001; Venema *et al* 2002). Ohnishi (2005) discovered that the Na^+/H^+ antiporter genes *InNHX1* and *InNHX2* can catalyse both Na^+ and K^+ transport into vacuoles in the Japanese morning glory. It is possible that TaNHX2 also plays a role in the tolerance of cells by transfering Na^+ or K^+ into vacuoles under high Na^+ or K^+ treatments.

The compartmentalization of Na^+ into vacuoles by Na^+/H^+ antiporters not only provides an efficient mechanism to avert the deleterious effects of Na^+ in the cytosol, but also allows plants to use NaCl as an osmoticum to maintain an osmotic potential (Blumwald *et al* 2000). In the present study, TaNHX2 has obvious roles in protecting *S. cerevisiae* cells during sorbitol stress (data not shown) and increasing the numbers of colonies that survive under freezing treatment (figure 5). These findings imply that TaNHX2 has the ability to improve the tolerance of yeast cells by transfering Na^+ or K^+ or other ions from the cytoplasm to vacuoles as an osmoticum under osmotic and freezing stress. We believe that TaNHX2 may also play an important role in maintaining the ion homeostasis of cells in the plant. Further studies will be done to more fully determine the function of TaNHX2 in transgenic plants.

Acknowledgements

We thank Professor Xuecheng Wang for kindly providing two yeast strains, W303 and *Δnhx1*::W303. This work was supported by National Basic Research Project (2006CB100102), Chinese Academic of Sciences ((KSCXZ-SW-327) and National 863 program (2001AA212121).

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MS received 11 December 2006; accepted 15 May 2007

*e*Publication: 30 July 2007

Corresponding editor: Man Mohan Johri