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Full Length Research Paper

Isolation and characterization of a vacuolar Na^+/H^+ antiporter gene from *Cucumis melo* L.

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We isolated a vacuolar Na^+/H^+ antiporter gene (*CmNHX1*) from melon using the rapid amplification of cDNA ends approach. Sequence analysis indicated that the full-length cDNA of *CmNHX1* was 2534 bp, including an open reading frame (ORF) of 1659 bp, which encoded a deduced polypeptide of 553 amino acids. The deduced protein contained conserved structural domains and shared a high degree of homology with putative vacuolar Na^+/H^+ antiporters from other higher plants. Subcellular localization revealed that this protein located to the vacuolar membrane. These results indicated that, *CmNHX1* is a vacuolar Na^+/H^+ antiporter. As shown by RT-PCR, expression of *CmNHX1* was detected in roots, stems and leaves. In addition, the expression increased in the root but decreased in the leaves with increasing NaCl concentration. Similarly, expression increased with increasing time in plants treated with 100 mM NaCl. Expression of *CmNHX1* in ATX3 yeast Na^+/H^+ antiporter mutants showed functional complementation. No differences in yeast cell growth were detected in the presence or absence of *CmNHX1* on a NaCl-free medium. However, control yeast growth was noticeably suppressed on medium containing NaCl, whereas, yeasts overexpressing *CmNHX1* showed increased population growth rates. These results indicate that the *CmNHX1* protein enhanced AXT3 salt tolerance.

Keywords: Melon (*Cucumis melo* L.), Na^+/H^+ antiporter, salt tolerance, vacuolar membrane, yeast.

INTRODUCTION

Soil salinity is one of the major factors that reduce plant growth and productivity in agricultural production. Sodium ions are the major toxic ions in saline soils and an excess of sodium ions is harmful to plants due to its negative effects on enzyme activities, photosynthesis and metabolism (Niu et al., 1995). To cope with salt stress, plants have evolved a variety of adaptation mechanisms, such as restricting the uptake of environmental Na^+, increasing the efflux of Na^+ from the cell and sequestering Na^+ into the large intracellular vacuole to reduce Na^+ accumulation. Compartmentation of Na^+ into vacuoles can be accomplished by the action of Na^+/H^+ antiporters in the vacuolar membranes, which catalyze the exchange of Na^+ for H^+ across tonoplast membranes under a proton electrochemical gradient generated by vacuolar H-ATPase and H-PPiase (Wyn Jones and Pollard, 1983; Blumwald et al., 2000). Thus, the Na^+/H^+ antiporter play a major role in improving the salt tolerance capacity of most plant species.

A vacuolar Na^+/H^+ antiporter was cloned from *Arabidopsis* as the first plant homologue of a vacuolar Na^+/H^+ exchanger in yeast (Gaxiola et al., 1999). Subsequently, a series of Na^+/H^+ antiporter coding genes have been identified and cloned from *Oryza sativa* (Fukuda et al., 1999), *Atriplex gmelini* (Hamada et al., 2001), *Beta vulgaris* (Xia et al., 2002), *Brassica napus* (Wang et al., 2003), *Gossypium hirsutum* (Wu et al. 2004), *Rosa hybrida* (Kagami and Suzuki, 2005), *Hordeum brevisubulatum* (Lü et al., 2005), *Aeluropus littoralis* (Zhang et al., 2008) and also from a halophytic plant (Hamada et al., 2001) and a salt-tolerant grass (Qiao et al., 2007; Rajagopal et al., 2007; Tang et al.,

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Abbreviations: CFP, Cyan fluorescent protein; **NHX,** vacuolar Na^+/H^+ antiporter; **ORF,** open-reading frame; **RACE,** rapid amplification of cDNA ends; **UPM,** universal primer mixture; **YPG,** yeast extract/peptone/galactose.

2010). However, to date, there has been limited research on horticultural plants, particularly melon.

Melon is an important horticultural crop, which is often cultivated in arid and semiarid regions of the world where salinity threatens to become, or already is, a problem (Navarro et al., 1999). Previous studies have shown that between fruit development and harvest, melon is salt tolerant, whereas, it is sensitive during the germination and seedling growth stages (Nukaya et al., 1984; Franco et al., 1993). In general, melon is known as a moderately salt-tolerant crop. It has been determined that salinity causes several types of damage, including growth inhibition (Franco et al., 1993; 1997; Mendlinger and Fossen, 1993; Carvajal et al., 1998), metabolic disturbances (Mavrogianopoulos et al., 1999; del Amor et al., 2000) and yield and quality losses (Mendlinger, 1994; Meiri et al., 1995; del Amor et al., 2000). Many efforts have been made to improve the salt tolerance of crops by traditional breeding programs; however, commercial success has been very limited (Santa-Cruz et al., 2002). Recently, attempts have been directed toward the genetic transformation of melons. However, more studies are needed to evaluate the key genes involved in melon salt tolerance. Therefore, it is very important to identify and clone a Na^+/H^+ antiporter gene in melon and to investigate its role in salt tolerance, thereby, paving the way to improvements in melon salt resistance by means of genetic engineering.

MATERIALS AND METHODS

Plant materials

Seeds of melon (*Cucumis melo* L. var. reticulatus Naud.), cultivar Chunli, were germinated and grown in perlite in an artificial climate chamber (16 h photoperiod, 25°C) until the cotyledons were fully expanded (approx. 10 days). Under the same growth conditions, the seedlings were then, transferred to a standard 1/2 Hoagland solution and grown until the first pair of true leaves was fully expanded (approx. 2 weeks). For salt treatments, the melon plants (approx. 2 weeks old) were randomly selected and then, grown in different 1/2 Hoagland solutions (pH 6.5 to 6.8) containing the indicated concentrations of NaCl (25, 50, and 100 mM), respectively. Treated plants, together with untreated control plants grown in parallel under the same conditions, were sampled as scheduled. The nutrient solutions were renewed at the time of sampling.

Cloning of the Na^+/H^+ antiporter cDNA

Total RNA (5 µg) was isolated using Trizol Reagent (Invitrogen, USA) from the roots of melon seedlings grown for 6 h in 1/2 Hoagland culture solution containing 200 mM NaCl. First-strand cDNA synthesis and RT-PCR were performed using a one step RNA PCR kit (Takara). A degenerate sense primer, 5′–GTGG (G/T)ATTGT(G/A/C)ATGTC(A/G)CATTA–3′, and an antisense primer, 5′–CAT(G/A/C)AG(A/C)CCAG(A/C)CCACCA(A/T)AT–3′, were designed based on the conserved regions of the Na^++/H^+ antiporter amino acid sequences from other plants. PCR amplification was performed for 35 cycles (94°C for 45 s, 56°C for 30 s and 72°C for 1 min) followed by a final extension step of 8 min at 72°C. The amplified PCR product was gel purified, cloned into a pGEM-T easy plasmid vector (Promega, USA) and sequenced using an ABI 377 sequencer (PerkinElmer, USA). A fragment of 332 bp was amplified from the root cDNA. The blasting of the fragment sequence in GenBank showed that this fragment shares high similarity with the sequences of other vacuolar antiporter genes.

In order to isolate the 3′ and 5′ regions of this gene, we used the RACE (Rapid Amplification of cDNA Ends) approach. For 3′ RACE, first-strand 3′-ready cDNA was synthesized according to the manual of the SMARTTM RACE cDNA amplification kit (Clontech, USA). 3′ RACE was performed using the 3′-ready cDNA as a template, the universal primer mix (UPM, provided in the kit) as the reverse primer and CMF1 (5′–AATACTGTTGGGATTGGTTC–3′) as the forward primer. A nested-PCR was then performed, with a nested universal primer (NUP, provided in the kit) as the reverse primer and n-CMF1 (5′–ACCAAGAAGTCCTCGCATGA–3′) as the forward primer. The PCR conditions were as follows: denaturation of the cDNA at 94°C for 3 min, 35 cycles of amplification (94°C for 45 s, 56°C for 45 s, 72°C for 90 s) and final extension at 72°C for 8 min. For the subsequent nested-PCR, the same PCR conditions were used as described earlier, except that the annealing temperature was decreased to 60°C. For 5′ RACE, first-strand 5′-ready cDNA was synthesized according to the kit manual using the 5′-RACE CDS Primer (provided in the kit). On the basis of the nucleotide sequences, we obtained the gene-specific reverse primer CMR2 (5′–GCTCTTCCAACCAGAACCAATCCCAAC–3′) and the nested gene-specific reverse primer n-CMR2 (5′–CAGCGACAAATGAAA GGGTGGCAAA–3′) were designed and synthesized. 5′ RACE-PCR was performed using the UPM as the forward primer under the following conditions: denaturation of the cDNA at 94°C for 3 min, 35 cycles of amplification (94°C for 45 s, 59°C for 45 s, 72°C for 2 min) and an extension step of 8 min at 72°C. Nested-PCR was subsequently performed with the NUP as the forward primer and n-CMR2 as the reverse primer using the following conditions; denaturation of the template at 94°C for 3 min, 35 cycles of amplification (94°C for 45 s, 60°C for 45 s, 72°C for 2 min) and an extension step of 8 min at 72°C.

The amplified fragments were sequenced and assembled to deduce the full length of the CmNHX1 cDNA. The full-length cDNA of M-GS2 was amplified with the 5'-ready cDNA as the template and CMF (5′–AACGCACGAACAGTCTCA–3′) and CMR (5′–CAACCCTCTATCAAACAA–3′) as gene specific primers, using the following conditions: denaturation of the cDNA at 94°C for 3 min, 35 cycles (94°C for 30 s, 56°C for 30 s, 72°C for 2 min), and an extension step of 10 min at 72°C.

Construction of plasmid pA7–CFP–*CmNHX1*

Plasmid pA7–CFP–*CmNHX1* driven by the CaMV (cauliflower mosaic virus) 35S promoter was constructed to investigate the subcellular localization of *CmNHX1* in onion epidermal cells. The encoded region of *CmNHX1* cDNA was amplified using the primer pair 5′–GTCGACATGGCGCCTGAAATGGTGGTCGAATCGGGCT TGTCGATGG–3′ and 5′–GACTAGTCTCTTCGAGTTTGTATAA GAATGGTTTTATTTTCATCAATGG–3′. Two restriction enzyme cleavage sites, *Sal*I and *Spe*I, were respectively contained within the primers, such that they could be fused in-frame to the C-terminal end of the CFP coding sequence. The pA7–CFP–*CmNHX1* was then, transformed into the epidermis cells of onion (*Allium cepa*), which had been maintained in the water for 3 days, using a PDS-1000/He particle gun (Bio-Rad). The onion epidermal cells were washed with 0.12 mol/L PIPES (pH = 7) and were visualized under a confocal microscope (Leica TCS SP5, Olympus).

Expression analysis of *CmNHX1*

Similar size cutting seedlings (2 weeks old) were transplanted into

individual pots filled with 1/2 Hoagland culture solution. We then, used two salt treatment protocols: (1) the seedlings were treated with 1/2 Hoagland culture solution containing 100 mM NaCl for 0, 3, and 6 h in a chamber; (2) the seedlings were treated with solutions containing 0, 25, 50, and 100 mM NaCl for 12 h.

Total RNAs separately extracted from the roots, stems and young leaves of plants were reverse transcribed as described earlier. We used the RT-PCR technique to examine the gene expression of *CmNHX1*. Amplification reactions were carried out using the gene-specific primers CMF3 (5′–ACTCTACTTTGGCAG ACATT–3′) and CMR3 (5′–CTGACCTGGTGAACTGATT–3′). As an endogenous control, we used ACTF (5′–GTGACAATGGAACT GGAATGG–3′) and ACTR (5′–AGACGGAGGATAGCGTGAGG–3′), the gene-specific primers for *M-actin* (Deng et al., 2010), the house keeping gene in melons. The RT-PCR conditions were as follows: 94°C for 3 min, 27 cycles (94°C for 30 s, 57°C for 30 s and 72°C for 1 min) and 72°C for 8 min. The PCR products (6 µl) were separated on 1% (w/v) agarose gels stained with ethidium bromide (10 mg/ml) and the quantity of products was analyzed with the gene analysis software package (Gene Company).

Expression of *CmNHX1* in yeast

For functional complementation analysis, *CmNHX1* cDNA was subcloned into the pYES2 yeast expression vector. The plasmids were transformed into the yeast strain AXT3 using an electro-transformation protocol. This AXT3 strain lacks the original Na^+/H^+ antiporter in the plasma membrane (NHA1) and the vacuolar membrane (NHX1) and also lacks the P-type ATPase involved in Na^+ exclusion (ENA1-4) (Quintero et al., 2000).

For growth tests, the yeast cells were grown on agar medium and in liquid YPG medium containing the indicated concentrations of NaCl and LiCl. According to the protocol of Yokoi et al. (2002), saturated yeast cultures were diluted to an absorbance of 0.8 at 600 nm with YPG solution. Aliquots (2 µl) aliquots of the OD 600 = 0.8 yeast cultures or tenfold serial dilutions were spotted onto different YPG plates and grown at 30°C for 48 h. Further, a 10 µl OD 600 = 0.8 culture was inoculated into 10 ml YPG medium, with or without 50 and 200 mM NaCl and the absorbance at 600 nm was measured after shaking at 30°C for 48 h.

RESULTS AND DISCUSSION

Characterization of *CmNHX1* cDNA and sequence analysis

The full-length cDNA of *CmNHX1* (accession no: FJ84-3078) was obtained using the RT-PCR and RACE methods. The 2534 bp cDNA of *CmNHX1* contained a 5′-untranslated region (UTR) of 322 bp and a 3′-UTR of 553 bp, including a putative poly (A) addition signal site (AATAAT) located 205 bp upstream of the tail of adenosine residues. The open reading frame (ORF) of 1659 bp encoded a protein of 553 amino acids with a theoretical molecular mass of 61 kDa and a theoretical pI of 6.96. Nucleotide sequence analysis of this cDNA revealed that, it corresponded to a Na^+/H^+ antiporter full-length cDNA and was thus, designated as *CmNHX1*. A hydropathy plot generated using the TMPRED program (http://www.ch.embnet.org/software/TMPRED_form.html) indicated that, *CmNHX1* consisted of 10 putative hydrophobic regions, which were different to the nine transmembrane domains in *AtNHX1* and *OsNHX1* (Fukuda et al., 2004b; Yamaguchi et al., 2003). As shown in Figure 1, the transmembrane domains of *CmNHX1* are highly conserved with those of other plants and the third transmembrane domain contains a putative amiloride binding domain (FFIYLLPPII) (Orlowski and Grinstein, 1997; Wu et al., 2004) that is common to vacuolar Na^+/H^+ antiporters. Further, there are regions of low conservation located in the N terminus (aa1~aa27) and C terminus (aa447~aa553).

The results of a BLAST search revealed a high degree of homology between the amino acid sequences of *CmNHX1* and the putative vacuolar Na^+/H^+ antiporters of other higher plants (Figure 2).The highest identity was found to be 77% similarity with *Vitis vinifera*. According to a phylogenetic analysis, the Na^+/H^+ exchanger family can be categorized into two distinct subgroups corresponding to plasma membrane and vacuolar transporters (Brett et al., 2005). The two subgroups are distinct from each other with respect to homology and functional properties. *CmNHX1* also shows a high degree of homology with the vacuolar antiporter proteins of monocot plants (*OsNHX1* in rice (73%)) and dicot plants (*AtNHX1* in *A. thaliana* (72%)) (Apes et al., 1999; Fukuda et al., 1999). However, *CmNHX1* shows considerably less homology with the plasma membrane Na^+/H^+ antiporter *AtSOS1* in *Arabidopsis* (10.34%) (Shi et al. 2000) and *OsSOS1* in rice (8.14%) (Martinez et al., 2007). On the basis of an amino acid sequence alignment, a phylogenetic tree of antiporter proteins was constructed using the MEGA 4.1 software program. The dendrogram confirmed that, the *CmNHX1* protein was closer to the vacuolar antiporters than to the plasma-membrane antiporters (Figure 2). *CmNHX1* forms a clade with the most closely related plant (*RhNHX1*) homolog, suggesting that *CmNHX1* is evolutionarily closer to dicot plants than to monocot plants.

In order to further determine the subcellular localization of *CmNHX1*, we fused the cDNA to the C terminus of CFP. In the control, the CFP was distributed throughout the cell (Figure 3a). As shown in Figure 3 b and c, the fluorescence was clearly observed on the vacuolar membrane in a transient transfection assay. It is although, very difficult to distinguish the vacuolar membrane and plasma membrane in mature onion epidermal cells. On the basis of the sequence alignment, phylogenetic analysis and subcellular localization of *CmNHX1*, we suggested that *CmNHX1* might be a vacuolar Na^+/H^+ antiporter.

Expression pattern of *CmNHX1* under salt stress

Previous studies have found that Na^+/H^+ accumulate in the root, shoot, leaf and flower tissues of *Arabidopsis* (Apes et al., 1998), wheat (Wang et al., 2002; Brini et al., 2005) and cotton (Wu et al., 2004) under conditions of salt stress. However, different results were obtained for *Agropyron elongatum*, in which *AeNHX1* is exclusively

Figure 1. Amino acid sequence analysis of Na^+/H^+ antiporters. *CmNHX1* is shown aligned to putative proteins from *Arabidopsis* (*AtNHX1*) and rice (*OsNHX1*). Dark letters reflect 100% sequence conservation.

expressed in the root tissues and is not induced by salt stress (Qiao et al., 2007). Previous reports have indicated that the expression of plant vacuolar Na^+/H^+ antiporters is discrepant in various tissues. Our results showed that, *CmNHX1* mRNA accumulates in root, shoot and leaf tissues and that transcript levels of *CmNHX1* are not induced by NaCl (Figure 4a,b). The expression of *CmNHX1* increased in roots and decreased in leaves when plants were subjected to 25, 50, and 100 mM NaCl for 12 h and the amounts of mRNA in roots was two times that in leaves under 100 mM NaCl treatment. Different results were obtained for rice, where expression of *OsNHX1* increased slightly in roots but markedly in leaves when plants were treated with 50 to 200 mM NaCl for 24 h (Fukuda et al., 2004a). In addition, a higher expression of *CmNHX1* in roots and a lower expression in leaves were observed under high NaCl concentrations. The transcript levels of *CmNHX1* varied slightly but were generally stable in shoots under salt stress. When subjected to different NaCl concentrations for 12 h, the higher the NaCl concentration, the greater the expression of *CmNHX1* in roots and the lower the expression in leaves. Similarly, higher expression of *CmNHX1* in roots and lower expression in leaves was observed with increasing time. Expression of *CmNHX1* peaked after 6 h of NaCl treatment in the roots; however, at this time point, expression in the shoot and leaf tissues was at its lowest (Figure 4d).

It has been found that a number of genes involved in stress tolerance are constitutively expressed with stress induction in *Arabidopsis* (Taji et al., 2004) or without stress induction in *A. elongatum* (Qiao et al., 2007). These results imply that stress inducible signaling path-ways are active in stress-tolerant plants under both stress and non-stress conditions. However, the results we obtained indicated an increasing expression of *CmNHX1* in roots with increasing time and NaCl

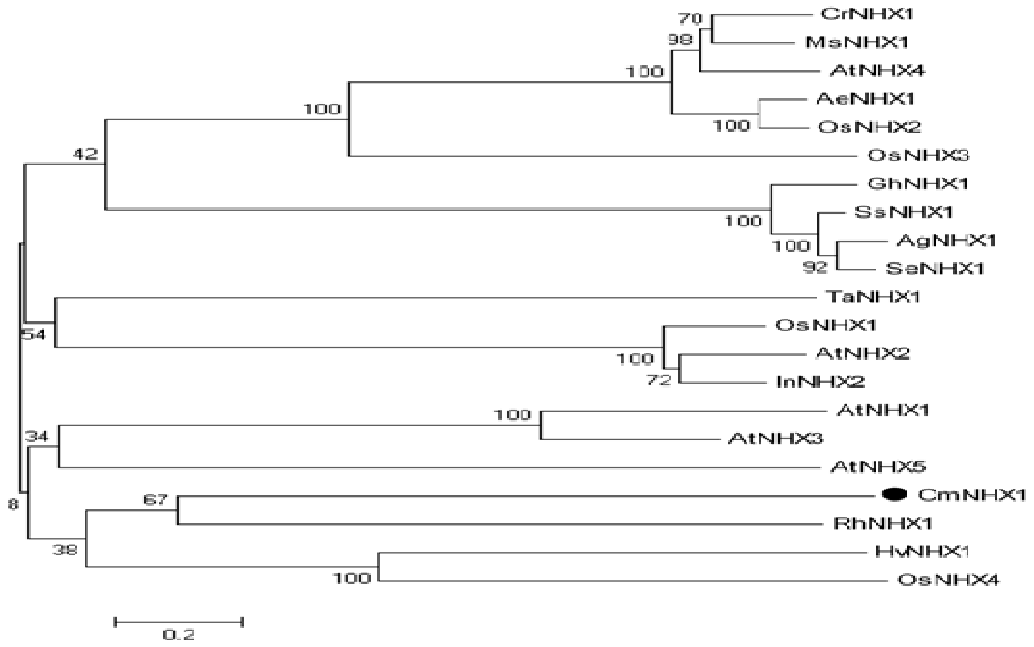

Figure 2. A phylogenetic tree of putative Na^+/H^+ antiporter proteins. *CmNHX1* (ACO90356, boxed) from *Cucumis melo* L; *TaNHX1* (AAS17949) from wheat, *HvNHX1* (AAS17948) from barley; *GhNHX1* (AAM54141) from cotton; *OsNHX1* (BAA83337), *OsNHX2* (AAQ63678), *OsNHX3* (ABA95118) and *OsNHX4* (BAD61599) from rice; *AtNHX1* (AAD16946), *AtNHX2* (AAG51408), *AtNHX3* (NP200358), *AtNHX4* (NP187288), *AtNHX5* (AAD25617), from *Arabidopsis*. *SsNHX1* (AAK53432) from *Suaeda salsa*; *CrNHX1* (AAT36679) from citrus reticulate; *MsNHX1* (AAS84487) from *Medicago sativa*; *AeNHX1* (AAQ07963) from *Agropyron; AgNHX1* (BAB11940) from *A. gmelini*; *SeNHX1* (AAN08157) from *Salicornia europaea*, *InNHX2* (BAD91201) from *Pomoea nil*; *RhNHX1* (BAD93487) from *R. hybrid*. The phylogentic tree was generated by using MEGA 4.1.

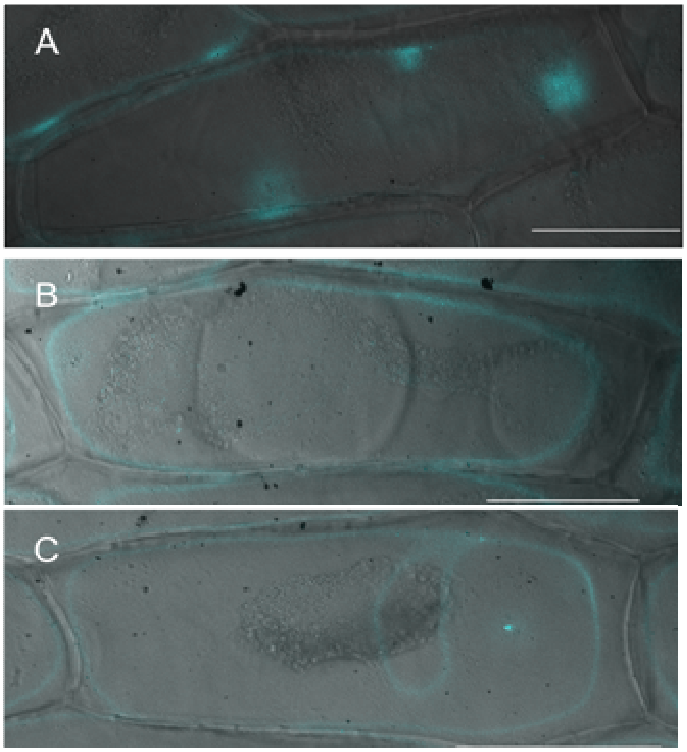

Figure 3. Subcellular localization of *CmNHX1* fused with CFP. Images of plant expression vectors including pA7–X–CFP (control) (A), pA7–CFP–*CmNHX1* (B), and (C) in onion epidermal cells, respectively. Scale bar 75 µm.

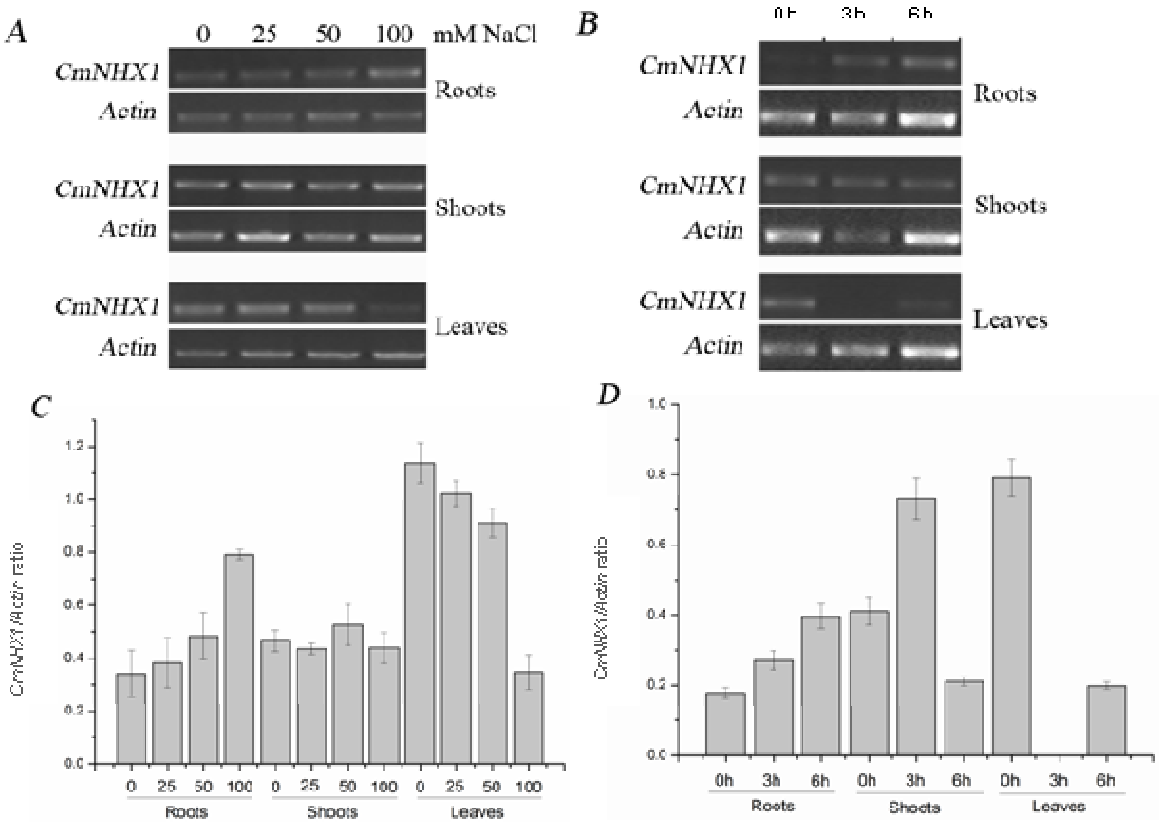

Figure 4. RT-PCR analysis of the *CmNHX1* genes from melon. A, RNA was extracted from the roots, shoots and leaves of plants after exposure to 0, 25, 50 and 100 mM NaCl for 12 h and exposure at 100 mM NaCl for 0, 3 and 6 h in chamber (B). The ratio of *CmNHX1* expression level to the *Actin* expression level in the roots, shoots and leaves were calculated by band intensities quantified with animage analyzer (C and D).

concentration. Thus, expression of *CmNHX1* in roots under salt stress might accumulate more Na^+ in the root vacuoles and this might result in a higher stress tolerance in melon and other salt-tolerant dicot plants.

Yeast complementation studies

In order to investigate the function of the protein encoded by *CmNHX1*, we introduced and expressed *CmNHX1* cDNA in yeast cells and analyzed their growth. Yeast expressing *CmNHX1* grew much better on medium containing NaCl than did the control strain (Figure 5a). Furthermore, *CmNHX1*-transformed yeast grew better than the control strain on plates containing 25 mM LiCl. In liquid medium containing 25 mM LiCl and 100 mM NaCl, *CmNHX1*-expressing yeast also grew better than the control yeast strain (Figure 5b). All the stated results show that *CmNHX1* mediated greater Na^+/Li^+ tolerance, implying that *CmNHX1* has a strong ion transport function.

In the yeast complementation test, the expression of *CmNHX1* markedly enhanced the growth ability of the nhx1 mutants in the presence of high NaCl and LiCl concentrations. *CmNHX1* protein has 10 predicted transmembrane domains in the N-terminal region and a long tail in the C-terminal portion. C terminus in NHXs is important for the cation selectivity and tolerance of yeast cells to ions. Some study found that, the C terminus in vacuolar Na^+/H^+ antiporters could regulate antiporter cation selectivity (Yamaguchi et al., 2003). In additon, partial truncation of the C terminus improved the tolerance of yeast cells to Na^+, Li^+, and Rb^+ or decreased Na^+ and Li^+ export activity, suggesting that the long cytoplasmic tail functions as a regulatory domain (Kinclova et al., 2001). Previous studies have demonstrated the importance of the C terminus. Several non-homologous regions, particularly in the C-terminal (528 to 553) region it have been observed in *CmNHX1*. This might explain the cation selectivity of *CmNHX1*. Accordingly, more studies should be conducted in the future in order to gain a better understanding of the Na^+/H^+ antiporters in melon.

Conclusions

A full-length cDNA Na^+/H^+ antiporter gene (*CmNHX1*) was isolated from melon. The cDNA was 2534 bp in

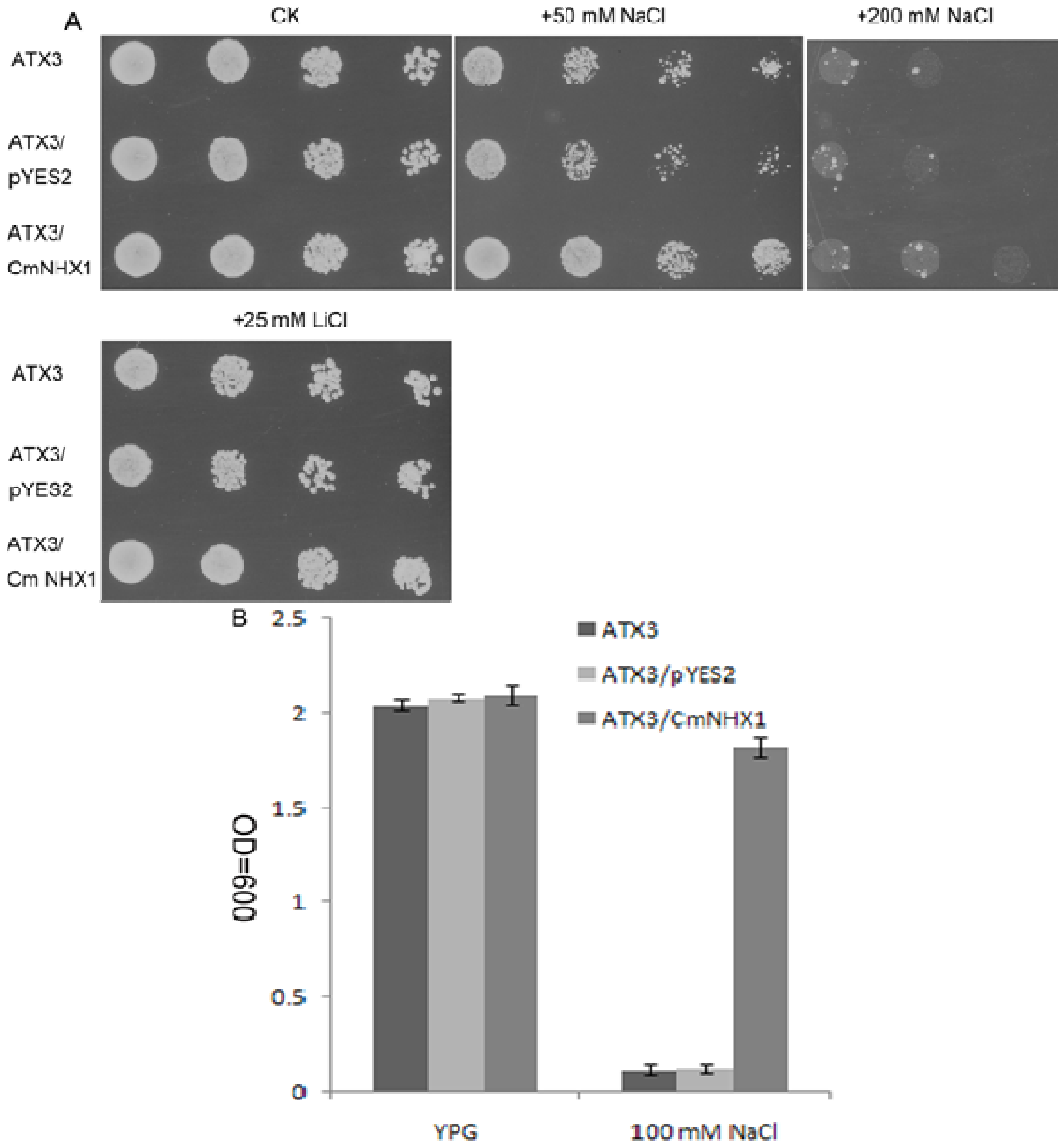

Figure 5. Expression of *CmNHX1* in the yeast nhx1 mutant suppresses salt-phenotypes. A,*CmNHX1* was subcloned into the yeast expression vector pYES2 and transformed into strain AXT3, the non-transformed and the empty pYES2 vector transformed mutant as control. A, 2 µl saturated yeast cultures or tenfold serial dilution were spotted onto YPG, and YPG containing 50 and 100 mM NaCl and 25 and 50 mM LiCl. B, the absorbance at 600 nm of each kind of cells is shown after shaking 24 h. The cells were grown in liquid YPG with or without 50 mM LiCl or 100 mM NaCl. Values are the average of three replicate samples.

in length, including an open reading frame (ORF) of 1659 bp, which encoded a predicted polypeptide of 553 amino acids. The *CmNHX1* protein shares high identity with other reported plant vacuolar Na^+/H^+ antiporters and is localized in the vacuolar membrane. Expression of *CmNHX1* is detected in roots, stems and leaves. In addition, the expression of *CmNHX1* in yeast cells markedly enhances salt tolerance in the salt-sensitive AXT3 mutant.

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