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Molecular cloning and functional characterization of *MdNHX1* reveals its involvement in salt tolerance in apple calli and *Arabidopsis*

Mei-Hong Sun, Qi-Jun Ma, Xiao Liu, Xiao-Ping Zhu, Da-Gang Hu*, Yu-Jin Hao*

State Key Laboratory of Crop Biology, National Research Center for Apple Engineering and Technology, Shandong Agricultural University, Tai'an, Shandong 271018, PR China

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ABSTRACT

Soil salinity is a major limited factor for apple production in saline-alkali land worldwide. Previous studies reported that sodium/proton exchangers (NHXs) play important roles in salt tolerance. Here, we isolated *MdNHX1*, a vacuole-localized Na^+/H^+ exchanger, from 'Gala' apple (*Malus* × *domestica* Borkh.). Ectopic expression of *MdNHX1* in yeast mutant strain *YDR456W* subsequently rescued the sensitivity of salt stress. qPCR assay demonstrated that *MdNHX1* was mainly expressed in the stems and flowers of apple; and it can be induced by NaCl and PEG, but not by KCl and mannitol. Finally, we found that overexpression of *MdNHX1* significantly improved salt tolerance in apple calli and *Arabidopsis*. In a word, our findings provide converging evidence that *MdNHX1* is involved in the regulation of salt tolerance in plants.

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1. Introduction

Soil salinity has severely detrimental effects on agricultural productivity, including water deficit resulting in osmotic stress, excessive accumulation of sodium ions and generation of reactive oxygen species; it significantly threats sustainable agriculture worldwide (Yadav et al., 2011; Isaienkov, 2012). To protect plants against salt stress, plants have developed a series of defense mechanisms (Shinozaki, 1999). For example, plants have evolved to use ions to adjust osmotic potential and to internally transport these ions outside the cytosol (Guo et al., 2001; Yadav et al., 2011). Plant vacuoles are effectively involved in transport and compartmentation of sodium ions into it (Munoz-Mayor et al., 2008). The vacuolar H^+-ATPase and H^+-PPase generate the electrochemical gradient of protons to drive the sodium transport (Bassil and Blumwald, 2014). In addition, the vacuolar transporters, Na^+/H^+ antiporters, play a decisive role in uptaking and distribution of Na^+ to maintain ion homeostasis in plant cells (Bassil et al., 2012).

In plants, the well-studied Na^+-H^+ exchanger (NHX) family belongs to CPA1 (Cation/proton Antiporter 1) subfamily (Ye et al., 2013). CPA members are thought to fall into two major families named as CPA1 and CPA2 (Chanroj et al., 2012). The CPA1 family is conserved in different species including bacteria, fungi, plants and animals, and contains multiple isoforms apart from the yeast *Saccharomyces Cerevisiae* (Yamaguchi et al., 2013). CPA1 superfamily is reported to regulate cation and pH homeostasis by exchanging Na^+, Li^+, or K^+ for H^+, and is also involved in the regulation of cell volume, expansion and differentiation (Cellier et al., 2004; Yamaguchi et al., 2013; Ye et al., 2013; Ashnest et al., 2015; Czerny et al., 2016).

NHX-type exchangers have particularly important role in the regulation of stress response (Reguera et al., 2014). For example, plasma membrane (PM)-localized SOS1-type antiporters enable them to sequestration of sodium ions outside the plant cells at the expense of the H^+-motive force driven by proton pumps (Shi et al., 2002; Shi et al., 2003; Katiyar-Agarwal et al., 2006; Olias et al., 2009); while vacuole-localized NHX1-type transporters transport sodium ions into the vacuolar compartments to avert ion toxicity in the cytosol and to adjust the cellular osmotic balance (Tester and Davenport, 2003; Leidi et al., 2010). The *Salt Overly Sensitive* (SOS) pathway was an classic pathway to regulate ion homeostasis and

Abbreviations: CPA1, Cation/proton Antiporter 1; MDA, Malondialdehyde; NHX, Na^+-H^+ exchanger; ORF, Open reading frame; PCR, Polymerase chain reaction; PBS, Phosphate buffered saline; SOS, *Salt Overly Sensitive*; TBA, Thiobarbituric acid; UV-2450, UV–vis spectrophotometer; WT, Wild Type.

* Corresponding authors.
E-mail addresses: fap_296566@163.com (D.-G. Hu), haoyujin@sdau.edu.cn (Y.-J. Hao).

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improve salt tolerance (Yang et al., 2009; Ji et al., 2013). The Ca^{2+} sensor SOS3 perceives cytosolic Ca^{2+} signal stimuli by high extracellular concentrations of salt, and interacts with and activates the protein kinase SOS2, which then targets and regulates the activities of ion transporters including the Na^+/H^+ antiporter SOS1, the vacuolar Na^+/H^+ exchangers NHXs, and the K^+ transporter HKT1 or transcriptionally regulates ion homeostasis or gene expression (Qiu et al., 2002; Zhu et al., 2007b; Mahajan et al., 2008; Huang et al., 2012).

The first biochemical characterization of cation/H^+ exchanger was reported in red beet tonoplast vesicles (Rea and Poole, 1985). Since then, with the completion of the plant genome sequencing, *NHXs* were subsequently cloned and found to be involved in salt tolerance in diverse species (Zorb et al., 2005; Rodriguez-Rosales et al., 2008; Ye et al., 2009; Li et al., 2010; Fukuda et al., 2011; Xu et al., 2013).For instance, Overexpression of *AtNHX1* significantly improved its salt tolerance in *Arabidopsis* (Chen et al., 2008; Asif et al., 2011; Zhou et al., 2011). The ion transport activity of vacuolar Na^+/H^+ exchanger *AtNHX1* can be regulated indirectly by the SOS pathway and directly by interacting with the calmodulin-like protein CaM15 (Yamaguchi et al., 2005). Remarkably, eight isoforms exist in *Arabidopsis* including AtNHX1 to AtNHX8. Among them, AtNHX1 to AtNHX6 are intracellular isoforms that located either in vacuoles or vesicles; while SOS1/AtNHX7 and AtNHX8 are two divergent members located in the PM (Yokoi et al., 2002; Asif et al., 2011; Qiu, 2016). Highly similar orthologues with *Arabidopsis* NHXs are found in different plant genomes, indicating that NHXs are ubiquitous and conserved during evolutionary process (Bassil and Blumwald, 2014).

Apple is one of the most important fruit tree in the world (Velasco et al., 2010; Zhang et al., 2013). As a non-halophyte, its growth is severely inhibited by salt stress. In this study, we isolated *MdNHX1* from apple (*Malus* × *domestica* Borkh.), and found that *MdNHX1* is mainly expressed in stems and flowers, and also significantly induced by NaCl and PEG treatment; *MdNHX1* restores the salt sensitivity phenotype in yeast *nha1* mutant. Overexpression of *MdNHX1* significantly enhanced its tolerance to salt stress in *Arabidopsis* and apple calli. Finally, the potential application of the *MdNHX1* gene in the genetic improvement of salt resistance is discussed.

2. Materials and methods

2.1. Plant materials, growth conditions and stress treatments

'Gala' apple (*Malus* × *domestica* Borkh.) was used for assay. The cultures of the 'Gala' apple were treated with NaCl, KCl, PEG, and mannitol at 25 °C under a maximum photoperiod of approximately 600 mmol m^{-2} s^{-1}. Samples collected at different time were used for analyzing the relative expression level of *MdNHX1*. Untreated sample was used as a control.

The apple calli used in this study were induced from the young embryos of 'Orin' apple (*Malus domestica* Borkh.). They were grown on MS medium supplemented with 0.5 mg L^{-1} indole-3-acetic acid (IAA) and 1.5 mg L^{-1} 6-benzylaminopurine (6-BA) at 25 °C in the dark. The calli were subcultured three times at 15-day intervals before being used for genetic transformation and in other assays. For salt stress, the apple calli were cultivated on MS medium supplemented with 0.5 mg L^{-1} indole-3-acetic acid (IAA), 1.5 mg L^{-1} 6-benzylaminopurine (6-BA) and 100 mM NaCl.

For the stress tolerance assay of *Arabidopsis*, firstly 7 days transgenic plants germinated on MS medium. Then transgenic plants were transferred to MS medium with 50, 100, 200 mM NaCl, respectively. Each treatment was obtained from three independent repeats.

2.2. Genetic transformation of *MdNHX1* into apple calli and Arabidopsis

The sequence of *MdNHX1* was obtained with RT-PCRs. Then the gene *MdNHX1* was digested with BamHI/SalI. It was then cloned into the binary vector pBI121 with Myc tag under the control of the CaMV35S-promoter. The primer sequences used were listed in Table S1. The gene accession number was MDP000041327.

The 20-day calli were used for gene transformation and the stress tolerance assay. The transformation of apple calli was performed as described by Hu et al. (2016a). For *Arabidopsis* transformation, Columbia-0 (Col-0) was applied. Construct *35S:MdNHX1* was transferred into WT (Col) by a floral dip method mediated with *Agrobacterium* strain GV3101 (Clough and Bent, 1998).

2.3. qRT-PCR assays

The total RNAs were extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). One microgram of total RNAs was used to synthesize first-strand cDNA using PrimeScript™ RT reagent Kit (TaKaRa, Dalian, China). qRT-PCR was performed to detect the expression level of *MdNHX1* in apple and *Arabidopsis* by using cDNA templates. Apple *18S rRNA* and *Arabidopsis AtACTIN2* genes were used as loading controls, respectively. The results of qRT-PCR were performed by three biological repeats. The primer sequences used were listed in Table S1.

2.4. Functional expression of *MdNHX1* in yeast

The following *Saccharomyces cerevisiae* strain was used in this study: YDR456w [MAT a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0;] and BY4741 [MATa; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0]. The yeast cells were grown at 30 °C in YPD medium. Transformation of yeast cells was performed. The lithium acetate method was used. The transferred strains were grown on selective Hartwell's complete (SC-U) medium.

The *MdNHX1* gene was inserted into the yeast expression vector pYES2. All plasmids were transformed into the yeast strain YDR456w. For the stress tolerance assays, yeast cells were normalized upon the absorbance of 0.12 at a wavelength of 600 nm. 4 mL aliquots of each 10-fold serial dilution were spotted onto SC-U plates supplemented with KCl, or NaCl as indicated, and incubated at 30 °C for 3 days. And the empty vector pYES2 was transformed into the same yeast strains as a control.

2.5. Western blotting and protein extraction assays

Western blotting and protein extraction assays were conducted as described by Hu et al. (2016a).

2.6. Transient expression in protoplasts of apple calli cells and fluorescence detection

Protoplasts isolated from apple calli cells were prepared and transformed as described by Hu et al. (2016b). The fluorescence in transformed cells was detected by the confocal laser scanning microscope (Zeiss LSM 510 META). The details were as described by Hu et al. (2016b).

2.7. Determination of malondialdehyde (MDA), Na^+ and K^+ contents

The determination of malondialdehyde (MDA), Na^+ and K^+ contents were as described by Dong et al. (2011).

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