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A vacuolar Na^+/H^+ antiporter gene, *IbNHX2*, enhances salt and drought tolerance in transgenic sweetpotato

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ABSTRACT

Plant vacuolar Na^+/H^+ antiporters (NHX) play a critical role in adaption to abiotic stresses by compartmentalizing Na^+ into vacuoles from the cytosol. In this study, a vacuolar Na^+/H^+ antiporter gene, named *IbNHX2*, was isolated and characterized from salt-tolerant sweetpotato (*Ipomoea batatas* (L.) Lam.) line ND98. IbNHX2 consisted of 542 amino acid residues with a conserved binding domain 'FFIYLLPPI' for amiloride and a cation/H^+ exchanger domain, and shared a high amino acid identity (73.72–96.13%) with the identified vacuolar Na^+/H^+ antiporters in other plant species. The genomic DNA of *IbNHX2* contained 14 exons and 13 introns. Expression of *IbNHX2* was induced by abscisic acid (ABA), NaCl and polyethylene glycol (PEG). Its overexpression significantly enhanced salt and drought tolerance in the transgenic sweetpotato. An significant increase of proline content and superoxide dismutase (SOD) and photosynthesis activities and significant reduction of malonaldehyde (MDA) and H_2O_2 content were found in the transgenic sweetpotato plants. Up-regulation of the stress-responsive genes encoding pyrroline-5-carboxylate synthase (P5CS), SOD, catalase (CAT), zeaxanthinepoxidase (ZEP), 9-*cis*-epoxycarotenoid dioxygenase (NCED), aldehyde oxidase (AO), late embryogenesis abundant protein (LEA), psbA and phosphoribulokinase (PRK) in the transgenic plants was also found under salt and drought stresses. The overall results demonstrate the explicit role of *IbNHX2* in conferring salt and drought tolerance of sweetpotato. The *IbNHX2* gene has the potential to be used for improving salt and drought tolerance of plants.

1. Introduction

Soil salinity and drought reduce the agricultural productivity worldwide (Munns and Tester, 2008; Hu and Xiong, 2014). Approximately 20% of the irrigated soils worldwide are suffering salt stress (Zhao et al., 2013). The problem of the global water scarcity caused by the increasing world population and worldwide climate change threatens sustainable traditional crop farming (Yang et al., 2010). Therefore, it is extremely important to develop crops with elevated levels of salt and drought tolerance.

Plants have evolved kinds of smart and precise mechanisms, comprising growth and development regulation, detoxification, ion homeostasis and osmotic adjustment, to deal with and adapt to salt and drought stresses (Bohnert et al., 1995; Zhu, 2001). In recent years, extensive studies have focused on the mechanism of ion homeostasis in plant cells (Yamaguchi et al., 2013; Reguera et al., 2014). It has been indicated that salt-tolerant plants have ability to adopt efficient strategies, including restricting the uptake of environmental Na^+, increasing the efflux of Na^+ from the cell and compartmentalizing Na^+ into vacuoles from the cytosol, to prevent superfluous accumulation of Na^+ in cytosol, so that plant cells can sustain the ion homoestasis. These biological processes might involve Na^+/H^+ antiporter (NHX) family (Xu et al., 2009). In *Arabidopsis*, the NHX family consists of eight members, six of which are intracellular (AtNHX1 to AtNHX6), located either to the vacuole (AtNHX1 to AtNHX4) or endosomes (AtNHX5 and AtNHX6) and the additional two more divergent members (AtNHX7/SOS1 and AtNHX8) at the plasma membrane (Bassil et al., 2012; Reguera et al., 2014).

In plants, *AtNHX1* is the first characterized gene of the vacuolar NHX type (Apse et al., 1999; Gaxiola et al., 1999). Subsequently, several *AtNHX1*-like Na^+/H^+ antiporter genes have been cloned and characterized from different plant species including *Oryza sativa*, *Brassica*, *Atriplex dimorphostegia*, *Chenopodium glaucum*, *Zygophyllum xanthoxylum*, *Solanum lycopersicum*, *Vigna radiate* and *Vigna*

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unguiculata (Fukuda et al., 1999; Zhang et al., 2001; Li et al., 2008; Wu et al., 2011; Gálvez et al., 2012; Mishra et al., 2014, 2015). *NHX1* has been shown to enhance salt and drought tolerance in several plant species (Apse et al., 1999; Xu et al., 2009; Banjara et al., 2012; Yarra et al., 2012; Chen et al., 2014; Mishra et al., 2014). However, there are only a few reports on isolation and characterization of the *NHX2* gene. The results of Yokoi et al. (2002) implicated *AtNHX2* and *AtNHX5*, together with *AtNHX1*, as salt tolerance determinants, and indicated that *AtNHX2* had a major function in vacuolar compartmentalization of Na^+. Bassil et al. (2011) found that *AtNHX1* and *AtNHX2* controlled vacuolar pH and K^+ homeostasis to regulate growth, flower development and reproduction in *Arabidopsis*. *AtNHX1* and *AtNHX2* were also found to mediate active potassium uptake into vacuoles to regulate cell turgor and stomatal function in *Arabidopsis* (Barragán et al., 2012). Overexpression of *AmNHX2* from *Ammopiptanthus mongolicus* enhanced salt and drought tolerance in transgenic *Arabidopsis* (Wei et al., 2011).

Sweetpotato, *Ipomoea batatas* (L.) Lam., is an important food crop in the world and its yield is often limited by salt and drought stresses (Xiao et al., 2009; Zang et al., 2009; Liu et al., 2015). Genetic engineering has been shown to have the potential for improving the tolerance to abiotic stresses in sweetpotato. Several salt tolerance-associated genes, *IbOr*, *IbNFU1*, *IbP5CR*, *IbMas* and *IbSIMT1*, have been isolated and characterized in sweetpotato (Kim et al., 2013a; Wang et al., 2013; Liu et al., 2014a,b,c, 2015). Lu et al. (2010) found that overexpression of *IbCu/ZnSOD* and *IbAPX* improved drought tolerance of transgenic sweetpotato plants. Overexpression of *LEA14* and down-regulation of *IbLCY-ε* enhanced salt and drought tolerance in transgenic sweetpotato calluses (Park et al., 2011; Kim et al., 2013b). In this study, we isolated a new vacuolar Na^+/H^+ antiporter gene, named *IbNHX2*, from sweetpotato and found that this gene significantly enhanced salt and drought tolerance in the transgenic sweetpotato.

2. Materials and methods

2.1. Plant materials

Sweetpotato line ND98 was used for gene cloning in this study. One expressed sequence tag (EST) clone, with 33.88% homology to *AtNHX2* (AT3G05030) of *Arabidopsis*, was selected from the ND98 EST library constructed at our laboratory and used to clone the gene. The cloned gene was further introduced into ND98 to characterize its function in responses of the transgenic plants to salt and drought stresses. This study was conducted at Experimental Station for Transgenic Crops of China Agricultural University, Beijing, China.

2.2. Cloning and sequence analysis of IbNHX2

Extraction and reverse-transcription of total RNA from fresh leaves of 4-week-old in vitro-grown plants of ND98 were conducted according to the method of Liu et al. (2014c). The 5′ and 3′ ends of the coding region were amplified using the 5′ and 3′-Full RACE Kit (TaKaRa, Beijing, China). Using the Primer 3 program (http://frodo.wi.mit.edu/primer3/), primers were designed based on the sequence of EST (Table 1). PCR amplifications were performed with an initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min and final extension at 72 °C for 10 min. PCR products were separated on a 1.0% (w/v) agarose gel. Target DNA bands were recovered, then cloned into PMD19-T, and finally transformed into competent cells of *Escherichia coli* strain DH5α for sequencing as described in detail previously (Liu et al., 2014c).

Table 1
Primers used for the cloning, transformation and identification of *IbNHX2* in sweetpotato.

Primer name	Primer sequence (5′-3′)
Primers for 5′/3′ RACE	
IbNHX2 3′ RACE primer 1	TATCATTTGGTGCGGTCAAA
IbNHX2 3′ RACE primer 2	CCGATTCTGTTTGCACATTG
IbNHX2 5′ RACE primer 1	GTGCCAATAGCTCCAAACAG
IbNHX2 5′ RACE primer 2	AACTGCTTCTTTTTCACCTG
Primers for genomic DNA	
IbNHX2 GD-F	AGTGATTTTTTATGTTTTGGGGAG
IbNHX2 GD-R	AGCCAAATTGATAATTCAGTCATTA
Primers for constructing expression vector	
IbNHX2-OE-F	GCTCTAGAATGGCGTTCGGATTATCTTC
IbNHX2-OE-R	GGAGCTCTCATCTAGGGCTCTGCTCAG
Primers for identifying transformants	
35S-F	TCAGAAAGAATGCTAACCCACA
IbNHX2-R	TTCGCTAAAGACGAGAAGATGTG
Primers for Southern blotting	
IbNHX2-probe-F	GTGTTCGGGTTGATGACGC
IbNHX2-probe-R	TGCCCCTAGAGCCCATTT
Primers for real-time quantitative PCR	
Actin-F	AGCAGCATGAAGATTAAGGTTGTAGCAC
Actin-R	TGGAAAATTAGAAGCACTTCCTGTGAAC
AO-F	GTCGTTTATGCGGGCTCCT
AO-R	CCTTTTCGTCCACCGATTTT
CAT-F	ACGCAATTCCCGGACGTGAT
CAT-R	AAGCCTTCCATGTGGCGGTA
LEA-F	CCCGTCACTGGGTACTAC
LEA-R	CAAGAATCCATCATAAGC
NCED-F	AGAAGCAGGGCAAATAAACAAG
NCED-R	CCGTCGCCGTACCTAAACTC
NHX2-F	TGTTCGGGTTGATGACGC
NHX2-R	GTTGGTTGTCCAGAAGTGGC
P5CS-F	GCCTGATGCACTTGTTCAGA
P5CS-R	GCCTGATGCACTTGTTCAGA
PRK-F	GCTCTCAACATAGATCAGCT
PRK-R	TGAAGGCTCTACTATCTCAT
psbA-F	CATCCGTTGATGAATGGTTA
psbA-R	GCAACAGGAGCTGAGTATGC
SOD-F	TCCTGGACCTCATGGATTTC
SOD-R	GCCACTATGTTTCCCAGGTC
ZEP-F	CCGTCTCGGGGCAGTTAAT
ZEP-R	GACTGATTTGCGTAGTAAACATGGT

Multiple sequence alignment, phylogenetic analysis and theoretical molecular weight and isoelectric point (*p*I) calculation of IbNHX2 were conducted according to the method of Liu et al. (2014a). The conserved domain was scanned by InterPro program (http://www.ebi.ac.uk/interpro/). Transmembrane domains ™ were predicted by TMHMM Sever 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Post-translational modification sites were predicted using ScanProsite (http://www.ebi.ac.uk/Tools/pfa/ps_scan/).

Genomic DNA was extracted from fresh leaves of 4-week-old in vitro-grown plants of ND98 using EasyPure Plant Genomic DNA Kit (Transgen, Beijing, China). The corresponding fragment was amplified using primers of *IbNHX2* (Table 1). PCR amplifications were conducted with an initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 6 min and final extension at 72 °C for 10 min. Cloning and sequencing of the corresponding product was done as described above. Exon-intron structure was constructed by alignment of cDNA sequence with the genomic sequence using Spidey tool (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/) and compared with the *NHX* gene family of *Arabidopsis* (http://www.arabidopsis.org/).