Cloning and Characterization of a Novel Vacuolar Na\(^+\)/H\(^+\) Antiporter Gene (Dgnhx1) from Chrysanthemum

Qing-Lin Liu\(^{1*}\), Ke-Dong Xu\(^2\), Ming Zhong\(^1\), Yuan-Zhi Pan\(^1\), Bei-Bei Jiang\(^1\), Guang-Li Liu\(^1\), Yin Jia\(^1\)

1 Department of Ornamental Horticulture, Sichuan Agricultural University, Chengdu, China, 2 Key Lab of Plant Genetics & Molecular Breeding of Department of Life Science, Zhoukou Normal University, Zhoukou, China

Abstract

Plant vacuolar Na\(^+\)/H\(^+\) antiporter genes play significant roles in salt tolerance. However, the roles of the chrysanthemum vacuolar Na\(^+\)/H\(^+\) antiporter genes in salt stress response remain obscure. In this study, we isolated and characterized a novel vacuolar Na\(^+\)/H\(^+\) antiporter gene DgNHX1 from chrysanthemum. The DgNHX1 sequence contained 1920 bp with a complete open reading frame of 1533 bp encoding a putative protein of 510 amino acids with a predicted protein molecular weight of 56.3 kDa. DgNHX1 was predicted containing nine transmembrane domains. Its expression in the chrysanthemum was up-regulated by salt stress, but not by abscisic acid (ABA). To assess roles of DgNHX1 in plant salt stress responses, we performed gain-of-function experiment. The DgNHX1-overexpression tobacco plants showed significant salt tolerance than the wild type (WT). The transgenic lines exhibited more accumulation of Na\(^+\) and K\(^+\) under salt stress. These findings suggest that DgNHX1 plays a positive regulatory role in salt stress response.

Citation: Liu Q-L, Xu K-D, Zhong M, Pan Y-Z, Jiang B-B, et al. (2013) Cloning and Characterization of a Novel Vacuolar Na\(^+\)/H\(^+\) Antiporter Gene (Dgnhx1) from Chrysanthemum. PLoS ONE 8(12): e83702. doi:10.1371/journal.pone.0083702

Introduction

Salinity is one kind of environmental stress affecting plant growth and productivity worldwide. Plant can initiate an array of morphological, physiological, and biochemical adaptations to salt stress. In these adaptations, many salt stress-related genes are induced [1,2]. Among numerous salt-induced genes, plant vacuolar Na\(^+\)/H\(^+\) antiporter genes (NHXs) received much attention [3-7]. Plant vacuolar Na\(^+\)/H\(^+\) antiporters play important roles in maintaining cellular ion homeostasis and mediating the transport of Na\(^+\) out of the cytosol and into the vacuole [8]. To date, a number of vacuolar Na\(^+\)/H\(^+\) antiporter genes have been isolated and characterized from different plant species. Many studies have been reported to improve salt stress resistance of plants by over-expression of vacuolar Na\(^+\)/H\(^+\) antiporter genes such as AtNHX1, GmNHX1, OcNHX1, and EgNHX1 etc [3,4,6,7]. All these results suggest that vacuolar Na\(^+\)/H\(^+\) antiporter genes play significant roles in salt tolerance.

Chrysanthemum is one of the most famous ornamental species in the world and its production is severely affected by high salinity conditions in the cutting-chrysanthemum industry [9-11]. So far, no research related to the vacuolar Na\(^+\)/H\(^+\) antiporter genes in chrysanthemum is available. In an objective of improving salt tolerance in chrysanthemum, we reported the cloning and characterization of a novel vacuolar Na\(^+\)/H\(^+\) antiporter gene, DgNHX1, and showed that it was induced by salt stress. By stress assays, overexpression of DgNHX1 in tobacco plants enhanced salt tolerance.

Materials and Methods

Plant materials and stress treatments

Chrysanthemum seedlings were grown in greenhouse. For salt and ABA treatments, seedlings at the six-leaf stage were incubated in 250 mM NaCl and 100 µM ABA solution respectively. Seedlings were sampled at 0, 3, 6, 12, 24, and 48 h after treatment, and immediately stored at −80°C for RNA extraction.

Cloning of DgNHX1 gene

Based on the transcriptomic data of chrysanthemum seedlings under normal conditions and salt treatment using 454 high throughput sequencing technique, numerous salt-induced transcripts were identified. Among these, one transcript, DgNHX1, was significantly induced by salt treatment. To obtain the full-length DgNHX1 sequence, seedlings at the six-leaf stage were incubated in 250 mM NaCl for 24 h were harvested. Total RNA was extracted following the manufacturer’s instructions (Mylab, Beijing). The full-length DgNHX1 sequence was obtained by PCR and using the gene specific primers (Table S1).

Gene expression analysis

For RT-PCR analysis, total RNA was extracted from plant samples following the manufacturer’s instructions (Mylab, Beijing) and treated with RNase-free DNase I in accordance with manufacturer’s instructions (Fermentas). Total RNA (1 µg) was reverse-transcribed in a 20 µl reaction mixture using the 1 µl superscript II enzyme (Invitrogen, USA). RT-PCR was performed...
Figure 1. Nucleotide and deduced amino acid sequences of \textit{DgNHX1}. 
doi:10.1371/journal.pone.0083702.g001
for 34 and 29 amplification cycles for *DgNHX1* and *NtUbiquitin*, respectively.

Seedlings at the six-leaf stage were incubated in 250 mM NaCl or 100 μM ABA solution respectively, and the leaves were collected for measurement. Extraction of total RNAs and reverse transcription were performed as described above. Quantitative real-time PCR (qRT-PCR) analysis was performed as described previously [10,11]. Relative expression levels were calculated by the 2-ΔΔCT method, where ΔΔCT = (CT, Target-CT, actin gene) - the indicated time treatment-(CT, Target-CT, actin gene)0h treatment [12]. Three replicate biological experiments were conducted.

**Generation of DgNHX1 transgenic tobacco**

The *DgNHX1* cDNA was cloned into pBI121 (Clotech) under the control of the cauliflower mosaic virus (CaMV) 35S promoter via BamHI and SacI sites. The recombinant plasmid was introduced into tobacco through *Agrobacterium tumefaciens* strains GV3101-mediated leaf disc method [13].

**Analysis of salt tolerance in the DgNHX1 transgenic tobacco plants**

The T2 generation plants of lines OE-6, OE-9, and OE-13 were used in the subsequent experiments. For survival experiments, three-week-old seedlings of *DgNHX1* transgenic T2 lines and wild type tobacco plants were irrigated with 400 mM NaCl for 7 days. The survival rate of the seedlings as scored after a re-watering regularly as a recovery process for 6 days. Three replicate biological experiments were conducted.

**Measurement of Na\(^+\) and K\(^+\) contents**

Three-week-old WT seedlings and T2 seedlings of *DgNHX1* transgenic tobacco plants lines OE-6, OE-9, and OE-13 were exposed to 400 mM NaCl for 96 h, and the leaves were harvested and then dried at 80°C for 24 h. The Na\(^+\) and K\(^+\) contents of the leaves were measured using atomic absorption spectrophotometry.

**Results**

**Cloning and sequence analysis of DgNHX1**

The *DgNHX1* sequence contained 1920 bp with a complete open reading frame of 1533 bp encoding a putative protein of 510 amino acids with a predicted protein molecular weight of 56.3 kDa (Figure 1). Sequence comparison by DNAMAN (Version 6.0) revealed that DgNHX1 shared high homology with other vacuolar Na\(^+\)/H\(^+\) antiporter proteins, such as OsNHX1 (60.56%), GhNHX1 (58.97%), AtNHX1 (58.44%), and AtNHX2 (58.67%), but it shared less homology with the plasma Na\(^+\)/H\(^+\) antiporter AtSOS1 (10.55%). The hydropathy plot generated by the SOSUI program revealed that DgNHX1 contained nine transmembrane domains, and the third transmembrane domain contained a putative amiloride-binding domain LFFITYLPPI.

**Figure 2. Amino acid sequences of DgNHX1 and other vacuolar Na\(^+\)/H\(^+\) antiporter proteins from selected plant species.** Alignments were performed using DNAMAN (version 6.0). Amino acid residues conserved in all four sequences were shaded in black, and those conserved in three sequences were shaded in light grey. The 9 transmembrane domains (labeled as I–IX) of DgNHX1 were indicated by lines above the sequences. doi:10.1371/journal.pone.0083702.g002
The phylogenetic analysis showed that DgNHX1 belongs to the vacuolar Na\(^+\)/H\(^+\) antiporter proteins and is more closely related to OsNHX1, and GhNHX1 (Figure 3).

Expression pattern of DgNHX1 in different tissues and response to salt stress

The tissue specificity of DgNHX1 transcript accumulation was examined by qRT-PCR method. As showed in Figure 4A, DgNHX1 was expressed strongly in seedling leaves, weakly in seedling roots, and seedling stems under non-stressed conditions.

The expression levels of DgNHX1 increased significantly under salt stress, but were not activated by ABA (Figure 4B and C). Under salt stress, DgNHX1 transcript increased gradually up to 24 h after NaCl treatment, and thereafter decreased slightly (Figure 4B).

Overexpression of DgNHX1 enhanced tolerance to salt stress

In order to analysis the function of DgNHX1, an overexpressing construct under the control of the CaMV 35S promoter, was transformed into tobacco plants. Among 22 lines of transformants, five independent transgenic lines (OE-4, OE-6, OE-9, OE-11, and OE-13) were confirmed by using RT-PCR analysis (Figure 5A). The T2 generation plants of lines OE-6, OE-9, and OE-13 were used in the subsequent experiments. Under normal growth conditions, no obvious differences were detected between the DgNHX1-overexpression and wild-type (WT) tobacco plants (Figure 5B). In the salt tolerance assay, three-week-old transgenic lines and WT were irrigated with 400 mM NaCl for 7 days. It was observed that the WT were more wilted than transgenic seedlings.
(OE-6, OE-9, and OE-13) (Figure 6A). After 6 days of recovery from salt stress, the three transgenic lines (OE-6, OE-9, and OE-13) showed significantly higher survival rates of 76%, 80%, and 75%, respectively, as compared to that of WT plants (28%) (Figure 6B).

Figure 6. Analysis of salt tolerance in DgNHX1-overexpression transgenic tobacco plants (OE-6, OE-9, and OE-13). (A) The seedlings in WT and DgNHX1-OE transgenic T2 lines were irrigated with 400 mM NaCl for 7 days to assess physical symptoms. (B) Survival rates of seedling in wild type and DgNHX1-OE transgenic T2 lines after 6 days recovery. About 100 seedlings were used for each treatment. Different letters above columns indicate (P<0.05) significant differences according to Duncan’s multiple range test between lines.

doi:10.1371/journal.pone.0083702.g006

**Analysis of accumulation of Na^+ and K^+ in DgNHX1 transformed tobacco plants under salt stress**

To investigate if overexpression of DgNHX1 enhanced the Na^+ and K^+ accumulation in tobacco, the Na^+ and K^+ contents were measured. Under normal conditions, there was no significant difference in the Na^+ and K^+ contents between WT and three
transgenic lines (Figure 7). Upon exposure to salt stress, there was a marked increase in Na\(^+\) content for both WT and the transgenic lines (Figure 7A). However, the accumulation of Na\(^+\) content was significantly higher in the three transgenic lines than WT in response to salt stress. In contrast, there was a marked decrease in K\(^+\) content for both WT and the transgenic lines, and the reduction of K\(^+\) content was significantly less in the three transgenic lines than WT in response to salt stress (Figure 7B).

**Discussion**

Plant vacuolar Na\(^+\)/H\(^+\) antiporter genes have been shown to play significant roles in salt tolerance [8]. A vacuolar Na\(^+\)/H\(^+\) antiporter gene termed DgNHX1 from chrysanthemum was cloned and characterized in this study. Sequence analysis showed that it contained nine transmembrane domains. The DgNHX1 was structurally similar to OsNHX1, which was isolated from *Oryza sativa* under salt stress [6], and GhNHX1, which was isolated from *Gossypium hirsutum* under salt stress [7]. These results indicate that DgNHX1 is a novel member of the vacuolar Na\(^+\)/H\(^+\) antiporter genes.

The phylogenetic analysis showed that DgNHX1 belongs to the vacuolar Na\(^+\)/H\(^+\) antiporter proteins, which consists of several well-characterized vacuolar Na\(^+\)/H\(^+\) antiporter genes, including OsNHX1, GbNHX1, and AtNHX1. In *Oryza sativa*, overexpression of OsNHX1 also enhanced the tolerance to salt stress in transgenic lines [6]. Overexpression of another salt-induced vacuolar Na\(^+\)/H\(^+\) antiporter protein gene, GbNHX1, has been reported to confer salt tolerance in tobacco plants [7]. In addition, overexpression AtNHX1 improved salt tolerance in many plants [3,14,15]. Transcript levels of DgNHX1 were increased by salt stress, and the 35S:DgNHX1 transgenic tobacco exhibited a markedly increased tolerance to salt. These results suggest that DgNHX1 might be involved in salt tolerance.

The putative protein encoded by the DgNHX1 gene shares 58.44% homology with AtNHX1, which may perform the similar function in salt tolerance improvement of the plants used. To overcome the issue of heterologous genetic transformation, DgNHX1 might be better than AtNHX1 for application in genetic engineering strategies aimed at improving salt stress tolerance in chrysanthemum.

DgNHX1-overexpression tobacco plants conferred salt tolerance and have no difference in phenotypes during all life cycles between the DgNHX1-overexpression and WT tobacco plants under normal conditions. It indicated that DgNHX1 might be a potentially excellent genetic resource for the improvement of salt tolerance in chrysanthemum.

Under salt stress, DgNHX1-overexpression plants accumulate more Na\(^+\) and K\(^+\), as compared to that of WT plants. The increased accumulation of Na\(^+\) and K\(^+\) may be correlated with an increase activity of the vacuolar Na\(^+\)/H\(^+\) antiporter, which plays important roles in the compartmentation of Na\(^+\) and K\(^+\) highly accumulated in the cytoplasm into the vacuoles [8,16]. Similar observations were reported for other vacuolar Na\(^+\)/H\(^+\) antiporter genes involved in salt stress, such as AtNHX1 and HcNHX1 [3,17]. These results indicate that DgNHX1 enhanced the accumulation of Na\(^+\) and K\(^+\) and resulted in the increased tolerance to salt stress.

ABA plays a crucial role in the adaptive response of plants to salt stress [18]. In this study, expression of DgNHX1 was not responsive to ABA treatment. This result suggests that DgNHX1 might be involved in an ABA-independent salt stress-responsive signal pathway.

In conclusion, this study cloned and characterized a vacuolar Na\(^+\)/H\(^+\) antiporter gene, DgNHX1, which was induced by salt stress, was isolated from chrysanthemum. DgNHX1-overexpression tobacco plants enhanced the accumulation of Na\(^+\) and K\(^+\) and resulted in the increased tolerance to salt stress. Therefore, DgNHX1 provides a promising tool for improving salt tolerance in chrysanthemum.

**Supporting Information**

Table S1 The primers used in the present study. (DOC)
Author Contributions
Conceived and designed the experiments: QLL, KDX. Performed the experiments: QLL, KDX, MZ. Analyzed the data: MZ, YZP, BBJ. Contributed reagents/materials/analysis tools: GLL, YJ. Wrote the paper: QLL, KDX.

References
1. Agarwal PK, Agarwal P, Reddy MK, Sopory SK (2006) Roles of DREB transcription factors in abiotic and biotic stress tolerance in plants. Plant Cell Rep 25: 1263–1274.
2. Zhu JK (2001) Cell signalingunder salt, water and cold stresses. Curr Opin Plant Biol 4: 401–406.
3. Asif MA, Zafar Y, Iqbal J, Iqbal MM, Rashid U, et al. (2011) Enhanced expression of AtNHX1, in transgenic groundnut (Arachis hypogaea L) improves salt and drought tolerance. Mol Biotechnol 49: 250–256.
4. Baltierra F, Castillo M, Gamboa MC, Rothhammer M, Krauskopf E (2013) Molecular characterization of a novel Na+/H+ antiporter cDNA from Eucalyptus globules. Biochem Bioph Res Co 430: 535–540.
5. Li C, Wei ZW, Liang D, Zhou SS, Li YH, et al. (2013) Enhanced salt resistance in apple plants overexpressing a Malus vacuolar Na+/H+ antiporter gene is associated with differences in stomatal behavior and photosynthesis. Plant Physiol Biochem 70: 164–173.
6. Fukuda A, Nakamura A, Tagiri A, Tanaka H, Miyao A, et al. (2004) Function, intracellular localization and the importance in salt tolerance of a vacuolar Na+/H+ antiporter from rice. Plant Cell Physiol 45: 146–159.
7. Wu CA, Yang GD, Meng QW, Zheng CC (2004) The cotton GhNHX1 gene encoding a novel putative tonoplast Na+/H+ antiporter plays an important role in salt stress. Plant Physiol 135: 600–607.
8. Wu K, Zhang H, Blumwald E, Xia T (2010) A novel plant vacuolar Na+/H+ antiporter gene evolved by DNA shuffling confers improved salt tolerance in yeast. J Biol Chem 285: 22999–23006.
9. Liu QL, Xu KD, Zhao LJ, Pan YZ, Jiang BB, et al. (2011) Overexpression of a novel chrysanthemum NAC transcription factor gene enhances salt tolerance in tobacco Biotechnol Lett 33: 2073–2082.
10. Liu QL, Zhong M, Li S, Pan YZ, Jiang BB, et al. (2013) Overexpression of a chrysanthemum transcription factor gene, DgWRKY3, in tobacco enhances salt tolerance to salt stress. Plant Physiol Biochem 69: 27–33.
11. Liu QL, Xu KD, Pan YZ, Jiang BB, Liu GL, et al. (2013) Functional analysis of a novel chrysanthemum WRKY transcription factor gene involved in salt tolerance. Plant Mol Biol Rep DOI 10.1007/s11105-013-0639-3.
12. Livak K, Schmittgen T (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-(Delta Delta C(T)) method. Methods 25: 402–408.
13. An G, Watson BD, Chang CC (1988) Transformation of tobacco, tomato, potato, and Arabidopsis thaliana using a binary Ti vector system. Plant Physiol 81: 301–305.
14. Chen LH, Zhang B, Xu ZQ (2008) Salt tolerance conferred by overexpression of Arabidopsis vacuolar Na+/H+ antiporter gene AtNHX1 in common buckwheat (Fagopyrum esculentum) Transgenic Res 17: 121–132.
15. Zhou SF, Zhang ZM, Tang QL, Lan H, Li YN, et al. (2011) Enhanced V-ATPase activity contributes to the improved salt tolerance of transgenic tobacco plants overexpressing vacuolar Na+/H+ antiporter AtNHX1. Biotechnol Lett 33: 375–380.
16. Rodriguez-Rosales MP, Jiang X, Galvez JF, Aranda MN, Cabero B, et al. (2008) Overexpression of the tomato K+/H+ antiporter LeNHX2 confers salt tolerance by improving potassium compartmentalization. New Phytol 179: 366–377.
17. Guan B, Hu YZ, Zeng YL, Wang Y, Zhang FC (2011) Molecular characterization and functional analysis of a vacuolar Na+/H+ antiporter gene (HcNHX1) from Halostachys capicata. Mol Biol Rep 38: 1809–1899.
18. Xiang Y, Tang N, Du H, Ye HY, Xiong LZ (2008) Characterization of OsBZIP23 as a key player of the basic leucine zipper transcription factor family for conferring abscisic acid sensitivity and salinity and drought tolerance in rice. Plant Physiol 148: 1938–1952.