Plant Gene and Trait 2022, Vol.13, No.2, 1-9
http://genbreedpublisher.com/index.php/pgt

Research Article Open Access

Isolation and Expression Analysis of *BvNHX1* from *Beta vulgaris* with High Sucrose

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Plant Gene and Trait, 2022, Vol.13, No.2 doi: 10.5376/pgt.2022.13.0002
Received: 12 Apr., 2022
Accepted: 21 Apr., 2022
Published: 30 May, 2022

Preferred citation for this article:
Li N.N., Sun Y.Q., and Li G.L., 2022, Isolation and expression analysis of *BvNHX1* from *Beta vulgaris* with high sucrose, Plant Gene and Trait, 13(2): 1-9 (doi: 10.5376/pgt.2022.13.0002)

Abstract In present study, the vacuolar Na^+/H^+ exchanger gene was isolated by homologous cloning technology from the high-sucrose *Beta vulgaris* ('BS02'), referred as *BvNHX1*, which contained an ORF of 1 659 bp, encoded 552 amino acids, the protein molecular weight was 61.31 kD, and the theoretical isoelectric point was 6.31. The protein encoded by *BvNHX1* gene had 12 transmembrane domains and the conserved domains of Nhap, Na_H_Exchanger and b_cpa1 superfamily, and grouped with various NHXs of Chenopodiaceae plants, such as *Salicornia europaea*, *Atriplex dimorphostegia*, *Suaeda salsa*, and belonged to Class I in the vacuolar Na^+/H^+ exchanger family. Under 400 mmol/L NaCl, 200 mmol/L KCl and 15 mmol/L ABA, the expression of *BvNHX1* reached the peaks in leaves and roots, respectively, and the expression of *BvNHX1* in leaves was significantly higher than that in roots, indicating that the expression of *BvNHX1* was induced by NaCl, KCl and ABA, and it may play a greater role in leaves than roots in response to abiotic stresses. This study will lay a foundation for the study of the salt tolerance molecular mechanism in *Beta vulgaris* with high sucrose, and provide a solid and reliable basis for the genetic improvement of salt tolerance in *Beta vulgaris* with high sucrose.

Keywords *Beta vulgaris* L.; *BvNHX1*; Gene cloning; Gene expression; Salt stress

Land salinization is one of the main abiotic factors affecting crop growth and yield. About 36.90×10^6 hm^2 of salinized land is mainly distributed in arid and semi-arid areas of Northeast, North and Northwest of China, among which 6.24×10^6 hm^2 of cultivated land is seriously affected by salinization. Moreover, due to unreasonable irrigation and surface evaporation, a large amount of salt is retained in the soil surface. This causes the increase of salinized arable land area in China year by year, eventually resulting in crop yield reduction of about 10%~50% (Zhang et al., 2017). Therefore, rational utilization of salinized land resources is particularly important. Salinization environment often causes crops to suffer from ion stress, osmotic stress and oxidative stress, resulting in crop yield reduction. In order to cope with the external salinization environment, plants have evolved a series of salt-tolerant mechanisms, including ion transport mechanism, osmotic regulation mechanism and reactive oxygen scavenging mechanism (Yigit et al., 2020). At present, studies on the mechanism of ion transport are in-depth, in which Na^+ and K^+ related transporters and channel proteins on plasma membrane and vacuole membrane act synergically to maintain ion homeostasis in crops under salt stress (Almeida et al., 2017), and genetic engineering technology is used to cultivate salt-tolerant crop varieties. This will lay a foundation for rational development and utilization of salinized land resources.

Vacuolar Na^+/H^+ exchanger is widely present in the plant kingdom and is involved in regulating many reactions in plant cells. For example, intracellular pH regulation, leaf development, flower development and coloring, cell enlargement, vesicle transport, signal transduction, and Na^+ and K^+ region isolation into vacuoles (Bassil et al., 2011a; Chanroj et al., 2012; Li et al., 2017). The first clone of *NHX* gene was from *Arabidopsis thaliana* and was named *AtNHX1*. It has been cloned in many crops since then such as cotton (*Gossypium hirsutum*) *GhNHX1* (Wu et al., 2004), maize (*Zea mays*) *ZmNHX* (Zörb et al., 2005), wheat (*Triticum aestivum*) *TaNHX1-2* (Yu et al., 2007), soybean (*Glycine max*) *GmNHX1* (Li et al., 2006), and these genes encode roughly 470~556 amino acids, with the

molecular weight of 47~179 kD or so, and these proteins have about 10~12 transmembrane domains. The research also showed that the expression characteristics of six members of Arabidopsis *NHX* family are different. *AtNHX1-2* was mainly expressed in roots, stems, leaves and flowers. *AtNHX3* was mainly expressed in flowers and *AtNHX4* was mainly expressed in roots. *AtNHX5-6* was slightly expressed in all tissues (Bassil et al., 2011b). The transcriptional level of *AtNHX1* gene in *Arabidopsis thaliana* was significantly upregulated under NaCl, KCl and ABA treatments, indicating that the expression of *AtNHX1* is regulated at the transcriptional level, and this regulation depends on ABA signal transduction pathway (Yokoi et al., 2002). At present, many studies also show that NHXs can insulate the excess intracellular Na^+ and K^+ regions into vacuoles, maintain the homeostasis balance of Na^+/K^+ ions in plants, improve the antioxidant and osmotic regulation ability of plants, and then improve the salt tolerance of plants. Therefore, this gene can be used as an important candidate gene for genetic improvement of crop salt tolerance.

Beta vulgaris L. belongs to *Beta* genus in the family of Chenopodium, mainly distributed in arid and semi-arid areas in Northwest, Northeast and North of China. It is one of the important sugar crops in China and also the important advantage of crop production in Inner Mongolia Autonomous Region. Because there is a large area of salinized land in Inner Mongolia Autonomous Region, if *Beta vulgaris* can be planted on the salinized land, it can not only effectively use the large area of salinized soil, but also improve the yield and quality of *Beta vulgaris* L.. Therefore, it is very important to study the molecular mechanism of *Beta vulgaris* L. salt tolerance and to breed high sucrose *B. vulgaris* with high salt tolerance. At present, most studies on salt tolerance of *Beta vulgaris* L. remain at the growth and physiological level (Yamada et al., 2009; Lü et al., 2019), but there are few reports on molecular biology. In this study, the Na^+/H^+ exchanger gene was isolated from the high sucrose *B. vulgaris* 'BS02', which was bred by the Beet Physiology Institute of Inner Mongolia Agricultural University. Bioinformatics analysis was conducted on the gene, and the tissue expression pattern of the gene was investigated under different salt stress. These results will provide a reliable basis for the study of molecular mechanism of *Beta vulgaris* L. salt tolerance and genetic improvement of high sucrose *B. vulgaris* salt tolerance.

1 Result and Analysis

1.1 Isolation of *BvNHX1* gene

Using cDNA of high sucrose *B. vulgaris* 'BS02' as template, PCR was performed with primers *BvNHX1*-F and *BvNHX1*-R, and the cDNA sequence of 1 659 bp was obtained by electrophoresis (Figure 1A). The PCR product was recovered by agarose gel, and the section was cloned into pEASY-T1 vector by TA cloning technology, and positive clones were obtained by colony PCR identification (Figure 1B). The positive clone was expanded and cultured for sequencing identification, and the sequence was finally identified as the cDNA sequence of beet *NHX* gene and named as *BvNHX1*.

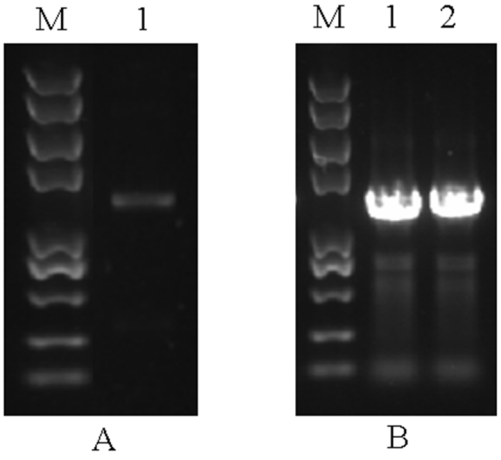

Figure 1 Cloning of *BvNHX1* genes from the high sucrose *B. vulgaris* ('BS02')

Note: M: Trans 2K Plus II DNA Marker; A: The PCR product for isolating *BvNHX1* fragment; B: Colony PCR product of *BvNHX1*

1.2 Bioinformatics analysis of *BvNHX1* gene

NCBI ORF finder software was used to analyze the sequence and it was found that the gene had 1 659 bp open reading frame encoding 552 amino acids (Figure 2). The molecular weight of the protein was 61.31 kD and the theoretical isoelectric point was 6.31. Functional domain prediction of BvNHX1 showed that there was an amiloride binding (Red box) site at 85~94 amino acids, which is a conserved sequence specific to Na^+/H^+ antiporter in plants; Conserved CaM binding sites (Blue box) were also present at amino acids 511 to 531.

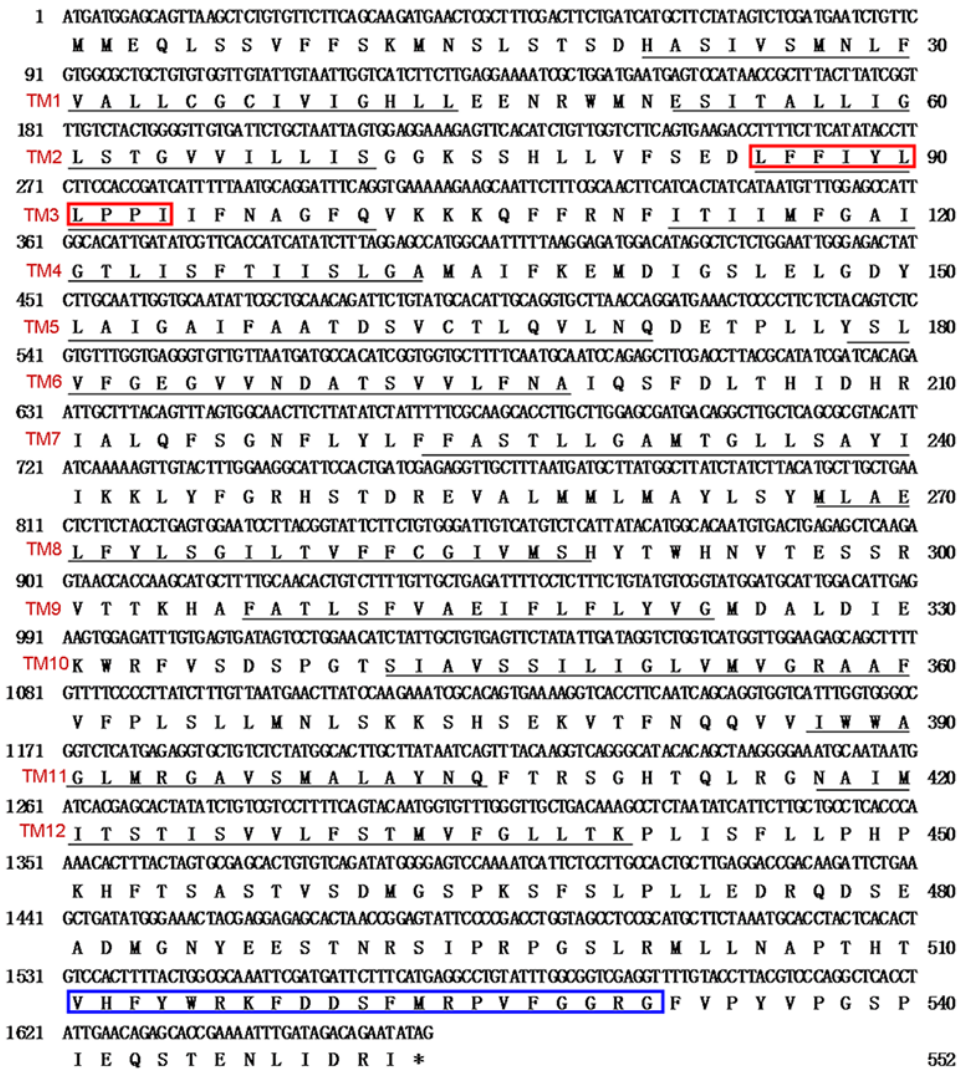

Figure 2 Alignment of cDNAs and amino acids of *BvNHX1*

Note: Red box: Amiloride binding site; Blue box: CaM binding site; Underline: The sites of the transmembrane region (TM1-12); *: The stop code

NCBI conserved domain prediction showed that the *BvNHX1* encoded protein was a member of the Na^+/H^+ exchanger family and had conserved sequence sites of Nhap, Na_H_Exchanger and b_cpa1 superfamilies (Figure 3A). The prediction of secondary structure of BvNHX1 protein showed that the α helix was about 243 aa (44.02%), the extended strand was about 93 aa (16.85%), the β turn was about 23 aa (4.17%), and the random coil was about 193 aa (34.96%). The results showed that the protein was dominated by α helix and random coil (Figure 3B). The signal peptide prediction indicates that the protein may have a 41 amino acid signal peptide present at the N-terminus of the sequence (Figure 3C). The prediction of transmembrane structure showed that the protein had 12 transmembrane domains, which were TM1~TM12 (Figure 2; Figure 3D).

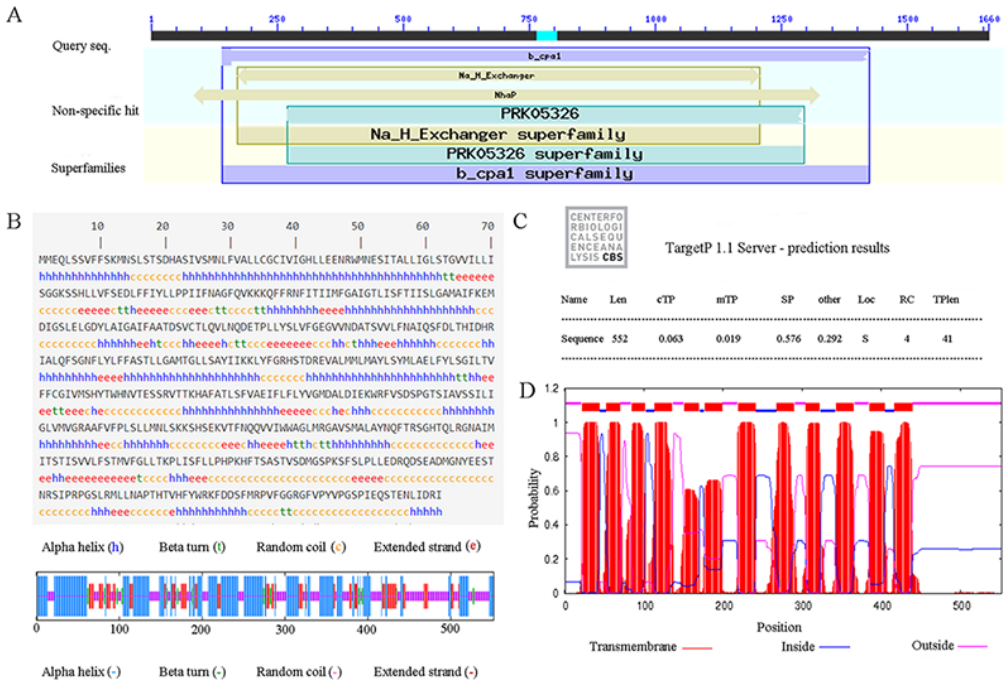

Figure 3 Bioinformatic analysis of *BvNHX1* from the high sucrose content *B. vulgaris* ('BS02')

Note: A: Conserved domain prediction; B: Secondary structure analysis; C: Signal peptide prediction; D: Transmembrane structure prediction

Based on multiple comparisons of 25 NHX amino acid sequences from 17 species, a phylogenetic tree was constructed. The results showed that Na^+/H^+ antiporter SOS1 could be clustered into one class. However, Na^+/H^+ antiporter NHXs on vacuolar membrane can be roughly divided into Class I and Class II. The protein encoded by *BvNHX1* gene of *Beta vulgaris* L. belongs to Class I, and is clustered into a group with NHXs of various Chenopodiaceae plants, such as *Salicornia salicornis*, *Ceratophora sp.* and *Salicornia salicornis*. Among them, BvNHX1 had the closest genetic relationship with NHX (Figure 4).

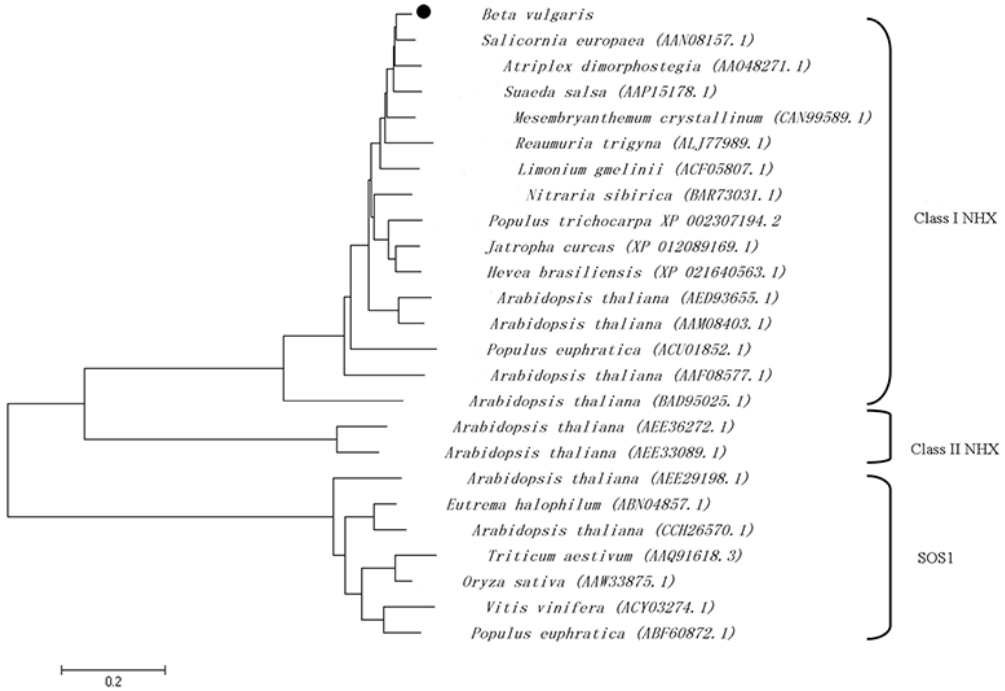

Figure 4 Neighbor joining phylogenetic tree of 25 NHX proteins from 17 species

1.3 Analysis of expression characteristics of *BvNHX1* gene

In order to investigate the expression pattern of *BvNHX1* gene under different stress conditions, beet seedlings were treated with different concentrations of NaCl, KCl and ABA at about 35 days. The relative expression levels of this gene in leaves and roots were detected by real-time quantitative PCR. The results showed that the expression level of *BvNHX1* gene in leaves was significantly higher than that in roots under the three treatment conditions (Figure 5). With the increase of NaCl concentration, the expression of *BvNHX1* gene gradually increased in both leaves and roots, reaching a peak at 400 mmol/L and then gradually decreased (Figure 5A). Under 200 mmol/L KCl stress, the expression of this gene reached the maximum in both leaves and roots, and then gradually decreased (Figure 5B). In addition, the expression level of this gene reached the maximum in both leaves and roots after 15 mmol/L ABA application, and then decreased gradually with the increase of ABA concentration (Figure 5C). These results indicated that *BvNHX1* gene could be induced by NaCl, KCl and ABA.

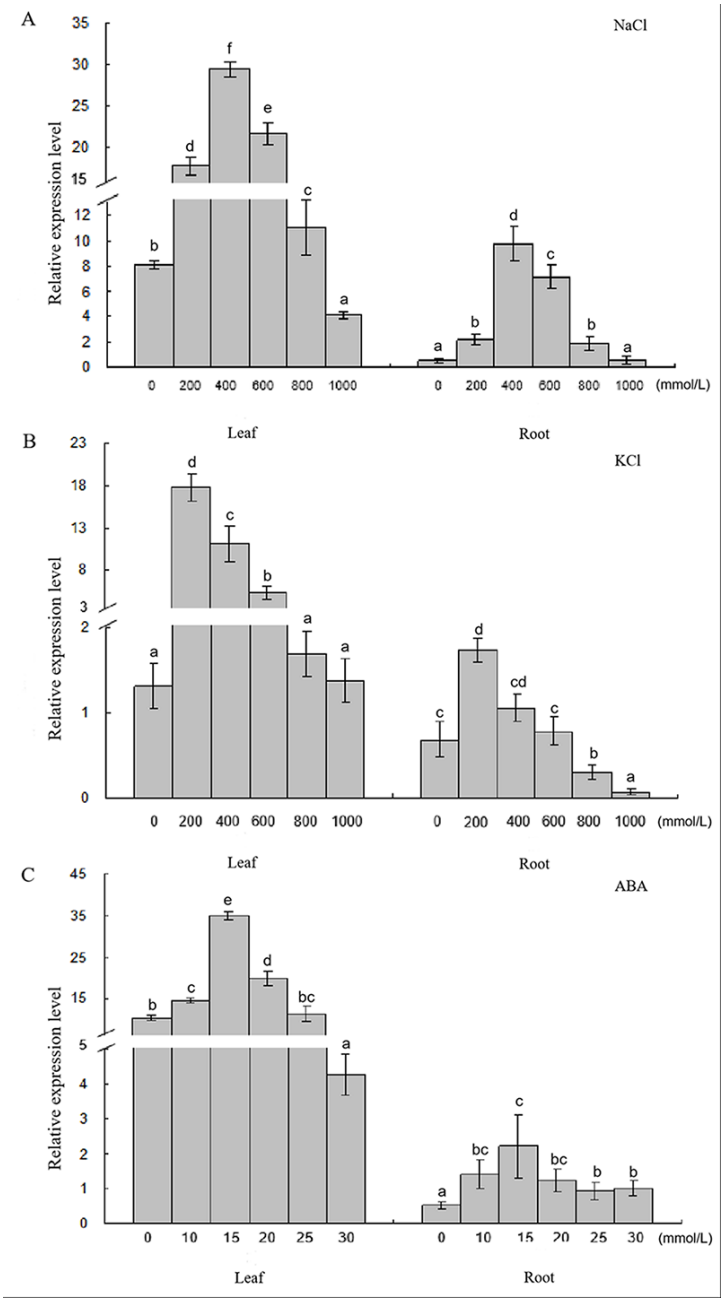

Figure 5 Expression levels of the *BvNHX1* in leaves and roots under different concentrations of NaCl (A), KCl (B) and ABA (C) conditions

2 Discussion

Plant vacuolar membrane Na^+/H^+ exchanger is a relatively large family of proteins widely distributed in a variety of plants, from flowering plants to algae (Bassil et al., 2011a; Chanroj et al., 2012). Current studies have confirmed that this protein may be involved in multiple physiological metabolic processes, including cytoplasmic pH regulation, Na^+ and K^+ compartmentalization into vacuoles, etc. (Li et al., 2017). The earliest cloned Arabidopsis *AtNHX1* was found to contain a highly conserved amiloride binding site (FF (I/L) (Y/F) LFLLPPI) at the N-terminal (Pardo et al., 2006) and a conserved action site of AtCaM15 at the C-terminal. This site plays an important role in the selective absorption of Na^+/K^+ (Yamaguchi et al., 2005), and these two conserved structural sites also exist in the beet BvNHX1 sequence. The secondary structure of *RtNHX1* encoded amino acids of *Reaumuria trigyna* is dominated by α helix and random coil, and has 12 transmembrane domains and 41 amino acid signal peptides (Li et al., 2017). These results were also found in the BvNHX1 sequence of beet. This suggested that the gene belonged to a member of the vacuolar membrane Na^+/H^+ antiporter family.

At present, a large number of studies have shown that the *NHXs* gene in plants can be up-regulated by high-salt environment and abscisic acid. For example, the transcription level of *Nitraria sibialis NsNHX1* gene was significantly increased under 200 mmol/L NaCl and 100 μmol/L ABA treatment (Wang et al., 2015). The expression level of *RtNHX1* gene was the highest at 200 mmol/L NaCl treatment for 3 h, while the transcription level reached the peak at 100 μmol/L ABA treatment for 6 h (Li et al., 2017). Under NaCl, KCl and ABA treatment, *AtNHX1* gene expression level was significantly induced, and its promoter activity was significantly increased. In addition, the expression level of *AtNHX1* gene was significantly decreased in ABA mutants (ABA2-1, ABA3-1) treated with NaCl, indicating that the response of this gene to salt stress depends on the ABA signal transduction pathway (Yokoi et al., 2002). Similar results were also found in this study. Under NaCl, KCl and ABA conditions, *BvNHX1* gene could be significantly induced expression in *Beta vulgaris* with high sucrose. In addition, the tissue expression characteristics of *NHXs* gene in plants show different patterns with different plants. For example, *AeNHX1* is mainly expressed in roots, stems, leaves and flowers in *Arabidopsis thaliana*. Japanese morning glory *RtNHX1* (Yamaguchi et al., 2001) is mainly expressed in flowers; *AeNHX1* (Qiao et al., 2007) is only expressed in the roots of *Elytrigia elongata*; Grape *VvNHX1* (Hanana et al., 2007) was only expressed in fruit; *RtNHX1* was mainly expressed in stems before salt stress, but in roots and leaves after salt stress (Li et al., 2017). In this study, *Beta vulgaris* L. with high sucrose *BvNHX1* genes in stress before and after processing are mainly expressed in leaves, show that the genes encoding proteins may exercise its function in the blade. When plants were subjected to salt stress, the protein may be the root absorption of excess Na^+ and K^+ segregation into vacuole, thereby maintaining stability in the cell osmosis, and maintaining the normal cellular water metabolism (Leidi et al., 2010). In this study, *NHX* gene was isolated from *Beta vulgaris* L. with high sucrose variety 'BS02', and through bioinformatics analysis and expression characteristics analysis, on the one hand, it laid a certain foundation for in-depth investigation of the gene's function and molecular mechanism of salt resistance, on the other hand, it provided a valuable candidate gene for genetic improvement of salt tolerance of *Beta vulgaris* with high sucrose.

3 Materials and Methods

3.1 Experimental materials

The seeds of *Beta vulgaris* L. 'BS02' were obtained from the Beet Physiology Institute of Inner Mongolia Agricultural University. The seeds were seeded in vermiculite and incubated in a climate chamber for about 35 days after germination. Beet seedlings with the same growth were selected and treated with 0, 200 mmol/L, 400 mmol/L, 600 mmol/L, 800 mmol/L, 1 000 mmol/L NaCl and KCl stress for 7 days. After application of 0, 10 mmol/L, 15 mmol/L, 20 mmol/L, 25 mmol/L and 30 mmol/L ABA treatment for 7 days, plant materials were collected, treated with liquid nitrogen, and stored at -80℃ for later use.

RNA Extraction Kit (TransZol Plant), Reverse transcription Kit (EasyScript® First-Strand cDNA Synthesis SuperMix), PCR kit (TransStart® *Taq* DNA Polymerase), Quantitative PCR Kit (TransStart® Tip Green qPCR SuperMix), *E. coli* receptive cells (Trans-T1) and TA clone vectors (pEASY-T1) were purchased from Beijing TransGen Biotech Co., Ltd.

3.2 Gene cloning

According to NCBI database, the mRNA sequence of sodium beet hydrogen exchanger was found, and the Genbank number was XM 010674170.2. According to this gene sequence, upstream and downstream primers containing open reading frame were designed, and the primer sequence was *BvNHX1*-F: ATGATGGAGCAGTTAAGCTCTG; *BvNHX1*-R: CTAGTCCTATATTCTGTCTATC. According to the instructions of TransZol Plant kit, the total RNA of *Beta vulgaris* with high sucrose 'BS02' was extracted and obtained, and the First Strand cDNA Synthesis SuperMix kit of TransGen was used to synthesize the first strand cDNA. The *NHX* gene of *Beta vulgaris* with high sucrose was amplified by PCR using cDNA as template and *BvNHX1*-F/R as primer. The reaction system was cDNA 1.0 μL, *BvNHX1*-F/R (10 μmol/L) 1.0 μL, TransStart *Taq* DNA Polymerase 0.5 μL, 10×TransStart Buffer 5 μL, dNTP (2.5 mmol/L) 4.0 μL, sterile water 37.5 μL; The reaction conditions were 94℃ for 3 min: 94℃ for 30 s, 58℃ for 30 s, 72℃ for 90 s, with 34 cycles. 72 ℃ for 5 min. PCR products were separated by electrophoresis and the target fragment was recovered, which was cloned into T-vector pEASY-T1. The recombinant bacteria detected positive by PCR were expanded for culture and sent to Beijing BGI Biotech Co., Ltd. for sequencing.

3.3 Sequence analysis

The conserved domain of *BvNHX1* gene was analyzed by Blast X of NCBI. The basic molecular characteristics of BvNHX1 protein were predicted by online software (https://web.expasy.org/protparam/); The secondary structure of BvNHX1 protein was analyzed by online software (http://expasy.org/tools/#secondary). The subcellular localization was analyzed by online software (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc/); At the same time, the transmembrane structure region of BvNHX1 protein was predicted by software (http://www.cbs.dtu.dk/services/TMHMM/); The amino acid sequences of *BvNHX* and *NHX* genes in different plants were analyzed with the help of ClustalW software. The phylogenetic tree was analyzed by neighbor-joining (N-J) method in MEGA 7.0 software.

3.4 Real-time fluorescence quantitative PCR analysis

In order to investigate the expression characteristics of *BvNHX1* gene under NaCl, KCl and ABA treatment, seedlings growing for about 35 days were treated with different concentrations of stress, and the aboveground and underground parts were collected on the 7th day after treatment. According to the cloned *BvNHX1* gene sequence, specific fluorescent quantitative PCR primers NHX-RT-F: ATGCTTATGGCTTATCTATC; NHX-RT-R: GCTTGGTGGTTACTCTTG were designed. Beet actin gene was used as internal reference (*Actin*-RT-F: TGCTTGACTCTGGTGATGGT; *Actin*-RT-R: AGCAAGATCCAAACGGAGAATG). TransZol Plant kit was used to extract total RNA from aboveground and underground tissues and reverse transcription into cDNA, which was diluted 10 times as template. Real-time quantitative PCR was performed according to the instructions of the TransStart Tip Green qPCR SuperMix (TransGen) kit (Bio-RAD, USA). The reaction system was NHX-RT-F/R or *Actin*-RT-F/R 0.40 μL, 2×TS Tip Super Mix 10 μL, cDNA 1.0 μL, deionized water 8.2 μL. The amplification procedure was 95°C for 2 min, 95°C for 10 s, 55°C for 10 s, 72°C for 20 s, with 40 times. Each treatment consisted of 3 biological replicates and 3 technical replicates. The relative expression of *BvNHX1* gene in *Beta vulgaris* L. was calculated by $2^{-\Delta\Delta Ct}$ method.

Authors' Contributions

LNN and SYQ are the experimental designer and executor of this study. LNN completed data analysis and wrote the first draft of the paper; LGL is the creator and principal of the experiment, directing the experiment design, data analysis, paper writing and revision. All authors read and approved the final manuscript.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (31760414), the Launching Fund for High-level Talents of Inner Mongolia Agricultural University (NDYB2018-11), and the Inner Mongolia Natural Science Foundation of China (2019BS03035).

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