Molecular cloning and expression analysis of \textit{RvNHX1} and \textit{RvVHA-c} genes related to salt tolerance in wild \textit{Rosa rugosa}

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Abstract  Salt stress is one important factor influencing the growth and development of plants, and salt tolerance of plants is a result of combined action of multiple genes and mechanisms. \textit{Rosa rugosa} is not only an important ornamental plant, but also the natural aromatic plant of high value. Wild \textit{R. rugosa} which is naturally distributed on the coast and islands of China has a good salt tolerance due to the special living environment. Here, the vacuolar Na⁺/H⁺ reverse transporter gene (\textit{NHX1}) and the vacuolar H⁺-ATPase subunit C gene (\textit{VHA-c}) closely related to plant salt tolerance were isolated from wild \textit{R. rugosa}, and the expression patterns in \textit{R. rugosa} leaves of the two genes under NaCl stress were determined by real-time quantitative fluorescence PCR. The results showed that the \textit{RrNHX1} protein is a constitutive Na⁺/H⁺ reverse transporter, the expression of the \textit{RrNHX1} gene first increased and then decreased with the increasing salt concentration, and had a time-controlled effect. The \textit{RrVHA-c} gene is suggestive of the housekeeping feature, its expression pattern showed a similar variation trend with the \textit{RrNHX1} gene under the stress of different concentrations of NaCl, and its temporal expression level under 200 mM NaCl stress presented bimodal change. These findings indicated that \textit{RrNHX1} and \textit{RrVHA-c} genes are closely associated with the salt tolerance trait of wild \textit{R. rugosa}.

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1. Introduction

Soil salinization is a widespread problem throughout the world. At present, about 20% of the farmland and nearly half of irrigated land are affected by high concentration salts. The high-concentration Na⁺ in soil will disrupt the ion balance in plants and cause metabolic disorder. As a result, the growth and development of the plants will be influenced, which finally leads to the crop yield decline. Salt stress has become one important factor influencing the growth and development of...
plants and reducing the crop yield (Waditee et al., 2006). Cultivating the plant species that can grow in alkali soil by improving the plant’s salt tolerance is an effective method to utilize and improve the alkali soil.

Salt tolerance of plants is a complex quantitative trait, which is a result of combined action of multiple genes and multiple salt tolerance mechanisms. Under salt stress, the plants will make a series of physiological and biochemical response, including the salt signal sensing and transduction, activation of specific transcriptional factors and expression of relevant genes (Zhang, 2003). Salt tolerance-related genes fall into two types: for one type, the encoded products act as the trans-mitting molecules or regulatory molecules in the signal transduction process; the representatives are a variety of protein kinases and transcriptional factors. For the other type, the encoded products are effectors, such as the genes regulating the synthesis of osmoregulation substances, genes for synthesizing active oxygen-scavenging enzymes, genes for synthesizing osmotin, genes for synthesizing the proteins related to transmembrane transport of ions and the genes encoding transcriptional factors. By transgenic engineering, the salt tolerance-related genes are introduced into recipient plants. This technique for acquiring plants with salt tolerance is the hot spot of research in related field.

Na+\text/H+ reverse transporter is involved in the regulation of intracytoplasmic pH and Na+ as well as the changes of cell volume. It is an electrically neutral Na+\text/H+ 1:1 transmembrane transporter, the generation of which depends on the Na+\text/H+ concentration gradient (Blumwald and Poole, 1985; Yang et al., 2009). Na+\text/H+ reverse transporter in advanced plants is further divided into vacuole membrane protein and plasma membrane protein. They perform the functions of compartmentalization of Na+ in the vacuole and the transport of Na+ out of the cell. These are the two key processes in osmoregulation and intracytoplasmic Na+ detoxification and also necessary for plants to resist the salt stress (Sun, 2008). It is demonstrated by a large amount of studies that the compartmentalization of Na+ in the vacuole is an important mechanism of salt tolerance. This process is jointly accomplished by Na+\text/H+ reverse transporter and H+\text-ATPase on the vacuole membrane.

Along with the progress in molecular biology and the research into the biological mechanism of salt tolerance of plants, Na+\text/H+ reverse transporter genes have been cloned from a variety of plants, including OsNHX1 from Oryza sativa (Fukuda et al., 1999), AgNHX1 from Atriplex gmelini (Hamada et al., 2001), ThNHX1 from Thellungiella halophila (Wu et al., 2009), LeNHX1 from Lycopersicon esculentum (Venema et al., 2003), and LmNHX1 from Lobularia maritima (Popova and Golldack, 2007). It is shown by recent studies that the overexpression of exogenous vacuole membrane and plasma membrane Na+\text/H+ reverse transporter genes can significantly increase the salt tolerance of recipient plants. For example, Apse et al. (1999) introduced the AtNHX1 gene from Arabidopsis thaliana into L. esculentum and Brassica napus, with the effect of increasing their salt tolerance. Rajagopal et al. (2007) isolated the vacuole membrane Na+\text/H+ reverse transporter gene PgNHX1 from Pennisetum glaucum and transferred it into Brassica juncea to induce its over-expression. The result showed that the salt tolerance level of B. juncea was improved. The transgenic B. juncea carrying the overexpressed PgNHX1 which could survive under 300 mM NaCl, bloomed and grew normal seeds. This indicates that Na+\text/H+ reverse transporter is an important component in the complex salt tolerance mechanism of plants. It has the value of further research and utilization.

H+\text-ATPase is widely present in the plasma membrane and various endomembrane systems of animals and plants, playing an important role in the metabolic pathways. Three major types of membrane H+-ATPase have been known: plasma membrane P-\text/H+-ATPase, mitochondrial F-\text/H+-ATPase and vacuolar V-\text/H+-ATPase. V-\text/H+-ATPase (or V-ATPase) mainly exists on the endomembrane system of eukaryotes. Under the stress of salt, drought, cold and heavy metals, the viability of plant cells greatly depends on the activity of V-ATPase. The regulation of V-ATPase gene expression and activity is the basis for plants to adapt to the adverse environment (Zhang and An, 2013). Studies show that under salt stress, the activity of V-ATPase in most salt-resistant plants and the gene expression of subunits presented correlation with salt stress. As a halophyte, Suaeda salsa can enhance the V-ATPase activity to propel ion storage in the vacuole to induce salt adaptability (Wang et al., 2001; Dong, 2012). The study on the response of V-ATPase to salt stress indicated that subunit C was more sensitive and the transcription level was increased significantly (Tsiantis et al., 1996). Under salt stress, the expression of V-ATPase subunit C in Tortula ruralis (Chen et al., 2002), Mesembryanthemum crystallinum (Low et al., 1996) and Limonium bicolor (Jiang et al., 2009) was significantly upregulated. Five homologous genes encoding the V-ATPase subunit C in A. thaliana presented obvious upregulation under salt stress. It is thus indicated that subunit C plays an important role in resisting salt stress for plants.

Rosa rugosa is a deciduous shrub belonging to genus Rosa, Rosaceae family. It is not only an ideal plant material for landscape greening, but also a natural aromatic plant of high value. The native range of R. rugosa includes Northeastern China, Northern Japan, the Korean Peninsula, and the Russian Far East (Fu, 1992; Feng et al., 2010). In China, wild R. rugosa is naturally distributed on the coast and islands of Southern Liaoning Province, Eastern Shandong Province, and Tumen River estuary in Jilin Province, and is classified as an endangered species (Fu, 1992; Bruun, 2005). Wild R. rugosa has a good salt tolerance due to the special living environment. It is usually used as the parental material for breeding new salt-resistant varieties of genus Rosa. In this study, the vacuolar Na+\text/H+ reverse transporter gene (NHX1) and vacuolar H+\text-ATPase subunit C gene (VHA-c) closely related to salt tolerance were isolated from wild R. rugosa produced in China. The expression patterns under salt stress were quantified. The result is of theoretical importance and application value for understanding the functions of the two genes and the salt-resistant mechanism of wild R. rugosa and for employing genetic engineering to breeding new salt-resistant varieties of genus Rosa.

2. Materials and methods

2.1. Plant materials and treatments

The experiment materials were wild R. rugosa from the seaside of Weihai, Shandong Province. The plants were cultivated in plastic pots of the same specification (33 cm × 19 cm × 22 cm) with one plant per pot. The salt stress treatment was performed depending on the research objective. In order to
prevent the influence of precipitation, the experiment was carried out in a smart greenhouse.

The following experiment was carried out to determine the influence of salt stress treatment of different concentrations on salt tolerance-related genes: Four treatments were performed with four pots per treatment, 100, 200 and 300 mM NaCl solutions were used to slowly irrigate the potted *R. rugosa* (water collection plate at the bottom to prevent the loss of effluent), and the control plants were irrigated with water without NaCl. After preliminary experiment, the dose was determined as 1 L. The young leaves of each plant were randomly collected after salt stress treatments for 6 h. The specimens were immediately stored in liquid nitrogen to take back to laboratory and pre-served in −80 °C ultralow temperature freezer for RNA extraction.

The experiment was carried out to detect the influence of treatment duration on the expression of salt tolerance-related genes: 1 L of 200 mM NaCl solution was used to irrigate the potted wild *R. rugosa* in a total of 16 pots. The first sampling (0 h) was done before the salt stress treatment, then the young leaves were collected after treatment for 6 h, 12 h, 24 h and 48 h, respectively. Four pots were randomly selected for sampling, and the young leaves were randomly collected from each plant. To maintain the stability of salt concentration in the treatment period and to prevent the influence of water evaporation on salt concentration, 1 L of 200 mM NaCl solution was irrigated every 6 h.

### 2.2. RNA extraction and purification

Total RNA was isolated from wild *R. rugosa* leaves according to a modified CTAB method (Zhao et al., 2011). RNA samples were performed with DNase using DNase I kit (TaKaRa, Japan) according to the manufacturer’s guidelines before reverse-transcription, and then quantified by a spectrophotometer (Eppendorf, Germany) at 230 nm, 260 nm and 280 nm.

### 2.3. Isolation of salt tolerance genes

The two key genes related to salt tolerance in wild *R. rugosa*, *RrNHX* and *RrVHA-c*, were isolated according to the experimental system established in our laboratory (Feng et al., 2014).

#### 2.3.1. 3' RACE

Total RNA of leaf tissue (1 mg) was used to synthesize the first strand cDNA according to the manufacturer’s instructions of the SMARTer™ RACE cDNA Amplification Kit (Clontech Laboratories, Inc., TAKARA). The gene-specific primers were designed and synthesized according to the above sequenced 3’ ends (Table 1). Then touchdown PCR was performed using the following program: 5 cycles of 30 s at 94 °C and 90 s at 72 °C, 5 cycles of 30 s at 94 °C, 30 s at 70 °C and 1 min at 72 °C, 30 cycles of 30 s at 94 °C, 30 s at 65 °C and 120 s at 72 °C (for *RrNHX*). Conditions of the other gene amplification are also listed in Table 1.

#### 2.3.2. 5' RACE

Total RNA of leaf tissue (1 mg) was used to synthesize the first strand cDNA according to the manufacturer’s instructions of the SMARTer™ RACE cDNA Amplification Kit User Manual (Clontech Laboratories, Inc., TAKARA). The gene-specific primers were designed and synthesized according to the above sequenced 5’ ends (Table 1). Then touchdown PCR was performed using the following program: 5 cycles of 30 s at 94 °C and 90 s at 72 °C, 5 cycles of 30 s at 94 °C, 30 s at 70 °C and 1 min at 72 °C, 30 cycles of 30 s at 94 °C, 30 s at 65 °C and 120 s at 72 °C (for *RrVHA*). Conditions of the other gene amplification are also listed in Table 1.

#### 2.4. Purifying, cloning and sequencing

The isolated PCR products were recovered from the 1% agarose gel, purified using the TaKaRa Agarose Gel DNA Purification Kit Ver. 2.0 (TaKaRa, Japan), cloned into the PMD18-T vector (TaKaRa, Japan) and transformed into competent *Escherichia coli* DH5a cells (Trans, China). The recombinant plasmids were identified with the restriction enzymes *BamH* and *Hind*III (TaKaRa, Japan) and the positive clones were sequenced in Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China).

#### 2.5. Sequence analysis

The full-length cDNA sequences were analyzed using the DNAMAN 5.0 software. Homology search was carried out online at the nucleotide level with BLASTn and at the amino acid level with BLASTp (http://www.ncbi.nlm.nih.gov/blast/).

#### 2.6. Gene expression analysis

mRNA relative expression of *RrNHX1* and *RrVHA-c* was analyzed by the method of real-time quantitative RT-PCR with a BIO-RAD CFX96™ Real-Time System (C1000™ Thermal Cycler) (Bio-Rad, USA). cDNA was synthesized from 1 μg RNA using PrimeScript® RT reagent Kit with gDNA Eraser.

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**Table 1 Primers used for cloning of the *RrNHX1* and *RrVHA-c* genes related to salt tolerance in wild *R. rugosa*.

| Primer         | Oligonucleotide sequence (5’-3’) | Application | Annealing condition |
|----------------|---------------------------------|-------------|---------------------|
| *RrNHX1*-1    | GGAGGCCGATACAACTGGC             | 1st of 3’RACE | 53 °C               |
| *RrNHX1*-2    | GGTATGGATGCTTGAGAC             | 2nd of 3’RACE | 53 °C               |
| *RrNHX1*-3    | GCTCGGGGAGGAAAGATGATGCAAC      | 5’RACE       | 65 °C               |
| *RrVHA-c1*    | GGGAAGGCTGGGATGGAGTGGT        | 1st of 3’RACE | 51 °C               |
| *RrVHA-c2*    | TGGTTATGCTCATCTCG              | 2nd of 3’RACE | 52 °C               |
| *RrVHA-c3*    | ACCATATTACCACCTTGCTGGAGGCT    | 5’RACE       | 66 °C               |
(TaKaRa, Japan). *Rosa hybrid* α-tubulin subunit actin gene (GenBank Accession No. AF394915.1) was used as an internal constitutively expressed control in the real-time quantitative RT-PCR analysis. PCR was performed using the primers shown in Table 2. Quantitative real-time PCR experiments were performed according to the instructions of the SYBR® Premix Ex Taq™ (Perfect Real Time) (TaKaRa, Japan) and contained 2× SYBR Premix Ex Taq™ 12.5 μl, 50× ROX Reference Dye II 0.5 μl, 2 μl cDNA solution as a template, 1 μl mix solution of target gene primers and 9 μl ddH2O in a total volume of 25 μl. The amplification was performed by an initial incubation at 50 °C for 2 min and at 95 °C for 5 min, then followed by 40 cycles of 15 s at 95 °C, 15 s at 51 °C, and 40 s at 72 °C. Expression levels for each gene were calculated by the 2−ΔΔCt comparative threshold cycle (Ct) method (Schmittgen and Livak, 2008). The Ct values were generated from the Bio-Rad CFX Manager V1.6.541.1028 software. All samples were repeated three times.

### 2.7. Statistical analysis

All data were average values of four replicates with standard deviations (SD). Statistical analyses were carried out by the SAS/STAT statistical analysis package (version 6.12, SAS Institute, Cary, NC, USA).

### 3. Results

#### 3.1. Isolation and sequence analysis of salt tolerance gene from wild *R. rugosa*

RNA extracted from the leaves of wild *R. rugosa* was taken as template, the full-length cDNA sequences were obtained for genes *RrNHX1* and *RrVHA-c* related to the salt tolerance by means of RACE, and the GenBank accession numbers were KC188664 and KF677023. The full-length cDNA sequence of the gene *RrNHX1* was 2358 bp. It contained start codon, complete open reading frame (1629 bp), stop codon, 5' non-coding region (462 bp), 3' non-coding region (267 bp) and poly (A) tail (12 bp), encoding 543 amino acids in total. The analysis of BLAST indicated that the amino acid sequences of the gene *NHX1* in plants such as *Rosa hybridra, Malus zami, Glycine max* and *Populus trichocarpa* had 81–99% identity and 90–99% similarity with the gene *RrNHX1* (Table 4). The full-length cDNA sequence of the gene *RrVHA-c* was 795 bp, containing start codon, complete open reading frame (498 bp), stop codon, 5' non-coding region (63 bp), 3' non-coding region (334 bp) and poly (A) tail (12 bp) and encoding 166 amino acids in total. The analysis of BLAST indicated that the amino acid sequences of the gene *VHA-c* in plants such as *A. thaliana, Cucumis sativus, Gossypium hirsutum* and *Populus trichocarpa* had 99% identity and 99–100% similarity with the gene *RrVHA-c* (Tables 3 and 4), this suggested that *VHA-c* is a highly conserved membrane protein.

#### 3.2. Expression analysis of *RrNHX1* and *RrVHA-c* genes under salt stress

According to the results of fluorescence quantitative PCR (Fig. 1), the expressions of *RrNHX1* and *RrVHA-c* genes could be detected in the leaves without salt stress. However, the salt stress treatment caused the upregulation of *RrNHX1* and *RrVHA-c* genes.

It is seen from Fig. 1 that the *RrNHX1* gene was obviously upregulated after 6 h of 100, 200 and 300 mM NaCl treatment. The expression levels were 2.46, 4.66 and 1.57 times that of the constitutively expressed control in the real-time quantitative RT-PCR analysis.
control samples without salt stress. Under 200 mM NaCl stress, the expression level of *RrNHX1* in leaves increased continuously within 24 h. At 6 and 12 h, the expression level was 3.57 and 4.96 times that of the control group. The highest expression level was achieved at 24 h, which was 12.16 times that of the control group. At 48 h, the expression level decreased to 2.27 times that of the control (Fig. 2).

The expression patterns of the *RrVHA-c* gene and the *RrNHX1* gene showed a similar variation trend under the stress of different concentrations of NaCl, but the variation of the expression level was milder. After 6 h of 100, 200 and 300 mM NaCl stress, the expression level of the *RrVHA-c* gene was 1.64, 2.35 and 0.42 times that of the control (Fig. 1). Under 200 mM NaCl stress, the expression level of *RrVHA-c* in leaves was 2.62, 2.11, 3.68 and 1.09 times that of the control at 6, 12, 24 and 48 h, respectively. That is, within 24 h, the general increasing trend was presented, with a slight reduction at 12 h. The highest expression level was detected at 24 h. And the expression decreased to the level comparable to the control at 48 h (Fig. 2).

### 4. Discussion

Vacuolar Na\(^+\)/H\(^+\) reverse transporter compartmentalizes Na\(^+\) in the vacuole as an effective mechanism to adapt to salt stress. V-ATPase is responsible for maintaining the ion homeostasis in cytoplasm and a normal cellular metabolism to make the plant more adaptable to the salt environment. In this article, the full-length cDNA sequence of the vacuolar Na\(^+\)/H\(^+\) reverse transporter gene *RrNHX1* and the vacuolar H\(^-\)-ATPase subunit C gene *RrVHA-c* from wild *R. rugosa* was obtained by using RT-PCR and RACE technique. The result showed that gene copy number of the vacuolar Na\(^+\)/H\(^+\) reverse transporter differed from one plant species to another. Blast analysis was performed for cDNA sequence of *RrVHA-c*. It was found that although most genes had similarity of over 80% with *RrVHA-c*, the identity was only about 60%. Blast analysis also performed to the predicted amino acid sequences. Among 100 amino acid sequences that were matched in GenBank, most had an identity and similarity of over 95% with the amino acid sequences of *RrVHA-c*. This indicates that vacuolar H\(^-\)-ATPase subunit C is a highly conserved membrane protein.

By analyzing the expression pattern of the *RrNHX1* gene in wild *R. rugosa*, it was found that the expression of the *RrNHX1* gene was detected in the control group without salt stress treatment. This indicates that the *RrNHX1* gene is a constitutive Na\(^+\)/H\(^+\) reverse transporter. And for wild *R. rugosa*, the *RrNHX1* gene was upregulated under salt stress within a certain range. This indicated that the *RrNHX1* gene was associated with the salt tolerance of wild *R. rugosa*. The *RrNHX1* gene was obviously upregulated after 6 h of 100 and 200 mM NaCl stress. However, the variation of expression under 300 mM NaCl stress was not obvious. The same variation trend was observed in the leaves of plantlets of *Malus zumi*: the expression level of the MaNHX1 gene first increased and then decreased with the increasing salt concentration, and the expression level of the MaNHX1 gene was lower than that of the control under high salt stress (200 mM) (Meng, 2010). Another study also found that Na\(^+\) began to be transported from the root of rice to the aboveground part and accumulated in leaves after 2 h of salt stress (Liu, 2006; Matushita and Matosh, 1991). Thus, after 6 h of 100 and 200 mM NaCl stress, the increase of *RrNHX1* on the transcription level may be caused by the accumulation of Na\(^+\) in leaves. Under 300 mM NaCl stress, the gene expression level was not high. This is probably because an excess salt concentration injures the leaves to some extent, and the expression of the *RrNHX1* gene was inhibited. Another possible reason is that the transport of Na\(^+\) from the root of wild *R. rugosa* to the aboveground part is inhibited by high salt concentration, and the efflux of Na\(^+\) from root took effect (Shi et al., 2002; Shi et al., 2003).

The *RrNHX1* gene showed a continuously increasing expression within 24 h under 200 mM NaCl stress. However, the expression amount dropped to the original level at 48 h. It was indicated that the expression of the *RrNHX1* gene had a time-controlled effect. This variation trend is similar.
gene was obviously upregulated after 6 h of 100 mM NaCl stress (Liu, 2006). The 

\( \text{RrNHX1} \) gene was still expressed continuously under high salt stress (200 mM NaCl), which indicates the strong salt tolerance of wild \( R. \ rugosa \).

V-ATPase is a biologically active holoenzyme assembled by subunits at a certain proportion. Subunit C is the core structure of V-ATPase \( V_{\text{r}} \) sector in the form of hexamer, and participates in the formation of proton channel. Therefore, the activity of subunit C directly influences the amount and activity of V-ATPase. A high expression of subunit C will cause the increase of the amount and activity of V-ATPase (Xu, 2005). This study shows that under normal conditions, \( \text{RrVHA-c} \) is expressed to some degree, which is suggestive of the housekeeping feature, \( \text{RrVHA-c} \) expression is necessary for maintaining the normal physiological action and life activities of plants. Under salt stress, \( \text{RrVHA-c} \) was upregulated, which was consistent with the upregulation of the V-ATPase subunit C in \( T. \ rurals \) (Chen et al., 2002), \( M. \ crystallinum \) (Low et al., 1996) and \( L. \ bicolor \) (Jiang et al., 2009). In addition, the \( \text{RrVHA-c} \) gene was obviously upregulated after 6 h of 100 and 200 mM NaCl stress. However, the expression level was lower than the control under 300 mM NaCl stress. The upregulation of the \( \text{RrVHA-c} \) gene may be explained by the fact that the cells need the energy provided by V-ATPase to support the metabolism under salt stress (Fu et al., 2009). The expression level of the \( \text{RrVHA-c} \) gene was lower than that of the control under 300 mM NaCl stress, which also may be related to the inhibited transport of \( \text{Na}^{+} \) to the aboveground part due to leaf damage and high salt concentration.

**Author contribution statement**

L.S. and L.F. designed the study. L.F. and H.D. drafted the manuscript. H.D. carried out gene isolation, sequence analysis, and gene expression analysis. J.W. and M.W. participated in complementation tests. W.X. and S.Z. helped to draft the manuscript. All authors read and approved the manuscript.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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