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Molecular cloning and expression analysis of *RrNHX1* and *RrVHA-c* genes related to salt tolerance in wild *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. *Rosa rugosa* is not only an important ornamental plant, but also the natural aromatic plant of high value. Wild *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 (*NHX1*) and the vacuolar H⁺-ATPase subunit C gene (*VHA-c*) closely related to plant salt tolerance were isolated from wild *R. rugosa*, and the expression patterns in *R. rugosa* leaves of the two genes under NaCl stress were determined by real-time quantitative fluorescence PCR. The results showed that the *RrNHX1* protein is a constitutive Na⁺/H⁺ reverse transporter, the expression of the *RrNHX1* gene first increased and then decreased with the increasing salt concentration, and had a time-controlled effect. The *RrVHA-c* gene is suggestive of the housekeeping feature, its expression pattern showed a similar variation trend with the *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 *RrNHX1* and *RrVHA-c* genes are closely associated with the salt tolerance trait of wild *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

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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 transferring 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^+/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^+/H^+ 1:1 transmembrane transporter, the generation of which depends on the Na^+/H^+ concentration gradient (Blumwald and Poole, 1985; Yang et al., 2009). Na^+/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^+/H^+ reverse transporter and H^+ -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^+/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 Gollmack, 2007). It is shown by recent studies that the over-expression of exogenous vacuole membrane and plasma membrane Na^+/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^+/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 over-expressed *PgNHX1* which could survive under 300 mM NaCl, bloomed and grew normal seeds. This indicates that Na^+/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^+ -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-H^+ -ATPase, mitochondrial F-H^+ -ATPase and vacuolar V-H^+ -ATPase. V-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^+/H^+ reverse transporter gene (*NHX1*) and vacuolar H^+ -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

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