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Cloning and functional analysis of *MxNRAMP1* and *MxNRAMP3*, two genes related to high metal tolerance of *Malus xiaojinensis*

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ABSTRACT

Metal stresses are common nutritional disorder in plants, especially in fruit trees grown in calcareous soil. The natural resistance-associated macrophage protein (NRAMP) family comprises integral membrane proteins that play an important role in regulating metal ion transport. Our research focused on an iron deficiency-resistant apple (*Malus xiaojinensis*) rootstock, as the study model. Based on conserved regions of NRAMP1 and NRAMP3 in other plants, we designed primers and cloned MxNRAMP1 and MxNRAMP3 genes from *M. xiaojinensis*, the functions of which were preliminarily characterized in yeast, a eukaryotic model organism. In this study, MxNRAMP1 is localized to the plasma membrane, whereas MxNRAMP3 is localized to the vacuolar membrane. In yeast, both proteins were able to transport Cd but not other metal ions. Upon Fe, Cd, Mn, and Zn stress treatments for *M. xiaojinensis*, MxNRAMP1 expression was specifically induced in roots. MxNRAMP3 was expressed in both roots and leaves, and was immediately expressed in roots at the onset of metal stress, whereas its expression was delayed in leaves. These results showed that MxNRAMP1 and MxNRAMP3 play an important role in metal absorption and transportation.

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1. Introduction

Mineral nutrients are essential for plant growth and development. They are not only plant constituents, but also play a key role in regulating physiological and biochemical functions. As we know, the common nutrition stresses are Fe, Cd, Mn, and Zn stress. Fe is one of the essential elements for plant growth and development, participating in the electron transport chain and enzymatic reactions of several physiological and metabolic processes, including photosynthesis, nitrogen fixation, respiration, and protein and nucleic acid synthesis (Thoiron et al., 1997; Colangelo and Guerinot, 2004). Mn is an activator of enzymes such as deoxygenases, which affect processes such as glycolysis and the TCA cycle (Saudagar et al., 2008). Zn is an essential element in chlorophyll biosynthesis. Heavy metal poisoning can be harmful to plants; for example, chlorophyll content and photosynthetic efficiency decrease with increased Cd concentration (Klobus and Buczek, 1985). During the course of evolution, plants have developed a unique mechanism that selectively absorbs necessary metal ions and eliminates toxic metal ions. Two groups of genes involved in metal uptake, *Irt/Zip* and *Dmt/NRAMP*, have been identified in plants (Eide et al., 1996; Belouchi et al., 1997; Curie et al., 2000; Thomine et al., 2000; Connolly et al., 2002; Vert et al., 2002; Bereczky et al., 2003).

NRAMP family genes encode a typical integral membrane polypeptide (Cellier et al., 1994) containing 10–12 transmembrane (TM) domains, 1–2 glycosylated extracellular loops, and an intracellular domain with structural characteristics of a transporter protein (Bairoch, 1993) that shares a high degree of amino acid sequence homology and displays a similar secondary structure. *NRAMP* genes are involved in the efficient transport of several metal ions, including Fe, Cd, Mn, and Zn; furthermore, these genes are also present in bacteria, fungi, and the plant and animal kingdoms (Gruenheid et al., 1995; Cellier et al., 2001).

Three *NRAMP* genes, *SMF1–SMF3*, have been identified in yeast and are responsible for regulating the absorption of Mn, Zn, Cd, and Fe (Liu et al., 1997; Chen et al., 1999). *NRAMP* genes are currently defined as a new gene family related to proteins responsible for transporting divalent metal ions. Further detailed studies suggest that *NRAMP* gene family members play an important regulatory role under iron deficiency stress; a new iron acquisition mechanism based on phagocytosis was accordingly proposed (Mori, 1999). In *Arabidopsis thaliana*, cDNA sequences of *NRAMP* gene family members have been successfully cloned. *AtNRAMP1*, *2*, and *6* are located on chromosome 1, whereas *AtNRAMP3*, *5*, and *4* are located on chromosome 2, 4, and 5 (Thomine et al., 2000; Curie et al., 2000), respectively. Expression of *AtNRAMP1* and *AtNRAMP3* was enhanced during low iron stress. When the mutated iron-sensitive yeast strain DDY4 was transformed with *AtNRAMP1*, growth resumed. *AtNRAMP3* releases metal ions stored in the vacuole into the cytoplasm near the vacuolar membrane (Thomine et al., 2003). In the wild tomato, expression of root-specific *LeNRAMP1* was strongly induced upon iron deficiency stress, whereas expression of *LeNRAMP3* was weakly induced under iron deficiency stress (Bereczky

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et al., 2003). In *fer* mutants, *LeNRAMP1* was not expressed, suggesting that *LeNRAMP1* is regulated by *fer*. In addition, three *NRAMP* genes have been cloned in rice. *OsNRAMP1* was mainly expressed in roots with very little expression in aerial parts; conversely, *OsNRAMP2* was mainly expressed in aerial parts. *OsNRAMP3* was expressed in both parts of the plants (Belouchi et al., 1995).

Malus xiaojinensis was selected as an apple rootstock that is resistant to iron deficiency (Han et al., 1998). In order to clarify the role of the *NRAMP* gene family in absorption and transport of metal ions in *M. xiaojinensis*, we cloned *NRAMP1* and *NRAMP3* from *M. xiaojinensis*, and performed bioinformatics analyses of the full-length gene sequences. The expression characteristics of these two genes in roots and leaves of *M. xiaojinensis* under Fe, Cd, Mn, and Zn stress were examined. In addition, *MxNRAMP1* and *MxNRAMP3* were transformed into yeast and preliminary analyses of their functions were performed.

2. Materials and methods

2.1. Experimental materials

M. xiaojinensis seedlings with strong growth were selected and cultured in a 2-L container, and supplied with a complete nutrient solution that was replaced once a week. These plantlets were hydroponically cultured in a greenhouse with a photoperiod of 16 h light under cool-white fluorescent light at 2000 lx. The temperature throughout the day was maintained at 25 ± 2 °C. The nutrient solution was replaced weekly (Gao *et al.*, 2011). After 3 weeks of culture, *M. xiaojinensis* seedlings were removed from the complete nutrient solution, washed three times with deionized water, and placed in a 500-mL container for subsequent experimental treatments: (1) normal treatment (Control: 40 μM EDTA-FeNa); (2) low Fe treatment (−Fe: 4 μM EDTA-FeNa); (3) high Fe treatment (++Fe: 120 μM EDTA-FeNa); (4) Cd supplement treatment (+Cd: 10 μM $CdCl_2$); (5) low Zn treatment (−Zn: 0.15 μM $ZnSO_4$); (6) high Zn treatment (++Zn: 3.5 mM $ZnSO_4$); (7) low Mn treatment (−Mn: 0 μM $MnSO_4$); and (8) high Mn treatment (++Mn: 1 mM $MnSO_4$). White newly grown roots and mature leaves were collected at 1 d, 2 d, 6 d and 9 d of treatments. Samples were immediately frozen in liquid nitrogen and stored at −80 °C until use. Each treatment included three replicates.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted using the CTAB method (Zhang et al., 2005). DNase (Takara, Dalian, China) was used to remove any DNA contamination. Purified RNA was detected by electrophoresis in 1% agarose gel and concentration was determined using an ultraviolet spectrophotometer (Thermo Scientific, NANO DROP2000, USA). RNA was stored at −80 °C until use. An aliquot of RNA (1 μg) was reverse-transcribed to cDNA using M-MLV reverse transcriptase (Takara, Dalian, China) and the cDNA was used in subsequent experiments.

2.3. Gene cloning

A BLASTp search within the GDR database (http://www.rosaceae.org/) was performed based on reported *MbNRAMP1* and *AtNRAMP3* gene sequences, and the coding sequence (CDS) with the highest homology was identified. Primers were designed for cloning the full-length gene, as shown in Table S1. However, cloning of *MxNRAMP1* has been reported in another study (Pan et al., 2015). The present study focused on the sequence of *MxNRAMP3*.

M. xiaojinensis cDNA was used as a template and PCR was performed using a high-fidelity DNA polymerase (Beijing TransGen Biotech Co., Ltd., Beijing, China). The reaction program was as follows: pre-denaturation at 94 °C for 4 min; 33 cycles of 94 °C for 45 s, 59 °C for 45 s, and 72 °C for 90 s; followed by 72 °C for 10 min. PCR products were recovered using TIANgel Midi Purification Kit (Tiangen Biotech Co., Ltd., Beijing, China) and inserted into a pEASY-T1 vector. Liquated products were transformed into *Escherichia coli* DH5α cells that were cultured at 37 °C for approximately 12 h. Colony PCR was used to identify positive clones. Sequencing was performed using BGI sequencing (Beijing, China) and multiple sequence alignment was performed using DNAMAN software (Lynnon Corp, Quebec, Canada). Phylogenetic trees were constructed using the neighborjoining method, and bootstrap resampling was performed 1000 times. These analyses were carried out using the Molecular Evolutionary Genetics Analysis (MEGA 4.1) program (Tamura et al., 2011). TMHMM-2.0 software (http://www.cbs.dtu.dk/services/TMHMM/) was used for prediction of TM regions.

2.4. qRT-PCR

An NCBI BLASTp (http://www.ncbi.nlm. nih.gov/) search using an NRAMP protein sequence query identified a conserved region of the superfamily. Nucleotide sequences in this region were avoided and primers for amplifying a 100–250-bp fragment of *MxNRAMP1* and *MxNRAMP3* were designed (Table S1). *M. xiaojinensis* cDNA was used to verify that the amplification efficiency of primer pairs was greater than 95%, indicating that the primers were suitable for use. *β-actin* was selected as a housekeeping control (Kürkcüoglu et al., 2007). The qRT-PCR 20 μL system included 1× SYBR Master Mix (Taraka Dalian, China) and 0.2 μM (final concentration) primers (Table S1). The settings of the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) were as follows: 95 °C for 30 s; 40 cycles of 95 °C for 5 s and 60 °C for 34 s; followed by 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. Each reaction included three replicates. The relative gene expression was calculated according to the $2^{-\Delta\Delta CT}$ method (Qi et al., 2010). Gene expression was shown in comparison to the value of the control in the first day to provide a relative quantification expression.

2.5. Vector construction

The yeast expression vector used was pYES2.0, provided by Dr. Hong-Qing Ling (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences). The vector of pYES2.0-eGFP was constructed by double digestion of pYES2.0 and pEZS-NL plasmids with *Hind*III and *Xba*I. The PCR products of *MxNRAMP1* with restriction enzyme digestion sites and a pYES2.0-eGFP plasmid were digested with *Kpn*I and *Bam*HI to construct a yeast expression vector, pYES2.0-MxNRAMP1-eGFP. *MxNRAMP3* PCR products carrying restriction enzyme sites and a pYES2.0-eGFP plasmid were digested with *Eco*RI and *Bam*HI to construct pYES2.0-Mx*NRAMP*3-eGFP. A heat shock protocol was used for *E. coli* DH5α (TransGen Biotech, Beijing, China) transformation. Recombinant plasmids were identified by colony PCR and confirmed by sequencing.

2.6. Yeast culture and transformation

YPD medium (containing 2% galactose) and SD-Ura medium were used (Gietz and Woods, 2002) for yeast culture. The recombinant plasmids were transformed into the *BJ2108* yeast strain using the PEG-LiAc method (Zhang et al., 2010). Transformed cells were spread onto SD-Ura plates for selection of positive clones. Single colonies were selected and cultured in liquid medium at 30 °C with constant shaking at 200 r/min for 48 h. Yeast cells were homogenized to disrupt cell walls and PCR was performed to identify positive clones.

2.7. Determination of subcellular localization

A small aliquot of yeast cells was cultured in YPD medium and placed on a shaker at 200 r/min for 48 h. Yeast cells (10 μL) were placed on a slide and covered with a cover slip, then observed under a confocal laser scanning microscope at × 100 magnification to determine the

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