Synchrotron X-ray Fluorescence Microtomography Profiling of *Malus xiaojinensis* Provides Insights into Mechanisms of Divalent Metals Transport Subjected to Iron Deficiency

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Abstract. Grafting has been widely used in orchard management because the rootstock can make the tree more tolerant to environmental stresses. Iron deficiency is one of the major limiting environmental factor in apple production worldwide. Systematic research has been made about iron-deficiency adaptive responses in the level of organs, cells, and subcells, whereas the interactions between Fe and other divalent cations in tissue level are little known. Synchrotron radiation X-ray fluorescence (SR-μXRF) was used to map the location of selected elements Fe, Zn, Mn, Ni, and Co in the longitudinal and latitudinal root samples of *Malus xiaojinensis*. Iron deficiency induced a significant increase in the relative contents of five micronutrients in epidermis and cortex. The ratio of element contents of roots under Fe-deficient condition and Fe-sufficient condition at same position increased obviously in the section of 1000- to 2000-μm distance from the root tip in xylem. Expression analysis of iron absorption- and transport-related genes in roots showed that *MdNramp3* and *MxCS1* increased significantly. These results indicated that iron deficiency promoted the long-distance transport of micronutrients in xylem, and *MdNramp3* and *MxCS1* might play an important role in this process. Importantly, this study directly provides visual divalent metals distribution in tissue level for an improved understanding of metal absorption process in apple rootstock.

As one of the important micronutrient, iron participates in many important physiological and biochemical processes. Although iron is abundant in the environment, plants often suffer from iron deficiency because of the alkaline and calcareous soils (Guerinot and Yi, 1994). In the long-term evolution process, plants have evolved into tight mechanisms such as reduction-based strategy I and chelation-based strategy II for iron uptake, transport, utilization, or storage (Marschner et al., 1986).

In the process of Fe absorption, intracellular or intercellular and long-distance transport, many genes are involved including

the root epidermal cell membrane transporters: iron-related transporter 1 (*IRT1*) and natural resistance-associated macrophage protein 1 (*Nramp1*) (Curie et al., 2000; Korshunova et al., 1999), vacuolar membrane transporter (*Nramp3*, *Nramp4*) and vacuolar iron transporter 1 (Kim et al., 2006; Lanquar et al., 2005), ferric reductase defective 3 (*FRD3*), citrate synthase 1 (*CS1*), nicotianamine synthase 1 (*NAS1*), and yellow stripe1-like (*YSL*) (Kobayashi and Nishizawa,

2012). Among these genes, the *ZIP* (ZRT, IRT-like proteins) metal transporter family and *NRAMP* family catch our attention all the time.

The absorption of metal elements in plants is a complicated process; they often use the same transport system for absorption, transportation, or storage (Rogers et al., 2000). Research suggests that there is a competition between Zn^{2+} and Fe^{2+} absorption in *Arabidopsis thaliana* (Fukao et al., 2011). Mn^{2+} deficiency can increase the transport of Fe^{2+} efficiently (Yang et al., 2008). Ni^{2+} accumulation may act as an iron-deficiency signal and induce the Fe-deficient response to upregulate Fe^{2+} absorption genes expression (Nishida et al., 2012). In addition, all of the previous studies were performed with the interaction between Fe^{2+} and other divalent metals. The mechanism of divalent metal ions absorption under iron deficiency in woody plants is little known.

Malus xiaojinensis is a native apple rootstock in China and has been characterized by its high efficiency for iron uptake (Han et al., 1998; Wu et al., 2012; Zha et al., 2014). In this study, we detected the spatial distribution of selected divalent metals in the root of *Malus xiaojinensis*, which belongs to the strategy I plant by using SR-μXRF as well as the expression of iron absorption- and transport-related genes in roots under iron-deficiency treatment, which will help to investigate the tissue-specific distribution of divalent metals when plants are subjected to iron deficiency.

Materials and Methods

Plant cultivation and sample preparation.

The seedlings of *Malus xiaojinensis* were propagated on Murashige and Skoog (MS) medium with 0.5 mg/L Indole-3-Butyric acid (IBA) and 0.5 mg/L 6-Benzylaminopurine for one month and transferred to one-half-strength modified MS medium with 0.5 mg/L IBA for rooting for one month and a half. The rooted seedlings were moved to one-half-strength modified Hoagland nutrient solution for 1 week and switched to Hoagland

Table 1. Primer sequences for the quantification of transcripts by real-time polymerase chain reaction.

Gene		Primer sequences
<i>MdIRT1</i>	Fwd	5' TTGACAAGGGAGAAAACGGAGAC 3'
	Rev	5' AACAACTGAATGGACAATGATACCC 3'
<i>MdCS1</i>	Fwd	5' GTTCTCGTCTCGGGCAACTGTC 3'
	Rev	5' TTCCTCTCATTCCACCAATCACC 3'
<i>MdFRD3</i>	Fwd	5' GTGGAAGATGCCTGTTGGTGT 3'
	Rev	5' CAATAGATACTCTGCTGCCGC 3'
<i>MdNAS1</i>	Fwd	5' CGATGTTTCCAAGTTATGCCAA 3'
	Rev	5' TTCTCCAAAAGTCCTTCTGCCT 3'
<i>MdNRAMP1</i>	Fwd	5' CCGAAGTTATTGGCACAGCGTT 3'
	Rev	5' TTCACATAACCCATTTCGCCGA 3'
<i>MdNRAMP3</i>	Fwd	5' GACGACGACTGCCAAACGCCG 3'
	Rev	5' CCACAGCACCATCTCGCCCA 3'
<i>MdNRAMP4</i>	Fwd	5' CGGCCCTAGTGATGGTAATC 3'
	Rev	5' GCTACATACCCGGCAGTAAA 3'
<i>MdYSL5</i>	Fwd	5' GTCCATGTTTGTGAGCCAAATC 3'
	Rev	5' GGATACGCACTTCCAGGTATTC 3'
<i>β-Actin</i>	Fwd	5' TGGTGAGGCTCTATTCCAAC 3'
	Rev	5' TGGCATATACTCTGGAGGCT 3'

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Fig. 1. Elemental maps of latitudinal sections of roots of *Malus xiaojinensis*, using the SR- μ XRF technique. The top pictures are the images of longitudinal sections of root samples observed by microscope. The SR- μ XRF signals for map were collected at 50- μ m steps. The areas mapped for +Fe and -Fe were 800 μ m $\times$ 800 μ m and 650 μ m $\times$ 650 μ m, respectively. The samples were treated under +Fe condition (40 μ M FeNaEDTA), or -Fe condition (0 μ M FeNaEDTA) for 3 d. Scale bars = 100 μ m. The color of the bars from blue to red means the iron content from low to high. SR- μ XRF = Synchrotron radiation X-ray fluorescence.

nutrient solution for 1 month with pH = 6.0 (Han et al., 1994). The nutrient solution was replaced once a week in greenhouse cultured under the condition of $25 \pm 2^\circ\text{C}$ and 60% to 70% humidity. When the seedlings showed 8–12 mature leaves, they were transferred to Hoagland nutrient solution with either 0 μM FeNaEDTA for iron-limitation treatment or 40 μM FeNaEDTA for iron-sufficient treatment. Roots were collected after +Fe condition (40 μM FeNaEDTA) or –Fe condition (0 μM FeNaEDTA) for 3 d for RNA extraction and freezing-drying microtomy.

Freezing-drying microtomy and SR- μXRF . Root samples of 1-cm length were cut off from the root tip and embedded quickly. The embedded root sample was sliced to 200- μm -thick longitudinal and latitudinal sections with a freezing microtome and placed on Kapton tape, then freeze-dry about 24 h for SR- μXRF analysis.

The SR- μXRF microspectroscopy experiment was performed at 4W1B end station, Beijing synchrotron Radiation Facility, which runs 2.5 GeV electron with current from 150 to 250 mA. The incident X-ray energy was monochromatized by W/B₄C Double-Multilayer Monochromator at 15 keV and was focused down to 50 μm in diameter by the polycapillary lens. The two-dimensional mapping was acquired by step mode: the sample was kept on a precision motor-driven stage, scanning 50 μm stepwise for latitudinal samples and 100 μm stepwise for longitudinal samples. The Si (Li) solid-state detector was used to detect X-ray fluorescence emission lines with live time of 60 s. The data reduction and process were performed using a PyMCA (<http://sourceforge.net/projects/pymca/>) package (Sole et al., 2007).

Gene-expression analysis by quantitative real-time polymerase chain reaction (PCR). Total RNA of root samples was extracted by Cetyltrimethylammonium bromide (CTAB; Sinopharm Chemical Reagent Co., Ltd., Beijing, China) method (Zhang et al., 2005). The first-strand cDNA was synthesized by the Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Takara Biotechnology Co., Ltd., Dalian, China). The quantitative measurement of the gene expression was performed with an AB7500 Real-time PCR System (Applied Biosystems, Foster City, CA). The real-time PCR program is 30 s at 95°C , 5 s at 95°C , and 34 s at 60°C for 40 cycles in a 20 μL total volume with the instruction of the SYBR Green fluorescence dye (Takara Biotechnology Co., Ltd.). The primers of *MxIRT1* (NCBI: AY193886.1), *MxNRAMP1* (NCBI: AY188777), *MxCSI* (NCBI: HM459855.1), *MxNAS1* (NCBI: DQ403256.1), and *MdFRD3* (NCBI: NM_111683.1) (Zha et al., 2014) are listed in Table 1. The primers of *MxYSL5* (NCBI: JF910129), *MdNRAMP3*, and *MdNRAMP4* were designed by Primer Premier 5 software (PREMIER Biosoft International, USA). The sequences of the genes in the apple genome were obtained from the website (<http://www.rosaceae.org/>) via its BLAST (basic local alignment search tool) service. The housekeeping gene β -Actin was detected as an internal reference. The relative expression of genes was calculated by using $2^{-\Delta\Delta\text{Ct}}$ method (Livak and Schmittgen, 2001).

Results

Spatial distribution of selected divalent metal in the root of *Malus xiaojinensis* under low iron condition. Synchrotron radiation X-ray fluorescence was used to map the location of selected elements Fe, Zn, Mn, Ni, and Co

in the longitudinal and latitudinal root samples of *Malus xiaojinensis*. Under sufficient iron condition (+Fe), most of the Fe, Zn, Mn, Ni, and Co were distributed in the epidermis and cortex. By comparing the relative content of each micronutrient in the root, we also found that the highest relative content was Fe, and then were Zn and Mn. The relative contents of Ni and Co were obviously lower than the other three divalent metals (Fig. 1). However, iron deficiency (–Fe) induced a significant increase in the relative contents of five micronutrients in epidermis and cortex. Moreover, the micronutrients in epidermis increased much more than in cortex (Fig. 1). According to this increase, IRT1 can be free to uptake other three divalent metals because of the iron deficiency, which is called the substrate nonspecificity of IRT1. The absorbed divalent metals gathered in epidermis at first and then moved to the cortex and stele to use.

For the latitudinal root samples, we scanned it from root tip in a line, the line just located on the vascular. We used the ratio of metal micronutrients content at the same position to describe the metal micronutrients absorption variation of iron-deficiency response. We found that the ratio of element content of roots under –Fe condition and +Fe condition at same position was all more than one which suggested that iron deficiency induced metal micronutrients accumulation, and Fig. 2 also showed that the ratio increased obviously in the section of 1000- to 2000- μm distance from the root tip. This result indicated that iron deficiency might promote the long-distance transport of micronutrients.

Expression analysis of iron absorption- and transport-related genes in roots under low iron condition. As described above, the contents of five micronutrients were all

Fig. 2. The ratio of element content of roots under –Fe condition and +Fe condition at the longitudinal sections of *Malus xiaojinensis*. The first picture is the image of latitudinal sections of root sample observed by microscope. The SR- μXRF signals for map were collected at 100- μm steps. X-axis presents the distance from detected location to root tip of the longitude sample. The longitude samples measured for +Fe and –Fe treatment were all 2100 μm . The samples were treated under +Fe condition (40 μM FeNaEDTA), or –Fe condition (0 μM FeNaEDTA) for 3 d. Scale bar = 200 μm .

increased and participated in long-distance transport. To explain the phenomenon better, we detected the relative expression of metal ions absorption and transport genes. Results showed that the expression of *MdNramp3*, *MdNramp4*, *MxCS1*, *MxNAS1*, and *MdFRD3* increased in response to iron deficiency, especially *MdNramp3* and *MxCS1*, they increased nearly 400% than the control (+Fe). However, *MxIRT1*, *MxNramp1*, and *MxYSL5* were all slightly decreased after treatment (Fig. 3). These results showed that iron deficiency could promote the long-distance transport of iron and other divalent metals.

Discussion

Iron deficiency becomes one of the major limiting environmental factor in apple production worldwide. In recent years, systematic research has made about iron-deficiency adaptive responses in the level of organs, cells, subcells, and transcription. However, the research about the interactions between Fe and other divalent cations in tissue level is still little. Based on these, this study has used the technology of SR-μXRF first on iron-efficiency-rootstock *Malus xiaojinensis* to explore the absorption and transport of divalent cations in response to iron deficiency in the level of tissue.

Figure 1 showed that the relative content of Zn, Mn, Co, and Ni increased apparently in root epidermis and cortex under iron-deficiency condition. Moreover, the results of latitudinal root map and relative gene expression suggested that Fe, Zn, Mn, Co, and Ni are participated in the long-distance transport after iron-deficiency treatment. It has been reported that nicotianamine (NA) and citric acid can chelate Fe and transport it through phloem or xylem, so CS1 and NAS1 are important for Fe transport (Han et al., 2013). FRD3 may play a role in iron localization in *Arabidopsis* and is likely to function in root xylem loading of an iron chelator or other factor (Green and Rogers, 2004). Yellow Stripe 1-Like family of proteins function as the transporter of NA-metal chelates for loading and unloading in vessels (Gendre et al., 2007). The relative gene expression of *MxCS1* and *MxNAS1* was upregulated which were the important enzymes involved in metal-ion chelator synthesis, and the expression of *MxCS1* was 400% higher than the control (+Fe), *MdFRD3* upregulated 110% and *MxNAS1* increased 85%, whereas *MxYSL5* downregulated (Fig. 3). All these results indicated that the existence of long-distance transport and the transporters make a contribution. At the same time, iron-deficiency can induce a local signal within root tissue or a long-distance signal from shoot to root (Vert et al., 2003). Iron-deficiency signal coming from shoot could transmit to roots to enhance the Fe uptake and transport (Enomoto et al., 2007). Our research also demonstrated that the iron deficiency induced a long-distance signal which could stimulate the long-distance transport of iron to meet the demand of shoot under iron-deficiency condition. Phylogenetic analysis showed that *MdNramp3* and *MdNramp4* were in the same class with

Fig. 3. Expression analysis of different genes for Fe absorption and transport in roots of *Malus xiaojinensis* under different iron condition. Values are means of three biological replications. Standard errors are labeled. Significant differences were determined by *t* test between +Fe and -Fe condition at a significant level of $*P < 0.05$. The samples were treated under +Fe condition (40 μM FeNaEDTA) or -Fe condition (0 μM FeNaEDTA) for 3 d. IRT1 = iron-related transporter 1; Nramp = natural resistance associated macrophage protein; CS1 = citrate synthase 1; NAS1 = nicotianamine synthase 1; FRD3 = ferric reductase defective 3; YSL5 = Yellow Stripe 1-Like 5.

Fig. 4. Phylogenetic analysis of *MdNramp3* and *MdNramp4* with Nramp family (AtNramp1–6). The proteins studied have the following accession numbers: AtNramp1, NP_178198.1; AtNramp2, AAD41078.1; AtNramp3, AAF13278.1; AtNramp4, NP_201534.1; AtNramp5, NP_193614.1; AtNramp6, NP_173048.3.

AtNramp3 and *AtNramp4* (Fig. 4). *AtNramp3* and *AtNramp4* have been demonstrated to play an important role in the intracellular transport of Fe from the vacuole and they are functional redundantly at both the subcellular and tissue level (Lanquar et al., 2005). *MdNramp3* and *MdNramp4* may have the similar function like them. What is more, we found that the relative gene expression of *MdNramp3* upregulated nearly 400% than the control (+Fe) after iron deficiency, but *MdNramp4* just upregulated 85% than the control (+Fe) (Fig. 3). We speculated that *MdNramp3* might play an important role in the process of intracellular Fe redistribution in apple.

We found that the relative contents of Zn, Mn, Co, and Ni increased obviously; however, the relative gene expression of *MxIRT1* and *MxNramp1* downregulated slightly. So, we speculated that they might not play the primary role in the absorption of these four metal ions.

Further research should be made to explore the main metal-ion transporter in this process. Similarly, Figs. 2 and 3 indicated that iron deficiency promoted the long-distance transport of the metal ions in xylem, but the relative gene expression of *MxYSL5* downregulated slightly. Hence, if these five metal ions can be transported in phloem, more researches about the metal-ion transporters for phloem loading of Fe, Zn, Mn, Co and Ni should be studied. All these will trigger a series of exciting research projects to elucidate the interaction between Fe and other divalent cations and the molecular mechanism of metal ions transport in plant.

Literature Cited

Curie, C., J.M. Alonso, M. Le Jean, J.R. Ecker, and J.F. Briat. 2000. Involvement of NRAMP1 from *Arabidopsis thaliana* in iron transport. *Biometrical J.* 347:749–755.

- Enomoto, Y., H. Hodoshima, H. Shimada, K. Shoji, T. Yoshihara, and F. Goto. 2007. Long-distance signals positively regulate the expression of iron uptake genes in tobacco roots. *Planta* 227:81–89.
- Fukao, Y., A. Ferjani, R. Tomioka, N. Nagasaki, R. Kurata, Y. Nishimori, M. Fujiwara, and M. Maeshima. 2011. iTRAQ analysis reveals mechanisms of growth defects due to excess zinc in *Arabidopsis*. *Plant Physiol.* 155:1893–1907.
- Gendre, D., P. Czernic, G. Conéjéro, K. Pianelli, J.F. Briat, M. Lebrun, and S. Mari. 2007. TcYSL3, a member of the YSL gene family from the hyper-accumulator *Thlaspi caerulescens*, encodes a nicotianamine-Ni/Fe transporter. *Plant J.* 49:1–15.
- Green, L.S. and E.E. Rogers. 2004. FRD3 controls iron localization in *Arabidopsis*. *Plant Physiol.* 136:2523–2531.
- Guerinot, M.L. and Y. Yi. 1994. Iron: Nutritious, noxious, and not readily available. *Plant Physiol.* 104:815–820.
- Han, D.G., Y. Wang, L. Zhang, L. Ma, X.Z. Zhang, X.F. Xu, and Z.H. Han. 2013. Overexpression of *Malus xiaojinensis* *CS1* gene in tobacco affects plant development and increases iron stress tolerance. *Sci. Hort.* 150:65–72.
- Han, Z.H., Q. Wang, and T. Shen. 1994. Comparison of some physiological and biochemical characteristics between iron-efficient and iron-inefficient species in the genus *malus*. *J. Plant Nutr.* 17:1257–1264.
- Han, Z.H., T. Shen, R.F. Korcak, and V.C. Baligar. 1998. Iron absorption by iron-efficient and -inefficient species of apples. *J. Plant Nutr.* 21:181–190.
- Kim, S.A., T. Punshon, A. Lanzirrotti, L. Li, J.M. Alonso, J.R. Ecker, J. Kaplan, and M.L. Guerinot. 2006. Localization of iron in *Arabidopsis* seed requires the vacuolar membrane transporter VIT1. *Science* 314:1295–1298.
- Kobayashi, T. and N.K. Nishizawa. 2012. Iron uptake, translocation, and regulation in higher plants. *Annu. Rev. Plant Biol.* 63:131–152.
- Korshunova, Y.O., D. Eide, W.G. Clark, M.L. Guerinot, and H.B. Pakrasi. 1999. The IRT1 protein from *Arabidopsis thaliana* is a metal transporter with a broad substrate range. *Plant Mol. Biol.* 40:37–44.
- Lanquar, V., F. Lelievre, S. Bolte, C. Hames, C. Alcon, D. Neumann, G. Vansuyt, C. Curie, A. Schroder, U. Kramer, H. Barbier-Brygoo, and S. Thomine. 2005. Mobilization of vacuolar iron by AtNRAMP3 and AtNRAMP4 is essential for seed germination on low iron. *EMBO J.* 24:4041–4051.
- Livak, K.J. and T.D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods* 25:402–408.
- Marschner, H., V. Römhild, and M. Kissel. 1986. Different strategies in higher plants in mobilization and uptake of iron. *J. Plant Nutr.* 9: 695–713.
- Nishida, S., A. Aisu, and T. Mizuno. 2012. Induction of IRT1 by the nickel-induced iron-deficient response in *Arabidopsis*. *Plant Signal. Behav.* 7:329–331.
- Rogers, E.E., D.J. Eide, and M.L. Guerinot. 2000. Altered selectivity in an *Arabidopsis* metal transporter. *Proc. Natl. Acad. Sci. USA* 97:12356–12360.
- Sole, V.A., E. Papillon, M. Cotte, P. Walter, and J. Susini. 2007. A multiplatform code for the analysis of energy-dispersive X-ray fluorescence spectra. *Spectrochim. Acta, B At. Spectrosc.* 62:63–68.
- Vert, G.A., J.F. Briat, and C. Curie. 2003. Dual regulation of the *Arabidopsis* high-affinity root iron uptake system by local and long-distance signals. *Plant Physiol.* 132:796–804.
- Wu, T., H.T. Zhang, Y. Wang, W.S. Jia, X.F. Xu, X.Z. Zhang, and Z.H. Han. 2012. Induction of root Fe(III) reductase activity and proton extrusion by iron deficiency is mediated by auxin-based systemic signalling in *Malus xiaojinensis*. *J. Expt. Bot.* 63:859–870.
- Yang, T.J., P.J. Perry, S. Ciani, S. Pandian, and W. Schmidt. 2008. Manganese deficiency alters the patterning and development of root hairs in *Arabidopsis*. *J. Expt. Bot.* 59:3453–3464.
- Zha, Q., Y. Wang, X.Z. Zhang, and Z.H. Han. 2014. Both immanently high active iron contents and increased root ferrous uptake in response to low iron stress contribute to the iron deficiency tolerance in *Malus xiaojinensis*. *Plant Sci.* 214:47–56.
- Zhang, Y., J.H. Cheng, Z.H. Han, X.F. Xu, and T.Z. Li. 2005. Comparison of methods for total RNA isolation from *Malus xiaojinensis* and cDNA LD-PCR amplification. *Biotechnol. Bul.* 4:50–53.