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Research article

Characterization of MxFIT, an iron deficiency induced transcriptional factor in *Malus xiaojinensis*

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ABSTRACT

Iron deficiency often results in nutritional disorder in fruit trees. Transcription factors play an important role in the regulation of iron uptake. In this study, we isolated an iron deficiency response transcription factor gene, *MxFIT*, from an iron-efficient apple genotype of *Malus xiaojinensis*. *MxFIT* encoded a basic helix-loop-helix protein and contained a 966 bp open reading frame. MxFIT protein was targeted to the nucleus in onion epidermal cells and showed strong transcriptional activation in yeast cells. Spatio-temporal expression analysis revealed that *MxFIT* was up-regulated in roots under iron deficiency at both mRNA and protein levels, while almost no expression was detected in leaves irrespective of iron supply. Ectopic expression of *MxFIT* resulted in enhanced iron deficiency responses in *Arabidopsis* under iron deficiency and stronger resistance to iron deficiency. Thus, MxFIT might be involved in iron uptake and plays an important role in iron deficiency response.

1. Introduction

Iron is a required component of numerous proteins and enzymes involved in photosynthesis and respiration. Iron deficiency often results in chlorosis which is a worldwide problem affecting a variety of crops in alkaline or calcareous soils. Fruit trees are one of the most affected crops by iron nutritional disorder (Tagliavini and Rombolà, 2001). Limited iron nutrition significantly decreases fruit yield and quality. One of the best solutions depends on the use of tolerant rootstocks to enhance resistance to this abiotic stress (Gogorcena et al., 2000; Gonzalo et al., 2011). *Malus xiaojinensis* is a native apple rootstock in China and has well developed roots and good grafting compatibility with apple cultivars (Han et al., 2011). Furthermore, under iron deficiency, *M. xiaojinensis* possesses typical tolerance to iron deficiency stress and the trait of delayed time of chlorosis is genetically stable (Han et al., 1998). As a potential genetic resource for apple rootstock breeding, study on the underlying mechanism in *M. xiaojinensis* of its high iron absorption efficiency is significant. In the long process of evolution, plants have developed two strategies to mobilize and uptake iron effectively from soils, Strategy I in nongraminaceous plants and Strategy II in graminaceous plants (Schmidt, 2003). *M. xiaojinensis* uses the Strategy I response (Han et al., 1998). In this response, H^+-ATPase releases protons to lower the pH of the soil and acidify the rhizosphere, thus making Fe (III) more soluble. Fe (III) is then reduced to Fe (II) at the root surface though the inducible Fe (III)-chelate reductase FRO2 (Robinson et al., 1999). Fe (II) is finally transported into roots by the major iron transporter IRT1 (Eide et al., 1996).

Gene regulation is crucial for plants to adapt to the iron-limiting stress. The basic helix-loop-helix (bHLH) proteins are a large family of diverse transcription regulators in plants. Some bHLH regulators in response to iron deficiency stress have been clarified in both non-graminaceous and graminaceous species in recent years. The *FER* gene, coding a bHLH transcriptional factor required for regulation of iron deficiency inducible genes, was first identified from the tomato T3238*fer* mutant (Ling et al., 2002; Brumbarova and Bauer, 2005). The *fer* mutant showed a defective Strategy I responses and was unable to enhance the expression of the iron reductase gene *LeFRO1* and the iron transporter gene *LeIRT1* in the roots under iron deficiency (Bereczky et al., 2003; Li et al., 2004). Reciprocal grafting of the *fer* mutant to wild type tomato suggested that the *FER* gene was required in iron uptake in roots but not in shoots (Brown et al., 1971). The *Arabidopsis* ortholog of the *FER* gene, *FIT*, was identified from the 161 predicted bHLH proteins by microarray analysis (Bauer et al., 2007). FIT also plays an important role in positively regulating various iron deficiency inducible genes, including *IRT1* and *FRO2* (Bauer et al., 2007; Yuan et al., 2008). The *fit* mutant plants were chlorotic and died 2–3 weeks post-germination without supplemental iron supply (Colangelo and Guerinot, 2004). Heterogeneous expression of *FIT* in tomato *fer* mutation enabled to regain the ability to activate iron deficiency responses and showed normal growth under iron deficiency stress

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(Yuan et al., 2005). The formation of homodimers or heterodimers is an important feature of bHLH transcription factors (Massari and Murre, 2000). Previously, it has been reported that FIT directly interact with AtbHLH38 or AtbHLH39 in cells, forming heterodimers to regulate the target genes *IRT1* and *FRO2*, and resulting in enhanced tolerance in iron deficient plates and higher iron content in shoots (Yuan et al., 2008). *AtbHLH38* and *AtbHLH39* belonged to the subgroup Ib *bHLH* genes and were strongly induced under iron deficiency in both leaves and roots (Wang et al., 2007). There are some linkages between FIT and several signaling molecules, including ethylene, nitric oxide (NO) and auxin. In *Arabidopsis*, FIT interacted with the ETHYLENE INSENSITIVE 3 (EIN3) and ETHYLENE INSENSITIVE 3-LIKE1 (EIL1) transcription factors which play vital roles in ethylene signaling (Lingam, 2011). It was reported that NO affected the FIT protein abundance and the post-transcriptional control of FIT in *Arabidopsis* (Meiser et al., 2011). The plants defective in auxin transport were unable to induce the expression of *FIT* or any of its target genes (Chen et al., 2010). Therefore, ethylene, NO and auxin were considered as signals involved in the regulation of iron deficiency responses. Besides FER and its orthologous protein FIT, other transcription factors related iron absorption have been reported, including AtPYE, OsIRO2, IDEF1, IDEF2 and so on (Long et al., 2010; Ogo et al., 2006; Kobayashi et al., 2007; Ogo et al., 2008).

Although the molecular mechanisms of iron deficiency response have been increasingly studied, little research has been done in apple trees. In this study, we isolated and characterized the *MxFIT* gene from *M. xiaojinensis*, and ectopically expressed *MxFIT* in *Arabidopsis*. *MxFIT* gene and MxFIT protein are up-regulated under iron deficiency in *M. xiaojinensis* and plays an important role in adaptation to iron deficiency stress in transgenic *Arabidopsis*. This study offers an important basis for in-depth investigating the mechanisms of iron deficiency response in *Malus xiaojinensis*

2. Materials and methods

2.1. Materials

Malus xiaojinensis seedlings were propagated on Murashige and Skoog (MS) medium containing 0.5 mg/L 6-Benzylaminopurine (6-BA) and 0.5 mg/L Indole-3-Butytric acid (IBA). After one month, the propagating seedlings were transferred to MS medium with 1.0 mg/L IBA for rooting for 1.5 months. The rooted seedlings were moved to Hoagland nutrient solution (Han et al., 1994), with the pH adjusted to 6.0 using sodium hydroxide. The nutrient solution was replaced once a week. After 8-12 leaves expanded, the plants in Hoagland nutrient solution were treated with either 0 μM (iron limitation) or 40 μM (sufficient iron supply) FeNaEDTA. Roots and young leaves were collected after 0 h, 12 h, 1-, 3-, 6- and 9-days of iron deficiency treatment for RNA and protein extraction.

Seeds of *Arabidopsis thaliana* (ecotype Columbia) were surface sterilized in 75% ethanol for 30 s, 25% sodium hypochlorite for 10 min and then washed 3 times with sterile distilled water. Seeds were germinated on MS medium supplemented with 2% sucrose, 0.6% agar and pH 5.8. Plates were cultured at 21 °C with a 16 h light and 8 h dark. For the iron deficient experiments, 12-day-old seedlings were transferred to iron-sufficient plates with 50 μM FeNaEDTA or iron-deficient plates without FeNaEDTA for four and seven days (Colangelo and Guerinot, 2004).

2.2. Cloning of MxFIT

Based on the gene sequences of *LeFER* and *AtFIT*, and the genome of the apple cultivar 'Golden Delicious' (http://genomics.research.iasma.it/) (Velasco et al., 2010), a pair of specific primers, (F) 5′-ATGGATTCGCTGGGAAACCA-3′ and (R) 5′- TTAGGCTGAGAATCCA-GAAGC-3′, was designed to amplify *MxFIT*. The PCR reaction was carried out at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min, with a final extension at 72 °C for 7 min. The amplification products were purified and subcloned into the pEasy-T1 vector (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. Three positive clones were sequenced respectively.

2.3. Intracellular localization analysis

The open reading frame (ORF) of *MxFIT* was subcloned into the pEZS-NL vector to generate the fusion gene *35S::MxFIT-eGFP*. The recombinant plasmid and the empty pEZS-NL vector (control) were each introduced into onion epidermal cells using particle bombardment device (Biolistic PDS-1000/He, Bio-Rad) according to the instruction manual. After incubating at 26 °C in darkness for 14–18 h, the transformed onion epidermis cells were observed by fluorescence microscope (Nikon Eclipse TE2000-E).

2.4. Transactivation experiment in yeast cells

The coding sequence of *MxFIT* was sub-cloned into the pGBKT7 vector at the *Eco*RI and *Sal*I insertion sites. The verified recombinant vector and the empty pGBKT7 vector (control) were respectively transformed into *Saccharomyces cerevisiae* AH109 yeast strain (*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4*Δ, *gal80*Δ, *LYS2::GAL1UAS-GAL1TATA-HIS3, GAL2UAS-GAL2TATA-ADE2, URA3::-MEL1UAS-MEL1TATA-lacZ*) using acetic acid lithium method (Gietz et al., 1992). The transformed yeast cells were cultivated on synthetic dextrose (SD) medium without tryptophan. Then the obtained colonies were tested for lacZ reporter activity using 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside (X-gal) as substrate.

2.5. Quantitative real-time PCR of MxFIT in roots and leaves of M. xiaojinensis

The relative expression level of *MxFIT* in roots and leaves was assayed with Applied Biosystems 7500 Real-time PCR device. Total RNA was isolated from the iron deficient roots using the CTAB method. Then RNA was digested by DNase (TaKaRa Biotechnology Co., Ltd., Dalian, China) and the first-strand cDNA was synthesized by PrimeScript RT reagent kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). Primers were designed to generate 100-200 bp amplification products by Primer Premier 5 software. The housekeeping gene *β-Actin* was used as a control. Specific primers were applied to amplify *MxFIT* (5′-GGGAAACCATCAAGGAGGTCATA-3′ and 5′-AGCCATTCATCATAAGGTCAGGA-3′) and *β-Actin* (5′-TGGTGAGGCTCTATTCCAAC-3′ and 5′-TGGCATATACTCTGGAGGCT-3′). The PCR reaction conditions were 30 s at 95 °C, 5 s at 95 °C, 34 s at 60 °C for 40 cycles. Each PCR reaction was carried out in triplicate. The relative expression was calculated by the $2^{-\Delta\Delta CT}$ method ([LivakandSchmittgen, 2001]).

2.6. Protein extraction and Western blot

Proteins were extracted according to trichloroacetic acid/acetone method (Damerval et al., 1986). In short, roots and leaves were fully ground in liquid nitrogen respectively, subsequently transferred into the extraction solution (containing 10% trichloroacetic acid in acetone) and left overnight at −20 °C. The extraction was centrifuged and precipitate was washed three times with ice-cold acetone. The sediment was then dried to powder by vacuum freezing and dissolved in lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS and 40 mM Tris-base). Nucleic acids were eliminated in an