Purification, immunological, and functional characterization of MxFIT in Malus xiaojinensis

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Abstract

MxFIT is a FER-like iron deficiency induced transcriptional factor in Malus xiaojinensis. Here, we described the heterologous expression of MxFIT in Escherichia coli BL21 (DE3) host cells. The E. coli harboring the recombinant construct pET-MxFIT was efficiently induced to express the MxFIT protein at a high level and the optimal profile for MxFIT expression was investigated. By inoculating a New Zealand rabbit with purified MxFIT-His fusion protein, a high specific anti-MxFIT antiserum was achieved. Western blot analysis showed MxFIT protein expression was induced in roots when iron supply was limiting and was inhibited when iron supply was excessive. In leaves, there was almost no expression irrespective of iron supply. The localization of MxFIT on transverse section of root indicated that MxFIT participated in iron deficiency response. Over-expression of MxFIT in transgenic tobacco suspension cells showed that MxFIT increased iron absorption under insufficient iron supply. This study provides a basis for further investigating the underlying mechanism of high iron absorption efficiency in M. xiaojinensis.

Keywords: Escherichia coli, iron uptake, MxFIT protein localization, tobacco suspension, Western blot.

Introduction

Iron is an essential microelement in plants as it is involved in many metabolic processes such as photosynthesis and respiration. Iron deficiency will impact plant growth, development, yield, and plant product quality (Briat et al. 2015). Although Fe is abundant on the earth, the available Fe is limited due to its complexation into insoluble Fe(III)-oxyhydroxide, especially in alkaline or calcareous soils (Kobayashi and Nishizawa 2012). In order to overcome Fe shortage, plants have evolved two strategies to mobilize Fe actively in the soil. Dicot and non-graminaceous monocot plants mobilize iron via strategy I response and the grasses use strategy II response to absorb iron efficiently (Marschner and Römheld 1994). In strategy I response, H\textsuperscript{+}-ATPase releases protons to acidify the rhizosphere, making more...
soluble Fe (III). Furthermore, most plant species secrete small molecules including carboxylic acids, coumarins, and riboflavin derivatives into the rhizosphere to chelate soil Fe\(^{3+}\) (Cesco et al. 2010, Rodríguez-Celma et al. 2013, Connorton et al. 2017). Fe\(^{3+}\) is thus reduced into Fe\(^{2+}\) by ferric reductase oxidase 2 (FRO2), and the released ferrous iron is taken up via iron-regulated transporter 1 (IRT1; Curie and Briat 2003, Hell and Stephan 2003). Strategy II plants chelate Fe\(^{2+}\) by secreting phytosiderophores, which have a high affinity for iron (Kobayashi et al. 2014).

Transcriptional regulation is a crucial way to regulate Fe homeostasis under Fe-deficient conditions. A number of basic helix-loop-helix (bHLH) transcription factors (TFs) have been identified to be positively involved in an intricate network of Fe deficiency response (Kobayashi and Nishizawa 2012). The upstream of the regulatory network in Arabidopsis is four IVc bHLH TFs, namely bHLH34, bHLH104, bHLH105, and bHLH115 (Gao et al. 2019). These four TFs interact in the form of homodimers or heterodimers in vivo to participate in plant response to Fe deficiency (Zhang et al. 2015, Li et al. 2016, Liang et al. 2017). Furthermore, these four TFs positively activate the transcription of genes encoding the Ib bHLH TFs bHLH38, bHLH19, bHLH20, and bHLH25 and regulates the accumulation of FIT protein. The interaction of IVa bHLHs with FIT promotes FIT degradation via the 26S proteasome pathway (Cui et al. 2018). Moreover, two closely related RING E3 ligases, BTL1 and BTL2, directly target FIT degradation and negatively regulate Fe deficiency responses (Sivitz et al. 2011, Hindt et al. 2017, Rodríguez-Celma et al. 2019). Therefore, there are different signaling pathways that either activate or inhibit FIT function to maintain Fe homeostasis in Arabidopsis.

In apple (Malus domestica), an IVc subgroup of bHLH TF gene MdHBHL104 was cloned and characterized. MdHBHL104 directly binds to the promoter of the MdAHA8, MdHBHL38, and MdHBHL39 genes and regulates plasma membrane H\(^{+}\)-ATPase activity under Fe-deficient conditions (Zhao et al. 2016a). Therefore, MdHBHL104 has a crucial function in iron acquisition and the tolerance to iron deficiency. Under high iron supply, E3 ubiquitin ligase MdCUL3 and BTB/TAZ protein MdBT2 complex target MdHBHL104 and promote the degradation of MdHBHL104 protein via the 26S proteasome pathway, thereby controlling the activity of H\(^{+}\)-ATPases and the acquisition of iron (Zhao et al. 2016b). Under Fe-deficient conditions, MdHBHL104 sumoylation, mediated by the SUMO E3 ligase MdSIZ1, promotes the stability of MdHBHL104 protein, thereby activating H\(^{+}\)-ATPases and enhancing iron acquisition (Zhou et al. 2018, Gao et al. 2019). In addition, MxFIT is also an important transcription factor for Fe homeostasis in apple (Malus xiaojinensis). In our previous study, we isolated Arabidopsis ortholog of the FIT gene, MxFIT, from Malus xiaojinensis (M. xiaojinensis). M. xiaojinensis is an iron deficiency resistant apple stock and its trait of delayed time of chlorosis under the absence of iron is genetically stable (Han et al. 1998, 2011). Under iron limiting conditions, MxFIT had up-regulated expression at mRNA level only in roots. Ectopic expression of MxFIT in Arabidopsis could activate iron deficiency responses by regulating IRT1 and FRO2 expression (Yin et al. 2014). The aim of this paper was to describe the expression of MxFIT gene in Excherichia coli BL21 (DE3) host cells, purification of recombinant proteins, and preparation of polyclonal antibody against MxFIT. Then the prepared antibody will be used to research the expression, distribution of MxFIT protein in tissues and to further elucidate its functions. This study could provide an important basis for in-depth study of the pathway of iron deficiency response in M. xiaojinensis.

Materials and methods

Growth conditions: Malus xiaojinensis

M.G. Cheng & N.G. Jiang seedlings were grown on Murashige and Skoog (MS) medium containing 0.5 mg dm\(^{-3}\) 6-benzylaminopurine (6-BA) and 0.5 mg dm\(^{-3}\) indole-3-butyric acid (IBA) for one month. Then the seedlings were moved to MS medium with 1.0 mg dm\(^{-3}\) IBA for rooting for 1.5 months. The rooted plants were grown in Hoagland nutrient solution which was replaced once a week (Han et al. 1994). When the plants had ten to twelve leaves, they were transferred to Hoagland nutrient solution with 4 μM (iron limitation), 40 μM (normal iron supply), and 160 μM (excessive iron supply) FeNaEDTA, respectively. Roots and leaves were collected after three days of treatments for MxFIT expression and immunohistochemical localization of MxFIT protein.

Prokaryotic expression of the recombinant MxFIT: The complete cDNA of MxFIT was sub-cloned into the pET-30a (+) to generate the recombinant plasmid pEET-MxFIT. Then the recombinant vector was subsequently introduced into E. coli BL21 (DE3). The E. coli BL21 (DE3) strain harboring the pEET-MxFIT vector was incubated in Luria-Bertani (LB) liquid medium with kanamycin (50 μg cm\(^{-3}\)) at 37°C in a shaking incubator until cells reached logarithmic growth period (the absorbance at 600 nm of 0.5). Then the cultured cells were induced with different isopropyl-β-D-thiogalactoside (IPTG) concentrations (0, 0.25, 0.5, 0.75, and 1 mM) for different times (1, 2, 3, 4, and 5 h) and at different temperatures (25, 30, and 37°C). After induction, the cells were harvested by centrifugation at 10 000 g and 4°C for 15 min. The collected cells were re-suspended in solution I (50 mM sodium phosphate, 300 mM NaCl, 1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride;
pH 8.0) and subsequently digested with lysozyme at a final concentration of 50 µg dm⁻³ at room temperature for 30 min. Then the cell suspension was sonicated five times on ice with interruption for 10 s every 10 s. The resulting cell lysate was centrifuged at 12,000 g and 4 °C for 20 min. The supernatant was discarded and the sediment was re-suspended in solution II (50 mM sodium phosphate, 300 mM NaCl, 0.5 mM EDTA, and 1 % Triton-100; pH 8.0). All samples were assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**Purification of recombinant MxFIT**: The sample containing recombinant MxFIT was loaded onto Ni-NTA His-Bind Resin (Novagen, Madison, USA) and the fusion protein was eluted according to instruction manual. In brief, the sample was instilled into the resin column equilibrated with phosphate buffer (50 mM sodium phosphate buffer, 300 mM NaCl and 20 mM imidazole, pH 8.0). After washing the column with a 10 column volumes binding buffer (300 mM NaCl, 50 mM sodium phosphate buffer, and 20 mM imidazole, pH 8.0), the fusion protein was eluted with 5 column volumes eluting buffer (300 mM NaCl, 50 mM sodium phosphate buffer, and 250 mM imidazole; all at pH 8.0).

**Antibody preparation**: Preparation of antibody was done according to Pan et al. (2005). Initially, New Zealand rabbit was immunized using 500 µg of the purified MxFIT recombinant protein after being emulsified with Freund’s complete adjuvant. After 1 month, the rabbit was boosted three times with 250 µg purified MxFIT recombinant protein each in incomplete Freund’s adjuvant at 2-weeks interval. Finally, the serum was obtained after the last bleeding. The rabbit IgG fraction was prepared by precipitation with 50 % saturated (NH₄)₂SO₄ and purified by DEAE-Sepharose column chromatography.

**Immunoblotting and antibody titer determination**: Immunoblotting was performed following the methods described by Wang et al. (2008). Antibody sensitivity was measured by protein dot blot and was defined as the minimum amount of antigen that could be visualized after coloration. Different protein quantities (614.4, 307.2, 153.6, 76.8, 38.4, 19.2, 9.6, 4.8, 2.4, and 1.2 ng) of purified MxFIT were diluted in 50 mM carbonate salt buffer (pH 9.6) and spotted on the nitrocellulose membranes printed with 96-well format. Then the membrane was left at room temperature until dry. Immunological assay on the nitrocellulose membranes was performed using anti-MxFIT antibody diluted 1 500-fold and anti-rabbit antibody conjugated with alkaline phosphatase as the secondary antibody. Finally, a color reaction was visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) alkaline phosphatase substrate solution.

Antibody titer was determined by ELISA according to previous method and was defined as the dilution times of the antibody corresponding to an absorbance of 0.500 at 490 nm (Wan et al. 2010). In brief, purified antigen was diluted to 10 µg cm⁻³ in coating buffer (15 mM Na₂CO₃ and 35 mM NaHCO₃, pH 9.6) and was assayed at 100 mm² per well of microtiter plates. The plates were kept at 37 °C for 2 h and then at 4 °C for 24 h. The diluted antibodies (1 000-, 2 000-, 4 000-, 8 000-, 16 000-, 32 000-, 64 000-, and 128 000-fold) reacted with MxFIT antigen for 3 h on the microtiter plates. The plates were washed and secondary antibody conjugated with horseradish peroxidase was used to incubate the plates for 1 h. The excess of the secondary antibody was then removed and the peroxidase activity was detected using phenylenediamine and H₂O₂ as substrates reacting for 20 min. The reaction was stopped with 2 M of sulfuric acid and the absorbance at 490 nm was detected using a Model 680 microtiter plate reader (Bio-Rad, Hercules, USA).

**Protein extraction and Western blot**: Proteins were extracted using trichloroacetic acid/acetone method (Damerval et al. 1986). Briefly, samples were fully ground with liquid nitrogen and transferred into extraction buffer (10 % trichloroacetic acid in acetone). The extract was left at -20 °C overnight. Then the extract was centrifuged, and sediment was collected and washed with pre-cooling acetone for three times. The washed pellet was then freeze dried by vacuum freezing apparatus. The dried powder was lysed in 40 mM Tris-base containing 7 M urea, 2 M thiourea, and 4 % (m/v) 3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonate (CHAPS) for 2 h on the ice. Finally, nucleic acids were degraded with ultrasonic processor and supernatant was collected.

Western blotting was performed as previously described (Cao et al. 2011). Briefly, total protein was loaded on the 12 % SDS-polyacrylamide gel for separation and subsequently transferred onto nitrocellulose (NC) membranes. Then membranes were blocked, incubated with antibody, washed, incubated with goat anti-rabbit antibody conjugated with alkaline phosphatase (Sigma, St. Louis, USA) as the secondary antibody for another 1 h, and washed. Finally, the membrane was developed with 10 cm² of BCIP/NBT in the dark, and the reactions were terminated by adding double-distilled water.

**MxFIT immunohistochemical localization on root cross-sections**: The MxFIT immunohistochemical localization was conducted essentially as described previously with some modifications (Hou and Huang 2005). Roots from *M. xiaojinensis* grown under normal iron (40 µM) and low iron (4 µM) supply conditions for three days were fixed overnight in 4 % paraformaldehyde and 2.5 % glutaraldehyde solution at 4 °C, dehydrated using graded ethanol, embedded in paraffin, and sectioned into 10 µm slices. Then the sections were deparaffinized with xylene and hydrated in an ethanol-water series. After blocked with blocking solution and washed using phosphate-buffered saline, the transverse sections were incubated using anti-MxFIT antibody (1:200) followed by anti-rabbit antibody conjugated with alkaline phosphatase as the secondary antibody (*Sigma*). The signals were visualized by a BCIP/NBT color reaction. Images were recorded using inverted microscope (Nikon-TI200, Japan).
Analysis of transgenic tobacco callus: The MxFIT full-length sequence was subcloned into pCAMBIA2300 to generate the pCAMBIA2300-35S:MxFIT recombinant vector. Then the construct was introduced into Agrobacterium strain EHi105, and these clones were finally transformed into tobacco callus. Transgenic callus was selected on MS plates with 30 mg dm$^{-3}$ kanamycin and 150 mg dm$^{-3}$ cephalosporin. Selected callus was cultured every 7 d until stabilization, for five generations. Positive transgenic tobacco callus was detected by PCR analysis and cultured in MS medium for three generations. The suspension cells with normal iron supply were used to detected MxFIT expression (by RT-PCR and Western blot). Active Fe content of the suspension cells under iron deficiency (4 μM) and iron sufficiency supply (100 μM) was measured using polarised Zeeman atomic absorption spectrophotometry (Z-5000, Hitachi, Tokyo) as previously described (Han et al. 1994).

Results

Escherichia coli BL21 (DE3) cells containing the recombinant construct pET-MxFIT were induced with different concentration of IPTG to express the MxFIT-His fusion protein. SDS-PAGE revealed that the recombinant polypeptide was found with a molecular mass around 50 kDa, while the corresponding band was not found in the cells without IPTG induction (Fig. 1A). To further confirm the band of MxFIT, Western blotting analysis with anti-His monoclonal antibody was performed. The results showed that a specific band appeared in the same place corresponding to SDS-PAGE analysis, while it was not found without IPTG induction (Fig. 1B). The results demonstrated that the MxFIT was successfully expressed in the E. coli cells.

We first studied the effect of IPTG concentration on the expression of MxFIT. The result in Fig. 1A showed that fusion protein yields were different with varying IPTG concentrations (0.25, 0.5, 0.75, and 1 mM). The signal intensity of the MxFIT expression was analyzed by the software Image J. The results showed that the maximum expression of MxFIT was obtained when the IPTG concentration was 0.5 mM (Fig. 1C). Then we investigated the optimal fermentation condition by varying induction time and temperature. The expression yield reached the maximum after induction for 4 h. Temperatures also had a certain influence and the yield was relatively higher at 37 °C than at 25 °C and 30 °C (Fig. 1D). Our results indicated that the maximum amount of the MxFIT protein was achieved at 37 °C using 0.5 mM IPTG induction for 4 h.

The purified MxFIT was used as antigen for inoculating New Zealand rabbit. The sensitivity of the obtained anti-MxFIT immunoglobulin fractions (IgG) was assayed by protein dot blot and enzyme-linked immunosorbent analysis. The results showed that antibody diluted 1 500-fold (final concentration of about 360 μg dm$^{-3}$) could detect 4.8 ng of antigen (Fig. 2A) and antibody diluted about 10 000 times (final concentration of about 54 μg dm$^{-3}$) was able to detect 1 μg of the antigen (Fig. 2B). These results suggested that the anti-MxFIT antibody had a high degree of detection sensitivity and could be used for further research.

Malus xiaojinensis seedlings were grown at deficient (4 μM), normal (40 μM), and excess (160 μM) iron supply for 3 d and the roots and leaves were collected. The total root and leaf protein extracts were subsequently used to analyse MxFIT expression (Fig. 3A). In root protein extracts, the band of MxFIT was immunologically detectable. This band was absent in leaf protein extracts regardless of iron supply. In roots, MxFIT protein content
was higher when plants were grown at deficient compared to normal iron supply. At 160 μM FeNaEDTA supply, the amount of MxFIT protein was undetectable. Thus, MxFIT protein expression was induced in roots when iron supply was limiting and inhibited when iron supply was excessive.

To investigate the cellular localization of MxFIT protein in response to iron supply, immunohistochemical localization of MxFIT was performed on day 3 in transverse root tip sections of mature root-hair zone under deficient and sufficient iron supply. The results showed that the signals were stronger in the stele than in epidermis and cortex cells under 160 μM Fe (Fig. 3B), and they become more intense in the whole region of the root section under 4 μM Fe (Fig. 3C). Compared with iron excess, MxFIT protein distribution showed no tissue specificity when iron supply was limited.

To further verify the function of MxFIT, pCAMBIA2300-35S:MxFIT recombinant vector was transformed into tobacco suspension cells. Three positive cell lines were generated and the expression of MxFIT in transgenic tobacco cells under normal iron supply was detected using RT-PCR and Western blot, respectively. The results showed that MxFIT expression and protein content in the transformed tobacco cells were detectable, while in the wild type cells were not detected (Fig. 4A). Under iron-deficiency supply (4 μM) of 1 day, active iron content was significantly higher in the three transformed cell lines than in the wild type cells. However, there was no significant difference between transformed tobacco cell lines and wild type cells under sufficient iron supply (100 μM) (Fig. 4B). Over-expression of MxFIT increased iron absorption under iron deficiency in tobacco suspension cells.

**Discussion**

Heterologous expression of recombinant proteins or protein fragments is vital for further analysis of protein structure and function. Prokaryotic expression in *E. coli*
has been widely used as the system for heterologous protein expression owing to its high expression, comparatively facile procedure, and the relatively low cost (Murby et al. 1996). The inducing conditions (concentrations of IPTG, temperatures, and times) have a direct effect on the form of the fusion protein. Low concentrations of IPTG might be unable to activate exogenous gene transcription, while high IPTG concentrations damage the host cells. Additionally, low induction temperatures affect the expression yields of the recombinant protein, while high temperatures might lead to insoluble inclusion bodies (Makrides 1996).

In this study, we optimized the induction conditions and the amount of MxFIT-His recombinant protein reached the highest content at 37 °C using 0.5 mM IPTG induction for 4 h. The yields were decreased when induced with 1 mM IPTG, inferring the damage to the host cells by higher IPTG concentration.

Our previous research showed that MxFIT responded to iron deficiency in roots at transcriptional level (Yin et al. 2014). In this study, MxFIT protein expression patterns were investigated. MxFIT protein content was higher in iron deficient roots than under normal iron supply and was hardly detectable in leaves regardless of iron supply. The MxFIT protein was consistent with MxFIT mRNA in response to iron deficiency. In tobacco, FER protein content is either similar or slightly lower when plants are grown at sufficient iron supply compared to plants grown at deficient iron supply and it is undetectable after excess iron supply (Brumbarova and Bauer 2005). These results suggest that MxFIT and FER are regulated in distinct cell types under different iron supply. The distribution of MxFIT in the root transverse sections showed MxFIT protein was located in the whole region of the root section in mature zone and was not tissue-specific in response to the iron supply. More intense signals were seen in the roots under iron deficiency than in the root under iron sufficiency. Previous study proved that AtFIT is expressed mainly in the outer cell layers of the mature zone in root, and that FER protein mainly concentrates in the parenchyma cells inside the vascular cylinder in the mature root hair zone (Colangelo and Guerinot 2004, Brumbarova and Bauer 2005).

Because the iron deficiency response genes FRO2 and IRT1 were localized in the outer layers of the root, it is speculated that AtFIT and MxFIT might regulate IRT1 and FRO2 in epidermis cells in a more direct manner than FER (Colangelo and Guerinot 2004). Generally, iron is transported from the root epidermis to the central cylinder, where it is translocated into xylem and then transported to shoot in the form of carbonic acids compound (Lopez-Millan et al. 2000). Therefore, we speculated MxFIT may also be involved in regulating of iron-transport in the vascular system because of its more intense signal in the stele in starved-root.

To further verify the biological functions of MxFIT, MxFIT was over-expressed in tobacco suspension cells. The study proved that over-expression of MxFIT increased iron content under iron deficiency in tobacco suspension cells. The previous results indicated that MxFIT over-expressing plants showed stronger resistance to iron deficiency by activating the transcript of IRT1 and FRO2 in roots (Yin et al. 2014). Therefore, over-expressed MxFIT both in cells and in plant demonstrated that MxFIT was closely related to iron absorption, and played an important role in rapid adaptation to changing iron environment to maintain iron homeostasis. Identification of the similar biological functions of AtFIT and MxFIT suggests that FIT might be a universal gene presented in Strategy I plants in controlling iron acquisition in roots. The plants over-expressing FIT together with A. thaliana H3/39/100/101 in Arabidopsis shows constitutive expression of the iron uptake genes FRO2 and IRT1 (Yuan et al. 2008). The single over-expression of AtFIT, A. thaliana H3/39 and A. thaliana H3/101 plants show increased expression of FRO2 and IRT1 only under iron deficiency, which agrees with the result of MxFIT over-expressing plants (Yuan et al. 2008, Wang et al. 2013, Jakoby et al. 2014). However, the single overexpression of A. thaliana H3/39 and A. thaliana H3/101 is able to enhance the expression of IRT1 and FRO2 and the plants accumulate more iron even under iron-sufficient conditions (Yuan et al. 2008, Wang et al. 2013). Our results showed over-expression of MxFIT in tobacco suspension cells increased iron content under iron deficiency. MxFIT overexpression in Arabidopsis plants also activates the transcription of IRT1 and FRO2 in roots (Yin et al. 2014). So, we speculated that there might be inducible factors, homologous of A. thaliana H3/39/100/101, together with MxFIT regulating iron absorption in Malus xiaojinensis, but this needs further study.

In conclusion, we heterologously expressed the MxFIT in BL21 (DE3) host cells, purified the MxFIT-His protein and prepared anti-MxFIT antiserum. The MxFIT protein expression characteristics and immunolocalization patterns in the root transverse sections indicate that MxFIT is related to iron absorption. Over-expression of MxFIT in transgenic tobacco suspension cells also proved this point. This study offers an important foundation for further research of iron stress response mechanisms in M. xiaojinensis.

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