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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^+-ATPase releases protons to acidify the rhizosphere, making more

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Abbreviations: 6-BA - 6-benzylaminopurine; BCIP/NBT - 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium; bHLH - basic helix-loop-helix; FRO2 - ferric reductase oxidase 2; IBA - indole-3-butytric acid; IPTG - isopropyl-β-D-thiogalactoside; IRT1 - iron-regulated transporter 1; LB - Luria-Bertani; *M. xiaojinensis* - *Malus xiaojinensis*; MS medium - Murashige and Skoog medium; TF - transcription factor; WB - Western blot; WT - wild type.
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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^{3+} 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* are 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 bHLH38/39/100/101(Wang *et al*. 2007, 2013, Gao *et al.* 2019). The bHLH38, bHLH39, bHLH100, and bHLH101 are the positive regulators of *FRO2* and *IRT1*. This activity relies on the heterodimer formed by their interaction with IIIa bHLH Fe-deficiency induced transcription factor FIT, an ortholog of the FER (Colangelo and Guerinot 2004, Yuan *et al*. 2008, Wang *et al*. 2013). FIT is proposed as an essential and central regulator to keep iron balance in roots in a changing environment (Bauer *et al*. 2007, Schwarz and Bauer 2020)**.** The main regulator *bHLH6/MYC2* of the jasmonic acid signaling pathway differentially affects the expression of IVa bHLH genes (*bHLH18*, *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, BTSL1 and BTSL2, directly target FIT degradation and negatively regulate Fe deficiency responses (Sivitz *et al*. 2011, Hindt *et al*. 2017, Rodriguez-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 *MdbHLH104* was cloned and characterized. MdbHLH104 directly binds to the promoter of the *MdAHA8*, *MdbHLH38*, and *MdbHLH39* genes and regulates plasma membrane H^+-ATPase activity under Fe-deficient conditions (Zhao *et al*. 2016a). Therefore, MdbHLH104 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 MdbHLH104 and promote the degradation of MdbHLH104 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, MdbHLH104 sumoylation, mediated by the SUMO E3 ligase MdSIZ1, promotes the stability of MdbHLH104 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 *Escherichia 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-butytric 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 pET-*MxFIT*. Then the recombinant vector was subsequently introduced into *E. coli* BL21 (DE3). The *E. coli* BL21 (DE3) strain harboring the pET-*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 $(NH_4)_2SO_4$ 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 Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.6) and was assayed at 100 mm^3 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 H_2O_2 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 cm^{-3} 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 EH105, 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

Esherichia 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. 1*A*). 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. 1*B*). 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. 1*A* 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. 1*C*). 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. 1*D*). 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. 2*A*) 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. 2*B*). 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. 3*A*). 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

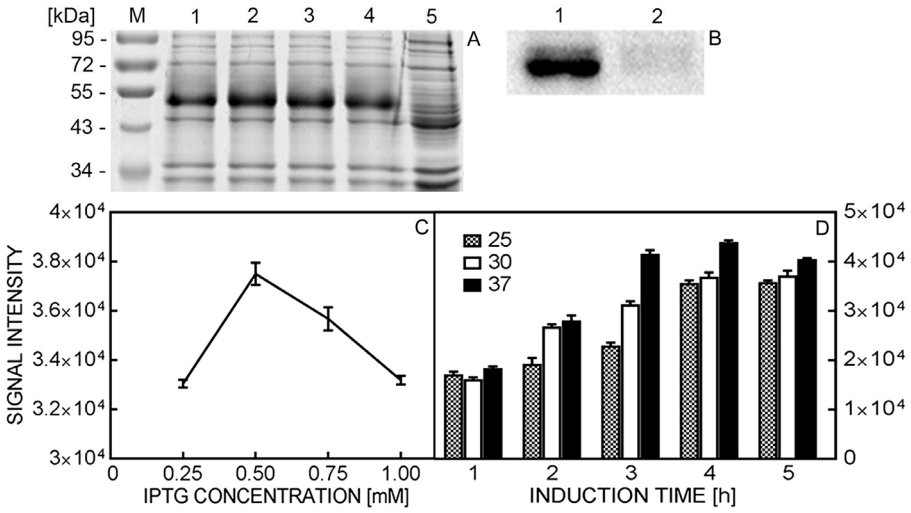

Fig. 1. Expression and optimization of recombinant *MxFIT*. *A* - SDS-PAGE analysis of *MxFIT* expression. *Lanes* 1 – 4 mean the *E. coli* induced with different IPTG concentrations (0.25, 0.5, 0.75, and 1.0 mM) and *Lane* 5 means the *E.coli* induced without IPTG.; M - standard protein molecular mass markers. *B* - Western blotting analysis of *MxFIT* expression with anti-His monoclonal antibody. *Lane*s 1 and 2 mean the result in the strain transfected with recombination plasmid pET-*MxFIT* and plasmid pET-30a (+). *C* - Effect of IPTG with concentrations 0.25, 0.5, 0.75, and 1.0 mM on MxFIT production. *D* - Effect of temperature (25 °C, 30 °C and 37 °C) and fermentation time on MxFIT production. The signal intensity was analyzed by the software *Image J*.

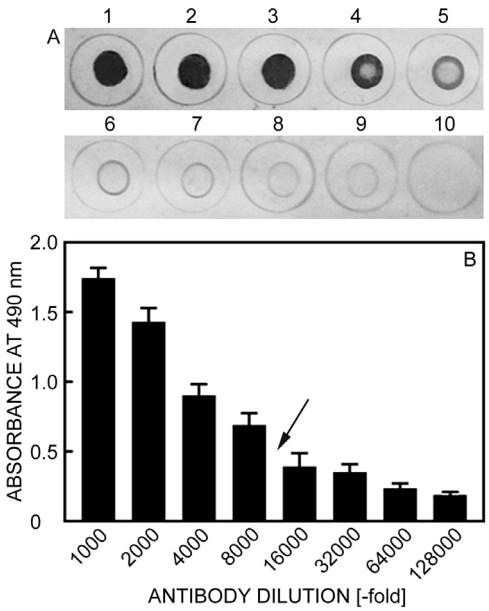

Fig. 2. Sensitivity detection of anti-MxFIT antibody. *A* - Protein dot blot with 1/1500 dilution of anti-MxFIT antibody. *Dots* 1 - 10 indicate purified MxFIT protein quantity of 614.4, 307.2, 153.6, 76.8, 38.4, 19.2, 9.6, 4.8, 2.4, and 1.2 ng, respectively. *B* - Analysis of anti-MxFIT titer. *Arrow* indicates antibody dilution of about 10 000-fold at an absorbance value of 0.5. Means ± SEs of three replicates.

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. 3*B*), and they become more intense in the whole region of the root section under 4 µM Fe (Fig. 3*C*). 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. 4*A*). 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. 4*B*). Over-expression of *MxFIT* increased iron absorption under iron deficiency in tobacco suspension cells.

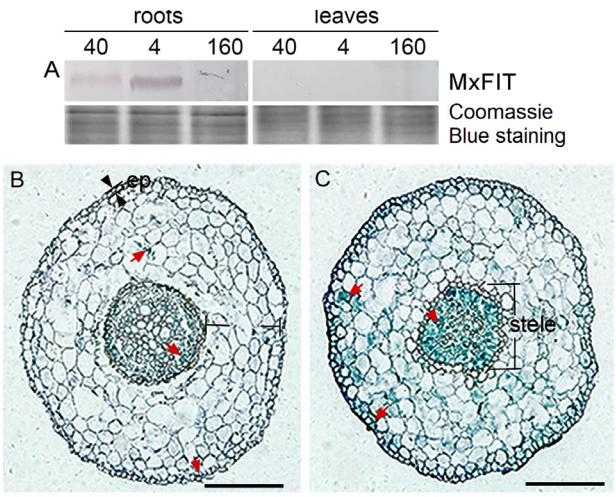

Fig. 3. Expression and immunohistochemical localization analysis of MxFIT. *A* - Western-blot analysis of MxFIT protein expression in roots and leaves of plants grown with different iron concentrations (4 µM, 40 µM and 160 µM). Coomassie Blue staining was used as a loading control. MxFIT immunohistochemical localization using anti-MxFIT antiserum on the 10 µm paraffin-embedded root transverse sections from mature root-hair zone in *M. xiaojinenesis* under iron-sufficient (160 µM) (*B*) and iron-deficient (4 µM) (*C*) supply. *Red arrowheads* indicate MxFIT expression.

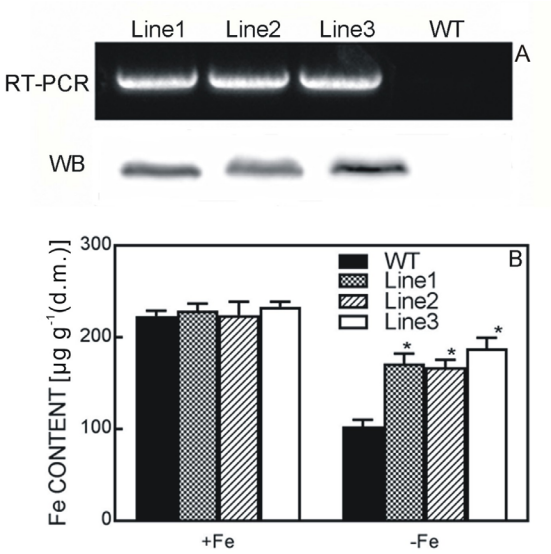

Fig. 4. Expression of *MxFIT* and function of MxFIT in transgenic tobacco suspension cells determined using RT-PCR and Western blot (WB). *A* - Expression of *MxFIT* in transgenic and wild type (WT) tobacco suspension cells determined using RT-PCR and Western blot (WB). *B* - Content of active iron in transgenic and WT tobacco suspension cells under iron-sufficiency (100 µM) (+) and iron-deficiency (4 µM) (-) conditions. Means ± SEs, n = 3; bars with * are significantly different (Duncan's multiple range test, P = 0.05).

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 recombination protein, while high temperatures might lead to insoluble inclusion bodies (Makrides 1996). In this study, we optimized the induction conditions and the mount 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 inducted 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 pattern was 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 tomato, 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 *AtbHLH38/39/100/101* in *Arabidopsis* show constitutive expression of the iron uptake genes *FRO2* and *IRT1* (Yuan *et al.* 2008). The single over-expression of *AtFIT*, *AtbHLH38* and *AtbHLH100* in 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 *AtbHLH39* and *AtbHLH101* is able to enhance the expression of *IRT1* and *FRO2* and the plants accumulate more iron even under iron-sufficiency 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 AtbHLH38/39/100/101, 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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