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Identification and expression analysis of multiple *FRO* gene copies in *Medicago truncatula*

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ABSTRACT. Iron (Fe) is an essential element for plant growth. Commonly, this element is found in an oxidized form in soil, which is poorly available for plants. Therefore, plants have evolved ferric-chelate reductase enzymes (FRO) to reduce iron into a more soluble ferrous form. Fe scarcity in plants induce the FRO enzyme activity. Although the legume *Medicago truncatula* has been employed as a model for FRO activity studies, only one copy of the *M. truncatula MtFRO1* gene has been characterized so far. In this study, we identified multiple gene copies of the *MtFRO* gene in the genome of *M. truncatula* by an *in silico* search, using BLAST analysis in the database of the *M. truncatula* Genome Sequencing Project and the National Center for Biotechnology Information, and also determined whether they are functional. We identified five genes apart from *MtFRO1*, which had been already characterized. All of the *MtFRO* genes exhibited high identity with homologous *FRO* genes from *Lycopersicon esculentum*, *Citrus junos* and *Arabidopsis thaliana*. The gene copies also presented characteristic conserved FAD and NADPH motifs, transmembrane regions and oxidoreductase signature motifs. We also detected expression in five of the putative *MtFRO* sequences by semiquantitative RT-PCR analysis,

performed with mRNA from root and shoot tissues. Iron scarcity might be a condition for an elevated expression of the *MtFRO* genes observed in different *M. truncatula* tissues.

Key words: *Medicago truncatula*; *FRO* genes; Iron

INTRODUCTION

Iron (Fe) is an essential element for plant growth; however, due to the low solubility of Fe in the soil, plants require effective mechanisms to obtain it. Based on the mechanism for Fe acquisition, plants can be grouped as either strategy I or strategy II plants (Römheld and Marschner, 1986). Strategy II plants include grasses whose roots secrete compounds known as phytosiderophores (PS) that chelate Fe^{3+} from the rhizosphere. Thereafter, the Fe^{3+}-PS complex is introduced into the cell via the Yellow Stripe 1 (YS1) carrier protein in the plasmalemma (Römheld and Marschner, 1986). In contrast, strategy I plants are all higher plants (except grasses) and the acquisition of Fe occurs in 3 main reactions: i) proton excretion via a self-phosphorylated-type adenosine triphosphate (ATP)ase, thus acidifying the surrounding soil to increase Fe solubility; ii) reduction of Fe^{3+} to Fe^{2+} by a ferric chelate reductase (FRO) (Robinson et al., 1999; Schmidt, 1999), and iii) transport of Fe^{2+} by the iron-regulated transporter (IRT) through the plasmalemma membrane (Römheld, 1987; Eide et al., 1996).

It is known that iron deficiency promotes the excretion of phenolic compounds, organic acids, and flavins, which also contribute to Fe reduction and solubility (Welkie and Miller, 1988; Susín et al., 1994). The *FRO* gene, is a key enzyme in Fe acquisition, and the first *FRO2* gene was identified in the model plant *Arabidopsis thaliana* (Robinson et al., 1999). Eight other copies of the *A. thaliana* (*At*)*FRO* gene family have also been identified and characterized; these genes exhibit tissue-specific expression (Wu et al., 2005), although there is some discrepancy in the expression patterns of different *AtFRO* copies in various tissues (Mukherjee et al., 2006). Other *FRO* genes have been reported in plants like *Pisum sativum* (Waters et al., 2002), *Solanum lycopersicum* (Holden et al., 1991), *Citrus junos* (Li et al., 2002), *Cucumis sativus* (Waters et al., 2002), *Arachis hypogaea* (Ding et al., 2009), and *Oryza sativa* (Gross et al., 2003).

Interestingly, it has recently reported the presence and expression of *FRO* genes in grasses such as barley and sorghum (Mikami et al., 2011), which raises the question of whether some other species of agricultural interest also present functional *FRO* genes, thus opening the possibility of improving their capabilities for iron acquisition. To date, no FRO activity has been observed in roots; this activity has only been detected in shoots and specific cellular organelles, thus likely contributing to Fe homeostasis and photosynthetic capacity (Mikami et al., 2011).

Medicago truncatula is a model legume that acquires Fe via strategy I. This plant has a small diploid genome, short generation time, is self-fertilizable, highly transformable, and its complete genome was recently reported (Cook, 1999; Trieu et al., 2000; Young et al., 2011). Additionally, it has the ability to establish symbiotic interactions with nitrogen-fixing bacteria of the genus *Shizorhizobium*, in addition to colonization by arbuscular mycorrhizal fungi (Harrison and Dixon, 1993). Recently it has been demonstrated that dimethyl hexadecylamine, an organic volatile compound produced by the rhizobacteria *Arthrobacter agilis*, induces the FRO activity in *M. truncatula* plants (Orozco-Mosqueda et al., 2012). So far only 1 copy of the *M. truncatula* (*Mt*)*FRO1* gene has been characterized in this model plant (Andaluz et al.,

2009). To explore the possibility that more copies of *FRO* genes are present and functional in the genome of *M. truncatula*, we carried out an *in silico* search for genes encoding FRO functions. Our results show that, apart from *MtFRO1*, there are at least 5 additional copies, which show highly conserved flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) motifs, transmembrane regions, and oxidoreductase signature motifs, a feature of characterized *FRO* genes (Mukherjee et al., 2006; Andaluz et al., 2009). Additionally, *MtFRO* gene expression was confirmed by semiquantitative real-time polymerase chain reaction (RT-PCR) in conditions of Fe sufficiency and deficiency, showing a differential expression pattern. The identification of the *MtFRO* gene family opens the possibility to better understand the Fe uptake mechanisms in strategy I plants.

MATERIAL AND METHODS

In silico search and identification of *FRO* gene copies

To search for unidentified *FRO* gene copies, we performed a homology Basic Local Alignment Search Tool (BLAST) search by employing the previously identified *FRO1* gene sequence as a probe, obtained from GenBank with accession No. AY439088. The BLASTs were carried out in the database of the *M. truncatula* Genome Sequencing Resources at the following webpages: http://www.medicagohapmap.org, the latest version of the genome of *M. truncatula* (Mt3.5) and http://blast.ncbi.nlm.nih.gov. Other *FRO* sequences from *P. sativum*, *S. lycopersicum*, *C. junos*, *C. sativus*, *A. hypogaea*, and *O. sativa* were also employed in BLAST searches, although better results were obtained with the *MtFRO1* sequence. We initially used Mt3.5 employing default parameters with an E-value of 0.01, which was modified to become either more or less restricted in various searches to open the possibility of detecting homologous genes with low or high identity. Five *FRO* putative sequences (open reading frames) were obtained with different identities, which were depurated of introns with help of the GENSCAN program, available at http://genes.mit.edu/GENSCAN.html (The Board of Regents of the University of Wisconsin System, 2003). Prediction of transmembrane domains was performed using the Hidden Markov Model for TOpology Prediction (HMMTOP) 2.0 transmembrane topology prediction program at http://www.enzim.hu/hmmtop. The obtained putative gene sequences were also analyzed to search for FAD and NADPH domains, transmembrane regions, and the oxidoreductase signature motifs through BLAST; Conserved Domain Architecture (CDART; National Center for Biotechnology Information).

Growth conditions of *M. truncatula* plants

We used plants of *M. truncatula* ecotype Jemalong (A17-1). The seeds were scarified with concentrated anhydrous sulfuric acid. The excess of acid was removed by 6 washes with sterile deionized water and sterilization was carried out with a 12% sodium hypochlorite solution for 2 min, rinsed with sterile distilled water, left for 3 to 4 days at 4°C, then placed in complete Murashige-Skoog (MS) medium (with 0.6% fitagar) on Petri dishes. The Petri dishes were transferred to a Percival growth chamber (16-h light/8-h dark cycle), with a light intensity of 200 $mol \cdot m^2 \cdot s^{-1}$ at 22°C for 7 days. Subsequently, plants were transferred to Petri dishes containing MS with Fe sufficiency (100 μM) and deficiency (1 μM) and were kept in these conditions for 24 h in a growth chamber.

RNA extraction

After 24 h of growth in Fe-sufficient or deficient media, total RNA extraction was carried out with the TRI reagent (Catalogue T9424, Sigma-Aldritch, St. Louis, MO, USA). The RNA was treated with RNase-free DNase I to remove residual genomic DNA (Mukherjee et al., 2006). The RNA samples were run on a 1.2% agarose gel, then stained with ethidium bromide to confirm the quality of the RNA. Finally, we carried out the cDNA synthesis with the SuperScript First-Strand Synthesis System for RT-PCR (Life Technologies/Gibco-BRL Grand Island, NY, USA).

Semiquantitative RT-PCR conditions

The oligonucleotides used in this study were designed with the Beacon Designer 4.02 program (Biosoft International Premier, Palo Alto, CA, USA). The PCR conditions were used as follows: initial denaturation at 95°C for 3 min; 1 min at 95°C for denaturation, 1 min at 60°C for annealing, and 2 min at 72°C for extension for 30 cycles, and a final extension step at 72°C for 10 min. PCR amplifications were performed with a TC-412 Techne Thermal Cycler (Keison Products Chelmsford, Essex, CM1 3UP, England). GoTaq® Master Mixes tubes (Promega Corporation Fitchburg, Madison, WI, USA) were used (tubes are supplied with enzyme, magnesium, dNTPs, and buffer). Only 0.1 µg template cDNA and 50 pM of each primer were added to each tube.

Sequencing of PCR products

The RT-PCR products were additionally purified by using the Wizard® SV Gel and PCR Clean-Up System (Promega), following manufacturer instructions and were sequenced at the Laboratorio Nacional para la Diversidad Genomica, Centro de Investigación y de Estudios Avanzados-Irapuato, Mexico. The sequencing results were employed to corroborate the homology of the *MtFRO* genes by using the BLAST program as previously described.

RESULTS

Identification of *MtFRO* gene copies

In this study, we conducted an *in silico* search for multiple copies of *FRO* genes in the genome of *M. truncatula*. To achieve this goal, we employed the previously identified *MtFRO1* sequence as a probe in BLAST searches at the Genome Sequencing *M. truncatula* Resources database. The results revealed 5 additional sequences with high identity to *FRO* genes (ranging from 67 to 76%), and contained the highly conserved motifs for FAD (HPFT), NADPH (GPYG), transmembrane regions, and the oxidoreductase signature motif (LVMVCGGSGITPFIS), which are known characteristics of *FRO* genes from diverse plants (Table 1). The number of transmembrane helices identified varied from 4 to 13, depending on the gene that was analyzed. The *FRO1* gene encodes a protein of 703 amino acids (aa), while *FRO2* and *FRO3* sequences showed similar lengths of 792 and 740 aa, respectively. Notably, we found other sequences with shorter lengths, which we also named *FRO4*

(476 aa), *FRO5* (417 aa), and *FRO6* (489 aa). *FRO* gene numbers were arbitrarily named according to the order in which they were found in our BLAST search. It is worth pointing out that we consider these smaller sequences as potential genes since they contain all of the motifs outlined above, as well as high identity with *FRO* genes.

Table 1. Summarized features of the *MtFRO* family open reading frames (ORFs) and other ORFs from *Arabidopsis thaliana* and *Pisum sativum* used as a reference.

Gene	Size (aa)	Transmembrane regions	FAD motif	NADPH motif	Oxidoreductase motif	Reference
MtFRO1	703	10	WHPFTI	EGPYGP	LVMVSGGSGITPFIS	Andaluz et al., 2009
MtFRO2	740	13	WHPFSV	EGPYGH	LILVAGGIGLSPFLA	This study
MtFRO3	792	13	WHPFTV	EGPYGP	IAMVSGGSGITPFIS	This study
MtFRO4	476	6	WHPFTV	EGPYGP	IVMVSGGSGVTPFIS	This study
MtFRO5	417	4	WHPFSV	EGPYGH	LILVAGGIGLSPFLA	This study
MtFRO6	489	6	WHPFSI	DGPYGA	ILLVGLGIGATPLIS	This study
AtFRO1	704	10	WHPFTI	EGPYGP	LVMVSGGSGITPFIS	Wu et al., 2005
AtFRO2	725	10	WHPFTI	EGPYGP	LVMVCGGSGITPFIS	Robinson et al., 1999
PsFRO1	712	10	WHPFTI	EGPYGP	LVMISGGSGITPFIS	Waters et al., 2002

GenBank accession Nos. for the ferric chelate reductase (FRO) putative sequences: MtFRO1 (AY439088.1), MtFRO2 (XM_003594382.1), MtFRO3 (AC121237.19), MtFRO4 (AC121237.19), MtFRO5 (AC151000.3), MtFRO6 (AC154391.1), AtFRO1 (NM_100041), AtFRO2 (NM_100040.2), and PsFRO1 (AF405422.2). aa = amino acid.

The above results suggest the possibility that the genome of *M. truncatula* contains multiple copies of the *FRO* gene, thus we decided to design primers to amplify them. The primer sequences that were used are found in Table 2. Once we amplified the putative *FRO* sequences, the RT-PCR products were sequenced; of note, 5 of the 6 possible genes gave a positive result, except the gene sequence identified as *FRO2*. The sequencing results demonstrated that all of the amplified and partially sequenced genes showed 100% identity with the *M. truncatula* genome, as well as high identity with *FRO* genes from other plant species, such as *A. thaliana*, *L. esculentum*, and *C. junos* (Table 3).

Table 2. List of primers designed and employed in this study.

Primer name	Nucleotide sequence (5'-3')	Size of the PCR expected product (bp)
FRO1UPRT	TGTGTCGGTAGTTGTTGTTG	207
FRO1LORT	TCAGGTCTTGTTCCATAATGC	
FRO2UPRT	GGTGGTATAGTGGTTGG	165
FRO2LORT	CATTTCTCTGACATTGATTC	
FRO3UPRT	AGGCGTTAGAGTGGAGCAAGAC	145
FRO3LORT	GAGAATGTAGAGATGGTGAGTGTAGAAG	
FRO4UPRT	ATAACAAGCATTCCACAAATAAGG	210
FRO4LORT	AAGGCGAGCAGATAGTAACC	
FRO5UPRT	TTATATTAGTGGCAGGTGGTATTGG	157
FRO5LORT	ACGGTTGAAAGAAGTGGAAGC	
FRO6UPRT	AAGACATAGAACAAGGAGTGGTAG	126
FRO6LORT	TGATGAAGTGATTGAAGCATAGTG	
MtACTINUPRT	CCAATAGGGACAACAACACTTTC	209
MtACTINLORT	ACCAAACAGCGGATAGTAAGC	

Table 3. Sequence identity of the fragments of *MtFRO* genes amplified by RT-PCR compared with other identified *FRO* genes[a].

Gene name	Closest match	GenBank accession No.	Identity
MtFRO1	*Medicago truncatula* Fe(III)-chelate reductase (*FRO1*) mRNA	AY439088.1	99%
MtFRO3	*Lycopersicon esculentum* Fe(III)-chelate reductase (*FRO1*) mRNA	AY224079.1	81%
MtFRO4	*Citrus junos* Fe(III)-chelate reductase mRNA	DQ985810.1	82%
MtFRO5	*Arabidopsis thaliana AtFRO6* Fe(III)-reductase mRNA	NM_124351.3	71%
MtFRO6	*Arabidopsis thaliana* Fe(III)-reductase-like mRNA	NM_114450.2	74%
MtACT2	*Medicago truncatula ACT2* gene, promoter and exon 1	AJ809891.1	100%

[a]All the sequences amplified by RT-PCR showed ≥99% identity with their own sequences found *in silico* (see Table 1).

The multiple *MtFRO* copies are modulated by iron availability

Once we identified the different copies of putative *MtFRO* genes and confirmed them by sequencing and BLAST identity, we explored whether these gene sequences are regulated. We performed semiquantitative RT-PCR to determine their expression in roots and shoots of plants grown under conditions of Fe sufficiency and deficiency. The results suggest that all of the sequences identified in this study, except *FRO2*, are functional at the level of expression. After designing 3 different sets of oligonucleotides to detect *FRO2* expression, we were unable to detect a band by semiquantitative RT-PCR (data not shown). In addition, we also observed that expression of the different *MtFRO* copies is inducible in Fe-deficient growth conditions and might be tissue-specific (Figure 1). In particular, *MtFRO1* was basally expressed in Fe-sufficient conditions, but when the plants are transferred to Fe-deficient media we noted an elevated expression level, especially in shoots. It was interesting to note that in conditions of Fe sufficiency, expression of the *MtFRO3*, *MtFRO4*, *MtFRO5*, and *MtFRO6* genes was detected in shoots but not in roots. However, under Fe deficiency conditions the expression of these genes was increased in both root and shoot parts. This suggests that the genome of *M. truncatula* contains multiple functional *FRO* gene copies, and that their expression pattern is likely tissue-specific and dependent on the concentration of Fe in the medium.

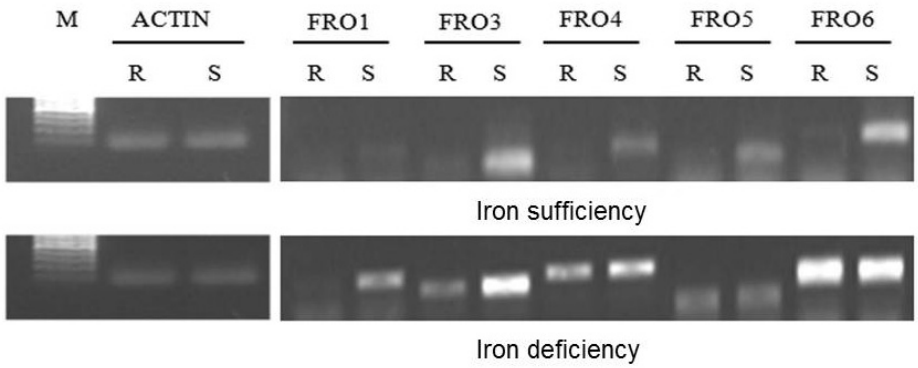

Figure 1. Semiquantitative RT-PCR detection of *MtFRO* genes in *Medicago truncatula* roots and shoots. *M. truncatula* plants were grown under iron sufficiency (100 µm) and deficiency (1 µm). The *actin* gene was employed as a control. *Lane M* = molecular marker; R = roots: S = shoots.

DISCUSSION

Plant genomics allows us to identify and further characterize genes that are involved in diverse cellular processes, such as Fe deficiency stress responses. Fe is one of the most abundant elements on earth, although its availability may be limited in plants; therefore, plants have developed efficient mechanisms for Fe acquisition (Römheld and Marschner, 1986). It is generally accepted that there are 2 strategies for Fe acquisition in plants. *M. truncatula* is a strategy I plant, which can acidify the rhizosphere and increase the bioavailability of Fe (Orozco-Mosqueda et al., 2012). In the present *in silico* study, we identified 5 putative sequences with high identity to *FRO* genes in the genome of *M. trucatula* (apart from *FRO1*, which was previously described; Andaluz et al., 2009). All sequences described herein showed highly conserved motifs characteristic of FRO proteins, such as FAD, NADPH, transmembrane regions, and the oxidoreductase signature motifs (Table 1). *FRO* genes are present in various plant species and are responsible for reducing Fe^3 to Fe^2, a more soluble form that can taken up by the roots (Hell and Stephan, 2003). Apart from FRO, other proteins such as IRT1, which is a high affinity transporter for Fe^{2+}, play an essential role in Fe acquisition in plants such as *A. thaliana* (Varotto et al., 2002). The expression of *FRO2* and *IRT1* is regulated by Fe deficiency conditions in strategy I plants (Connolly et al., 2003).

The *FRO1* gene was the first to be characterized in *A. thaliana* (Robinson et al., 1999), 7 other functional copies of the same family have been characterized (Wu et al., 2005; Mukherjee et al., 2006). In this sense the question arises: why do plants such as *A. thaliana* or *M. truncatula* contain multiple *FRO* copies in their genomes? One answer has been widely discussed in various studies, suggesting that the existence of multiple copies of the *FRO* gene are required for Fe reduction, uptake, and homeostasis in different plant tissues or organs, which are differentially regulated by the availability of Fe (or other factors) (Wu et al., 2005; Mukherjee et al., 2006). Likewise, it has been postulated that the expression of *FRO* genes in plant roots may play other roles, apart from reduction of Fe (Muherjee et al., 2006), since other elements can regulate their gene expression.

The presence of *FRO* genes is not unique to strategy I plants, as other grasses such as rice contain 2 *FRO* genes (Ishimaru et al., 2007). It has been argued that *O. sativa* (*Os*)*FRO2* and *OsFRO1* may not code for ferric chelate reductase functions, even when these activities have been reported only in mesophyll cells in other plant grasses such as barley and sorghum (Mikami et al., 2011). Interestingly, the expression of *FRO1* and *FRO2* genes in barley and sorghum was detected in leaves but not in roots. The above results suggest the possibility of detecting more gene copies in other grasses, and that the *FRO* genes may encode for FRO proteins with functions not only in leaves or shoots but in roots, which would break the exclusivity of Fe acquisition strategy I for non-grass plants.

In this study, we report evidence that the multiple copies of *FRO* genes in *M. truncatula* are expressed in low Fe concentrations and are present in root and/or shoots. This agrees with other studies in *A. thaliana*, which demonstrated that genes such as *AtFRO2* and *AtFRO3* are expressed under low Fe conditions mainly in roots, while *AtFRO5*, *AtFRO6*, *AtFRO8*, and *AtFRO7* are exclusively found in shoots and not regulated by this condition (Wu et al., 2005). Besides, the expression of each gene is tissue specific; for example, *AtFRO2* and *AtFRO3* are mainly expressed in roots, while *AtFRO5* and *AtFRO6* were detected in shoots and flowers, *AtFRO6* and *AtFRO7* in cotyledons and trichomes, and *AtFRO8* is specifically expressed in

veins of leaves (Wu et al., 2005), although slight differential expression has been proposed by other authors (Mukherjee et al., 2006). In *M. truncatula*, we also observed a differential expression pattern for each *MtFRO* gene with respect to the Fe condition. In conditions of Fe sufficiency, *MtFRO1*, *MtFRO4*, and *MtFRO5* showed expression that can be considered basal, but *MtFRO3* and *MtFRO6* showed a strong expression level at the shoots. In Fe-deficient conditions, all of the *MtFRO* genes showed increased expression at both the roots and the shoots. It was very interesting to note the strong expression level of the *MtFRO3* and *MtFRO6* in the shoots, since experiments suggest that the ferric-chelate reduction is performed in aerial tissues. The role of the chelate Fe^{3+}-nicotianamine on the plant Fe transport via the phloem has been demonstrated (Takahashi et al., 2003), and it has been suggested that Fe could be internalized in the cells via the Fe^{3+}-nicotianamine transporter in both strategy I and strategy II plants (Koike et al., 2004). Our results provided molecular evidence that the FRO is strongly expressed in aerial tissues and indicates that Fe is reduced before it can be internalized in the plant cells as the results of Wu et al. (2005) and Mukherjee et al. (2006) suggest. Our study also showed that different members of the *MtFRO* genes are expressed in the same organs (roots, leaves, and stems), thus their physiological functions could be redundant. Further studies regarding the role of FRO for plant iron homeostasis utilizing loss-of-function mutants must now consider these redundant genes. We are currently conducting experiments to determine the iron-reducing functions of the multiple *MtFRO* copies in different tissues, as well as their regulation by different factors, including the presence of plant growth-promoting rhizobacteria.

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