Identification and Expression Analysis of Multiple Ferric Chelate Reductases in *Citrus junos*

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Abstract. Ferric chelate reductase (FRO) is a critical enzyme for iron absorption in strategy I plants, reducing Fe$^{3+}$ to Fe$^{2+}$. To identify FRO family genes in the local *Citrus junos* cultivar Ziyang Xiangcheng and to reveal their expression model, the citrus (*Citrus* sp.) genome was searched for homologies of the published sequence CjFRO1. Five FROs were found, including CjFRO1; these were named CjFRO2, CjFRO3, CjFRO4, and CjFRO5, respectively, and cloned via reverse transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) PCR. The deduced amino acid sequences of five CjFROs contained flavin adenine dinucleotide (FAD)-binding motifs, nicotinamide adenine dinucleotide (NAD)-binding motifs, and 6–10 transmembrane domains, with isoelectric points between 6.73 and 9.46, and molecular weights between 67.2 and 79.9 kD. CjFRO1 and CjFRO2 were predominantly found in the aboveground parts of *C. junos*, with CjFRO1 highly expressed in leaves, and CjFRO2 largely expressed in stems and leaves. CjFRO3 was less expressed in roots, stems, and leaves. CjFRO4 and CjFRO5 were predominately found in roots. Under iron-deficient conditions, CjFRO4 was significantly and specifically increased in the roots of *C. junos*, whereas CjFRO1 was upregulated in the roots and leaves.

Iron is an essential element for plant growth, which is involved in numerous vital biological processes, including chlorophyll biosynthesis, photosynthetic electron transfer, DNA synthesis, and nitrogen reduction. Although abundant in soil, iron is one of the most common nutrients to limit plant growth and development because of its particularly low solubility in aerobic soils at neutral and alkaline pH (Mimmo et al., 2014). Based on their mechanism for Fe acquisition, plants can be categorized into strategy I or strategy II plants (Eide et al., 1996; Robinson et al., 1999; Romheld, 1987; Romheld and Marschner, 1986). Strategy II plants include grasses with roots that secrete compounds known as phytosiderophores (PS), which chelate Fe$^{3+}$ from the rhizosphere. Subsequently, the yellow stripe I carrier protein in the plasmalemma introduced the Fe$^{3+}$-PS complex into the root cell. By contrast, strategy I plants are all dicots and non-graminaceous monocots, and the acquisition of Fe$^{3+}$ occurs in reducing reaction processes: 1) proton excretion via a self-phosphatated type of adenosine triphosphatase, thus acidifying the surrounding soil to increase iron solubility; 2) reduction of Fe$^{3+}$ to Fe$^{2+}$ via FRO; and 3) transport of Fe$^{2+}$ via iron-regulated transporter through the plasmalemma membrane.

The FRO protein is a key enzyme for Fe$^{3+}$ acquisition from soil, which has been confirmed through its functionality for chlorosis tolerance and RNA-interference in plants (Gama et al., 2017; Lee et al., 2016; Martinez-Cuenca et al., 2016).

The first FRO gene was identified in the model plant *Arabidopsis thaliana* (Robinson et al., 1999). Further FRO genes have been reported in a variety of plants such as *Arachis hypogaea* (Ding et al., 2009), *Cucumis sativus* (Waters et al., 2007), *Lotus japonicus* (Klein et al., 2012), *Malus xiaojinensis* (Wu et al., 2012), *Medicago truncatula* (Andaluz et al., 2009; Orozco-Mosqueda et al., 2012), *Oryza sativa* (Gross et al., 2003), *Pisum sativum* (Waters et al., 2002), and *Solanum lycopersicum* (Kong et al., 2013; Li et al., 2004). Eight FROs have been described in the genome of *A. thaliana* and shown to exhibit tissue-specific expression (Mukherjee et al., 2006; Wu et al., 2005). FRO family members regulate metal ion homeostasis in different locations in the plant. *AtFRO2* is mainly expressed in epidermal cells of roots where it participates in the reduction of Fe$^{3+}$ to Fe$^{2+}$ in the rhizosphere. *AtFRO3* is predominantly expressed in the vascular cylinder of roots where it might be involved in the transport of Fe. *AtFRO7* is located in the chloroplast membrane, retaining iron homeostasis in the chloroplast (Connolly et al., 2003; Jeong et al., 2008; Jeong and Connolly, 2009; Mukherjee et al., 2006). Furthermore, FROs have been reported to have different regulatory mechanisms, although their overexpression in plants can rescue lime-induced chlorosis (Connolly et al., 2003; Li et al., 2011). *AtFRO2* has been reported to be positively regulated by transcriptional factor FIT (FER-like iron-deficiency-induced transcription factor), and POPEYE (bHLH047) has been shown to upregulate the expression of *AtFRO3* (Hindt and Guerinot, 2012; Long et al., 2010).

The local *Citrus junos* cultivar Ziyang Xiangcheng was reported to be tolerant to lime-induced iron chlorosis in both field and laboratory experiments, and it could efficiently uptake iron from calcareous soils. Until now, only CjFRO1 (GenBank...
performed using the ClustalX2.1 software package (Thompson et al., 2017). Multiple alignments of sequences were predicted with the TMHMM program (Technical University of Denmark, 2016). Transmembrane domains of putative CjFRO proteins were predicted with the software Protparam (Gasteiger et al., 2005) and Pfam (Finn et al., 2009; Mukherjee et al., 2006). In addition, CjFRO gene expression was confirmed via real-time PCR in different tissues and in conditions of Fe sufficiency and deficiency. The identification of the CjFRO gene family enables an improved understanding of Fe uptake, translocation, distribution, and usage mechanisms in citrus plants.

Materials and Methods

In silico search and identification of FRO gene copies. To identify unknown FRO members, a homology Basic Local Alignment Search Tool (BLAST) search was performed, employing the previously identified CjFRO1 gene (GenBank no. DQ985810). The BLASTs were conducted in the database of the Citrus sinensis Genome Sequencing Resources (Xu et al., 2012). Based on the predicted cDNA sequences of FROs in the database, sense and antisense primers for each CjFRO were designed, and the coding sequences were amplified from the total RNA of 1-year-old ‘Ziyang Xiangcheng’ seedlings via RT-PCR or RACE (634858; Clontech, Beijing, China). They were purified with the Biospin Gel Extraction Kit (BSC02M1; Biotech Technology Co., Hangzhou, China), subsequently cloned into a pMD19-T vector (6013; TaKaRa, Beijing, China), subsequently cloned into the Beijing Genomics Institute (Shenzhen, China). For analyzing the CjFROs expression in different tissues, the root tips (1.5 cm), stems (between fifth and sixth leaves), and the first fully developed leaves were collected from five plants.

RNA extraction. Total RNA was extracted with the RNA Extraction Kit (RN0902; Aidlab Biotechnologies Co., Beijing, China) and the RNA was treated with RNase-free DNase I to remove residual genomic DNA. RNA samples were run in a 1.2% agarose gel and stained with ethidium bromide to verify RNA quality. Each RNA sample was quantified with a spectrophotometer (NanoDrop 2000c; Thermo Fisher Scientific, Shanghai, China) at 260 nm.

Full-sequence cloning of CjFROs. Full sequences were obtained via PCR using primer pairs listed in Supplemental Table 1. The PCR conditions were 95 °C for 1 min; 94 °C for 30 s, 58 °C for 30 s, 72 °C for 2 min 30 s for 40 cycles; followed by 72 °C 10 min. The PCR products were cloned into the pMD19-T vector and transformed into competent Escherichia coli DH5α cells. PCR confirmed positive clones were sequenced in the Beijing Genomics Institute (Shenzhen, China).

Real-time PCR. cDNA was prepared from 1 μg total RNA with the iScript™ cDNA Synthesis Kit following the manufacturer’s instructions (170–8891; Bio-Rad Laboratories, Hercules, CA). Amplification with the primers in Supplemental...
Table 1. Summarized features of the ferric chelate reductase genes of *Citrus junos* (*CjFROs*) from ‘Ziyang Xiangcheng’ according to their nucleic acid sequences and amino acid sequences.

| Name     | Nucleic acids (no.) | Amino acids (no.) | MW (kDa) | pI | Transmembrane regions (no.) | Introns (no.) | Exons (no.) |
|----------|---------------------|-------------------|----------|----|-----------------------------|---------------|-------------|
| CjFRO1   | 2103                | 700               | 79.7     | 9.23 | 10                         | 13            | 14          |
| CjFRO2   | 2097                | 698               | 77.3     | 6.73 | 6                          | 7             | 8           |
| CjFRO3   | 2112                | 703               | 79.9     | 9.46 | 10                         | 7             | 8           |
| CjFRO4   | 1809                | 602               | 67.2     | 9.10 | 6                          | 5             | 6           |
| CjFRO5   | 2112                | 703               | 79.2     | 9.06 | 9                          | 7             | 8           |

*MW* Molecular weight.  
*pI* Isoelectric point.

Table 2 was performed using the SYBR Green PCR Master Mix (1725271; Bio-Rad Laboratories) and the iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories). The following protocol was used for amplification: 95 °C for 1 min; 94 °C for 10 s, 60 °C for 15 s, and 92 °C for 15 s, for 40 cycles. After completion of the amplification reaction, melt curves were compared to verify the amplification of a single peak. Each experiment was performed in at least three technical replications and three biological replicates were run per sample (using three independent sets of RNA). The data analysis used the 2ΔCt method, and *actin* was used as a reference gene.

Results

Identification and bioinformatic analysis of *CjFROs*. The genome sequence of citrus was searched through BLASTx against the amino acid sequence of *CjFRO1*. Five predicted sequences of *C. sinensis* (Cs3g01120, Cs9g19350, Cs5g06040, orange1.100399, and Cs9g19360) were found. Cs9g19350 corresponds to *CjFRO1*, and the other genes of ‘Ziyang Xiangcheng’ were designated as *CjFRO2* (Cs3g01120), *CjFRO3* (Cs5g06040), *CjFRO4* (orange1.100399), and *CjFRO5* (Cs9g19360). These revealed high-sequence identity among each other at the amino acid level (Supplemental Table 3).

Based on the predicted open reading frame sequences of the citrus genome database, sense and antisense primers were designed for all four novel *CjFRO* genes, and the cDNAs of these genes were amplified with the primers listed in the Supplemental Table 1, using total RNA that was isolated from 1-year-old ‘Ziyang Xiangcheng’ seedlings. Via RT-PCR, *CjFRO2* was obtained with the primer pairs FROasF/S and FROasR of leaves, and *CjFRO4* was obtained with FROasF/S and FROasR of roots. For the remaining two *FRO* genes, it was difficult to obtain the whole open-reading-frame sequence. The upstream nucleic acid sequence of *CjFRO5* was obtained via PCR with the primer pair FROasF/S and FROasR, and the downstream sequence was obtained with the primer pair FROasF/S and FROasR. For *CjFRO3*, the upstream nucleic acid sequence was obtained via PCR, using the primer pair FROasF/S and FROasR, and the downstream sequence was obtained via 3′ RACE with the primer pairs FROasF/S and FROasR. The complete predicted protein sequences of the five *CjFROs* in *Citrus junos* were aligned with the other plant FROs through MEGA 5.0 (Tamura et al., 2011). *AtFRO* from *Arabidopsis thaliana* (*AtFRO1*, NM_100041; *AtFRO2*, NM_100040; *AtFRO3*, NM_102150; *AtFRO4*, NM_122303; *AtFRO5*, NM_122304; *AtFRO6*, NM_124351; *AtFRO7*, NM_124352; *AtFRO8*, NM_124395), *MtFROs* from *Medicago truncatula* (*MtFRO1*, AY439088; *MtFRO2*, XM_003594382), *LjFRO1* from *Lotus japonicus* (JF290367), *MxFRO* from *Malus xiaojinensis* (EF577061), and *PsFRO1* from *Pisum sativum* (AF405422). The bootstraps P values were obtained by the neighbor-joining method and indicate the divergence of each branch. FROs from *C. junos* were highlighted with diamonds on the tree.
AtFRO4 and AtFRO5; CjFRO3 grouped with AtFRO8; CjFRO2 shared a group with AtFRO6 and AtFRO7.

**Expression profiles of CjFROS in citrus tissues.** Real-time PCR was performed to evaluate the expression of all five CjFROS in roots, stems, and leaves of 1-year-old ‘Ziyang Xiangcheng’ seedlings. The sense terminal primers and anti-sense terminal primers for real-time PCR (Supplemental Table 2) were designed in different exons, to avoid genomic DNA contamination. According to tissue expression (Fig. 3), CjFRO2 and CjFRO1 were expressed at higher levels in stems and leaves. Compared with CjFRO2 expression in roots, its accumulation increased 396-fold in stems and 189-fold in leaves. CjFRO1 expression in stems and leaves was 21-fold and 296-fold higher than in roots. However, CjFRO4 expression in roots was 31-fold higher than in stems and 7-fold higher than in leaves. CjFRO5 was a gene with a root-specific expression and was barely detectable by real-time PCR in stems and leaves. CjFRO3 showed lower expression in roots, stems, and leaves.

**CjFROS response to the Fe deficient condition.** To analyze CjFROS expression in both Fe sufficiency and Fe deficiency conditions, citrus roots and leaves of 2-month-old seedlings, cultured in Hoagland’s nutrient solution were tested via real-time PCR (Fig. 4). After 2 d of iron deficiency stress, CjFRO1 was upregulated 3.0-fold in roots and 1.9-fold in leaves. The expression CjFRO2 and CjFRO3 remained unchanged in roots and leaves (change fold ≤ 1.5). CjFRO4 showed a specifically and significantly higher accumulation in roots (4.5-fold) under the Fe starvation and not changed in the leaves. CjFRO5 were not detectable in leaves and roots of 2-month-old seedlings under Fe-sufficient and Fe-starvation conditions.

**Discussion**

Online plant genomics offer greatly valuable databases for the identification and further characterization of genes that are involved in diverse cellular processes, such as Fe-starvation responses. Eight FROs had been found in the A. thialiana genome and six FROs in the M. truncatula according their genomic databases (Orozco-Mosqueda et al., 2012; Wu et al., 2005). Just like A. thialiana and M. truncatula, C. junos belongs to the plants using strategy I for Fe acquisition, which can reduce rhizospheric iron, thus increasing the bioavailability of iron. In the present in silico study, four putative sequences with high identity to FROs of other plants were identified and cloned (apart from CjFRO1, which was previously described.). All sequences described herein showed highly conserved characteristics of FRO proteins, such as FAD and NAD binding domains, transmembrane regions, and oxidoreductase signature motifs. FROs are present in various plant species and are
responsible for the reduction of Fe$^{3+}$ to Fe$^{2+}$, a more soluble form that can be absorbed by the roots. Gene expression and posttranslational effects on Fe-deficient conditions (Du et al., 2015; Hindt and Guerinot, 2012; Kabir et al., 2012; Yang et al., 2016; Yuan et al., 2008; Zhai et al., 2016).

In plants, AtFRO2 was the first FRO gene that was characterized (Robinson et al., 1999). Subsequently, seven other functional members of the same family have been identified in the model plant Arabidopsis (Mukherjee et al., 2006; Wu et al., 2005). Similarly, six MtFROs were reported for M. truncatula (Orozco-Mosqueda et al., 2012) and five CjFROs for C. junos in our experiment. The reason why plants contain multiple FRO genes in their genomes has already been summarized in excellent reviews: FROs are well known for their role in iron uptake by roots and furthermore, may play critical roles in the iron uptake by leaves as well as for iron homeostasis in chloroplasts and mitochondria (Jain et al., 2014; Jeong and Connolly, 2009). Moreover, FROs are not only found in strategy I plants, but also in strategy II plants, such as O. sativa that contains two FRO genes (Ishimaru et al., 2007). The O. sativa FRO1 mutant revealed that FRO might have a new function and might be involved in iron trafficking under Fe-toxic conditions (Siriphat et al., 2015).

In this study, multiple members of FROs in C. junos were found to be present in roots and shoots and are induced by Fe deficiency. These results agree with FRO studies in A. thaliana, which demonstrated that genes such as AtFRO2 and AtFRO3 are mainly expressed in roots under low-Fe conditions, whereas AtFRO5, AtFRO6, AtFRO7, and AtFRO8 are exclusively found in shoots and not regulated by Fe supply (Wu et al., 2005). Furthermore, the expression of each gene is tissue specific; for example, AtFRO2 and AtFRO3 are mainly expressed in roots, whereas AtFRO5 and AtFRO6 were detected in shoots and flowers, and AtFRO7 in cotyledons and trichomes. AtFRO6 and AtFRO7 were located on plasma membranes and chloroplast membranes, respectively, whereas AtFRO8 was specifically expressed in the veins of leaves (Jeong et al., 2008; Wu et al., 2005). In C. junos, CjFRO4 and CjFRO5 were clustered with the well-characterized plant FROs, which were highly or specifically expressed in the roots. CjFRO1–3 was clustered with the shoot-specific-expressed AtFROs (Fig. 2). The real-time PCR analysis for the five CjFROs confirmed the result of cluster grouping (Fig. 3). CjFRO4 accumulated significantly higher levels in roots under iron starvation (Fig. 4). The lack of CjFRO5 observation in leaves and roots was unexpected, although it was highly expressed in the roots of 1-year-old ‘Ziyang Xiangcheng’ seedlings. This phenomenon has been described in arabidopsis for AtFRO3, which was expressed at high levels in seedlings, but at lower levels in mature, soil-grown plants (Mukherjee et al., 2006).

In conclusion, at least five FRO genes have been found within the citrus genome. Here, we detected the four novel genes CjFRO2, CjFRO3, CjFRO4, and CjFRO5, which had the transmembrane domain, FAD-binding domain, and NAD binding domain of ferric reductase. The genes CjFRO5 and CjFRO4 were highly expressed in roots, whereas CjFRO2 and CjFRO1 were highly expressed in leaves. CjFRO3 had lower expression in roots, stems, and leaves. Fe stress significantly increased CjFRO4 expression in roots.

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Supplemental Table 1. Primers used for full cDNA sequence cloning of ferric chelate reductase genes of *Citrus junos* (*CjFROs*). FROafS and FROafR were used for *CjFRO2* PCR. Primer pairs FROcfS2/FROcfR2 were used to obtain the upstream sequence of *CjFRO3*, FROcfS3'/3' RACE outer primer and FROcfS4'/3' RACE inner primer for the downstream sequence of *CjFRO3*. FROdfS and FROdfR were used for *CjFRO4* PCR. Primer pairs FROefS/FROeqR3 and FROeqS3/FROefR were used to obtain the upstream and downstream sequences of *CjFRO5*, respectively.

| Genes | Sense primers | Antisense primers |
|-------|---------------|-------------------|
| *CjFRO2* | FROafS: 5'-ATGTGGAACCTGAGGACTT-3' | FROafR: 5'-CTACGATCAAATGCTGG-3' |
| *CjFRO3* | FROcfS2: 5'-ATGGTATTTTATGATGCGCTG-3' | FROcfR2: 5'-AATCTGTATTACCGTTGCTCCTGTC-3' |
|        | FROcfS3: 5'-AGCATCAGGCTTAAAGTTTCACCACT-3' | 3' RACE outer primer: 5'-TACCGTGTTCCACTAGTGATT-3' |
|        | FROcfS4: 5'-CGAGTATTTCCAAATTTCACTGGCA-3' | 3' RACE inner primer: 5'-CGCGGATCCTTTCCACTAGTGATTTCACTATAGG-3' |
| *CjFRO4* | FROdfS: 5'-ATGCTGCTGAAAGGCCACT-3' | FROdfR: 5'-TCACGCTCAAAGCTGATAGATT-3' |
| *CjFRO5* | FROefS: 5'-ATGGAGACCATTAAATCAGCTATAAAG-3' | FROeqR3: 5'-ATTATGGGCCCAAGAGACGACG-3' |
|        | FROeqS3: 5'-CCATTTTACAGGGTGACGAC-3' | FROeqR: 5'-TCACGCTAAAGGCTATGGATT-3' |

PCR = polymerase chain reaction; RACE = rapid amplification of cDNA ends.

Supplemental Table 2. Primers used for analyzing the expression of *Citrus junos* ferric chelate reductase genes (*CjFROs*) in different tissues and responding to the iron starvation.

| Genes | Sense primers | Antisense primers | cDNA length (bp) |
|-------|---------------|-------------------|-----------------|
| *CjFRO2* | 5'-AGGCACCCTTTTACTACCTACCTAC-3' | 5'-GACAAAGACTTCCCTTGTGATA-3' | 181 |
| *CjFRO1* | 5'-CATGCTGCGCATTTTCCTCTCTTT-3' | 5'-GTTTCGCTCCATTTAGACACCT-3' | 202 |
| *CjFRO3* | 5'-CCAGCTTTGAGATTGGTGTGTT-3' | 5'-GGTACTTTGAGTTGGCCATGGTT-3' | 135 |
| *CjFRO4* | 5'-GACCTGAGCTAAGAAGGATGCT-3' | 5'-GCCCAGACCGATGAGACGATTG-3' | 115 |
| *CjFRO5* | 5'-CATTATTTACAGGGACGAC-3' | 5'-ATTATTGGCCAAAGAGAGACGACG-3' | 100 |
| *actin* | 5'-CATCCCTCAGGACGACTCC-3' | 5'-CCAAACCTTACGCTTCC-3' | 198 |

Supplemental Table 3. Distributions of percentage protein sequence identity for pairs of *Citrus junos* ferric chelate reductases (*CjFROs*).

|  | *CjFRO1* | *CjFRO2* | *CjFRO3* | *CjFRO4* | *CjFRO5* |
|------|---------|---------|---------|---------|---------|
| *CjFRO1* | 35      | 30      | 58      | 54      |         |
| *CjFRO2* | 32      | 35      | 33      |         |         |
| *CjFRO3* | 32      | 31      |         |         |         |
| *CjFRO4* | 69      |         |         |         |         |
| *CjFRO5* |         |         |         |         |         |