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ORIGINAL ARTICLE

Rhizobacterium *Arthrobacter agilis* UMCV2 increases organ-specific expression of *FRO* genes in conjunction with genes associated with the systemic resistance pathways of *Medicago truncatula*

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Abstract
Ferric-chelate reductase (*FRO*) genes are essential for iron uptake in strategy I plants. In this study, *Medicago truncatula* plants were grown in a greenhouse under iron-sufficient and iron-deficient conditions with inoculation of the beneficial rhizobacterium *Arthrobacter agilis* UMCV2. The expression of five *MtFRO* genes and the marker genes *MtDef2.1* and *MtPR1*—involved in systemic resistance pathways—was quantified by RT-qPCR in plant organs. *MtFRO1* and *MtFRO3* were expressed in roots, and *MtFRO2* was expressed in leaves, flowers, and pods. *MtFRO4* was expressed in leaves and roots, and *MtFRO5* was expressed in roots, stems, and leaves. *A. agilis* UMCV2 and iron deficiency induced the expression of all *MtFRO* genes and systemic defense marker genes. The defense genes exhibited expression profiles similar to those of the *MtFRO* genes, and strong synergistic gene induction was observed in *A. agilis* UMCV2-inoculated plants grown under iron-deficient conditions. Our data supported the existence of a relationship between systemic defense responses and systemic iron deficiency responses.

Keywords *Medicago truncatula* · *Arthrobacter agilis* UMCV2 · *MtFRO* · ISR · SAR

Introduction

Iron plays a central role in several essential biochemical reactions that use oxygen, hydrogen, or water as substrates, including electron transport during respiration and photosynthesis and as a cofactor for metalloproteins (Johnson et al. 2005; Sánchez et al. 2017). In plants, iron functions in vital metabolic reactions, and its imbalance affects cellular metabolism (Vigani et al. 2013).

Although iron abundance is commonly high in the geosphere, its low solubility in the soil restricts its availability for plant roots (Mimmo et al. 2014). Approximately 30% of the world's soils are considered iron-limited (Wu et al. 2002). To address the low iron availability, plants use two mechanisms aimed at mobilizing this metal from the rhizosphere and transporting it through the plasma membrane of root cells. Strategy I, which is used by all dicotyledons and non-grass monocotyledons, depends on rhizosphere acidification to increase iron solubility via proton release through an ATPase enzyme (Curie and Briat 2003). Subsequently, there is a secretion of phenol compounds, carboxylates, and flavonoids from the pleiotropic drug resistance 9 (PDR9) protein that chelates Fe(III) (Ito and Gray 2006). Free or chelated Fe(III) is reduced to Fe(II) by the action of the enzyme ferric-chelate reductase that is encoded by the ferric reductase oxidase 2 gene (*FRO2*) (Robinson et al. 1999). Finally, reduced iron is internalized to root cells through the iron regulated transporter 1 (IRT1) protein (Eide et al. 1996). In strategy II, used by grasses, there is a release of phytosiderophores [members of the mugineic acid (MA) family] through the transporter of MA (TOM) to solubilize iron in the rhizosphere (Nozoye et al. 2011) and the subsequent transport of the Fe(III)–phytosiderophore complex through the plasma membrane of root epidermal cells via the yellow stripe 1 (YS1) carrier protein (Curie et al. 2001).

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Iron is required in each type of plant tissue, and its transport inside the plant involves the formation of chelates with the non-proteinogenic amino acid nicotianamine, as well as with citrate (Morrissey and Guerinot 2009). Ferric-chelate reductase activity is found in aerial tissues (Feng et al. 2006; Solti et al. 2014), and it is essential for plant survival under Fe-limited conditions (Jeong et al. 2008).

In addition to the strategies used by plants for iron uptake, there are soil microorganisms involved with the mineralization and transformation of nutrients inside the rhizosphere. Among these microorganisms are the so-called plant growth-promoting rhizobacteria (PGPRs), which are able to modify the availability of nutrients through changes in the environment (Pii et al. 2015a; Terrazas et al. 2016). For instance, PGPRs alter the secretion of molecules with chelating and reducing capacities, and these capacities allow plants to uptake nutrients, including iron (De Nobili et al. 2001; Pii et al. 2015b). Therefore, beneficial soil microorganisms (fungus and rhizobacteria) play a central role in plant iron acquisition (de Santiago et al. 2013; Masalha et al. 2000) through different mechanisms, including the mobilization of soil iron via siderophore release (Pii et al. 2015b; Zhao et al. 2014), direct iron reduction in soil (Valencia-Cantero et al. 2007; Zhao et al. 2014), and induction of plant iron uptake mechanisms such as ferric-chelate reductase activity in plant roots (Orozco-Mosqueda et al. 2013a; Zhang et al. 2009) and leaves (Castulo-Rubio et al. 2015; Zhao et al. 2014).

PGPRs also eliminate or reduce the damage to plants caused by deleterious microorganisms antagonizing them (Berendsen et al. 2012) or triggering plant systemic defense pathways "induced systemic resistance" (ISR) mediated by jasmonic acid (JA), and "systemic acquired resistance" (SAR) mediated by salicylic acid (SA) (Farag et al. 2013; Pieterse et al. 2014).

Recent studies suggest that plant iron deficiency responses and systemic resistance responses are related. The JA and SA response genes, *PR1* and *PDF1.2*, are induced in *Arabidopsis* plants that are subjected to iron deficiency, and the effect is synergistic in the presence of pathogens (Koen et al. 2014). Volatile compounds released by *Trichoderma* spp. stimulate iron uptake responses in roots resulting in priming of JA-dependent defenses in shoots of *A. thaliana* and *Solanum lycopersicum*, in vitro (Martínez-Medina et al. 2017).

We observed that volatile compounds emitted from the beneficial rhizobacteria *Arthrobacter agilis* UMCV2 also induce the marker genes *SbCOI1* and *SbPR-1*, involved in systemic resistance pathways in *Sorghum bicolor* plants, simultaneously with the induction of the iron transporter genes *SbYS1* and *SbIRT1* (Hernández-Calderón et al. 2017). Volatile organic compounds (VOC) produced by *A. agilis* UMCV2, mainly dimethylhexadecylamine (DMHDA), increased the length and biomass of shoots and roots as well as the chlorophyll content in *Medicago truncatula* (Orozco-Mosqueda et al. 2013b) and *S. bicolor* (Castulo-Rubio et al. 2015). Both a VOCs cocktail and DMHDA increased the expression of five *M. truncatula FRO* genes in shoots and roots (data not published) and a *FRO* gene in *S. bicolor* (Castulo-Rubio et al. 2015) under in vitro conditions. Furthermore, plants grew equally well under iron-sufficient and iron-deficient conditions, but the effect of *A. agilis* UMCV2 inoculation on plants grown under greenhouse conditions is unknown.

In the present work, we evaluated the expression of *MtPR1* (Peleg-Grossman et al. 2012) and *MtDef2.1* (Hanks et al. 2005) genes, which are markers of SAR and ISR, respectively. At the same time, we evaluated the *MtFRO* gene expressions under greenhouse conditions. Moreover, these markers were used to explore the crosslinks between iron deficiency and defense pathways. We hypothesized that *A. agilis* UMCV2 increases the organ-specific expression of *M. truncatula FRO* under conditions of iron deficiency and sufficiency in the same way that it induces elements of the *M. truncatula* systemic resistance pathways.

Materials and methods

Plant materials and growth conditions

Plants of *M. truncatula* ecotype Jemalong A17 were used. Seeds were subjected to chemical scarification as described by Orozco-Mosqueda et al. (2013b). Briefly, seeds were immersed in a vial containing 1 mL of sulfuric acid and were constantly shaken until small black spots appeared in the tegument (approximately 8 min). The acid was removed, and the seeds were rinsed seven times with sterile deionized water. Sterilization was carried out with 12% sodium hypochlorite solution for 2 min, and the seeds were subsequently rinsed five times with sterile deionized water. Seeds were germinated in Petri dishes with 0.2 × MS medium (Murashige and Skoog Basal Salts Mixture, Sigma-Aldrich catalogue no. M5524) at 6.5 pH with 6 g of agar/L. Seeds were then transferred to greenhouse conditions in pots containing sterile peat moss (sterilized twice in a pressure vessel for 1 h) 5 days after germination.

Greenhouse assays

Three seedlings that had been germinated for 6 days were placed in pods containing 1 kg of peat moss under greenhouse conditions, and three pots per treatment were used. Seedlings were inoculated (with the exception of control specimens) with 3 mL of an *A. agilis* UMCV2 suspension with an approximate density of 1×10^9 CFU mL^{-1} three times, at 15 days after transplantation, 30 days after

transplantation, and 145 days after transplantation. The plants were watered twice per week with deionized water and once per week with complete Hoagland nutrient solution (Hoagland and Arnon 1950), which had the following composition (all concentrations are in mmol L^{-1}): $NH_4H_2PO_4$ (1), KNO_3 (6), $Ca(NO_3)_2$ (4), $MgSO_4$ (2), H_3BO_3 (0.05), $MnCl_2$ (0.0015), $ZnSO_4$ (0.0015), $CuSO_4$ (0.0005), H_2MoO_4 (0.00015), and 100 µM Fe(III)-EDTA (treatments corresponded to iron sufficient conditions for controls) or with Hoagland solution minus iron (treatments corresponded to iron deprivation). Plants were harvested and samples were obtained 150 days after transplantation.

Chlorophyll quantification

Chlorophyll quantification was conducted as previously described (Castulo-Rubio et al. 2015) using a CCM-200 chlorophyll meter (Opti-Sciences, Inc.; Hudson, NH, USA) to measure the chlorophyll concentration based on the rates of transmitted radiation (940 and 660 nm) through a leaf in arbitrary units.

RNA extraction

RNA extraction was performed in three biological composite samples of roots, stems, leaves, flowers, and pods; each biological sample was composed of the mixture of the organ of interest from the three plants in each pot. Total RNA extraction was conducted with the TRI reagent (Catalogue T9424, Sigma-Aldrich, St. Louis, MO, USA), and RNA was treated with DNaseI to remove residual genomic DNA. Finally, the samples were run on a 1% agarose gel at 90 V to determine specimen integrity.

Bacterial DNA quantification

Bacterial DNA quantification in seeds was conducted as described by Aviles-Garcia et al. (2016) with minimum modifications. In brief, seeds were aseptically extracted from mature pods and ground in liquid nitrogen, DNA from 100 mg of powdered seeds was extracted employing the methodology reported by Mahuku (2004), and then specific primers 2CV2F and 2CV2R designed to amplify an amplicon in the *A. agilis* UMCV2 16S-23S ITS were used to perform absolute quantifications of bacterial DNA by qPCR.

Phylogenetic analysis

The phylogenetic tree and molecular evolutionary analyses of the *MtFRO* genes and related sequences were performed using MEGA v6 software (Tamura et al. 2013) employing the "maximum parsimony" algorithm with 1000 repetitions for bootstrapping. Sequences of previously reported *FRO* genes were obtained from GenBank (https://www.ncbi.nlm.nih.gov/genbank/). Sequences of previously reported *MtFRO* genes (Orozco-Mosqueda et al. 2012) were identified on the *M. truncatula* Genome Database (http://www.medicagogenome.org/), with the exception of *MtFRO5*, for which the predicted products were located on GenBank.

Gene expression analysis

Reverse transcription quantitative real-time PCR (RT-qPCR) were performed in triplicate for each organ and gene using an ABI StepOne ™ System thermocycler (Applied Biosystems, Foster City, CA, USA). Oligonucleotides designed by Orozco-Mosqueda et al. (2012) were used to amplify *MtFRO1, MtFRO3*, and *MtFRO4*, genes and *MtACT* as normalizer gene. The expression of *MtPR1* was evaluated using the oligonucleotides reported by Peleg-Grossman et al. (2012). In addition, oligonucleotides MtFRO2F (5′ACT ATG CAG GCT CTT GCA GC3′), MtFRO2R (5′TAA TCC AAT CCC GCC CGA AC3′), MtFRO5F (5′GAT ATA TTA GCT CAT AGT GGT TGG C3′), MtFRO5R (5′ATC TTC ACT TGA CGA AAT TGG C3′), MtDef2.1F (5′ACT TTA ATA CAC ACA CCC ATT TGC 3′), and MtDef2.1R (5′TCA GTT AAG ATC TAG AGT CCC ACA3′) were used to amplify *MtFRO2* and *MtFRO5* (Orozco-Mosqueda et al. 2012) and *MtDef2.1* (Hanks et al. 2005), and the primers were designed using NCBI's Primer Designing Tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast). *MtPR1* and *MtDef2.1* expression was only measured in leaves. The RT-qPCR analysis was performed using the SYBR-Green kit (Applied Biosystems) and the following protocol: 10 µL SYBR-Green, 1 µL each of forward and reverse oligo's, 1 µL cDNA, and 7 µL water. Samples were run using the following protocol: 95 °C for 4 min, 40 cycles at 95 °C for 15 s, and 60 °C for 30 s. To prepare the melting curve, samples were run at 95 °C for 15 s, 60 °C for 1 min, and the temperature was subsequently raised to 95 °C at a rate of 0.3 °C/s. Gene expression was evaluated using the comparative ΔΔCt method according with Livak and Schmittgen (2001).

Statistical analysis

The results were analyzed using the Student's *t* test or with analysis of variance and Duncan's means separation test for multiple comparisons ($p \leq 0.05$).

Results

Phylogenetic relationships of *MtFRO* genes

To date, no studies have evaluated the organ-specific expression of *M. truncatula FRO* genes. However, the expression

of these genes has been observed in other strategy I plants, and *FRO* genes were also reported in strategy II plants. Aiming to identify possible orthological relationships between *MtFRO* genes and previously reported *FRO* genes, a phylogenetic analysis was performed using the maximum parsimony algorithm (Fig. 1), and three clusters were identified. The first cluster included genes expressed in shoots: *HvFRO1* and *HvFRO2* (Mikami et al. 2011); *OsFRO1* and *OsFRO2* (Ishimaru et al. 2006); *SbFRO1* (Castulo-Rubio et al. 2015); *AtFRO7* and *AtFRO6* (Mukherjee et al. 2006); and *MtFRO2* and *MtFRO5* (Orozco-Mosqueda et al. 2012). *MtFRO2* and *MtFRO5* were most closely related to *AtFRO7*, which codes for a protein that is located in the chloroplast. The second cluster was composed of the following genes with their expression localized in the roots: *AtFRO5, AtFRO4, AtFRO2, AtFRO1, AtFRO3* (Mukherjee et al. 2006); *LsFRO1* (Li et al. 2004); *CsFRO1* (Waters et al. 2007); and *MtFRO1, MtFRO3*, and *MtFRO4* (Orozco-Mosqueda et al. 2012). A sequence reported by Wang et al. (2017) as *MtFRO2* (GeneBank Accession number KX641482.1) was grouped together with *MtFRO1* (Andaluz et al. 2009). Both sequences are 99% homologues, they were reported to be expressed in roots, and their expression is induced by iron deficiency. With these bases, both were considered as the same gene. The third cluster contained *AtFRO8, gp91phox, FRE1*, and *MtFRO6* next to *AtRBOHA*. *AtRBOHA* codes for a respiratory burst-oxidase that differs structurally from most FRO reported proteins, although it contains the motifs needed to perform iron reduction (Baxter et al. 2014). On this basis, we concluded that the *MtFRO6* gene is more closely related to respiratory burst-oxidase genes than to ferric-chelate reductases and was not included in further experiments.

Arthrobacter agilis UMCV2 modulates the organ-specific expression of *M. truncatula FRO* genes

Plants were inoculated three times (with the exception of control specimens), at 15 days after transplantation, 30 days after transplantation, and 145 days after transplantation. Plants were harvested and samples were obtained 150 days after transplantation. Chlorophyll has been reported as an indicator of the iron nutritional status of plants (Masalha et al. 2000; Radhamani et al. 2016). Therefore, from iron-deficient (or iron-sufficient) *M. truncatula* plants that were inoculated (or not) with *A. agilis* UMCV2, the chlorophyll was quantified (Fig. 2). Plants inoculated with *A. agilis* UMCV2 and under iron-sufficient conditions had a chlorophyll content that was 9.7 arbitrary units (au) (approximately 25%) higher than that of control plants grown under iron-sufficient conditions. Plants grown under iron-deficient conditions had a chlorophyll content that was 11.8 au (approximately 40%) lower than that of iron-sufficient controls.

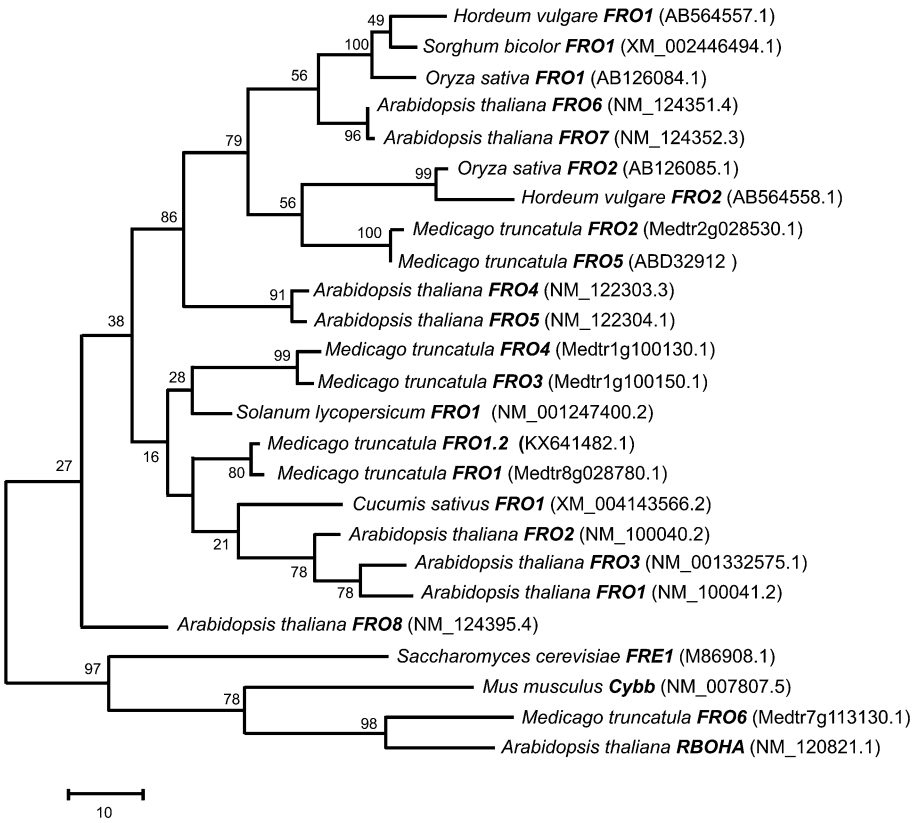

Fig. 1 Phylogenetic analysis of *MtFRO* genes. A phylogenetic tree of *MtFRO* genes and related genes from various species was constructed using maximum parsimony. Numbers on nodes represent bootstrap values that are based on 1000 replicates. GenBank Accession Numbers are indicated in parentheses. *MtFRO* genes are referred to in *Medicago truncatula* Genome Database, with exception of *MtFRO5*, which predicted product was located on GenBank

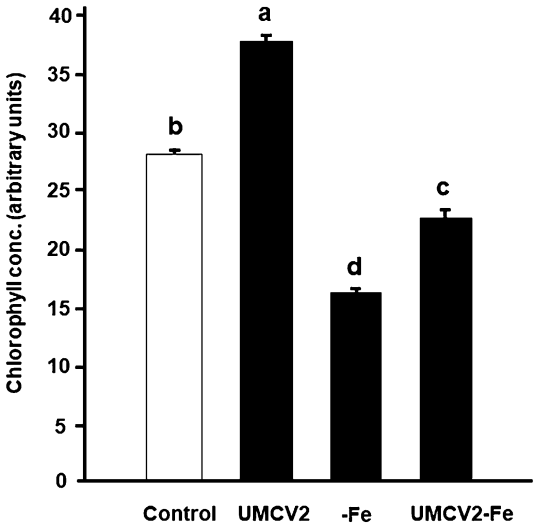

Fig. 2 Effects of A. *agilis* UMCV2 inoculation in plants grown under iron-sufficient and iron-deficient conditions on leaf chlorophyll content. Chlorophyll content was measured in leaflets, and values represent data from triplicate tests of nine 5-month-old *M. truncatula* plants from each treatment. Control represents iron-sufficient conditions without bacteria; UMCV2 indicates iron-sufficient conditions in presence of bacteria; -Fe refers to iron-deficient conditions without bacteria, and UMCV2-Fe indicates iron-deficient conditions with bacteria. Letters are used to indicate significant statistical differences determined by Duncan's multiple range test ($p \leq 0.5$; $n = 9$)

However, *A. agilis* UMCV2-inoculated plants that were grown under iron-deficient conditions had a chlorophyll content that was 6.4 au (approximately 25%) higher compared to that of plants that were not inoculated and grown under iron-deficient conditions.

Furthermore, the successful colonization of *A. agilis* UMCV2 in plants was confirmed through the quantification of DNA from *A. agilis* UMCV2 in seeds. Seeds of plants inoculated with *A. agilis* UMCV2 under iron-sufficient and iron-deficient conditions contained (medium ± standard error) 0.61 ± 0.10 and 0.12 ± 0.02 ng of *A. agilis* DNA/g seed, respectively. None of the *A. agilis* DNA was detected from the seeds harvested from non-inoculated control specimens.

Previous research has shown that *FRO* gene expression and ferric-chelate reductase activity are not constant but induced by a stimulus and followed by a down regulation (Andaluz et al. 2009; Connolly et al. 2003), with this in mind, plants were reinoculated 5 days prior to analyzing *MtFRO* expression.

The organ-specific expression patterns for *MtFRO* genes were as follows: *MtFRO1, MtFRO3, MtFRO4*, and *MtFRO5* were expressed in the roots; *MtFRO5* was expressed in the stems; *MtFRO2, MtFRO4*, and *MtFRO5* were expressed in the leaves; and *MtFRO2* was expressed in the flowers and pods (Fig. 3).

Inoculation with *A. agilis* UMCV2 induced the expression of all *MtFRO* genes in their corresponding tissues. In the roots, the expression of *MtFRO1, MtFRO3, MtFRO4*, and *MtFRO5*, ranged from 1.4- to 3.1-fold higher than controls, while in the stems, *MtFRO5* was expressed 3.7-fold higher than controls. In the leaves, the expression of *MtFRO2, MtFRO4*, and *MtFRO5* were 4.4-, 2.5-, and two-fold higher than controls, respectively. In flowers and pods, *MtFRO2* was induced between 6 and threefold higher than controls, respectively (Fig. 3).

Iron-deficient conditions produced a more conspicuous effect with regard to the induction of *MtFRO* genes in their corresponding tissues than that observed after bacterial inoculation. In the roots, the expression of *MtFRO1, MtFRO3, MtFRO4*, and *MtFRO5* ranged from 18- to 35-fold higher than in the controls. In the stems, *MtFRO5* was expressed 19-fold higher than in the controls. In the leaves, *MtFRO2, MtFRO4*, and *MtFRO5* expression was 24-, 12-, and 10-fold higher than in the controls, respectively. In the flowers and pods, the expression of *MtFRO2* was between 29 and ninefold higher than in the controls, respectively (Fig. 3).

Interestingly, when plants were grown under iron-deficient conditions and inoculated with *A. agilis* UMCV2, the *MtFRO* induction effect was synergistic. In this way, the expression of *MtFRO1, MtFRO3, MtFRO4*, and *MtFRO5* ranged from 48- to 548-fold higher than in the controls. *MtFRO5* expression in the stems was 49-fold higher than in the controls. *MtFRO2, MtFRO4*, and *MtFRO5* in the leaves ranged between 36- and 89-fold higher than in the controls, and *MtFRO2* expression in the flowers and pods ranged from 72- to 126-fold higher than in the controls, respectively (Fig. 3).

Inoculation of *A. agilis* UMCV2 induces the expression of defense pathways genes in *M. truncatula*

Previously, we reported that volatile compounds from *A. agilis* UMCV2 induced SAR and ISR responses in *S. bicolor* (Hernández-Calderón et al. 2017). With this in mind, the following qPCR assays for defense-related genes were performed: *MtDef2.1* (a JA response gene involved in ISR) and *MtPR1* (an SA response gene involved in SAR). Following *A. agilis* UMCV2 inoculation, an increase of 12.0-fold was observed in the expression of *MtDef2.1* with respect to the control. However, iron deficiency also induced the expression of the gene, which increased fourfold without bacteria and 29.1-fold under iron-deficient conditions and in the presence of *A. agilis* UMCV2 (Fig. 4a). The expression of *MtPR1* also increased but was lower compared to that of *MtDef2.1*. A 12.7-, 3.2-, and 14.4-fold increases were

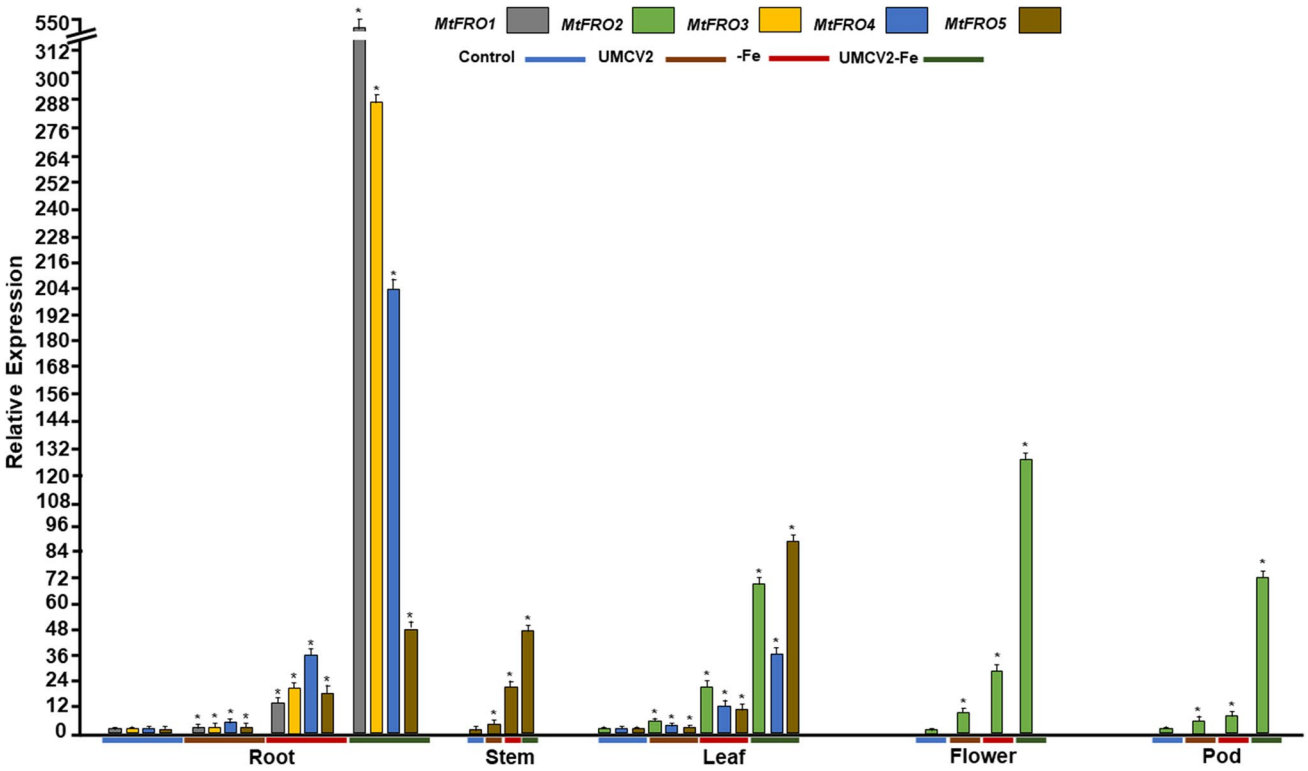

Fig. 3 RT-qPCR analysis of *MtFRO* gene expression in plants inoculated with *A. agilis* UMCV2 and grown under iron-sufficient and iron-deficient conditions. Using cDNA from different organs, RT-qPCRs were performed to evaluate effects of *A. agilis* UMCV2 inoculation on *MtFRO* gene expression. Control represents iron-sufficient conditions without bacteria; UMCV2 indicates iron-sufficient conditions in presence of bacteria; -Fe refers to iron-deficient conditions without bacteria, and UMCV2-Fe indicates iron-deficient conditions with bacteria. Asterisks represent significant statistical differences with respect to control, based on Student's *t* test ($p \leq 0.05$; $n = 3$)

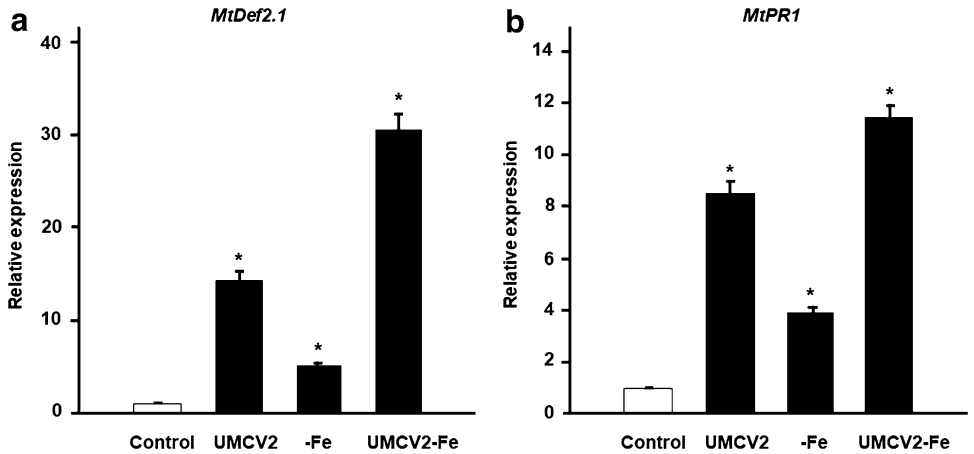

Fig. 4 RT-qPCR analysis of *MtDef 2.1* and *MtPR1* gene expression in *M. truncatula* plants inoculated with *A. agilis* UMCV2 and grown under iron-sufficient and iron-deficient conditions plants. Using cDNA from leaves, RT-qPCRs were performed to evaluate effects of *A. agilis* UMCV2 on *MtDef 2.1* and *MtPR1* gene expression. **a** *MtDef2.1* expression in different treatments. **b** *MtPR1* expression in different treatments. Control represents iron-sufficient conditions without bacteria; UMCV2 indicates the iron-sufficient conditions in presence of bacteria; -Fe refers to iron-deficient conditions without bacteria, and UMCV2-Fe indicates iron-deficient conditions with bacteria. Asterisks represent significant statistical differences with respect to control, based on Student's *t* test ($p \leq 0.05$; $n = 3$)

observed in the presence of bacteria under iron-deficient conditions and iron-deficient conditions combined with *A. agilis* UMCV2 inoculation, respectively (Fig. 4b).

Discussion

It has been established that some but not all bacteria are able to trigger the iron deficiency responses on plants, even in plants grown under iron-sufficient conditions (Scagiola et al. 2016; Orozco-Mosqueda et al. 2013b). The mechanisms that mediate this phenomenon are unknown, but recent observations have contributed to the clarification of the question.

Since *FRO* genes are key elements in the iron uptake process in strategy I plants, they have been studied in several plants, including *A. thaliana* (Robinson et al. 1999). In *M. truncatula*, six *FRO* genes have been identified (Orozco-Mosqueda et al. 2012), and the expression of these genes has been observed in both shoots and roots but not in an organ-specific form. In the present study, we evaluated the organ-specific expression of these genes and the effects of the beneficial rhizobacterium *A. agilis* UMCV2 on these expression profiles.

Prior to evaluating the organ-specific expression of *M. truncatula FRO* genes, a phylogenetic tree was constructed to compare the *MtFRO* genes with previously reported *FRO* genes and to detect possible orthological relationships. Three clusters were identified, including one with genes expressed in the roots, another with genes expressed in the shoots, and one with genes that expressed proteins that differed structurally from other *FRO* genes. *MtFRO6* was found in the third cluster, thus suggesting that the gene was related to *AtRBOHA* and was not considered in further experiments. In addition, the *FRO* genes of monocotyledonous plants are related to among them as well as to the *FRO* genes of dicotyledonous plants that are expressed in the shoots, indicating that these genes arose before the separation of the plant lineages.

Although no previous studies related to the organ-specific expression of the *FRO* genes in *M. truncatula* have been conducted, Wu et al. (2005) and Mukherjee et al. (2006) examined *FRO* gene expression profiles in the different organs of *A. thaliana*. While they did not obtain completely similar results, both works indicated that *AtFRO2* and *AtFRO3* were expressed in the roots; *AtFRO3, AtFRO6, AtFRO7*, and *AtFRO8* were expressed in the aerial tissues; and *AtFRO6, AtFRO7*, and *ATFRO8* were expressed in the flowers.

The following was discovered in the present study: *MtFRO1* and *MtFRO3* were expressed in the roots; *MtFRO2* was expressed in the leaves, flowers, and pods; *MtFRO4* was expressed in the roots and leaves; and *MtFRO5* was expressed in the roots, stems, and leaves. These data suggested that functional redundancy may exist between members of the *MtFRO* gene family. However, *A. thaliana* research has shown that different *FRO* genes are expressed in different tissues in the same organ. For instance, *AtFRO2* is expressed in the epidermal cells of roots, and *AtFRO3* is expressed in the vascular cylinder in roots (Mukherjee et al. 2006). Our data also show that at least one *MtFRO* gene is expressed in each studied organ, and that the genes are likely involved in iron reduction and homeostasis throughout the plant.

In all of the evaluated organs of the plants grown under greenhouse conditions, *MtFRO* expression increased under iron-deficient conditions, and the increased expression ranged from 9- to 35-fold higher than that observed in controls. *FRO* genes induced by iron deficiency have been detected in *A. thaliana*. Connolly et al. (2003) and Ye et al. (2015) observed that, when plants were grown under iron deficiency in vitro, *AtFRO2* was induced 2720- and 147-fold higher, respectively, compared to the expression levels in the controls. The induction levels observed in our iron deficiency treatments were clearly lower, and this was likely due to the use of a different species and differences between the iron-deficient conditions in our greenhouse and the in vitro conditions of Connolly et al. (2003) and Ye et al. (2015).

Under iron-sufficient conditions, inoculation with the rhizobacterium *A. agilis* UMCV2 also increased *MtFRO* gene expression by 1.3- to sixfold with respect to the control plants, but the induction of gene expression was lower than that observed under iron-deficient conditions. Moreover, the chlorophyll content in inoculated plants grown under iron-sufficient conditions was approximately 25% higher than that observed in control plants, thus suggesting a better iron nutrimental status than control plants (Radhamani et al. 2016). Inoculation with *A. agilis* UMCV2 induced *MtFRO* genes throughout all plant tissues, strongly suggesting that the bacteria not only enhanced iron uptake, as was previously shown *Phaseolus vulgaris* plants (Valencia-Cantero et al. 2007), but that it also promoted iron reduction across all plant tissues, thus modulating plant iron homeostasis.

In our laboratory, we have conducted several experiments with *A. agilis* UMCV2 and observed that the bacteria use VOCs to promote plant growth in *M. sativa* (Velázquez-Becerra et al. 2011), *M. truncatula* (Orozco-Mosqueda et al. 2013b), and *S. bicolor* (Castulo-Rubio et al. 2015) under in vitro conditions. The effect is mainly attributed to the aminolipid DMHDA. We observed an increase in the expression of the *S. bicolor SbFRO1* gene (Castulo-Rubio et al. 2015) in the presence of *A. agilis* UMCV2 VOCs or pure DMHDA when in a closed system, and the greatest effect occurred when plants grew with iron deficiencies.

In the present study, we also observed that the iron-deficient condition together with *A. agilis* UMCV2 inoculation produced a synergistic effect on *MtFRO* gene induction, and

the expression values ranged from 44- to 493-fold relative to the control plants. A greenhouse system was used in this study, so the increased expression of *MtFRO* genes cannot be explained by an accumulation of VOCs in a confined space, such as that used by Orozco-Mosqueda et al. (2013a, b) and Castulo-Rubio et al. (2015). Therefore, the expression of genes in the aerial tissues requires another explanation. One possibility that we explored is the systemic effect related to the induction of defense genes such as *MtDef 2.1* and *MtPR1*.

It is known that PGPR bacteria induce ISR defenses, so the expression of related genes (*PDF1.2* and *Hel*) (Pieterse et al. 2014; Van Loon et al. 1998) is also known to induce the parallel expression of SAR- and ISR-related genes (García-Gutiérrez et al. 2013). Conn et al. (2008) reported that endophytic actinobacteria isolated from wheat induce the SAR-related genes *PR1* and *PR5* and the ISR-related genes *PDF1.2* and *Hel* in *Arabidopsis*. These results are in agreement with those of the present study, since markers of both ISR and SAR responses were induced by the endophytic actinobacteria *A. agilis* UMCV2. Interestingly, PGPRs also induce a phenomenon called induced systemic tolerance (IST), which triggers resistance to drought, salinity, and high or low temperatures (Yang et al. 2009). ISR, SAR, and IST are related responses. In a model proposed by Farag et al. (2013), the VOCs of PGPRs are thought to trigger SAR and ISR responses by increasing the synthesis of ethylene and salicylic acid, thus inducing the expression of *PR* and *DEF* genes and establishing such responses. On the other hand, VOCs induce the expression of *FIT1* that codes for a transcription factor throughout the plant, thus inducing the expression of *FRO2* and *IRT1*, improving the iron deficiency response, and triggering IST (Farag et al. 2013). Based on our results, the relationship between iron systemic responses and systemic resistance was observed because of an increase in the expression of *MtDef 2.1* and *MtPR1* under iron-deficient conditions and a synergistic effect associated with iron deficiency and *A. agilis* UMCV2 inoculation. Kobayashi et al. (2016) proposed a model in strategy II plants that related ISR to iron uptake (IST), and they observed that iron deficiency increased the expression of inducible JA genes. Furthermore, the transcription factor *IDEF1* that modulates the expression of iron responsive genes also increased the expression of JA response genes, and this, in turn, increased the expression of the aforementioned *IDEF1* factor, indicating that the signaling pathway response to iron deficiency and the jasmonate signaling pathway are connected. Because iron-deficient conditions resulted in an induction of *MtDef2.1* expression, which responds to jasmonate, it can be concluded that a regulation phenomenon occurs both at the defense level and in the iron uptake of the strategy I plant, *M. truncatula*. Furthermore, this phenomenon is similar to what happens in the strategy II plant, *Oryza* sativa. Koen et al. (2014) demonstrated that iron deficiency in *A. thaliana* induced the expression of *PR1* and *DEF1.2*. Moreover, the presence of the phytopathogenic fungus *Botrytis cinerea* produced an additive effect in the induction of *PR1* and *DEF1.2*. Our results demonstrate that the induction of the expression of both defense genes also occurs under iron-deficient conditions and that the effect is enhanced in the presence of the PGPR *A. agilis* UMCV2. Recently, Raya-González et al. (2017) found that DMHDA induced the expression of the jasmonate synthesis gene *LOX2* in *A. thaliana*. Furthermore, mutant plants affected in the conjugation (*Jar1*), perception (*coi1-1*), and expression of genes responding to JA (*myc2*), were resistant to DMHDA. Based on this observation, it is probable that DMHDA is involved in the induction of *MtDef2.1* via *A. agilis* UMCV2 inoculation, and that it is involved in the iron deficiency responses in *M. truncatula* (Orozco-Mosqueda et al. 2013a, b).

In conclusion, our data demonstrate that *MtFRO* genes are expressed virtually throughout the *M. truncatula* plant, and the PGPR *A. agilis* UMCV2 systemically induced the expression of *MtFRO* genes (likely through associated VOCs such as DMHDA). Furthermore, the bacteria induced marker genes of the ISR and SAR pathways, thus triggering resistance responses to biotic stresses, and these effects were considerably greater under iron-deficient conditions (Fig. 5). Therefore, our data support the existence of a relationship between systemic iron deficiency responses and systemic defense responses.

Author contribution statement VMR: Data collection, data analysis and interpretation, drafting the article. RMC: Data collection. EGP: Data analysis and interpretation. EVC: Conception or design of the work, data analysis and interpretation. Final approval of the version to be published.

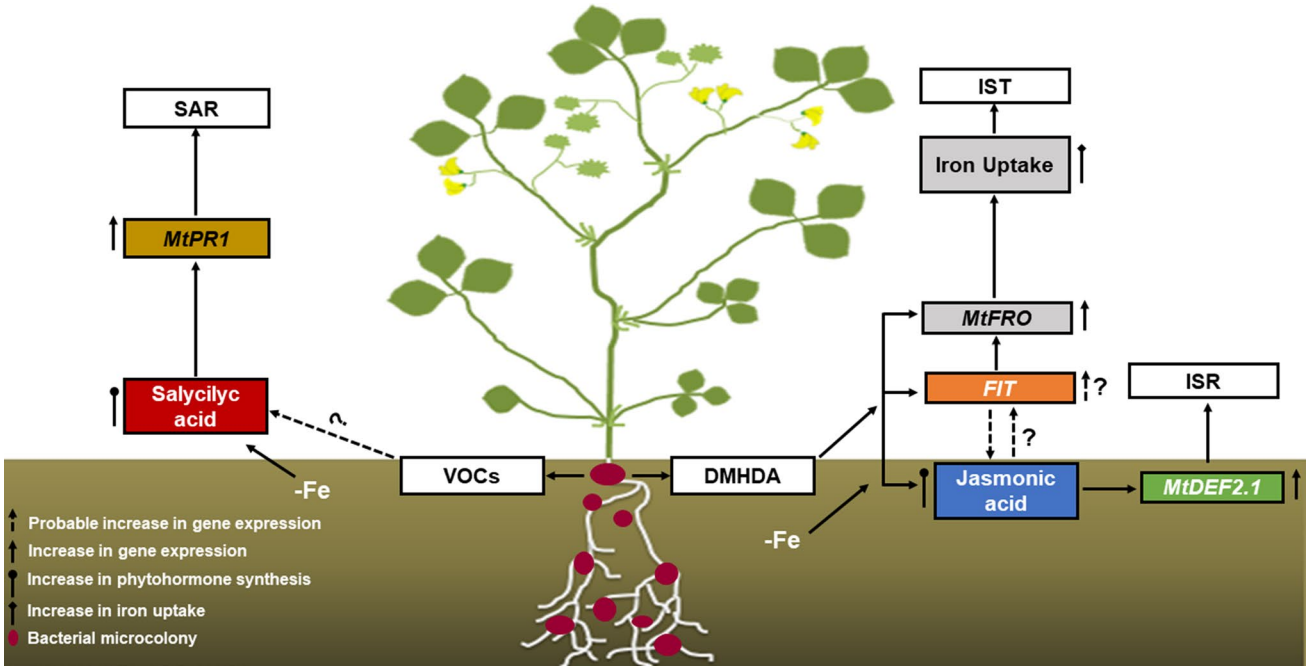

Fig. 5 General model of iron deficiency and *A. agilis* UMCV2 inoculation effects on iron uptake and resistance pathways in *M. truncatula*. Based on the results and background information, we propose a model in which elements of the ISR pathway are related to elements of the iron uptake pathway

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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