SCAP
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Comparative analysis of Iron Deficiency Chlorosis responses in soybean (*Glycine max*) and barrel medic (*Medicago truncatula*)

Análise comparativa das respostas à Clorose por Insuficiência de Ferro em soja (*Glycine max*) e luzerna-cortada (*Medicago truncatula*)

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ABSTRACT

Legume grains have an important socio-economical role, being highly utilized in human and animal nutrition. Although iron (Fe) is abundant in the earth's crust, its limited solubility makes it poorly bioavailable for plants, contributing to iron deficiency chlorosis (IDC). In this work the physiological and molecular mechanisms associated with IDC were studied, namely, the mechanisms involved on Fe deficiency response, as well as a new Fe metabolism related gene in two important legume crops, *Glycine max* and *Medicago truncatula*. Fe deficient plants developed: decreased root and shoot length, increased number of secondary roots and lower chlorophyll levels. Fe shoot content decreased six- and 11-fold for *G. max* and *M truncatula* in Fe-deficiency. Whilst in *G. max* roots no significant differences were detected, in *M. truncatula* roots Fe decreased nine-fold in Fe-deficiency. Genes involved in Fe uptake (*FRO2*-like and *IRT1*-like), were over-expressed in roots of Fe-sufficient *G. max* and in Fe-deficient *M. truncatula*. *VIT1*-like, *YSL1*-like and *ferritin* presented higher expression levels in Fe-sufficient shoots and roots, whereas *NRAMP3*-like and *GCN2*-like showed higher expression values in Fe-deficiency.

Key Words: Ferric reductase, *Glycine max*, *Medicago truncatula*, morphological analysis, RT-PCR.

RESUMO

As leguminosas têm um importante papel socio-económico, pela sua utilização na nutrição humana e animal. Apesar do ferro (Fe) ser um elemento abundante na crosta terrestre, a sua solubilidade limitada diminui a disponibilidade para as plantas, contribuindo para o desenvolvimento da Clorose por Insuficiência de Ferro (CIF). No presente trabalho, mecanismos fisiológicos e moleculares associados à CIF foram estudados, nomeadamente, os mecanismos de resposta à insuficiência de Fe e um novo gene associado ao metabolismo do Fe, em duas espécies cultivadas com relevância económica, *Glycine max* e *Medicago truncatula*. Plantas deficientes em Fe apresentaram: tamanho diminuído, maior número de raízes secundárias e baixos níveis de clorofila. Em insuficiência de Fe, o conteúdo de Fe na parte aérea diminuiu seis e onze vezes para *G. max* e *M. truncatula*, respetivamente; nas raízes de *G. max* não houve diferenças significativas e nas de *M. truncatula* o conteúdo de Fe diminuiu nove vezes. Genes envolvidos na absorção de Fe (*FRO2-like* e *IRT1-like*) foram sobre-expressos nas raízes de *G. max* em suficiência de Fe e, nas raízes de *M. truncatula*, quando em insuficiência. *VIT1-like*, *YSL1-like* e *ferritina* apresentaram níveis de expressão mais elevados em suficiência de Fe, ao contrário dos genes *NRAMP3-like* e *GCN2-like*, cuja expressão foi aumentada em insuficiência de Fe.

Palavras-chave: Análise morfológica, luzerna-cortada, reductase férrica, RT-PCR, soja.

INTRODUCTION

Legumes represent one of the most important foods, for both humans and animals (Vasconcelos and Grusak, 2006), providing an important source of protein and oil (Libault *et al*., 2010). One of the world's top commodity production is soybean (*Glycine max* L.). In fact, much of the world's protein and oil comes from soybean and this legume contains more protein (40%) and oil (20%) than any other ordinary food source, including meat, cheese and fish (Krishnan, 2005; Bolon *et al*., 2010). The appropriate addition of soy to different products, results in lower calorie alternative food products, with high content of protein, dietary fiber and minerals, preserving the physical and sensory characteristics of the product (Dhingra and Jood, 2001). The genome of soybean was sequenced, assembled and published (Schmutz *et al*., 2010), making it a good model crop to study genetic and molecular mechanisms. Barrel medic (*Medicago truncatula*) has been chosen as a model species for molecular studies in view of its growth and genomic characteristics (Trieu *et al*., 2000). To be convenient as a model for legume genomics, it is also essential that *M. truncatula* exhibit genome conservation with other crop legumes. Detailed comparisons between *M. truncatula* and *M. sativa* – a high feeding value crop used in animal nutrition – have reported that marker relationships were uniformly syntonic and that genes from *M. truncatula* share very high sequence identity to their counterparts from *M. sativa*, so it serves as an excellent model organism for soybean and other economically important legumes (Bell *et al*., 2001; Choi *et al*., 2004).

Besides protein and oil, legumes are also an important source of micronutrients, such as iron (Fe) (Vasconcelos and Grusak, 2006). This mineral is involved in the production of chlorophyll, and is also a component of many enzymes associated with the antioxidant system, energy transfer and nitrogen reduction and fixation. Legumes are very susceptible to Fe deficiency, when grown in adverse conditions, like calcareous soils, due to the low solubility of the oxidized form of Fe (Fe^{3+}) at near neutral and alkaline soil pH (Waters *et al*., 2002; Andaluz *et al*., 2009). Insufficient Fe uptake leads to Fe-deficiency chlorosis (IDC) symptoms, such as yellowing of the younger leaves, interveinal chlorosis and stunted growth, as well as reduction of crop yields (Prasad, 2003; Kim and Guerinot, 2007). IDC lowers the concentrations of Fe in the seeds and other harvested tissues (Grusak, 1999), affecting both farmer profit and the nutritional value of plant products (Vasconcelos and Grusak, 2013).

In order to uptake Fe from the soil, dicotyledonous plants such as soybean and barrel medic, utilize Strategy I, where Fe^{3+} is reduced to Fe^{2+} through the action of a membrane-bound Fe^{3+}-chelate reductase, like the ferric reduction oxidase (FRO). Fe^{2+} is then transported into the plant by specific membrane transporters (Grotz and Guerinot, 2006), such as the Iron-Regulated Transporter 1 (IRT1) (Waters *et al*., 2002). A broad spectrum of transporters have been characterized, such as the Natural Resistance Associated Macrophage (NRAMP) proteins, involved in Fe import into the cytoplasm, the Vacuolar Iron Transporter (VIT), involved in the uptake of Fe^{2+} into the vacuole for storage (Brear *et al*., 2013), and the Yellow Stripe 1-Like (YSL), involved in the transport of Fe^{2+}-NA complexes (Kim *et al*., 2006). Free Fe is toxic since it facilitates the generation of highly reactive oxygen species (ROS). ROS can damage cellular constituents and, therefore, Fe homeostasis needs to be strictly controlled to avoid iron deficiency and toxicity (Liao *et al*., 2012). Therefore, storage proteins, such as Ferritin, play an important role in iron homeostasis, since they assure that ferric Fe is bio-available in case of cellular needs but yet nonreactive with oxygen (Briat *et al*., 2010).

Even though much has been learned about the physiology of Fe uptake in *Arabidopsis*, there is still a limited understanding of the physiology of tolerance to Fe deficiency in soybean and barrel medic, and this has hampered breeding programs (Vasconcelos and Grusak, 2013). There have been few works focusing in the comparative study between these two species (Yan *et al*., 2004), however more information is needed to understand the mechanisms at a molecular level, such as which genes have been selectively conserved or lost between both species. Since increasing the Fe uptake in the roots can augment Fe concentrations in the leaves, it is possible that some of this additional Fe may be remobilized to the grains, which would help in biofortification efforts that aim at enhancing Fe seed levels (Santos *et al*., 2013,

2015). However, the increased Fe translocation from shoots to seeds still remains one of the major bottlenecks in most biofortification programs (White and Broadley, 2005), and the answer to this may be in the identification of new candidate genes. GCN2 is a protein kinase present in several organisms such as mammals and yeasts (Lageix *et al*., 2008) and is activated in plants by amino acid deprivation conditions (Zhang *et al*., 2008), as well other stress stimuli, such as purine deprivation, UV light, cold shock and wounding (Lageix *et al*., 2008). To this date, there are no published studies on the role of *GCN2* on Fe uptake in plants growing in Fe deficiency, which makes the study of this gene an important innovation in Fe nutrition in plants. However, its regulation is still not well known (Liu *et al*., 2015), which makes it relevant to study this gene, in order to understand how its expression if is affected by Fe deficiency and which mechanisms it may be associated with.

The present study describes the common mechanisms underlying the response to Fe deficiency at a physiological and molecular level, in *G. max* and *M. truncatula* grown hydroponically under Fe deficiency and Fe sufficiency. It also describes further analysis on the role of a novel candidate gene, *GCN2*, on Fe metabolism.

MATERIALS AND METHODS

Plant material and growth conditions

Medicago truncatula cultivar "Luzerna revilheira" and *Glycine max* cultivar "Williams 82" were grown in a growth chamber (Aralab Fitoclima 10000EHF) with 16 h day / 8 h night photoperiod. The temperature was kept at 20 ºC during the light period, with 350 µmol s^{-1} m^{-2} of photon flux density, and at 18 ºC during the dark period, with 75 % of relative humidity. Seeds of *M. truncatula* and of *G. max* were germinated for seven days in the dark and then transferred to hydroponic solutions with 20 µM FeEDDHA (Fe+) or with no FeEDDHA (Fe-) supply. The standard solution for hydroponic growth of *M. truncatula* contained as macronutrients: 3 mM KNO_3, 1 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4.7H_2O$, 0.5 mM $NH_4H_2PO_4$, 0.75 mM K_2SO_4, 25 µM $CaCl_2$; and as micronutrients: 25 µM H_3BO_3, 2 µM $MnSO_4$, 2 µM $ZnSO_4.H_2O$, 0.5 µM $CuSO_4.H_2O$, 0.5 µM MoO_3, 0.5 µM $NiSO_4$. The conditions used for *G. max* included as macronutrients: 1.2 mM KNO_3, 0.8 mM $Ca(NO_3)_2$, 0.3 mM $MgSO_4.7H_2O$, 0.2 mM $NH_4H_2PO_4$, 25 µM $CaCl_2$; and as micronutrients: 25 µM H_3BO_3, 0.5 µM $MnSO_4$, 2 µM $ZnSO_4.H_2O$, 0.5 µM $CuSO_4.H_2O$, 0.5 µM MoO_3, 0.1 µM $NiSO_4$. Both hydroponic solutions were buffered by the addition of 1mM MES, pH 5.5. The assay ended at the 14th day of hydroponic growth.

Morphological and biochemical evaluations

At the end of the experimental time period, five plants of each species and treatment were harvested and the length and fresh weight of shoots and roots was measured. Also, the number of secondary roots was counted and the chlorophyll concentration was quantified accordingly to Abadía *et al*. (1984).

Fe reduction was measured in the roots of five intact plants via the spectrophotometric measurement of Fe^{2+} chelated to BPDS, as described in Vasconcelos and Grusak (2006). Rates of reduction were determined using the molar extinction coefficient of 22.14 mM^{-1} cm^{-1}. Roots and shoots were dried at 70 ºC and 200 mg of each sample was analyzed for the determination of Fe content using the ICP-OES Optima 7000 DV (PerkinElmer, Massachusetss, USA) with radial configuration, according to Roriz *et al*. (2014).

Gene expression analysis

Additional five replicates of each species and treatments were pooled and the RNA from leaves and roots was extracted following manufacturer's instructions, using the Qiagen RNeasy Plant Mini Kit (USA, #74904). cDNA was synthesized using First Strand cDNA Synthesis Kit (Fermentas).

Candidate genes were selected according to their established (*FRO2*-like, *IRT1*-like, *NRAMP3*-like, *VIT1*-like, *YSL1*-like, *ferritin*) or possible (*GCN2*-like) role on Fe metabolism. In order to identify orthologs for these genes, known sequences from *Arabidopsis* were blasted, and the most homologous sequence (E_{value} < 10^{-20}) was selected (Table 1). Quantitative Real-Time PCR (qPCR) reactions were performed on a Chromo4 thermocycler (Bio-Rad). Amplifications were carried out using 1.25 µM of the specific primers and mixed to 12.5 µL of 2xPCR iQ SYBR Green Supermix (Bio-Rad) and 100 ng of cDNA in a final volume of 25 µl. Three technical replicates were performed for each gene tested in qPCR reactions, as well as for controls.

Table 1 - Gene accession numbers and forward and reverse primer sequences used in quantitative Real-Time PCR analysis

Gene	Species	Accession numbers	Primer sequences
18S rRNA	-	X75080.1	F 5'- TTAGGCCATGGAGGTTTGAG -3' R 5'- GAGTTGATGACACGCGCTTA -3'
FRO2-like	*G. max*	XM_003548612.1	F 5'- TGCTTGGACTCACACCAGAG -3' R 5'- AGAGGTAGAAACCGGGGAGA -3'
	M. truncatula	XM_003622457.1	F 5'- CACTTGTGATGGTGAGTGGA -3' R 5'- GATGGTGTGCCAGAAATAGG -3'
IRT1-like	*G. max*	XM_003520096.2	F 5'- GATTGCACCTGTGACACAAA -3' R 5'- CAGCAAAGGCCTTAACCATA -3'
	M. truncatula	XM_003630873.1	F 5'- GACAAAGGAACCGGAACAAA -3' R 5'- TTGATGGAAGCAAAGTGCAG -3'
YSL1-like	*G. max*	XM_003536126.2	F 5'- GCTTTTGGAGCAGGTCTCAC -3' R 5'- AGACCACAACCCACAAGTCC -3'
	M. truncatula	XM_003602267.1	F 5'- GATCTTGGCCCACAACAAGT -3' R 5'- ACTGCAGGAACCATCAAACC -3'
VIT1-like	*G. max*	XM_003525172.2	F 5'- TTGTTAGCTTGGCGTGACAG -3' R 5'- TGCAACCAAGGTAACCACAA -3'
	M. truncatula	XM_003630932.1	F 5'- GGGTGGAATTGTTCCTCTCA -3' R 5'- AGCACTCCTGATTGGCTTGT -3'
ferritin	*G. max*	U31648.1	F 5'- CCCCTTATGCCTCTTTCCTC -3' R 5'- GCTTTTCAGCGTGCTCTCTT -3'
	M. truncatula	XM_00362331.1	F 5'- GTAAGAAATGGGGTGGTGGA -3' R 5'- CGAGCCAAAGAAACTTGAGG -3'
NRAMP3-like	*G. max*	XM_003524624.2	F 5'- TGTTCAGTCAAGGCAGGTTG -3' R 5'- CCAGCATTTACAAGGCCAAT -3'
	M. truncatula	XM_003611600.1	F 5'- TTTGGATCCTGGAAACTTGG -3' R 5'- GCTGAATCAAAAGCCCCATA -3'
GCN2-like	*G. max*	XM_006592086.1	F 5'- ATCCTTGCCTCATCACCAAC -3' R 5'- ATGGGGAACTGTGTTTGAGC -3'
	M. truncatula	XM_003636896.1	F 5'- GTAACCGAGGTCCGAGATGA -3' R 5'- CTCCACCATGGGTCAGAAGT -3'

The amplification of all genes was performed accordingly to Han *et al*. (2013). The comparative CT method (ΔΔCT) (Livak and Schmittgen, 2001) was utilized for the relative quantification of gene expression value of Fe stress related genes using the 18S rRNA gene as the housekeeping gene (Opticon Monitor 3 Software, Bio-Rad).

RESULTS AND DISCUSSION

For several organisms, Fe represents a cofactor in vital metabolic pathways such as the electron transport chain of respiration. Plants have an additional need for Fe because photosynthesis and chlorophyll biosynthesis both require this micronutrient (Jeong and Guerinot, 2009). Thus, how plants maintain Fe homeostasis and the anatomical modifications concerning Fe absence is a biologically relevant question. In the current work, when Fe was absent, both *G. max* and *M. truncatula* behaved similarly, developing characteristic IDC symptoms, such as impaired growth, observed by the reduction in plant weight and length (Table 2). More specifically, *G. max* had 2.2- and 2.1-fold lower fresh weight in shoots and roots, respectively, under Fe deficiency, which was more pronounced than *M. truncatula*, that had a reduction of 1.5- and 1.8-fold (Table 2).

Another important characteristic associated with the absence of Fe is the development of secondary structures. Here, plants submitted to -Fe conditions showed swelling of root tips and increased number of secondary structures, namely, an average of 60 % more for *G. max* and 69 % more for *M. truncatula* (Table 2). The increased number of secondary structures helps the plant in augmenting the absorbable area for Fe uptake, and the scavenging of Fe in the rhizosphere (Schmidt,

Table 2 - Fresh weight (FW) (g), length (cm) and number (#) of secondary roots of G. max and M. truncatula grown hydroponically in Fe-sufficient (Fe+) and Fe-deficient (Fe-) conditions. Data are means ± SE of five independent replicates. For each parameter analyzed, different letters represent significant differences between samples ($p < 0.05$)

		G. max		*M. truncatula*	
		Fe+	**Fe-**	**Fe+**	**Fe-**
Shoot	FW	6.44 ± 0.53 a	2.92 ± 0.32 b	0.82 ± 0.09 c	0.55 ± 0.07 d
	Length	30.5 ± 0.70 a	16.92 ± 0.65 b	10.75 ± 0.49 c	7.33 ± 0.50 d
Root	FW	5.38 ± 0.46 a	2.61 ± 0.24 b	1.03 ± 0.11 c	0.57 ± 0.09 d
	Length	49.75 ± 1.16 a	27.08 ± 0.78 b	35.42 ± 1.51 c	28.75 ± 1.84 d
# Secondary Roots		34.2 ± 3.09 a	57.00 ± 5.14 b	17.4 ± 1.62 c	25.2 ± 2.95 d

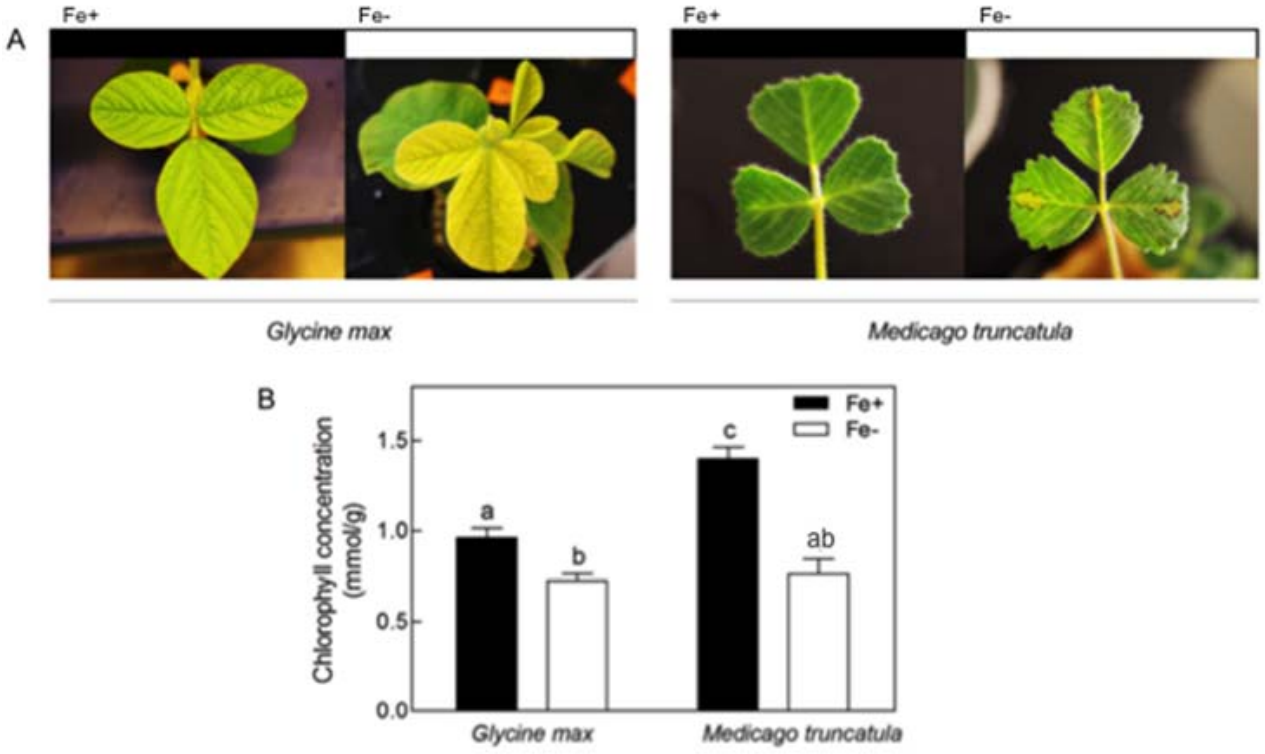

Figure 1 - Visible chlorosis symptoms (A) and chlorophyll concentration (B) of G. max and M. truncatula plants grown hydroponically in Fe-sufficient (Fe+) and Fe-deficient (Fe-) conditions. Data are means +SE of five independent replicates. Different letters represent significant differences between samples ($P < 0.05$).

1999). Since the surface of root hairs can represent up to 70% of the total root surface area (López-Bucio *et al*., 2003), the relevance of root hairs in nutrient uptake is crucial.

At the shoot level, the absence of Fe is known to inhibit chloroplast biogenesis and chlorophyll biosynthesis, leading to the development of chlorosis, especially in younger leaves (Henriques *et al*., 2002). Also, Fe starved plants may be more prone to oxidative damage (Kumar *et al*., 2010), leading to the accumulation of ROS, to oxidative stress, and to lower chlorophyll levels and increased chlorosis symptoms (as seen in Figure 1).

In this work, chlorosis symptoms appear to be more severe in *G. max* plants when compared to *M. truncatula* plants (Figure 1A), but the absolute values of chlorophyll concentration in Figure 1B seem to be contradictory. However, this is due to the fact that *G. max* plants, even under Fe sufficiency, weren't as green as *M. truncatula* plants under the

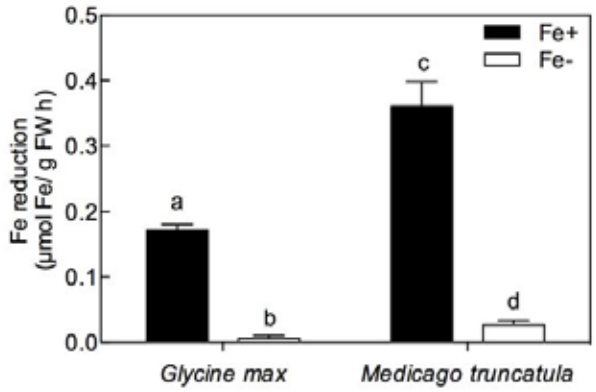

Figure 2 - Root Fe reductase activity of G. max and M. truncatula plants grown hydroponically in Fe-sufficient (Fe+) and Fe-deficient (Fe-) conditions. Data are means +SE of five independent replicates. Different letters represent significant differences between samples (P < 0.05).

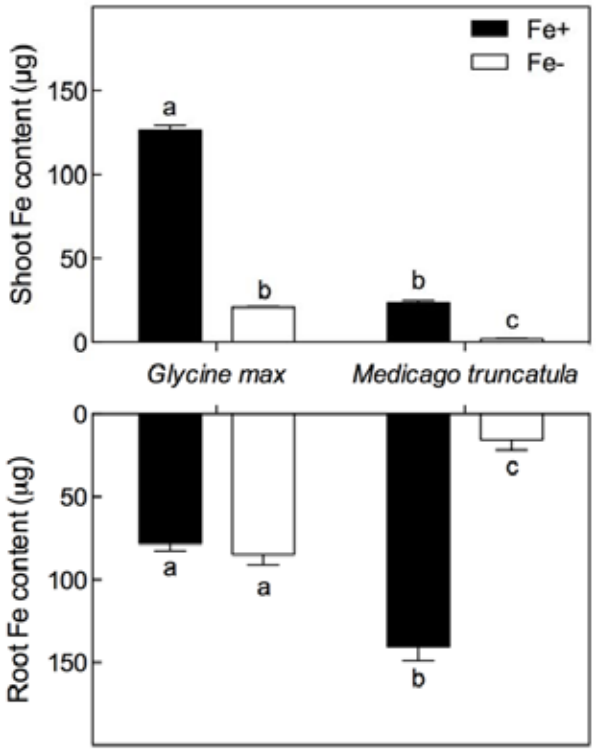

Figure 3 - Fe content of shoots and roots of G. max and M. truncatula grown hydroponically in Fe-sufficient (Fe+) and Fe-deficient (Fe-) conditions. Data are means +SE of five independent replicates. Different letters represent significant differences between samples (P < 0.05).

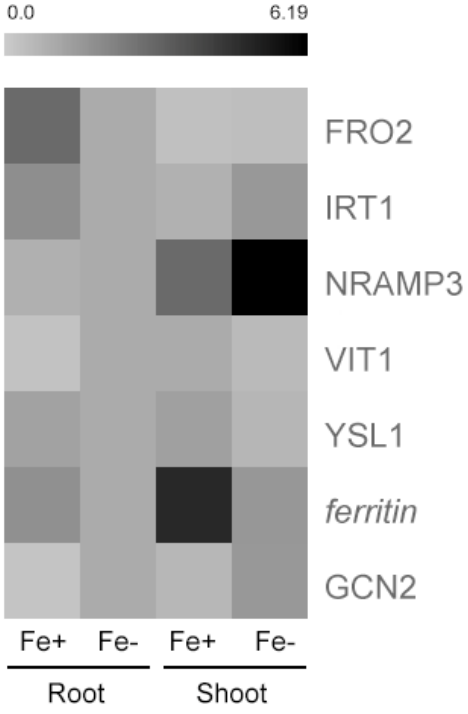

Figure 4 - HeatMap of the expression patterns of FRO2-, IRT1-, NRAMP3-, VIT1- and YSL1-like genes and ferritin and GCN2-like genes in root and shoot tissues of G. max plants grown hydroponically in Fe-sufficient (Fe+) and Fe-deficient (Fe-) conditions. "Fe- Root" was the reference sample; expression was normalized with 18S rRNA housekeeping gene. In black: increased gene expression; in light grey: lower gene expression. Total RNA was extracted from a pool of five independent replicates. Corresponding values are presented in Table 3.

Table 3 - Fe deficiency-related genes relative expression values of G. max plants grown under Fe-sufficient (Fe+) and Fe-deficient (Fe-) hydroponic conditions. Total RNA was extracted from a pool of five independent replicates

	Root		**Shoot**	
	Fe+	**Fe-**	**Fe+**	**Fe-**
FRO2-like	2.98	1	0.37	0.41
IRT1-like	1.87	1	0.81	1.60
NRAMP3-like	0.84	1	2.98	6.19
VIT1-like	0.28	1	0.98	0.55
YSL1-like	1.28	1	1.32	0.68
ferritin	1.81	1	4.95	1.62
GCN2-like	0.23	1	0.64	1.57

same treatment, leading to an acuter decrease in chlorophyll concentration.

Root Fe uptake capacity is linked with the solubilisation of Fe in the rhizosphere by the plant's root Fe reductase activity, which is necessary to convert the less soluble Fe^{3+} to the more soluble Fe^{2+} (García *et al*., 2013). Here, for both species, the enzyme was more active in Fe+ conditions and was higher in *M. truncatula* plants (Figure 2). It has been hypothesized that, for some genotypes, Fe is necessary for the functioning of the reductase enzyme itself (Blair *et al*., 2010). Although most studies imply that Fe reduction is induced under Fe deficiency (Wang *et al*., 2013; Zha *et al*., 2014), it has already been described that this is not always this way (Vasconcelos and Grusak., 2006; Santos *et al*., 2015).

In order to understand how Fe deficiency affects the mineral composition of Fe in *G. max* and *M. truncatula*, root and shoot tissues were analyzed by ICP-OES. When *G. max* was faced with the lack of Fe, it appeared to accumulate its internal Fe storage in the roots and the shoot Fe content decreased six-fold (Figure 3). It has been seen before that in response to shortage in mineral nutrition plants usually allocate more resources to the roots (Hermans *et al*., 2006; Santos *et al*., 2015). On the other hand, *M. truncatula* plants had a general reduction in Fe content in both tissues under Fe deficiency.

To further understand the mechanisms triggered by Fe shortage, it is crucial to comprehend the key conserved molecular players involved in nutrient uptake (e.g. *FRO2* and *IRT1*), transport (e.g. *NRAMP3*, *VIT1* and *YSL1*) and storage (e.g. *ferritin*), as well as identify novel candidate genes, that could have important roles in Fe metabolism (*GCN2*). When plants are faced with stress situations, the rate of nutrient uptake needs to increase, in order to compensate the lack of Fe. Thus, root Fe uptake related genes *FRO2* and *IRT1* are extremely important since they participate in this critical step concerning the plant response to Fe deficiency, and which control the efficiency of Fe uptake.

The results obtained for *G. max* plants show that in Fe- the expression of *FRO2*-like was decreased by three-fold (Figure 4), accordingly to the Fe reductase activity previously described (Figure 2). On the contrary, *M. truncatula* roots over-expressed *FRO2*-like gene under Fe deficiency (Figure 5), as previously obtained in *A. thaliana* (Robinson *et al*., 1999), tomato (Li *et al*., 2004) and soybean (Santos *et al*., 2016). When Fe was present in sufficient amounts, *M. truncatula* had almost null *FRO2*-like expression (Figure 5); since from the beginning of the trial, plants were in optimal conditions, they captured sufficient Fe to meet their daily requirements, thus inhibiting *FRO2*-like expression in order to avoid Fe toxicity. However, the Fe reductase activity was higher under Fe+ conditions (like in *G. max*). The Fe reduction is thought to be the rate-limiting step for Fe transport since Fe transporters, such as *IRT1*, do not reach saturation at normally achieved concentrations of Fe^{2+} (Grusak *et al*., 1990). If there is no Fe being reduced, *IRT1*-like should consequently present lower activity, which was clearly observed in *G. max* Fe- roots (Figure 4). In both species, the levels of *IRT1*-like expression were very similar to those obtained for *FRO2*-like (Figures 4 and 5), suggesting that *IRT1*-like is co-regulated with this gene, as previously seen in *Arabidopsis thaliana* (Vert, 2002; Kim and Guerinot, 2007).

After Fe is transported into the roots by IRT1, the transport of this nutrient across the plant is another crucial step that needs to be well known to efficiently develop an IDC mitigation or a biofortification strategy. Fe transporter families, such as VIT, NRAMP and YSL, are extremely important in Fe metabolism, as they assure that Fe is efficiently delivered to shoots, and other plant edible parts and storage organs. *NRAMP3* and *VIT1* have contrasting functions: while the first is responsible for the remobilization from the vacuole (Lanquar *et al*., 2005), the second is responsible for the Fe loading in the vacuole (Kim *et al*., 2006). Studies in *A. thaliana* demonstrate that *NRAMP3* is an H^+ metal symporter responsible for Fe and Mn remobilization from the vacuole, a crucial step during early seedling development (Lanquar *et al*., 2010). Accordingly, under Fe deficiency, as plants need more remobilization of Fe to respond to their needs, *NRAMP3*-like was more expressed (Figures 4 and 5) and *VIT1*-like was repressed, because plants activate *VIT1*-like in Fe sufficient conditions to increase Fe^{2+} uptake into the vacuole for storage

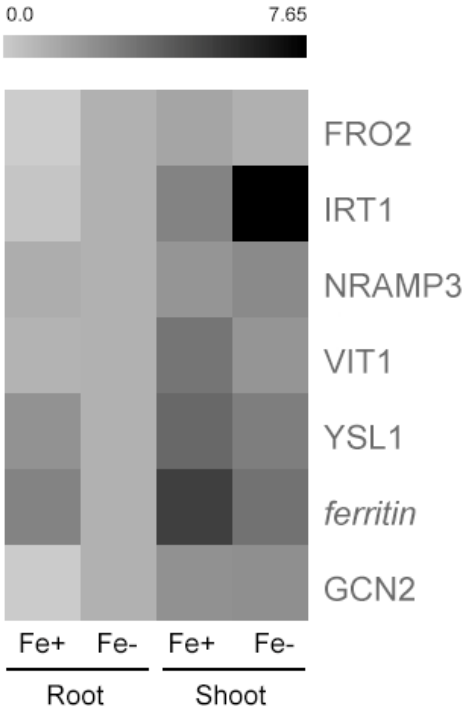

Figure 5 - HeatMap of the expression patterns of FRO2-, IRT1-, NRAMP3-, VIT1- and YSL1-like genes and ferritin and GCN2-like genes in root and shoot tissues of M. truncatula plants grown hydroponically in Fe-sufficient (Fe+) and Fe-deficient (Fe-) conditions. "Fe- Root" was the reference sample; expression was normalized with 18S rRNA housekeeping gene. In light grey: lower gene expression; in black: increased gene expression. Total RNA was extracted from a pool of five independent replicates. Corresponding values are presented in Table 4.

Table 4 - Fe deficiency-related genes relative expression values of M. truncatula plants grown under Fe-sufficient (Fe+) and Fe-deficient (Fe-) hydroponic conditions. Total RNA was extracted from a pool of five independent replicates

	Root		Shoot	
	Fe+	Fe-	Fe+	Fe-
FRO2-like	0.01	1	1.45	1.05
IRT1-like	0.25	1	2.75	7.65
NRAMP3-like	1.15	1	2.10	2.46
VIT1-like	0.94	1	3.28	2.10
YSL1-like	2.17	1	3.77	2.91
ferritin	2.73	1	5.25	3.37
GCN2-like	0.05	1	2.19	2.31

purposes (Brear *et al*., 2013). Studies in *A. thaliana* (Kim and Guerinot, 2007) demonstrated that *AtNRAMP3* and *AtVIT1* mutants present arrested seedling growth when grown on Fe deficient soils. Moreover, Zhang *et al*. (2012) reported that the disruption of the rice *VIT* orthologues (*OsVIT1* and *OsVIT2*) increased Fe and Zn accumulation in rice seeds and decreased Fe and Zn in the leaves.

As well as *NRAMP3*-like and *VIT1*-like, the *YSL1*-like transporter may also play a crucial role in the control of the amount of Fe translocated to the seeds of *G. max* and *M. truncatula*. Both species had similar expression patterns (Figures 4 and 5), where both tissues presented higher levels in Fe+ conditions, suggesting a role in Fe translocation at diverse plant organs, as seen before (Kim *et al*., 2006). This gene is involved in the transport of the Fe^{2+}-NA complexes (Kim *et al*., 2006) that are hypothesized as the main transportable Fe form in the phloem (Jean *et al*., 2005; Waters *et al*., 2006; Chu *et al*., 2010). Jean et al. (2005) used *A. thaliana* lines with a knock out mutation in *AtYSL1*, and the levels of NA and Fe in leaves and seeds decreased, as well as germination rates, even when plants were grown in Fe excess, showing that Fe and NA levels in seeds rely in part on *YSL1* function.

Storage proteins such as ferritin play an important role in Fe homeostasis, assuring that Fe in excess is in a bio-available way in case of cellular needs but yet nonreactive with oxygen (Briat *et al*., 2010). Thus, the higher expression levels of this gene in Fe sufficient soybean and barrel medic plants are understandable (Figures 4 and 5) and are coherent with previous studies (Santos *et al*., 2016). This protein manages the insolubility and potential toxicity of Fe in the presence of oxygen, being involved in oxidative protection by sequestering free Fe (Lobreaux *et al*., 1995).

Even though several gene families are known to be involved in the Fe uptake mechanism, transport and storage, there are still many undiscovered genes that may have important roles in these processes. Therefore, it is worthwhile to find candidate genes that could have an important role in Fe metabolism. To this end, a novel gene was studied in the current

work: *GCN2*-like. Both *G. max* and *M. truncatula* plants over-expressed *GCN2*-like under Fe deficient conditions (Figures 4 and 5), particularly at the root level, and it seems to indicate a role for *GCN2*-like in alleviating Fe stress, for both legume species. Lageix *et al*. (2008) showed that *AtGCN2* was strongly activated following wounding and exposure to key hormones, and suggested that this enzyme plays a role in plant defense responses to insect pathogens, representing a key player linking biotic and abiotic stresses. Moreover, no studies have looked at the possible role of *GCN2* and Fe nutrition, which highlights the importance of the current work. Further studies to link its role on Fe metabolism are under way.

The current work compared the responses of two legume species, soybean and barrel medic, to Fe deficiency. Taken together, the results described above suggest a conservation of anatomical and biochemical responses in the two legume species. Also, it is apparent that for genes such as *FRO2*-like and *IRT1*-like the regulation differs between these two legumes and is not conserved with other plants such as *A. thaliana*. It shows that generalizations in Fe uptake processes should not be lightly done. Finally, a novel sequence showing up-regulation under Fe deficiency was identified, opening doors to future studies looking at the role of this gene under Fe deficiency.

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