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Transcriptomic analysis of iron deficiency related genes in the legumes

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ABSTRACT

Among the mineral elements required by humans, iron (Fe) is the most common cause of nutritional deficiencies, particularly anaemia. Legume plants are extremely important in the world's diet and they are major sources of mineral nutrients. However, when these plant foods are grown in calcareous soil, their production is severely affected by Fe deficiency chlorosis (IDC), and when less Fe is available for absorption, less amount of this element will be available for accumulation in the edible plant parts. As Fe plays critical roles in photosynthesis and respiration, when lacking this element, plants develop chlorosis and their growth is drastically reduced. IDC morphological symptoms were monitored in soybean (*Glycine max*), common bean (*Phaseolus vulgaris*) and the model crop barrel medic (*Medicago truncatula*). When compared to the other two legumes, *G. max* presented lower Fe-reduction rates and severe chlorosis, associated with lower SPAD values. Transcriptome analysis was performed in roots of the three species when grown in Fe deficiency and Fe sufficiency, and 114,723 annotated genes were obtained for all samples. Four IDC-related genes were up-regulated in common by the three species and can be considered key players involved in the IDC response, namely, metal ligands, transferases, zinc ion binding and metal ion binding genes. With regards to the genes most highly expressed under iron deficiency individually by each species, we found that the most highly expressed genes were a defensin in *P. vulgaris*, a phosphatase in *M. truncatula* and a zinc ion binding gene in *G. max*.

1. Introduction

Out of the world's 6 billion people, one third of them suffer from mineral deficiencies. Since most of the world's population does not ingest enough Fe to meet daily dietary requirements, Fe deficiency is one of the most common nutritional deficiencies and the leading cause of anaemia (Zimmermann & Hurrell, 2002). Among the main risk groups are pregnant women and women of childbearing age (Krafft, Murray-Kolb, & Milman, 2012), as well as children, both infants and teenagers (Toutain, Le Gall, & Gandemer, 2012). This constitutes a serious problem, since anaemia can cause poor pregnancy outcome and children morbidity, as well as diminished work productivity in adults (WHO, 2001).

Grain legumes are cultivated primarily for their seeds which are rich in starch and dietary protein. Legumes have an important socio-economical role in the Mediterranean diet. Their benefits on human health are diverse, ranging from their high protein content, to high concentration in micronutrients, such as Fe and zinc (Vasconcelos & Grusak, 2006). However, the low bioavailability of Fe in alkaline soils, where this nutrient is often insoluble, together with the cultivation of susceptible genotypes causes drastic economic damage due to the reduced crop viability (Zamboni et al., 2012). When Fe lacks in plant metabolism, several processes are affected, like photosynthesis, respiration, nitrogen fixation, DNA synthesis, hormone production, chlorophyll formation (Vasconcelos & Grusak, 2006), among others. This generally leads to the development of Fe deficiency chlorosis (IDC), characterized by the yellowing of the upper leaves, interveinal chlorosis and stunted growth, with the plant's yield severely affected (Prasad, 2003). To cope with this, non-grass plants use a two-step mechanism for Fe uptake: firstly, Fe(III) is reduced to Fe(II) by a plasma membrane-bound ferric reductase, and the latter is subsequently released from the chelate and then transported into the cytoplasm via a transport protein (Jeong & Connolly, 2009). 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 re-mobilized to the grains, which would help in biofortification efforts that aim at enhancing Fe seed levels. However, the increased Fe translocation from shoots to seeds still remains one of the major bottlenecks in most biofortification programs (White & Broadley, 2005).

Common bean (*Phaseolus vulgaris*) and soybean (*Glycine max*) are rich in protein, which makes them valuable crops for worldwide consumption, and they are both susceptible to IDC. Specifically, *P. vulgaris* total production exceeds 23 million metric tonnes (MT) and consists a major staple of eastern and southern Africa, as well as of Latin America (Broughton et al., 2003). Moreover, *G. max* is a good crop to study IDC molecular mechanisms since, in 2010, its genome was sequenced, assembled and published (Schmutz et al., 2010). This species had a production yield of 2567 kg/ha in 2002 in Brazil

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and, besides contributing for vegetable oil production, they are also rich sources of dietary protein for chicken and pork industries (Graham & Vance, 2003). On the other hand, barrel medic (*M. truncatula*) represents a good model for Strategy I plants, since when this plant is challenged with Fe shortage, the most important root physiological responses induced by Fe deficiency are developed, including the yellowing of root tips (Andaluz, Rodríguez-Celma, Abadía, Abadía, & López-Millán, 2009).

Different sequencing technologies have given us some insight regarding the legume plants response to Fe deficiency. A few examples include: 1) genome-wide transcriptional analysis in tomato roots, that identified genes potentially involved in Fe starvation and root response to nutrient deficiency (Zamboni et al., 2012); 2) microRNA (miRNA) survey of genes related to Fe deficiency in *Arabidopsis*, where 24 miRNA genes were found to contain Fe deficiency responsive cis-Element 1 and 2 in their promoter regions (Kong & Yang, 2010); 3) Solexa sequencing, a high throughput sequencing technology that allowed to isolate 1,563,959 distinct *M. truncatula* sequences and to predict target genes for novel miRNAs (Szittya et al., 2008); 4) high-throughput sequencing analysis of miRNA associated with stress response in *G. max*, from which 133 expressed conserved miRNAs were identified, putatively inducible in response to certain stresses like alkalinity (Li et al., 2011).

In this study, specific genes associated with plant mineral metabolism were identified by high throughput sequencing (Illumina Hiseq 2000). Root samples of *Glycine max*, *Phaseolus vulgaris* and *Medicago truncatula* grown hydroponically under Fe-sufficiency and Fe-deficiency were analyzed to further our knowledge on legume nutrition and abiotic stress. Identification of these genes can help us understand the common and individual regulatory mechanisms of iron uptake in the legumes and assist in plant biofortification programs.

2. Materials and methods

2.1. Plant growth conditions

All plants (*Medicago truncatula* cultivar Luzerna revilheira, *Glycine max* cultivar Williams 82 and *Phaseolus vulgaris* ecotype PMB-0121 [Rodiño, Monteagudo, Santalla, & De Ron, 2001]) were grown in an Aralab Fitoclima 10000EHF with 16 h day/8 h night photoperiod. The temperature was kept at 20 °C during the light period, with 70% of relative humidity and 350 μmol s^{-1} m^{-2} of photon flux density, and at 18 °C during the dark period, with 80% of relative humidity.

Scarified seeds of *M. truncatula* were germinated in 1.2% Agar inside the chamber, and seeds of *G. max* and *P. vulgaris* were rolled in filter paper and placed vertically in a solution of 250 mM $CaCl_2$, for 7 days in the dark. The 7 day old seedlings were transferred to hydroponic solution with different Fe treatments.

The standard solution for hydroponical growth of *M. truncatula* contained: 3 mM KNO_3; 1 mM $Ca(NO_3)_2$; 0.5 mM $MgSO_4 \cdot 7H_2O$; 0.5 mM $NH_4H_2PO_4$; 0.75 mM K_2SO_4; 25 μM $CaCl_2$; 25 μM H_3BO_3; 2 μM $MnSO_4$; 2 μM $ZnSO_4 \cdot H_2O$; 0.5 μM $CuSO_4.H_2O$; 0.5 μM MoO_3; 0.5 μM $NiSO_4$. The conditions used for *G. max* and *P. vulgaris* included: 1.2 mM KNO_3; 0.8 mM $Ca(NO_3)_2$; 0.3 mM $MgSO_4 \cdot 7H_2O$; 0.2 mM $NH_4H_2PO_4$; 25 μM $CaCl_2$; 25 μM H_3BO_3; 0.5 μM $MnSO_4$; 2 μM $ZnSO_4 \cdot H_2O$; 0.5 μM $CuSO_4.H_2O$; 0.5 μM MoO_3; 0.1 μM $NiSO_4$. All hydroponic solutions were buffered with the addition of 1 mM MES, pH 5.5.

Five plants of each species were maintained for 14 days in Fe sufficient (10 μM Fe(III)-EDDHA [ethylenediamine-N,N′bis(o-hydroxyphenyl) acetic acid]) and Fe deficient (0 μM Fe(III)-EDDHA) conditions. During the time of the experiment, pH and conductivity were measured daily and solutions were changed every 2 days.

Soil and Plant Analyzer Development (SPAD) readings were taken on the last day of the assay with a chlorophyll meter (Konica Minolta SPAD-502Plus; Minolta, Osaka, Japan) from at least four random trifoliate leaves.

2.2. Fe reductase localization in roots

As previously performed by Vasconcelos et al. (2006) a gel composed by nutrient solution (6 mM KNO_3; 4 mM $Ca(NO_3)_2$; 1.5 mM $NH_4H_2PO_4$; 1 mM $MgSO_4$; 25 μM $CaCl_2$; 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 $NiSO_4$), 100 mM agarose (SeaPlaque, Duchefa Biochemie, The Netherlands), 100 mM MES Buffer, 100 μM Fe(III)-EDTA and 100 μM BPDS (bathophenanthroline disulfonic acid) was prepared. Intact roots were carefully laid in the mixture and left for 45 min in the dark, before visualization of a pink coloration around the roots was observed, indicating Fe(II)-$BPDS_3$ formation. These assays were performed using 2-week-old plants grown hydroponically as described above.

2.3. Root Fe reductase measurements

Reduction was measured in intact roots via the spectrophotometric measurement of Fe^{2+} chelated to BPDS. To measure Fe reduction, roots were submerged in assay solution containing: 1.5 mM KNO_3, 1 mM $Ca(NO_3)_2$, 3.75 mM $NH_4H_2PO_4$, 0.25 mM $MgSO_4$, 25 μM $CaCl_2$, 25 μM H_3BO_3, 2 μM $MnSO_4$, 2 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.5 μM H_2MoO_4, 0.1 μM $NiSO_4$, 100 μM Fe(III)-EDTA and 100 μM BPDS. All nutrients were buffered 1 mM MES, pH 5.5. The assays were conducted under low light conditions at 20–22 °C and were terminated after 45 min by removal of the roots. Absorbance values were obtained spectrophotometrically at 535 nm, and an aliquot of free-roots solution was used as blank. Rates of reduction were determined using the molar extinction coefficient of 22.14 mM^{-1} cm^{-1}.

2.4. Statistical analysis

Student's *T*-test corrected for multiple comparisons using the Holm–Sidak method was used to analyse statistical significant differences between samples (Prism 6 — GraphPad Software, Inc).

2.5. RNA extraction

The roots of the five plants of each treatment were polled together and grounded with liquid nitrogen, until a fine powder was obtained. To extract the RNA, Qiagen RNeasy Plant Mini Kit (USA, #74904) was used. Possible DNA contamination was removed using the Turbo DNA-free kit (Ambion, Austin, TX, USA), according to manufacturer's instructions. RNA quality and quantity were checked with UV-spectrophotometry, using a nanophotometer (Implen, Isaza, Portugal).

The RNA was sent for transcriptome analysis with high throughput sequencing (Illumina Hiseq 2000, Fasteris, Switzerland).

2.6. Bioinformatic analysis

After Illumina sequencing, high-quality small RNA reads were extracted from raw reads through filtering out the low quality tags and eliminating contamination of adaptor sequences. Each sample

Table 1
Forward and reverse primer sequences used in quantitative real time PCR analyses.

Gene	5′-3′ Forward primer	5′-3′ Reverse primer
18S-rRNA	TTAGGCCATGGAGGTTTGAG	GAGTTGATGACACGCGCTTA
Metal ion binding	ACTAACGGTGACGGGAGAGA	GACATCTGGTGGCTTCGTTT
Glucan 1, 3-β-glucosidase	TACGCCGCTCTTGAAAAAGT	CAATTGCTCCGGGTCTCTTA
Phosphotransferase	GCAAGCACGTTCACAGAAAA	TCTGCTGCAACGACCTAATG
UGT	CAACACCACCAGATCATTGC	TTCCCAAACTCCAGGTCTTG