Title
Ferric Reductase Transcription and Activity in Pisum sativum Accessions

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Introduction

Under conditions of low iron (Fe) availability, dicots and non-graminaceous monocot plants induce a three part Fe acquisition response to mobilize Fe within the rhizosphere and increase transport to roots. This process, commonly referred to as Strategy I, includes activation of proton pumps to acidify the rhizosphere, reduction of ferric iron to the ferrous form through a plasma membrane bound ferric reductase, and the uptake of ferrous iron by transporters localized to the plasma membrane of the root epidermis (Marschner and Römheld, 1994). Physiological studies completed in pea (*Pisum sativum* L.) suggest that iron reduction from the ferric to ferrous form is the rate-limiting physiological process in Fe acquisition (Grusak et al, 1990). The pea mutants bronze (*brz*) and degenerative leaflet (*dgl*) have been used by a number of studies to better understand the regulation of ferric reductase activity in plants (Grusak et al, 1990; Kneen et al, 1990; Grusak, 1994; Grusak and Pezeshgi, 1996; Waters et al, 2002; Marentes and Grusak, 1998). These pea mutants display a constitutive iron deficient root phenotype (high levels of ferric reductase activity, increased Fe transport into root tissue) even under iron replete growth conditions (when these processes are normally downregulated) leading to excessive iron accumulation in shoot tissue (Kneen et al, 1990; Grusak, 1994; Marentes and Grusak, 1998). Grafting experiments conducted with these mutant lines demonstrate constitutive expression of *PsFRO1*, the ferric reductase in roots of pea plants, suggesting that shoot based signals play a role in the regulation of Fe uptake in roots (Waters et al, 2002). Molecular and physiological studies in *Arabidopsis thaliana* have also demonstrated the importance of shoot-based signals in regulating ferric reductase activity (Vert et al, 2003; Connolly et al, 2003), but the specific shoot signal and the mechanisms leading to reductase activity regulation have not yet been determined.

We are interested in further dissecting the regulation of the ferric reductase in *P. sativum* and are examining the levels of reductase transcription and rates of enzyme activity across a range of pea accessions. We have selected twenty-nine different pea accessions from diverse geographic locales, and have included both commercial varieties and wild collected lines in order to incorporate a range of physiological and genetic backgrounds. To better understand the regulation of ferric reductase activity, these accessions have been evaluated for *PsFRO1* transcription and ferric reductase activity when grown at low (0.5 μM) or high (15 μM) Fe hydroponic concentrations. The difference in transcript levels and reductase activity within each line following low or high Fe treatment is described, as is the correlation between relative differences in transcript abundance and activity.

Methods and Materials

Twenty-nine different pea accessions were obtained from the USDA-ARS Pisum Germplasm Collection (Pullman, Washington, USA). Seeds were germinated on filter paper with dH_2O. On day 4, plants were transferred to tubs and grown hydroponically in modified Johnson's solution under low or high Fe conditions (0.5 or 15μM Fe(III)-EDDHA). Plants were harvested at day 14 for root Fe reductase activity assays or analysis of *PsFRO1* transcript abundance via real time quantitative RT-PCR.

The reductase activity assay was run with whole intact root systems; roots were rinsed, submerged in a bathophenanthroline disulfonic acid (BPDS) assay solution [0.2 mM $CaSO_4$, 5 mM MES at pH 5.5, 0.1 mM Fe(III)-EDTA, and 0.2 mM BPDS]. After 20 min, an aliquot of the assay solution was removed and A_{535} was determined. Fe(II)-$BPDS_3$ concentration was calculated using the molar extinction coefficient: 22.14 mm^{-1} cm^{-1}.

Real time RT-PCR was run as two-step RT-PCR procedure. The RT reaction was run with High Capacity RNA-to cDNA Master Mix and the PCR was run with SYBR® Green PCR Master Mix (both from Applied Biosystems, Foster City, California, USA). *PsFRO1* primers were chosen from a highly conserved region and transcription was analyzed as relative quantification following comparative cycle time (ΔCt) analysis with *18S* as the reference sequence.

Brief Results

This study is part of a larger effort to understand how ferric reductase activity is regulated at the transcriptional and post-transcriptional levels. To that end, an initial survey of ferric reductase transcription levels and ferric reductase activity was conducted across a range of geographically diverse pea plants grown under low and high Fe media to identify variation in iron acquisition responsiveness.

The range of relative *PsFRO1* transcription levels, (listed as ΔCt values; Figure 1) and reductase activity (Figure 2) is presented for the twenty-nine pea accessions maintained on low or high Fe treatment. *PsFRO1* transcript levels were below detectable levels in accession 29 when grown at 15 μM Fe. Both relative transcript abundance and root-weight normalized reductase activity varied dramatically across the accessions. For low Fe grown accessions, the measured *PsFRO1* transcript numbers varied by 9 PCR cycles (ΔCt), equivalent to a 512-fold (2^9) range in transcript amounts. Reductase activity for the same accessions grown under low Fe conditions varied only 10-fold. For high Fe grown accessions, the measured *PsFRO1* transcript numbers varied by 7.5 PCR cycles (ΔCt), equivalent to a 180-fold ($2^{7.5}$) range in transcript numbers. Reductase activity for the same plants varied 4.4-fold in activity level. From these results, it appears that reductase activity and *PsFRO1* transcript levels vary significantly even in iron replete (15 μM) growth conditions across the pea accessions examined in this study (Figure 1, 2). To determine whether transcript and activity differences seen between low and high Fe grown plants vary equally across the accessions, the relative change in transcript and activity values for each accession were determined. The correlation between changes in relative transcript levels ($2^{-\Delta\Delta Ct}$) and differential reductase activities between low- and high-Fe grown plants are illustrated in Figure 3. Our results suggest that across the twenty-nine pea accessions studied, there is no tendency for a change in transcript abundance to accurately predict the change in reductase activity.

Implications

This study demonstrates that across a range of naturally occurring pea accessions, *PsFRO1* transcription and reductase activity varies significantly. While greater differences are seen in transcription level across the accessions studied, these differences do not strictly correlate with reductase activity.

The range of reductase activity and ferric reductase transcription seen across these lines combined with the lack of correlation suggests a degree of variation in how pea accessions regulate ferric reductase activity. Further study of those pea accessions with altered regulation between transcription and activity may provide additional information of interest to identify the factors regulating ferric reductase in Strategy I plants. We are most interested in identifying those lines that show large changes in transcriptional levels but lack large differences in reductase activity and lines that show minimal changes in transcriptional levels between low and high Fe growth conditions but have very large changes in reductase activity. These two subsets

of pea accessions will be further examined for differences in reductase transcripts, promoter regions and other factors that may explain the disconnect between *PsFRO1* transcription and ferric reductase activity.

Acknowledgements

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Figure 1. Relative *PsFRO1* transcript levels in two week old pea accessions grown under low [0.5 µM Fe(III)EDDHA] and high [15 µM Fe(III)-EDDHA] Fe media. Values presented are cycle time numbers, relative to those of *18S* mRNA.

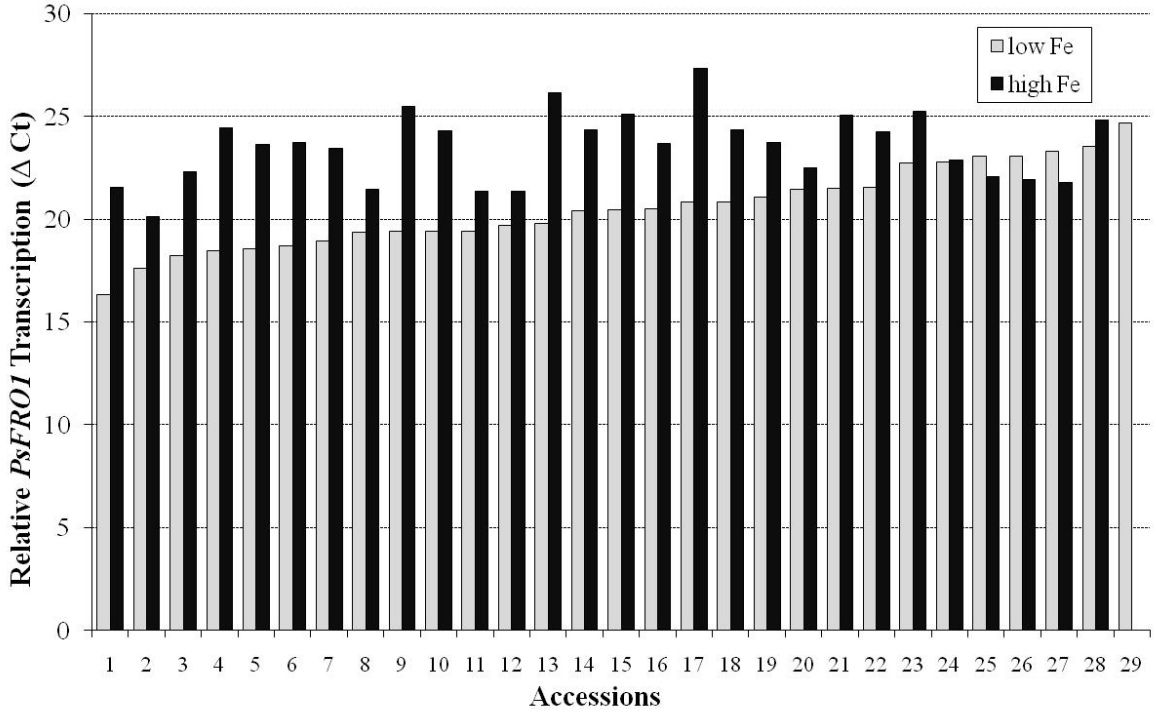

Figure 2. Ferric Reductase activity in two week old pea accessions grown under low [0.5 µM Fe(III)EDDHA] and high [15 µM Fe(III)EDDHA] Fe media. Reductase activities were measured with 100 µM Fe(III)EDTA.

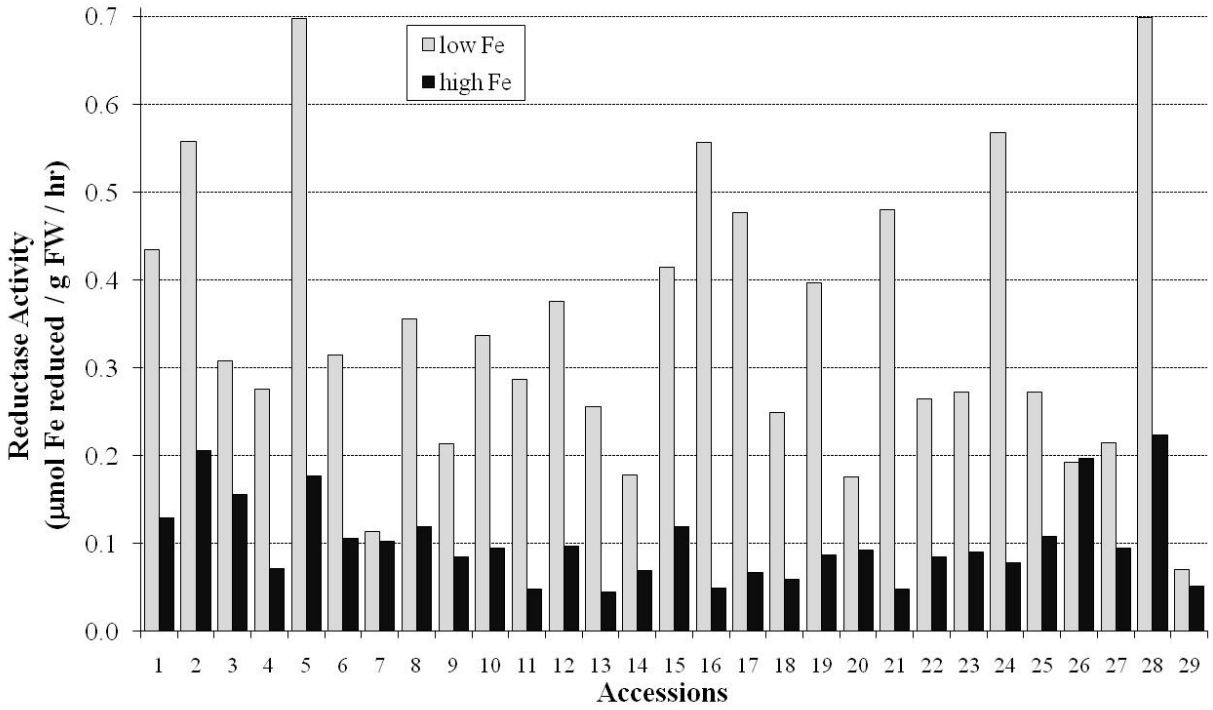

Figure 3. The relative difference of *PsFRO1* transcript abundance in low [0.5 µM Fe(III)EDDHA] vs. high [15 µM Fe(III)EDDHA] Fe grown pea accessions compared to relative changes in ferric reductase activity under the same conditions.