

RESEARCH PAPER

Use of natural variation reveals core genes in the transcriptome of iron-deficient *Arabidopsis thaliana* roots

Ricardo J. Stein¹ and Brian M. Waters*

Department of Agronomy and Horticulture, University of Nebraska-Lincoln, Lincoln, NE 68583-0915, USA

¹ Present Address: Department of Plant Physiology, Fakultät für Biologie und Biotechnologie - Ruhr Universität Bochum, Germany

* To whom correspondence should be addressed. E-mail: bwaters2@unl.edu

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Abstract

Iron (Fe) is an essential mineral micronutrient for plants and animals. Plants respond to Fe deficiency by increasing root uptake capacity. Identification of gene networks for Fe uptake and homeostasis could result in improved crop growth and nutritional value. Previous studies have used microarrays to identify a large number of genes regulated by Fe deficiency in roots of three *Arabidopsis* ecotypes. However, a large proportion of these genes may be involved in secondary or genotype-influenced responses rather than in a universal role in Fe uptake or homeostasis. Here we show that a small percentage of the Fe deficiency transcriptome of two contrasting ecotypes, Kas-1 and Tsu-1, was shared with other ecotypes. Kas-1 and Tsu-1 had different timing and magnitude of ferric reductase activity upon Fe withdrawal, and different categories of overrepresented Fe-regulated genes. To gain insights into universal responses of *Arabidopsis* to Fe deficiency, the Kas-1 and Tsu-1 transcriptomes were compared with those of Col-0, Ler, and C24. In early Fe deficiency (24–48 h), no Fe-downregulated genes and only 10 upregulated genes were found in all ecotypes, and only 20 Fe-downregulated and 58 upregulated genes were found in at least three of the five ecotypes. Supernode gene networks were constructed to visualize conserved Fe homeostasis responses. Contrasting gene expression highlighted different responses to Fe deficiency between ecotypes. This study demonstrates the use of natural variation to identify central Fe-deficiency-regulated genes in plants, and identified genes with potential new roles in signalling during Fe deficiency.

Key words: *Arabidopsis*, iron deficiency, microarray, natural variation, transcriptional profiling.

Introduction

Iron (Fe) is an essential micronutrient for plants and most other forms of life. Iron is utilized in a wide variety of proteins that are involved in oxidation/reduction biochemical reactions, such as cytochrome- and iron-sulphur cluster-containing proteins of the electron transport chains of photosynthesis and respiration, and in enzymes involved in ameliorating oxidative stress, such as certain peroxidases, catalases, and superoxide dismutases (Hansch and Mendel, 2009). Excess Fe in cells or unbound Fe can stimulate the production of free radicals that are sources of oxidative stress damage (Halliwell and Gutteridge, 1992). Thus, the uptake, distribution, and storage of Fe, for example in

ferritin proteins (Briat *et al.*, 2010), must be tightly regulated (Walker and Connolly, 2008; Giehl *et al.*, 2009).

Iron deficiency in dicotyledons results in increased capacity for roots to take up Fe by physiological responses including increased Fe(III) reduction, Fe(II) transport, and rhizosphere acidification. In *Arabidopsis*, these responses result from Fe-deficiency-regulated gene expression of the ferric reductase *FRO2* (Robinson *et al.*, 1999), the iron transporter *IRT1* (Henriques *et al.*, 2002; Varotto *et al.*, 2002; Vert *et al.*, 2002), and the H⁺-ATPase *AHA2* (Santi and Schmidt, 2009). The transcription factor *FIT* is a key regulator of Fe-deficiency responses (Colangelo and

Abbreviations: ABRC, Arabidopsis Biological Resource Center; SNP, single-nucleotide polymorphism; EDDHA, ethylenediamine-*N,N'*-bis(2-hydroxyphenylacetic acid); MES, 2-(*N*-morpholino)ethanesulphonic acid; RT, reverse transcriptase; C_t, cycle threshold; GO, Gene Ontology; MIPS, Munich Information Center for Protein Sequences; bHLH, basic helix-loop-helix.

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Guerinot, 2004; Jakoby *et al.*, 2004; Yuan *et al.*, 2005), and is essential for FRO2 and IRT1 activity. To help understand additional genes that are involved in Fe-deficiency responses, several studies have used the Affymetrix ATH1 microarray (Buckhout *et al.*, 2009; Colangelo and Guerinot, 2004; Dinnyen *et al.*, 2008; Garcia *et al.*, 2010; Long *et al.*, 2010; Yang *et al.*, 2010) or previous versions of microarrays (Thimm *et al.*, 2001; Wintz *et al.*, 2003) to identify genes with altered transcript levels in Fe-deficient *Arabidopsis* roots. A proteomic study (Lan *et al.*, 2010) has also identified Fe-regulated proteins in *Arabidopsis* roots. However, in these screens, typically hundreds to thousands of genes have increased or decreased expression, and many of these genes may not be involved directly in Fe uptake or homeostasis. Growth patterns or metabolic pathways may be altered by Fe limitation (Thimm *et al.*, 2001; Lan *et al.*, 2010), and it is possible that much of the Fe regulon may be involved in these processes. An improved method of filtering genes involved in early Fe-deficiency responses would be useful to help focus future research.

A large number of ecotypes of *Arabidopsis thaliana* have been collected from natural habitats in different regions of the world. These ecotypes represent homozygous inbred lines that are adapted to specific environments (Mitchell-Olds and Schmitt, 2006). Naturally occurring polymorphisms in DNA, such as single-nucleotide polymorphisms (SNPs), insertions, and deletions, are a source of genetic variability and can result in different alleles of genes or differences in promoter activity (Wray *et al.*, 2003) that can give rise to differences in growth and responses to the environment between ecotypes (Alonso-Blanco *et al.*, 2009). Research based on genotype \times environment differences resulting from natural genetic variation among *Arabidopsis* ecotypes has led to identification of genes for uptake or accumulation of several minerals, such as sodium (Rus *et al.*, 2006), molybdenum (Baxter *et al.*, 2008a), and cobalt (Morrissey *et al.*, 2009), and quantitative trait loci related to traits such as seed mineral concentration (Vreugdenhil *et al.*, 2004; Waters and Grusak, 2008a; Ghandilyan *et al.*, 2009), nitrogen use efficiency (Loudet *et al.*, 2003), and water use efficiency (McKay *et al.*, 2008). In bean (*Phaseolus vulgaris*), natural variation for root ferric reductase activity was the basis for mapping quantitative trait loci associated with this trait (Blair *et al.*, 2010).

For analysis of the Fe regulon, one approach to filter the number of genes that are involved specifically in early Fe-deficiency responses may be to compare the Fe-regulated transcriptomes of different ecotypes (Schmidt and Buckhout, 2011). Most *Arabidopsis* resources have been developed for the ecotype Columbia-0 (Col-0), and the majority of microarray studies in *Arabidopsis* have utilized this ecotype (Colangelo and Guerinot, 2004; Dinnyen *et al.*, 2008; Garcia *et al.*, 2010; Long *et al.*, 2010; Yang *et al.*, 2010). Two other ecotypes have been studied for Fe-deficiency-regulated gene expression thus far: Landberg *erecta* (Ler) (Buckhout *et al.*, 2009), and C24 (Yang *et al.*, 2010). Col-0 and Ler originated from regions with similar temperate climates in the central USA and Germany, whereas C24 originated from the

somewhat warmer climate of Coimbra, Portugal. Although fold change of expression under Fe deficiency differed for many genes between Col-0 and C24, there were only six genes that were Fe-regulated in one ecotype but not the other (Yang *et al.*, 2010).

In this work we compare root Fe-regulated transcriptomes of two divergent ecotypes, Kashmir-1 (Kas-1) and Tsushima-1 (Tsu-1), with each other and those of Col-0, Ler, and C24. Kas-1 and Tsu-1 are relatively large ecotypes that evolved in greatly contrasting climate zones (McKay *et al.*, 2008). Kas-1 [Arabidopsis Biological Resource Center (ABRC), stock no. CS28376] was collected in the cold and dry high elevations of Kashmir, India (latitude 35 °N, longitude 77 °E; www.naturalvariation.org), whereas Tsu-1 (ABRC stock no. CS28781) was collected from a warm and humid region of Tsushima, Japan (34/35 °N, 136/137 °E; ABRC). These ecotypes have been genotyped with 149 genome-wide SNPs (www.naturalvariation.org/hapmap) and 48 microsatellites (McKay *et al.*, 2008). Phenotypic differences between Kas-1 and Tsu-1 have been demonstrated for carbon isotope discrimination (McKay *et al.*, 2003), drought adaptation (McKay *et al.*, 2008), gene expression under drought (Juenger *et al.*, 2010), flowering time (Werner *et al.*, 2005), and mineral accumulation (Buescher *et al.*, 2010), and Kas-1 was shown to be resistant to powdery mildew, in contrast to Col-0 (Wilson *et al.*, 2001). We show that Kas-1 and Tsu-1 respond to Fe deficiency on different timescales than each other or Col-0. We hypothesized that these differences in timing could be exploited to refine genes that are likely involved specifically in early Fe uptake and distribution responses while excluding genes that are differentially regulated under Fe deficiency but are not specifically related to Fe uptake. Here we describe a relatively small number of genes that respond to Fe deficiency in all or most ecotypes studied and discuss categories of genes that differ between ecotypes.

Materials and methods

Plant material and growth

Seeds of the three *A. thaliana* ecotypes used in this study, Col-0, Kas-1, and Tsu-1, were imbibed in 0.1% agar at 4 °C for 72 h. Seeds were planted onto rockwool loosely packed into 1.5 ml centrifuge tubes with the bottoms removed. The tubes were floated in foam rafts in containers of nutrient solution, composed of 0.8 mM KNO₃, 0.4 mM Ca(NO₃)₂, 0.3 mM NH₄H₂PO₄, 0.2 mM MgSO₄, 50 μM Fe(III)-ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid) (EDDHA), 25 μM CaCl₂, 25 μM H₃BO₃, 2 μM MnCl₂, 2 μM ZnSO₄, 0.5 μM CuSO₄, 0.5 μM Na₂MoO₄, and 1 mM 2-(N-morpholino)ethanesulphonic acid (MES) buffer (pH 5.5). Lighting was provided at a photoperiod of 16 h of 150 μmol m⁻² s⁻¹ 4100K fluorescent light (on at 06:00 and off at 22:00). After 10 days, seedlings and the tubes were transferred to holes in lids of containers containing 0.75 l of the same nutrient solution with constant aeration for an additional 14 days before plants were transferred to treatments. The +Fe solution contained 50 μM Fe(III)-EDDHA (Sprint 138; Becker-Underwood, Ames, IA, USA); Fe was omitted from the -Fe solution. All the experiments were initiated at 14:00, 8 h before the end of the photoperiod.

Root ferric reductase assays were performed for 30 min on individual roots, using 10 ml of an assay solution of 0.1 mM ferrozine (Sigma-Aldrich, St. Louis, MO, USA), 0.1 mM Fe(III)-ethylenediaminetetra-acetic acid, and 5 mM MES buffer (pH 5.5) (Fisher Scientific, Fair Lawn, NJ, USA). Reduced Fe was calculated using absorbance at 562 nm with the extinction coefficient of $28.6 \text{ mM}^{-1} \text{ cm}^{-1}$.

Real-time reverse transcriptase-PCR

Total RNA was extracted from roots using the Plant Total RNA kit (IBI Scientific, Peosta, IA, USA). RNA quality and concentration was determined by UV spectrophotometry. DNase-treated RNA (1 μg ; RNase-free DNase I, New England Biolabs, Ipswich, MA, USA) was used for cDNA synthesis, using the High Capacity cDNA Reverse Transcription kit (ABI, Foster City, CA, USA) with random hexamers at 2.5 μM final concentration. cDNA corresponding to 50 ng of total RNA was used in a 15 μl real-time PCR reaction performed in a MyIQ (Bio-Rad, Hercules, CA, USA) thermal cycler using SYBR GreenER qPCR SuperMix (Invitrogen Technology, Carlsbad, CA, USA) and 0.2 μM gene-specific primers (see below). The following standard thermal profile was used for all PCRs: 50 °C for 2 min, 95 °C for 8 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. At the end of each reaction, a dissociation curve was performed to ensure primer specificity. The C_t values for all genes were calculated using BioRad IQ5 System Software version 2.0 (BioRAD, Hercules, CA, USA) and normalized to the C_t value of ubiquitin (*UBQ10*) using the equation $Y = 2^{-\Delta C_t}$, where $\Delta C_t = C_{tUBQ10} - C_{t\text{target gene}}$. The PCR efficiency from the exponential phase (E) was calculated for each individual amplification plot using the equation $(1+E) = 10^{\text{slope}}$ calculated by the LinRegPCR program (Ramakers *et al.*, 2003). PCR efficiency ranged from 97 to 99.5%, with correlation coefficient (r^2) ranging from 0.98 to 1.0. The calculated PCR efficiency was then used to normalize each obtained C_t value.

The primers used were as follows: *AHA2* (At4g30190; 5'-TGACTGATCTTCGATCCTCTCA-3', 5'-GAGAATGTGCATGTGCCAAA-3'), *FIT* (At2g28160; 5'-GGAGAAGGTGTTGCTCCATC-3', 5'-TCCGGAGAAGGAGAGCTTAG-3'), *FRO2* (At1g01580; 5'-CCACATCTGCGTATCAAGTT-3', 5'-TCCCAAACAAGTACGACCA-3'), *NRAMP4* (At5g67330; 5'-AGCGGACACATCGGTCTTGC-3', 5'-CGGGA AAAACCCACCCCGT-3'), *OPT3* (At4g16370; 5'-CTCGATGCAGGGACCGCGTT-3', 5'-TTCCAGGAGCCGTGGGACAGG-3'), and *UBQ10* (At4g05320; 5'-TGGTGGTATGCAGATTTTCG-3', 5'-GGCTTTCAGTTATCAATGG-3'). To correct for SNPs and amplify the same amplicons, different reverse primers were used for Kas-1 and Tsu-1 for the following: *FRO3* (At1g23020; forward 5'-GATTC-TACTGGCTTCTCTTGG-3', reverse 5'-CTAATCCGGCCTTCAC-TAAC-3' for Kas-1, reverse 5'-TAACCCTAATCCCTTCACTAAC-3' for Tsu-1), *IRT1* (At4g19690; forward 5'-CGGTTGGACTTCTAAATGC-3', reverse 5'-CGATAATCGACATTCCACCG-3' for Kas-1, reverse 5'-CGATAATGGACATTCCACCG-3' for Tsu-1). All primer pairs generated amplicons ranging from 100 to 300 bp.

Microarrays and analysis

Total RNA was isolated from roots using the RNeasy Plant Kit (Qiagen, Chatsworth, CA, USA), and quality was assessed using the Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). The Affymetrix GeneChip Arabidopsis ATH1 Genome Array was used for microarray analysis, with three biological replicates for each treatment and time point (+Fe, -Fe 24 h, -Fe 48 h), and each ecotype (Kas-1 and Tsu-1), for a total of 18 arrays. DNase I (5 μg ; Qiagen)-treated RNA was used and all further procedures (hybridization, washing, staining, and scanning) were carried out at the Genomics Core Research Facility of the University of Nebraska according to manufacturer's instructions. For data analysis, the probe intensity files (.cel files) were imported into GeneSpringX software (Agilent), and the set of nine arrays from

each ecotype was normalized using the MAS5 algorithm, with only the entities present or marginal in at least three replicates. To identify differentially expressed genes, an analysis of variance (using $P \leq 0.05$) was performed on the filtered and normalized dataset, and genes that showed significant differences as up- or downregulated by at least 1.5-fold were considered to be differentially expressed. Averages from three biological replicates for each sample were used for analysis. The complete dataset is available as GEO Accession GSE29086 (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE29086).

Bioinformatics

Overrepresented functional categories of Fe-regulated genes were identified for Gene Ontology (GO) and Munich Information Center for Protein Sequences (MIPS) classification using BioMaps software on the Virtual Plant (Katari *et al.*, 2010) website. Sets of Fe-upregulated or -downregulated genes were used for multifactor comparison to generate five-way Venn diagrams using Sungear software on the Virtual Plant website. Supernode networks were constructed with Gene Networks software on the Virtual Plant website based on MIPS functional classification with settings specifying direct associations and primary enzymatic reactions. MapMan software (Thimm *et al.*, 2004) was used with fold-change values to categorize expression of Fe-regulated genes from this study and previous studies (Colangelo and Guerinot, 2004; Garcia *et al.*, 2010; Long *et al.*, 2010; Yang *et al.*, 2010) into metabolic functional bins. Output tables of these bins were combined and selected genes were graphically represented as heatmaps using the Matrix2png interface (www.bioinformatics.ubc.ca/matrix2png/index.html).

Results

Several *A. thaliana* ecotypes that are parents of recombinant inbred line populations (Col-0, Ler, Bur-0, Bay-0, Est-1, Sakhara, Cvi, Kondara, Kas-1, and Tsu-1) were tested for suitability of growth in our hydroponic system. Kas-1 and Tsu-1 were chosen for further study. Over a time course of Fe-deficiency-induced root ferric reductase activity, ecotypes Col-0, Kas-1, and Tsu-1 exhibited substantially different timing and magnitude of response (Fig. 1A–C). None of the ecotypes had increased root ferric reductase activity at 24 h. At 48 h of Fe deficiency, Kas-1 ferric reductase activity was increased by 10-fold, whereas Col-0 and Tsu-1 had increased activities of 2.4-fold and 2.9-fold, respectively. By 72 h, Kas-1 activity declined markedly, then recovered slightly at 96 h, and remained elevated throughout the remainder of the experiment. This decline and recovery pattern was observed in multiple replications of this experiment. In contrast, Col-0 reached peak activity at 72 h, with total ferric reductase activity 20% less than that of Kas-1 at 24 h. Fe-deficient Col-0 ferric reductase activity declined to levels equivalent to those of Fe-replete plants at 120–168 h (5–7 days). Tsu-1 reached its peak activity at 96 h, at a magnitude 27% less than that of Kas-1 on 24 h, and root ferric reductase activity remained elevated in Fe-deficient plants until 144 h. The pattern of expression of the *FRO2* gene was very similar to total root reductase activity in Kas-1 and Tsu-1 over the time course (Fig. 1D). *FRO2* gene expression was slightly but significantly upregulated in Kas-1 after 16 h Fe deficiency; however, the most

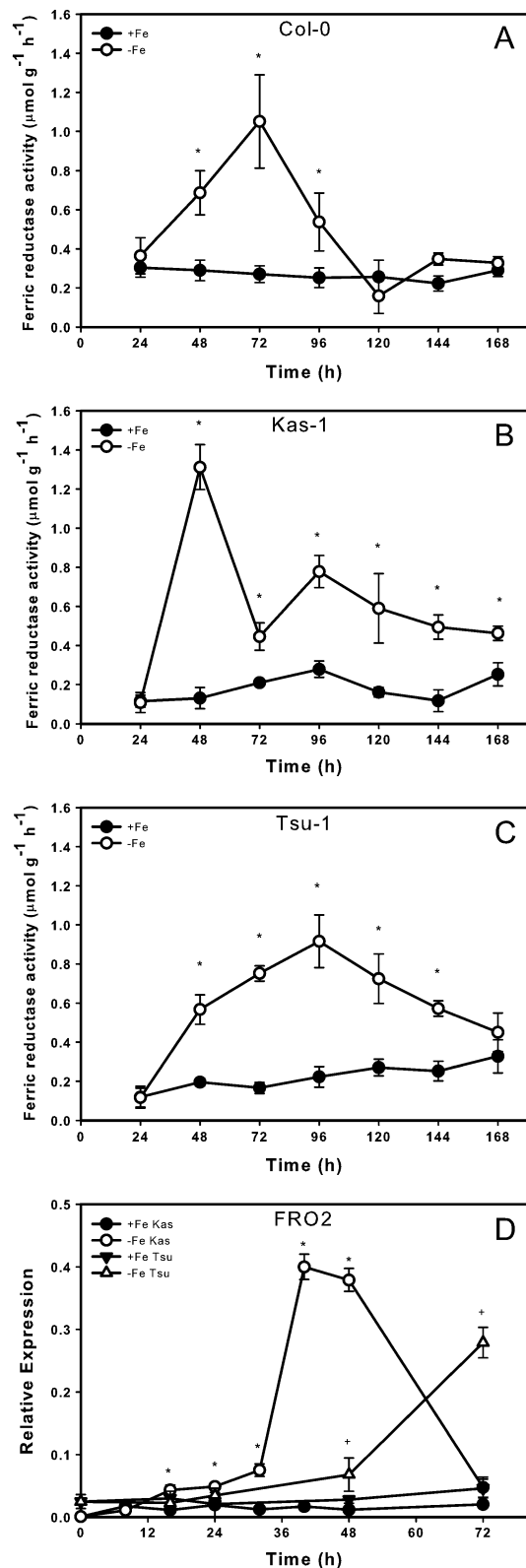


Fig. 1. Time course of root ferric chelate reductase activity and *FRO2* gene expression. Ferric reductase activity of (A) Col-0, (B) Kas-1, and (C) Tsu-1. Expression of *FRO2* (D) in Kas-1 and Tsu-1 roots over time. Values of a representative experiment, $n = 3 \pm \text{SD}$. * Denotes statistical significance for Kas-1, + denotes statistical significance for Tsu-1 ($P \leq 0.05$) between treatments at each time point. +Fe, 50 μM Fe, -Fe, no added Fe.

substantial increases were observed at 40 and 48 h, with a 20-fold induction, which coincided with the highest root ferric chelate reductase activity observed in this ecotype. The earliest significant upregulation of *FRO2* in Tsu-1 was at 48 h, and expression continued to increase afterwards, reflecting the pattern of whole-root ferric reductase activity.

Based on the differences in timing of ferric reductase activity and *FRO2* expression after transfer of plants to Fe-deficiency solution, we conducted microarray transcriptional profiling of Kas-1 and Tsu-1 at 24 and 48 h of -Fe treatment. A total of 1504 entities were Fe-regulated ≥ 1.5 -fold in Kas-1, and 630 were Fe-regulated ≥ 1.5 -fold in Tsu-1. A notable pattern of expression was that the majority of Tsu-1 genes were expressed at one but not both time points, exclusively at the earlier (41% of all downregulated, 51% of all upregulated) or later (45% of all downregulated, 30% of all upregulated) time points (Fig. 2), whereas in Kas-1 a greater portion of genes was differentially expressed at both time points (45% of all downregulated, 35% of all upregulated) or only at the later time point.

All genes that were upregulated or downregulated in Kas-1 or Tsu-1 (at either time point) were analysed for overrepresented GO and MIPS categories. Kas-1 Fe-deficiency-downregulated genes had 20 overrepresented GO categories and 18 overrepresented MIPS categories (Supplementary Table 1). Many of these GO and MIPS categories were for genes that respond to various chemical, biotic or abiotic stress, wounding, or certain plant hormones. There were also genes categorized as involved in amino acid, aromatic compound, and secondary metabolite metabolism. For Fe-deficiency-upregulated genes,

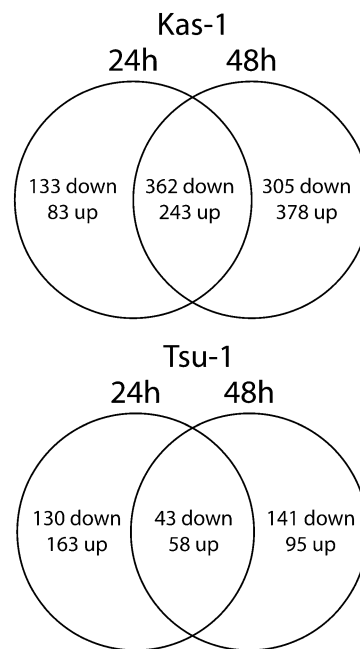


Fig. 2. Venn diagram of microarray expression of *Arabidopsis* root transcripts in response to Fe deficiency. Numbers of differentially regulated genes in Kas-1 (A) and Tsu-1 (B) ecotypes 24h, 48 h, or at both 24 and 48 h after transfer to control (+Fe, 50 μM Fe) or Fe-deficiency (no added Fe) conditions.

three GO- and five MIPS-overrepresented categories were primarily in cell-wall metabolism (Supplementary Table S1). The results for Tsu-1 Fe-regulated genes were very different, in that there were no enriched GO categories and only one category of ‘unannotated’ for MIPS classification for Fe-downregulated Tsu-1 genes (Supplementary Table 1). Similarly, Fe-deficiency upregulated genes were enriched in only one GO category, for suberin biosynthesis, and only one MIPS category of ‘mitochondrion’.

To compare the Fe regulon in the rapid responding Kas-1 and the more slowly responding Tsu-1 with that of other ecotypes, we utilized the Sungear software on the Virtual Plant website to create a five-way Venn diagram between our datasets and those of Col-0, C24, and *Ler*. There have been several microarray studies for Col-0 previously, so to account for possible variation between experiments and growth conditions differences, we created a union set of all the up- or downregulated Fe-regulated transcripts from Colangelo and Guerinot (2004), Garcia *et al.* (2010), Dinneny *et al.* (2008) (Fe-deficiency time course dataset only, not including any salt stress, root zone, or cell-specific datasets), Long *et al.* (2010), and Yang *et al.* (2010). When comparing all five ecotypes (Fig. 3), surprisingly, no genes were significantly downregulated by Fe deficiency in all ecotypes. Only two genes (At5g49770 and At2g36885) were significantly downregulated by Fe deficiency in four of the five ecotypes, and only 18 genes were downregulated in three of the five ecotypes (Table 1), a very small percentage of the total number (1310) of Fe-deficiency-downregulated genes. We considered genes found in at least three of the five ecotypes to be a set of ‘common’ Fe-regulated genes (Table 1). The genes not included in this table can be found

in Supplemental Dataset 1. The Venn set analysis also revealed that the greatest percentage of the total set of Fe-downregulated genes was observed in only one ecotype.

In a 5-way Venn diagram for genes that were upregulated under Fe deficiency, similar to Fe-deficiency downregulated genes, by far the largest sets were those that were upregulated in only one of the ecotypes (Fig. 4). However, there was a small set of 10 genes that was upregulated by Fe deficiency in all five ecotypes, which we refer to as the ‘core’ Fe-deficiency genes (Table 2). There were also relatively small sets of ‘common’ Fe-upregulated genes in four of the five ecotypes (16 genes, Table 2) and three of the five ecotypes (42 genes, Table 2). The genes not included in this table can be found in Supplemental Dataset 1.

To gain insight into possible functional networks of these core and common genes, hierarchical supernode networks based on MIPS functional categories were constructed. For downregulated common genes (Fig. 5), ion homeostasis within subcellular compartments comprised a major network. A smaller network of unclassified proteins included signalling genes, while standalone nodes included cell-wall, phenylpropanoid, and secondary product metabolism. For the core and common upregulated genes supernode networks (Fig. 6), in addition to cellular transport networks, metabolic networks for amino acids and nitrogen and sulphur compounds, cell rescue, defense, and virulence, protein fate, and energy were found to be associated with these highly conserved Fe-regulated genes.

To validate the microarray results and further characterize the molecular responses of Kas-1 and Tsu-1 to Fe deficiency, we carried out a time course of gene expression by real-time reverse transcriptase (RT)-PCR for some of the genes detected on the microarrays and some other genes known to respond to Fe deficiency. Our microarray analysis placed *IRT1* in the core set of Fe-deficiency-upregulated genes (Table 2), and indicated that *IRT1* was more highly expressed in Kas-1 than Tsu-1 at each time point. Our time course results were similar, indicating that *IRT1* was significantly upregulated in Kas-1 but not in Tsu-1 at 24 h, and was upregulated in both ecotypes at 48 h and afterwards (Fig. 7A). *OPT3* is another member of the core Fe responsive genes, and was upregulated earlier in Kas-1 than Tsu-1 (Fig. 7B), although in Tsu-1 this gene was upregulated at both time points by microarray analysis. Another core gene is *FRO3*, expression of which was significantly upregulated in Fe-deficient Kas-1 and Tsu-1 roots at 48 and 72 h as shown by real-time RT-PCR (Fig. 7C), and at both time points in both ecotypes by microarray (Table 2). By microarray, *NRAMP4* was upregulated by Fe deficiency in four of five ecotypes. By RT-PCR, *NRAMP4* was significantly upregulated after 16 h of Fe deprivation and afterwards in Kas-1 by RT-PCR, and in Tsu-1 at 24 h and later (Fig. 7D). The common Fe-responsive transcription factor *FIT* was detected in single microarray experiments for C24 and *Ler*, and in some but not all experiments using Col-0 (Table 2). *FIT* transcripts were not significantly upregulated in our microarray experiments, but by real-time RT-PCR we observed upregulation

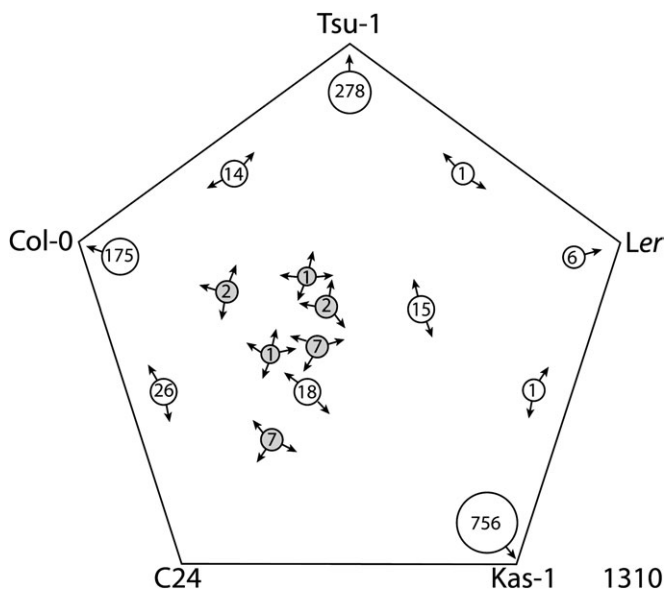


Fig. 3. Five-way generalized Venn diagram of Fe-deficiency-downregulated genes from *A. thaliana* ecotypes Tsu-1, Kas-1, *Ler*, Col-0, and C24. Arrows on the circles indicate ecotypes in which genes of that set were significantly downregulated. Common gene sets are shaded. Total number of genes for all ecotypes is indicated in the lower right corner.

Table 1. Common set of root Fe-deficiency-downregulated genes found in at least three of five *A. thaliana* ecotypes (Tsu-1, Kas-1, Ler, Col-0, and C24). Fold change for –Fe as compared to +Fe is provided when available in original reference, otherwise ‘up’ indicates upregulation in –Fe as compared to +Fe, and ‘down’ indicates downregulated in –Fe as compared to +Fe.

Gene locus	Description	Fold change (–Fe versus +Fe)												
		Tsu-1 24 h	Tsu-1 48 h	Kas-1 24 h	Kas-1 48 h	Ler 24 h ^a	Col-0 24 h ^b	Col-0 24 h ^c	Col-0 24 h ^d	Col-0 48 h ^d	Col-0 72 h ^d	Col-0 72 h ^e	Col-0 72 h ^f	C24 72 h ^f
At5g59520	ZIP2, metal transporter			–7.9	–12.7								–5.0	–5.0
At2g32270	ZIP3, metal transporter			1.5	2.4	Down			Down	Down	Down		–2.9	–2.4
At3g56240	CCH, copper chaperone			–2.3	–2.7								–1.7	–2.0
At5g01600	FER1, ferritin					Down		–2.5	Down	Down	Down		–6.7	–4.8
At3g56090	FER3, ferritin					Down					Down		–2.1	–1.6
At2g40300	FER4, ferritin					Down		–1.5					–3.4	–2.6
At3g09220	LAC7, putative laccase				–2.0					Down	Down		–3	–3.8
At3g25190	Nodulin-like 21			2.3		Down	7.4		Down	Down	Down		–3.8	–5.3
At1g21140	Nodulin-like 1					Down	6.2						–5.9	–5.0
At5g49360	BXL1, bifunctional β-D-xylosidase/ α-L-arabinofuranosidase	–1.7	1.8	–2.4	–3.6						Down			
At5g18670	BMY3, putative β-amylase	–1.8		–2.5	–3.5						Down			
At4g36220	F5H, ferulate 5-hydroxylase			1.6	–2.0								–2.3	–1.8
At5g49780	Leucine-rich repeat transmembrane protein kinase				–1.8								–2.1	–2.5
At5g49770	Leucine-rich repeat		–2.2		–1.7								–2.7	–1.8
At5g49760	transmembrane protein kinase Leucine-rich repeat				–1.5								–2.0	–2.4
At1g79320	Caspase/cysteine-type endopeptidase		–4.1	2.4	2.5			–1.9		Down			–3	–3.7

Table 1. Continued

Gene locus	Description	Fold change (-Fe versus +Fe)											
		Tsu-1 24 h	Tsu-1 48 h	Kas-1 24 h	Kas-1 48 h	Ler 24 h ^a	Col-0 24 h ^b	Col-0 24 h ^c	Col-0 48 h ^d	Col-0 72 h ^d	Col-0 72 h ^e	Col-0 72 h ^f	C24 72 h ^f
At4g13250	Similar to short-chain dehydrogenase/reductase (SDR) family protein			-1.9	-2.4								
At4g18550	Lipase	-1.6	-1.5										-2.8
At2g36885	class 3 family protein		-1.7			Down							-2.5
At1g68650	Unknown protein					Down		Down					-2.3

^a Buckhout et al. (2009)
^b Garcia et al. (2010)
^c Long et al. (2010)
^d Dinnenny et al. (2008)
^e Colangelo and Gueriot (2004)
^f Yang et al. (2010).

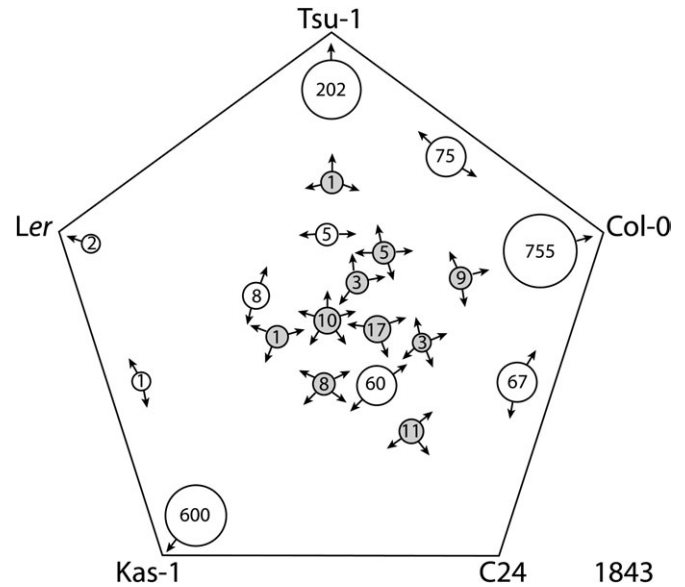


Fig. 4. Five-way generalized Venn diagram of Fe-deficiency-upregulated genes from *A. thaliana* ecotypes Tsu-1, Kas-1, Ler, Col-0, and C24. Arrows on the circles indicate ecotypes in which genes of that set were significantly upregulated. Core and common gene sets are shaded. Total number of genes for all ecotypes is indicated in the lower right corner.

to nearly 3-fold (Fig. 7E). Kas-1 upregulated this gene before Tsu-1 did, by 24 h. The H⁺-ATPase *AHA2* gene was 1.5-fold-upregulated in the 48 h Kas-1 arrays. Expression of *AHA2* by real-time RT-PCR resembled that of *FRO3*, in that expression was similar between Kas-1 and Tsu-1, and was upregulated in both ecotypes at 48 h and later (Fig. 7F).

Discussion

We have established a genotype × environment interaction for timing of maximal induction of ferric reductase activity using a time-course approach with three diverse *Arabidopsis* ecotypes, Kas-1, Tsu-1, and Col-0, where Kas-1 responded the most rapidly, Tsu-1 the most slowly, and Col-0 was intermediate. Our overall hypothesis for this study was that the most important early Fe-responsive genes could be determined by comparing the Fe-regulated transcriptomes of these diverse *Arabidopsis* ecotypes at fixed time points early in the progression of responses to the onset of Fe deficiency. We chose 24 and 48 h to sample our plants, because in Kas-1 24 h is prior to ferric reductase activity upregulation, and 48 h is at the peak, while both of these time points are prior to the peak for Tsu-1. This analysis revealed a small overlapping set of genes from among the many with altered expression under Fe deficiency. This filtering method could narrow the focus of follow-up studies to determine the roles of universally Fe-regulated genes by avoiding genes that respond to Fe deficiency in a genotype-influenced manner; that is, in some but not in all ecotypes. We also propose that this analysis could offer insight into new transcription factor and/or signalling proteins that

Table 2. Core and common set of root Fe deficiency upregulated genes from *Arabidopsis thaliana* ecotypes (Tsu-1, Kas-1, Ler, Col-0 and C24). Fold change for –Fe as compared to +Fe is provided when available in original reference, otherwise ‘up’ indicates upregulation in –Fe as compared to +Fe, and ‘down’ indicates downregulated in –Fe as compared to +Fe.

Gene locus	Description	Fold change (–F versus +Fe)												Col-0 ^g	
		Tsu-1 24 h	Tsu-1 48 h	Kas-1 24 h	Kas-1 48 h	Ler 24 h ^a	Col- 0 24 h ^b	Col- 0 24 h ^c	Col- 0 24 h ^d	Col- 0 48 h ^d	Col- 0 72 h ^d	Col- 0 72 h ^e	Col- 0 72 h ^f		C24 72 h ^f
Core set of root upregulated genes (observed in five of five ecotypes)															
At4g19690	IRT1, metal transporter	3.8	6.3	29.3	61.7	Up	27.4	29.3	Up	Up	Up	16.3	13.7	6.6	
At4g16370	OPT3, metal transporter	1.7	5.2	–1.6	1.7	Up		5.2	Up	Up	Up		7.3	5.7	
At1g23020	FRO3, metal transporter	2.8	5.4	2.0	2.8	Up		4.8	Up	Up	Up		7.8	6.7	
At3g58810	MTP3/MTPa2, metal transporter	3.4	7.1	3.4	6.5	Up	4.8	5.4	Up	Up	Up	11.9	9.4	6.7	Up
At3g56980	bHLH039, transcription factor	5.4	10.2		2.6	Up		9.5	Up	Up	Up		24.5	20.3	Up
At3g12900	2OG-Fe(II) oxygenase family protein	2.4	9.7	6.0	19.2	Up	24.9	11.2	Up	Up	Up	18.5	95.5	35.3	
At3g07720	Galactose oxidase/kelch repeat-containing protein	1.6	10.5	4.2	4.0	Up	8.3	11.2	Up	Up	Up	12.9	11.3	12.1	Up
At5g53450	ORG1, OBP3 responsive gene (protein kinase)	1.7	3.4		2.4	Up		4.4	Up	Up	Up		5.3	3.5	
At1g47400	Unknown protein	3.0	5.2	2.7	5.9	Up		5.2	Up	Up	Up		13.6	6.6	
At1g74770	Unknown protein	2.0	3.7		2.4	Up				Up	Up		5.4	4.9	
Common set of root upregulated genes (observed in four of five ecotypes)															
At4g19680	IRT2, metal transporter			4.3	5.6	Up	3.0	3.8				21.0	14.3	7.1	Up
At5g67330	NRAMP4, metal transporter		3.0			Up		2.0	Up	Up	Up		2.2	2.0	
At3g58060	MTPc3, metal transporter			3.5	3.9	Up		3.0	Up	Up	Up	8.9	21.2	22.4	
At5g02780	Glutathione S-transferase (flavonoid synthesis)	3.7	7.7	3.8	7.4		7.0	11.2	Up	Up	Up	19.1	27.4	21.6	
At3g50740	UGT72E1, UDPG:coniferyl alcohol glucosyltransferase	2.4	3.6	4.0	4.7			2.6	Up	Up	Up	8.6	4.2	3.9	Up
At1g09560	GLP5 germin-like protein			2.3	3.7	Up	3.8	3.0	Up	Up	Up		3.0	2.4	
At1g34760	GRF11/GR14, 14-3-3 protein, binds H ⁺ -ATPase (root hairs)			2.0	2.9	Up	2.3	3.7	Up	Up	Up	18.4	9.4	10.9	
At3g06890	Similar to oxidoreductase/transition metal ion binding				1.7	Up		2.7		Up	Up	2.8	3.7	4.6	
At3g47640	PYE, bHLH transcription factor		1.9			Up		1.5	Up	Up	Up		2.6	2.2	
At3g18290	BTS, E3 ligase	1.6	2.7			Up		2.2	Up	Up	Up		2.7	2.5	
At2g20030	RING-H2 finger protein ATL2D precursor			1.6		Up	2.0					3.0	3.0	2.5	
At5g05250	Unknown protein	2.5	7.2			Up		3.0	Up	Up	Up		6.0	4.4	
At3g56360	Unknown protein	2.2	1.6			Up		2.7	Up	Up	Up		2.3	1.6	
At1g49000	Unknown protein	3.3	–1.5	1.5	1.9		2.7			Up	Up		5.8	3.4	
At2g29995	Unknown protein			1.6	1.8	Up							2.3	3.3	
At3g61930	Unknown protein			3.3	8.7	Up	11.8	6.9	Up	Up	Up	32.5	23.9	11.7	
Common Set of root upregulated genes (observed in three of five ecotypes)															

Table 2. *Continued*

Gene locus	Description	Fold change (–F versus +Fe)													Col-0 ^g
		Tsu-1 24 h	Tsu-1 48 h	Kas-1 24 h	Kas-1 48 h	Ler 24 h ^a	Col- 0 24 h ^b	Col- 0 24 h ^c	Col- 0 24 h ^d	Col- 0 48 h ^d	Col- 0 72 h ^d	Col- 0 72 h ^e	Col- 0 72 h ^f	C24 72 h ^f	
At4g30120	HMA3, P1B-type Zn-ATPase			1.7			1.6			Up		5.5	5.1	4.3	
At1g80830	NRAMP1, metal transporter			1.5								4.2		2.8	
At5g13740	ZIF1, zinc transporter					Up	2.5	Up	Up	Up			2.6	2.3	
At3g46900	COPT2, Cu transporter					Up	3.7	4.0	Up	Up	Up	5.8	15.4	7.5	
At5g03570	FPN2/IREG2, metal transporter					Up		3.0	Up	Up	Up	6.6	6.4	4.2	
At5g26820	IREG3, transporter					Up				Up			2.3	1.9	
At5g38820	Amino acid transporter family protein			1.7	3.3	Up	3.7	4.5	Up	Up	Up	6.8	16.3		
At3g60330	AHA7, H ⁺ -ATPase			1.7	2.0		2.1					3.0	2.6	2.8	
At5g07390	RBOHA	2.6								Up	Up		2.3	1.6	
At5g47910	RBOHD	1.5	2.7					2.9	Up	Up	Up	3.8	4.2	4.4	
At3g12820	MYB10, transcription factor					Up	3.5	2.6	Up	Up	Up	11.7	24.0	24.2	
At1g56160	MYB72, transcription factor					Up	2.2	3.4	Up	Up	Up	23.4	84.5	72.2	
At2g28160	FIT, transcription factor					Up	2.8					3.5	2.4	2.8	
At5g04150	bHLH101, transcription factor					Up		4.1	Up	Up	Up		13.9	6.1	
At4g01250	WRKY22, transcription factor	1.6	2.2				2.0						1.6	2.0	
At2g14210	MADS box gene, transcription factor			2.0	2.0								2.7	2.8	
At1g02500	SAM1, S-adenosylmethionine synthetase				1.8			2.1	Up	Up	Up		2.6	2.2	
At5g04950	NAS1, nicotianamine synthase			1.9	2.2		7.5	5.9	Up	Up	Up	3.5	3.1	3.1	
At5g13910	LFY, ethylene response factor subfamily B-1		1.8							Up	Up		3.1	3.7	
At2g45400	BEN1, dihydroflavinol 4-reductase-like protein					Up		2.3		Up			2.7	3.3	
At1g14185	Glucose-methanol-choline (GMC) oxidoreductase family protein				2.6		2.1						9.9	7.1	
At2g16060	HB1/GLB1, nonsymbiotic haemoglobin	15.9	6.2		2.4					Up	Up				
At5g61250	Heparanase-like protein 2			1.7	1.8					Up			2.6	2.2	
At4g36010	Pathogenesis-related thaumatin family protein	2.6	2.0		1.8					Up	Up				
At5g16570	GLN1, glutamine synthetase				1.7						Up		2.4	2.0	
At5g36890	Glycosyl hydrolase family 1 protein				1.6		3.4	4.1	Up	Up	Up	6.2	8.7	5.7	
At4g17260	Lactate/malate dehydrogenase family protein	6.3	1.6		1.5					Up	Up				
At2g46710	rac GTPase activating protein family		2.5										2.2	2.1	
At3g21240	Isoform of 4-coumarate:CoA ligase (4CL)		1.8					2.2	Up	Up	Up	3.0	3.9	3.7	
At2g42750	4Fe-4S ferredoxin, iron-sulphur-binding family		1.7			Up		1.8	Up	Up	Up				

Table 2. Continued

Gene locus	Description	Fold change (-F versus +Fe)												Col-0 ^g
		Tsu-1 24 h	Tsu-1 48 h	Kas-1 24 h	Kas-1 48 h	Ler 24 h ^a	Col- 0 24 h ^b	Col- 0 24 h ^c	Col- 0 24 h ^d	Col- 0 48 h ^d	Col- 0 72 h ^d	Col- 0 72 h ^e	Col- 0 72 h ^f	
At4g30490	AFG1-like ATPase family protein		1.5					1.7				3.3	2.5	
At4g10510	Subtilase family protein	1.7								Up	Up	2.1	6.2	4.8
At4g12910	Serine carboxypeptidase family	-1.5	1.6							Up	Up	2.3	2.4	2.1
At1g18910	Zinc finger (C3HC4-type RING finger) family protein					Up		1.7		Up	Up	2.8	3.2	3.0
At3g53280	CYP71B5, cytochrome P450 monooxygenase					Up	3.5			Up	Up	19.4	40.1	33.8
At3g61410	U-box domain-containing protein kinase					Up		2.1		Up	Up	3.6	4.2	4.8
At4g29220	PFK1, phosphofructokinase family protein					Up		1.9		Up	Up	2.7	2.5	2.8
At4g38950	Similar to microtubule motor; similar to kinesin heavy chain				-1.5	Up						2.1	2.0	2.1
At4g19160	Unknown protein				1.5					Up	Up		3.3	2.6
At5g55620	Unknown protein					Up	2.1			Up	Up		11.6	12.3
At5g67370	Unknown protein					Up		2.6	Up	Up	Up		7.3	13.3
At1g01570	Similar to fringe-related protein					Up							2.9	3.3

^a Buckhout *et al.* (2009)^b Garcia *et al.* (2010)^c Long *et al.* (2010)^d Dinnenny *et al.* (2008)^e Colangelo and Guerinot (2004)^f Yang *et al.* (2010)^g Ivanov *et al.* (2011).

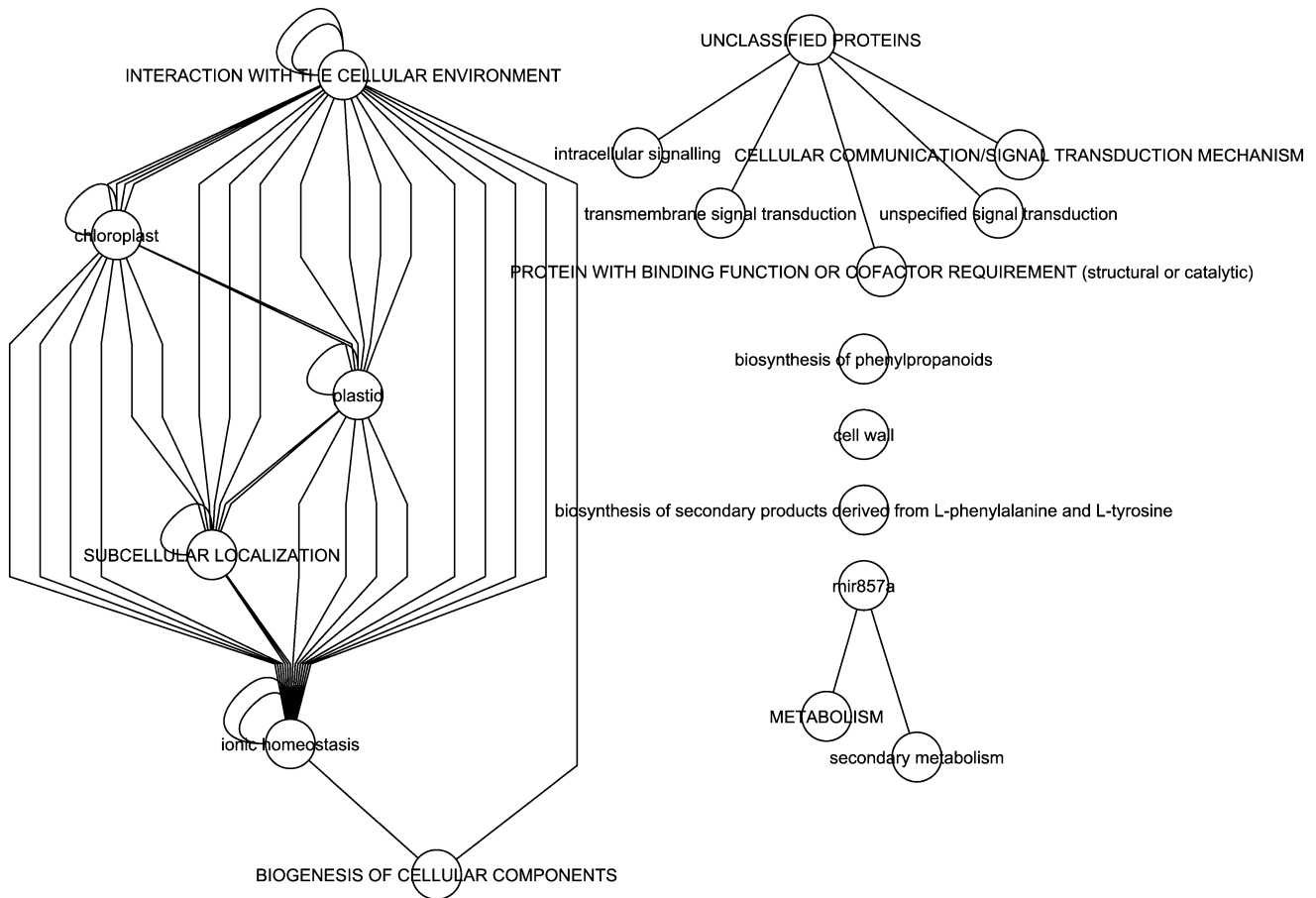


Fig. 5. Hierarchical super node gene networks of Fe-deficiency-downregulated genes common among *A. thaliana* accessions Tsu-1, Kas-1, Ler, Col-0, and C24. Each super node represents a group of genes with common protein function based on MIPS categories. Each edge represents interactions between a gene in each super node.

might be upstream regulators of conserved Fe-deficiency responses.

Diversity in root ferric reductase activity at a single time point was described for a number of ecotypes (Saleeba and Guerinot, 1995). Under our time-course conditions, Kas-1 and Tsu-1 were one day sooner or later to reach maximum root ferric reductase activity than Col-0, offering the potential for detecting diverse molecular responses between these ecotypes that are involved in perception and signalling of Fe-deficiency responses. Root ferric reductase activity is carried out by *FRO2* (Robinson *et al.*, 1999), which is regulated by *FIT* (Colangelo and Guerinot, 2004). Consistent with ferric reductase activity, *FIT* and *FRO2* were upregulated in Kas-1 one day sooner than in Tsu-1, suggesting that Kas-1 has genetically determined differences in Fe status perception or signalling. Similarly, *IRT1* and *NRAMP4* were upregulated in Kas-1 earlier than in Tsu-1. Other, later-responding genes, such as *FRO3* and *AHA2*, did not show significant differences in timing of induction, suggesting that multiple regulatory pathways are required for the complete Fe regulon.

We hypothesize that genes that do not respond to Fe deficiency in multiple ecotypes are less likely to be primary Fe-responsive genes and may represent genotype × environment

responses that differ between ecotypes. To produce our dataset, Kas-1 and Tsu-1 were grown side by side in the same batch of nutrient solution and collected simultaneously; however, only 17 downregulated (Fig. 3) and 24 upregulated (Fig. 4) genes were in common between these ecotypes, demonstrating that genotype effects were important in the same environment. This is in contrast to the study of Yang *et al.*, (2010), which observed a small number of differentially expressed genes between Col-0 and C24 ecotypes. A surprisingly small number of genes, 10 in total, were upregulated at one or more early time points in all ecotypes studied, which we termed the ‘core’ Fe-deficiency-response genes. Although not present on the Affymetrix ATH1 Genome Array, *FRO2* probably also belongs to this set of core genes, since this gene has consistently been upregulated in this and previous *Arabidopsis* Fe-deficiency studies (Colangelo and Guerinot, 2004; Wu *et al.*, 2005; Mukherjee *et al.*, 2006; Buckhout *et al.*, 2009; Garcia *et al.*, 2010). The core genes include *IRT1*, known to be important for Fe uptake (Vert *et al.*, 2002). These genes also include *MTP3*, which is thought to sequester Zn into the vacuole under Fe deficiency (Arrivault *et al.*, 2006), *OPT3*, potentially involved in translocation of Fe into or from vascular tissues (Stacey *et al.*, 2008), and *FRO3*, a ferric reductase

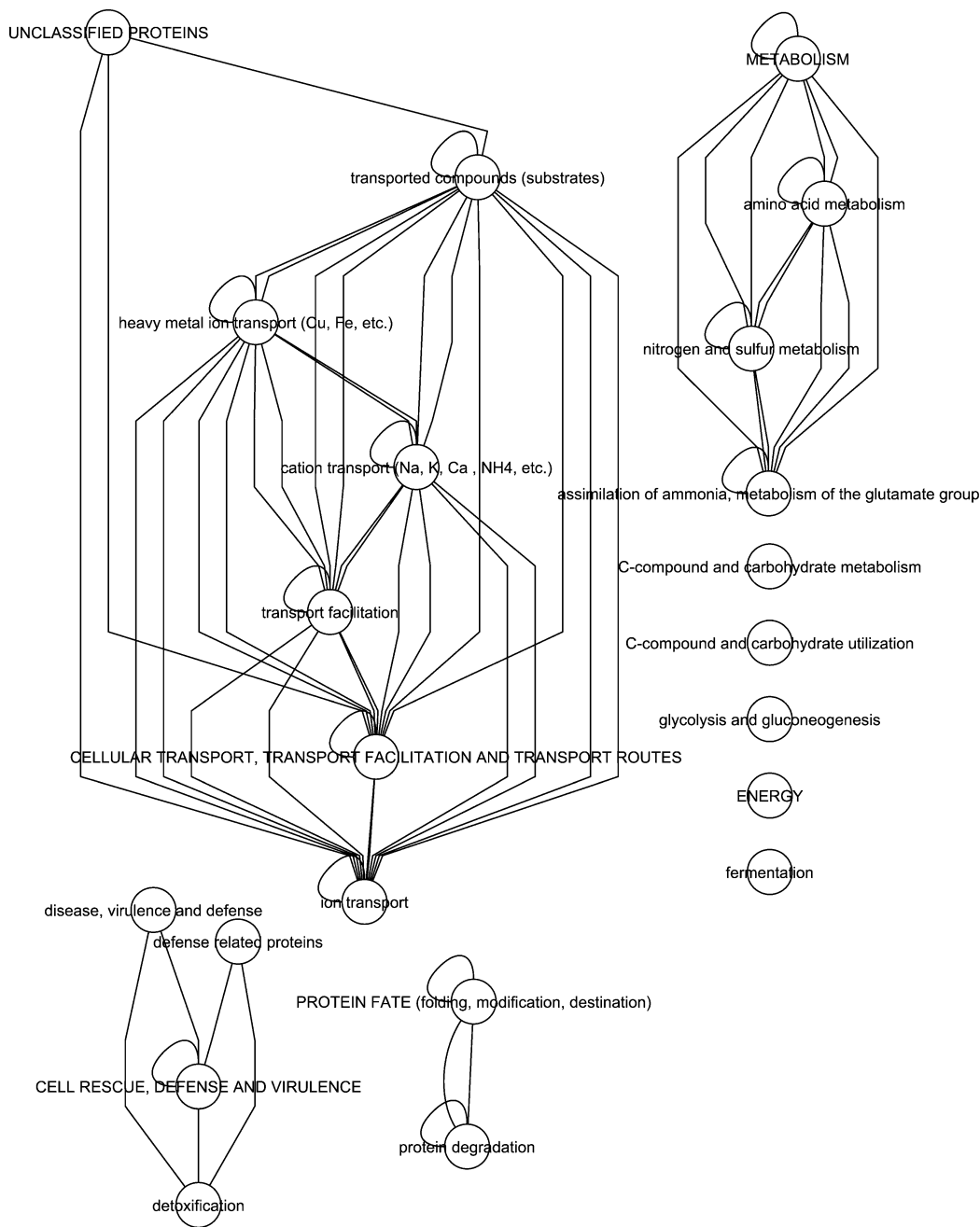


Fig. 6. Hierarchical supernode gene networks of core and common Fe-deficiency-upregulated genes from *A. thaliana* accessions Tsu-1, Kas-1, *Ler*, Col-0, and C24. Each super node represents a group of genes with common protein function based on MIPS categories. Each edge represents interactions between a gene in each super node.

with undefined function. Only four out of the 10 core genes are FIT-dependent, suggesting that transcription factors in addition to FIT take part in the Fe-deficiency regulation. Some of the core genes were not significant in all of the previous Col-0 microarray experiments. Although our Tsu-1 and Kas-1 plants were grown in identical conditions, to allow for some degree of experimental variability between labs, we also considered ‘common’ genes that were Fe-regulated in three or four of the five ecotypes, which is still a relatively small number of only 58 out of 1842 genes. One striking feature of the core and common Fe-deficiency genes is that there are several genes annotated as ‘unknown’,

or their specific function in the Fe-deficiency response has not been determined. This result illustrates that there are crucial processes yet to be discovered in the early responses and adaptation to Fe deprivation in plants. By far, the largest number of Fe responsive genes, both upregulated and downregulated, for Kas-1, Tsu-1, and the Col-0 combined set, were not observed in any of the other ecotypes. Thus, it seems likely that there is a small set of universal Fe responsive genes and a large number of genes that represent genotype-influenced responses in different ecotypes, based on their evolutionary history. A similar result was observed for the transcriptome response to salicylic acid, where most

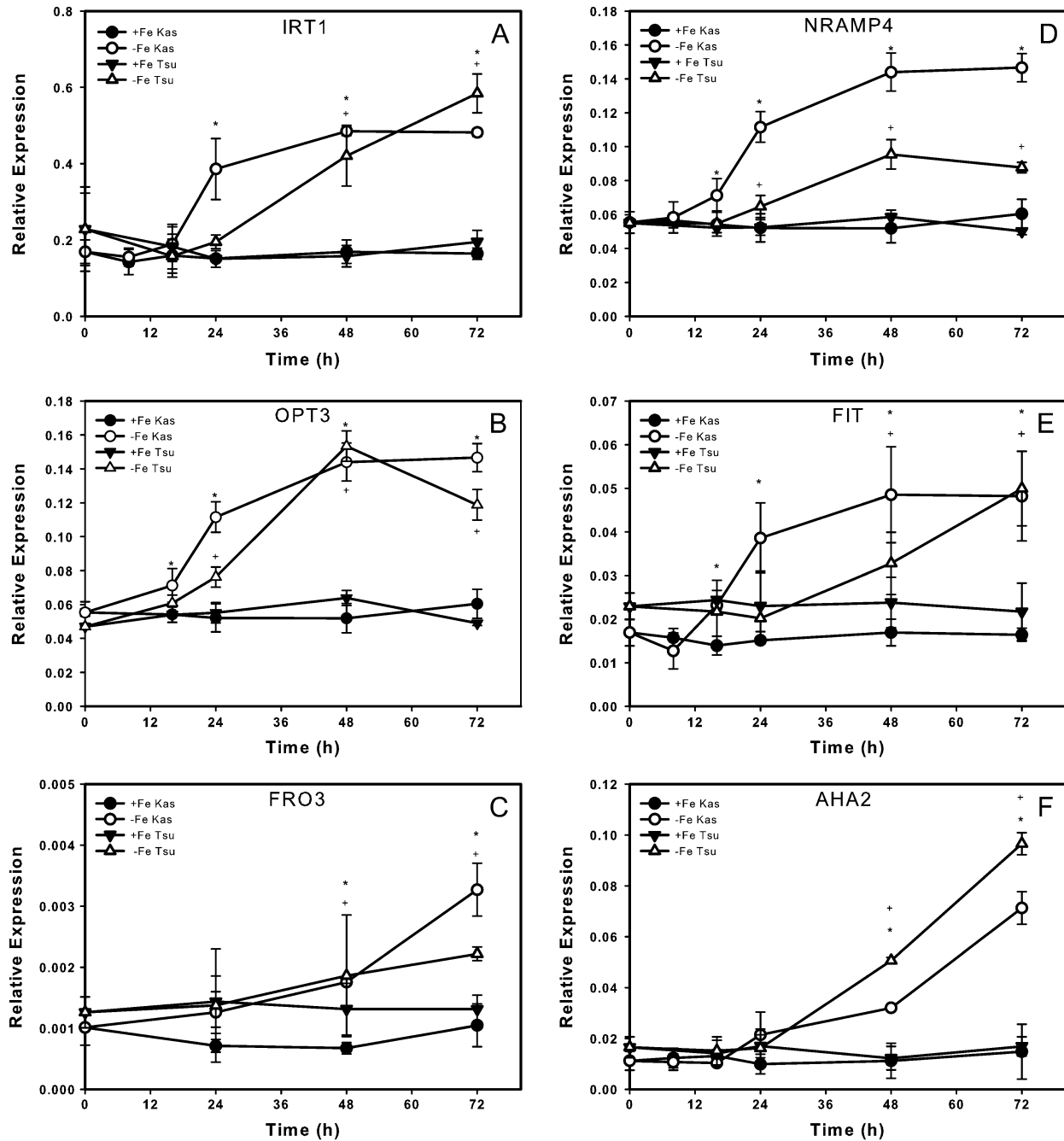


Fig. 7. Time course expression of Fe-uptake-related genes in Kas-1 and Tsu-1 roots. (A) *IRT1*, (B) *OPT3*, (C) *FRO3*, (D) *NRAMP4*, (E) *FIT*, (F) *AHA2*; $n = 3 \pm \text{SD}$. * Denotes statistical significance for Kas-1, + denotes statistical significance for Tsu-1 ($P \leq 0.05$) between treatments at each time point. +Fe, 50 μM Fe; -Fe, no added Fe.

responsive genes were observed in only one or a few of seven different *Arabidopsis* ecotypes, and only a small set of genes (38 in total) were commonly regulated in the majority of ecotypes (van Leeuwen *et al.*, 2007).

It is not clear at this point what roles the ‘ecotype-specific’ Fe-regulated genes play in the root adaptation to Fe deprivation. We note that since ecotypes can respond to Fe deficiency in different timescales, if each ecotype were sampled at the same relative stage of response, the number of genes in the core set may increase. A caveat is that the

number of secondary stress-responsive genes would also increase. Thus, we believe our approach to compare diverse ecotypes at temporally chosen time points allows identification of the earliest and most robust primary Fe-response genes. Potentially, the genes observed in only one ecotype are not involved with Fe uptake *per se*, but with ecotype-specific growth patterns that may be perturbed by Fe deficiency. For example, we have observed that Kas-1 seedlings produce more lateral roots than Tsu-1 seedlings on both Fe-replete and Fe-deficient agar medium (Supplementary Fig. S1). The

notable number of genes involved in cell-wall processes by GO or MIPS categorization in Fe-deficient Kas-1 may reflect initiation or elongation of additional lateral roots. Another contrasting trait between Kas-1 and Tsu-1 is water-use efficiency, with Kas-1 showing superior drought avoidance and constitutive lower stomatal conductance (McKay *et al.*, 2008; Juenger *et al.*, 2010). Whether constitutive differences in water-use efficiency among ecotypes could impact the sensitivity and tolerance to mineral deficiencies remains to be tested, but it is known that water deficit has a significant effect on mineral accumulation in *Arabidopsis* (Ghandilyan *et al.*, 2009).

Another interesting observation from the common Fe-deficiency genes is the dissimilarity in genes expressed in Kas-1 and *Ler*. In genes downregulated in four ecotypes, seven genes that were downregulated in *Ler* were not significant in Kas-1, and there were 10 downregulated genes in Kas-1 that were not significant in *Ler* (Table 1). Only one gene out of this set, *ZIP3*, was significant in both Kas-1 and *Ler*, but this gene was upregulated in Kas-1, and in contrast was downregulated in *Ler* and Col-0. In the common upregulated Fe-responsive genes upregulated in three of the five ecotypes (Table 2), a similar lack of overlap between Kas-1 and *Ler* was observed. There was much higher agreement between *Ler* and Col-0, which may reflect their adaptations to similar climate zones that are quite different than the native Kas-1 climate.

To gain additional insight into ecotype-specific metabolic responses, we compared gene expression as fold change among Kas-1, Tsu-1, Col-0, and C24 ecotypes for MapMan (Thimm *et al.*, 2004) functional categories, an approach that has also been taken to compare Col-0 and an Fe homeostasis mutant (Schuler *et al.*, 2011), with a number of similar categories observed. Within the transport bin (Supplementary Fig. S2), differences in expression of transporters for minerals other than Fe may indicate that the different ecotypes have differing homeostasis mechanisms for handling excess non-Fe metals that may be taken up by IRT1 (Korshunova *et al.*, 1999; Rogers *et al.*, 2000) or other upregulated transporters. Additionally there were several transporters for oligopeptides, phosphate, potassium, sulphate, nitrate, amino acids, and calcium that were primarily Fe-regulated in Kas-1 and/or Tsu-1 but not Col-0 or C24. These differences may explain variability in mineral concentrations between *Arabidopsis* ecotypes that have been reported (Baxter *et al.*, 2008b; Waters and Grusak, 2008b; Buescher *et al.*, 2010). In the metal-handling bin, all four ferritin (*FER*) genes were downregulated, but only in Col-0 and C24 (Supplementary Fig. S3). Ferritin protein is important for Fe storage in plants, and differences in expression may be representative of differences in internal metal accumulation. Under non-stress growth conditions, Tsu-1 had higher accumulation of Mn (+23% in relation to Col-0), Fe (+21%), Na (+127%), and K (+20%), whereas Kas-1 showed a decreased accumulation of Zn (−33% in relation to Col-0), Mo (−59%), and Cd (−26%) (Buescher *et al.*, 2010). Similarly, in the redox bin (Supplementary Fig. S4), the Fe superoxide dismutase *FSD1* was downregulated in Col-0 and C24, but not in

Kas-1 or Tsu-1. Kas-1 had Fe regulation of several genes involved in sulphur-containing redox processes that were not observed in the other ecotypes, suggesting differences in metabolism that may interact with responses to Fe deficiency. Further suggestions of fundamental metabolic differences are seen when the cytochrome P450 and peroxidase genes from the ‘miscellaneous’ MapMan bin are displayed (Supplementary Fig. S5). There are several down-regulated and upregulated genes in both of these categories that were Fe-regulated in only one ecotype.

Transcription factors of selected families are shown in Supplementary Fig. S6. Some *MYB* family genes are important for regulating flavonoid synthesis under mineral nutrient deficiency (Lillo *et al.*, 2008). Fifteen *MYB* family transcription factors were Fe-regulated only in Kas-1. Similarly, there were several *NAC* and *WRKY* family transcription factor genes that were Fe-regulated only in Kas-1, while three *NAC* genes had opposite directions of regulation in Kas-1 and Col-0 or Tsu-1, suggesting a genotype-influenced response. The NAC family proteins regulate a wide array of plant development and responses to stress (Olsen *et al.*, 2005), while WRKY proteins regulate diverse responses to pathogens, development, and abiotic stress (Rushton *et al.*, 2010). Regulation of a number of *WRKY* genes was enriched in genes differentially expressed between WT and *nas4x-1* mutants (Schuler *et al.*, 2011). It is possible that differences in Fe regulation of these transcription factors result in genotype-influenced Fe-deficiency responses.

As with other transcription factor families, several basic helix-loop-helix (bHLH) family genes were Fe-upregulated in Kas-1 only. Several *bHLH* genes were previously shown to be Fe-regulated and/or have been implicated in regulation of Fe-deficiency responses, including the core Fe-response gene *bHLH039* and common Fe-response genes *bHLH101*, *PYE*, and *FIT*. Based on phenotypes of mutants, *FIT* has been the most important Fe-responsive transcription factor gene described; however, it is not the most robustly Fe-regulated, suggesting that fold-change regulation does not necessarily correlate to functional importance. Overexpression of *Arabidopsis* *bHLH039* in tobacco roots resulted in production of riboflavin (Vorwieger *et al.*, 2007), a common root Fe-deficiency response. *bHLH039* has been reported to act as a heterodimer with *FIT* in binding to *FRO2* and *IRT1* promoters (Yuan *et al.*, 2008). Constitutive overexpression of *FIT*, *bHLH038*, or *bHLH039* alone had no effect on Fe reduction under Fe-replete conditions, but co-overexpression of *FIT* and *bHLH038* or *FIT* and *bHLH039* resulted in a constitutive reduction of ferric iron at the roots (Yuan *et al.*, 2008). Like many transcription factors, *PYE* protein can also interact with other bHLH proteins (Long *et al.*, 2010), and WRKY and NAC proteins are known to form heterodimers (Olsen *et al.*, 2005; Rushton *et al.*, 2010). An extensive network of genes is associated with *PYE* (Ivanov *et al.*, 2011), including metal homeostasis genes. These results suggest that differences in expression of partner *bHLH* genes between ecotypes for genes that interact with *FIT* (e.g. *bHLH039*, *bHLH038*, other unknown proteins) or other key

transcription factors may contribute to different Fe regulons in different ecotypes.

As with other MapMan functional categories, there were a number of signalling genes that were Fe-regulated in some, but not all ecotypes (Supplementary Fig. S7). However, several interesting gene-expression patterns were conserved among several ecotypes. Two leucine-rich repeat protein kinases and one serine/threonine kinase were downregulated in multiple ecotypes, and one (At5g49760) was also downregulated at the protein level (Lan *et al.*, 2010), suggesting that the proteins encoded by these genes may be involved in early Fe-deficiency responses. Decreased expression of these kinases could modulate the activity of a number of undetermined proteins that carry out physiological responses to Fe deficiency.

An additional gene of interest that was conserved across ecotypes is GRF11/GR14, a 14-3-3 protein, expression of which depended on presence of a functional *FIT* gene (Colangelo and Guerinot, 2004). The 14-3-3 proteins activate or deactivate other proteins by binding phosphorylated motifs in the target protein and changing its conformation (Oecking and Jaspert, 2009). GRF14 was demonstrated to activate the AHA2 H⁺-ATPase protein (Jahn *et al.*, 1997), which has been identified as the primary source of rhizosphere acidification under Fe deficiency (Santi and Schmidt, 2009). *AHA2* gene expression increases under Fe deficiency, but at time points later than *FIT*, *IRT1*, or *FRO2*. Activation of the protein could be a more rapid and additional level of regulation for rhizosphere acidification activity, and modification by 14-3-3 proteins may be a mechanism for activation of other Fe-deficiency responses. We searched the *Arabidopsis* proteome for potential 14-3-3-binding sites (RSX(S/T)XP) using Patmatch, then compared these putative target proteins to the core and common Fe response genes. This identified additional potential targets in metabolic (At3g21240, 4-coumarate:CoA ligase in the phenylpropanoid pathway), signalling (rac GTPase activating protein), and regulatory (bHLH101, WRKY22) targets, suggesting that GRF11 may be a key control point in the cascade of Fe-deficiency responses in *Arabidopsis* by modulating protein activity prior to transcriptional upregulation, which occurs for these genes in most or all of the ecotypes studied. Proteomic profiling has demonstrated that 14-3-3 proteins can interact with *WRKY* family transcription factors (Chang *et al.*, 2009). A model of the potential GRF11 network is presented in Supplementary Fig. S8.

Our study suggests that comparison of transcriptomes of diverse germplasm using similar experiments can reveal robustly differentially Fe-regulated genes and exclude a substantial number of genes that are more slowly or non-Fe-responsive in some ecotypes. We have also identified conserved signalling networks that may play key roles in universal Fe responses at the level of protein activity. Future studies can further exploit natural variation between ecotypes to further understand the biology of different ecotypes on a systems level and uncover the factors that control sensing of and responses to plant Fe status.

Supplementary material

Supplementary material is available at *JXB* online.

Supplementary Table S1. Overrepresented GO and MIPS categories in Kas-1 and Tsu-1.

Supplementary Fig. S1. Number of lateral roots of Kas-1 and Tsu-1 seedlings.

Supplementary Fig. S2. Heat map of the MapMan 'transport' functional bin.

Supplementary Fig. S3. Heat map of the MapMan 'metal-handling' functional bin.

Supplementary Fig. S4. Heat map of the MapMan 'redox' functional bin.

Supplementary Fig. S5. Heat map of the MapMan 'miscellaneous' functional bin.

Supplementary Fig. S6. Heat map of the MapMan 'regulation' functional bin.

Supplementary Fig. S7. Heat map of the MapMan 'signalling' functional bin.

Supplementary Fig. S8. Model of the involvement of GF14/GRF11, a 14-3-3 protein, in a potential network of root Fe-deficiency-regulated responses.

Supplementary Dataset. Iron-regulated genes from sets represented in Fig. 3 and Fig. 4.

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