Mutually Exclusive Alterations in Secondary Metabolism Are Critical for the Uptake of Insoluble Iron Compounds by Arabidopsis and Medicago truncatula\textsuperscript{1[C][W]}

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The generally low bioavailability of iron in aerobic soil systems forced plants to evolve sophisticated genetic strategies to improve the acquisition of iron from sparingly soluble and immobile iron pools. To distinguish between conserved and species-dependent components of such strategies, we analyzed iron deficiency-induced changes in the transcriptome of two model species, Arabidopsis (Arabidopsis thaliana) and Medicago truncatula. Transcriptional profiling by RNA sequencing revealed a massive up-regulation of genes coding for enzymes involved in riboflavin biosynthesis in M. truncatula and phenylpropanoid synthesis in Arabidopsis upon iron deficiency. Coexpression and promoter analysis indicated that the synthesis of flavins and phenylpropanoids is tightly linked to and putatively coregulated with other genes encoding proteins involved in iron uptake. We further provide evidence that the production and secretion of phenolic compounds is critical for the uptake of iron from sources with low bioavailability but dispensable under conditions where iron is readily available. In Arabidopsis, homozygous mutations in the Fe(II)- and 2-oxoglutarate-dependent dioxygenase family gene \textit{F6′H1} and defects in the expression of \textit{PLEIOTROPIC DRUG RESISTANCE3}, encoding a putative efflux transporter for products from the phenylpropanoid pathway, compromised iron uptake from an iron source of low bioavailability. Both mutants were partially rescued when grown alongside wild-type Arabidopsis or \textit{M. truncatula} seedlings, presumably by secreted phenolics and flavins. We concluded that production and secretion of compounds that facilitate the uptake of iron is an essential but poorly understood aspect of the reduction-based iron acquisition strategy, which is likely to contribute substantially to the efficiency of iron uptake in natural conditions.

Due to its ability to transport electrons, iron is an essential component in respiration and photosynthesis and, thus, an irreplaceable element in virtually all living organisms. The abundance of iron on Earth is close to that of oxygen, but the bioavailability of iron is severely restricted by its low solubility in the presence of the latter. In soils, iron forms immobile complexes with phosphate and other soil constituents, causing severe yield losses in crops and decreasing the nutritional value of crop plants. As a consequence, iron deficiency anaemia has become the largest nutritional disorder worldwide, affecting approximately two billion people (de Benoist et al., 2008). To counteract the low solubility of iron under these conditions, graminaceous plant species have evolved a mechanism that involves the synthesis of iron chelators that bind Fe(III) with high affinity (phytosiderophores [PSs]). PSs are excreted via the \textit{TRANSPORTER OF MUGINEIC ACID (TOM1)} transporter, and following binding of iron, they are taken up by a high affinity transporter of the Yellow Stripe family without prior splitting of metal-ligand chelate (Curie et al., 2001; Nozoye et al., 2011). PSs of the mugineic acid family are synthesized from L-Met (Negishi et al., 2002) and form strong, hexadentate complexes with Fe(III) that are stable over a wide pH range. This mechanism of iron acquisition is restricted to grasses (Strategy II; Römheld and Marschner, 1986), while all other species rely on a reduction-based iron acquisition mechanism (Strategy I), which involves an obligatory split of Fe(III)-chelate complexes by reduction of Fe(III) via the plasma membrane-bound FERRIC REDUCTION OXYGENASE2 (FRO2; Robinson et al., 1999) and the subsequent uptake of Fe(II) by IRON-REGULATED TRANSPORTER1 (IRT1), a transporter of the ZIP family (Eide et al., 1996). The acquisition of iron is supported by rhizosphere acidification mediated by the proton-translocating ATPase AHA2. Acidification increases the free activity of iron at low

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pH (Santi and Schmidt, 2009). Strategy I species do not synthesize PSSs; instead, iron deficiency triggers exudation of root-specific substances, often in large quantities. Chemically, iron deficiency-induced root exudates include a wide range of molecules, including organic acids, phenolics, flavins, and flavonoids, among others (Schmidt, 1999; Dakora and Phillips, 2002; Cesco et al., 2010; Abadía et al., 2011; Rodríguez-Celma et al., 2011b). The composition of root exudates appears to be genetically determined (Rovira, 1969; Dakora and Phillips, 2002). In most cases, the physiological significance of iron deficiency-induced root exudates has not been determined. Phenolics have both chelating and reducing properties, and it has been speculated that excreted phenolic compounds could contribute to the obligatory reduction of iron. However, the reductive power of excreted flavonoids/phenolics is much lower than the rates of enzymatic reduction (Barrett-Lennard et al., 1983; Grusak et al., 1990), an observation that draws into question the biological relevance of such a mechanism. Phenolics secreted by red clover (Trifolium pratense) roots enhanced the utilization of iron pools bound to negatively charged residues in the cell wall and provided clear-cut evidence for a role of iron deficiency-induced secretion of phenolics in iron acquisition (Jin et al., 2007). However, the mechanism(s) by which phenolics improve iron acquisition remains obscure. A possible scenario involves Fe(III) mobilization via chelation, reduction by FRO2, and subsequent uptake by IRT1. Alternatively, in analogy to the PS-uptake machinery, a phenolic compound transferred to the cytoplasmic side of the plasma membrane could be internalized by a putative transporter without prior reduction. In rice (Oryza sativa) roots, a phenolics efflux transporter has been identified from a reverse genetic screening for Cd-accumulating mutants (Ishimaru et al., 2011). In the phenolics efflux zero1 (pez1) mutant, precipitated iron accumulates in the apoplast, whereas plants overexpressing PEZ1 grow better on calcareous soil in which iron bioavailability is extremely low. These results indicate that, similar to what has been reported for red clover, excreted phenolics in rice play a role in the utilization of apoplastic iron. While these data clearly imply that secretion of phenolics aids in solubilizing precipitated iron, genetic evidence for such a mechanism in strategy I plants is lacking. Moreover, the variety of compounds that are excreted by plant roots makes an assessment of the underlying mechanism difficult.

Besides phenolics, some species synthesize and secrete large quantities of flavins. In particular, derivatives of riboflavin (Rbfl) are secreted in response to iron deficiency and are visible as yellowing of roots and the growth media. This response has been described over several decades for a variety of species, but a physiological role of flavin secretion is still under debate (Weinstein et al., 1954; Higa et al., 2010; Rodríguez-Celma et al., 2011b). Similar to the production of phenolics, the dominating Rbfl derivatives appear to differ between species. In sugar beet (Beta vulgaris) and spinach (Spinacia oleracea), excreted flavins have been identified as Rbfl 3’- and 5’-sulfates (Sustin et al., 1993, 1994), whereas in exudates of Medicago truncatula, 7-hydroxy-Rbfl, 7α-hydroxy-Rbfl, and 7-carboxy-Rbfl were detected (Rodríguez-Celma et al., 2011b). Neither the transporter that mediates the root export of phenolics or flavins nor the regulation of the synthesis of these compounds is understood in molecular terms.

In this article, we dissect iron deficiency-induced changes in the transcriptome of two model species, Arabidopsis (Arabidopsis thaliana) and M. truncatula and show that induction of the Rbfl and phenylpropanoid synthesis pathways is species dependent and mutually exclusive. We further provide genetic evidence that the production and secretion of phenolic compounds are critical for the assimilation from poorly soluble iron pools but are dispensable when iron is readily available. Taken together, our results show that the production and secretion of iron-binding compounds constitute an iron mobilization module that is an integral component of the reduction-based iron acquisition machinery.

RESULTS

Differential Gene Expression in Arabidopsis and M. truncatula Roots

Transcriptional profiling using the RNA sequencing (RNA-seq) technology identified transcripts from approximately 26,000 and approximately 44,000 genes in Arabidopsis and M. truncatula roots, respectively; subsets of 2,679 and 2,592 genes were differentially expressed between iron-sufficient and iron-deficient plants (Fig. 1; Supplemental Data Set S1). An ortholog search using the InParanoid algorithm (http://inparanoid.sbc.su.se) revealed
that 1,518 of 2,679 differentially expressed genes in Arabidopsis had orthologs in the M. truncatula genome, and 952 differentially expressed M. truncatula genes were orthologous to Arabidopsis genes (Supplemental Table S1). The overlap between these two subsets comprised 266 genes. Among this group, some well-described iron-responsive genes were found, such as FRO2, FE-DEFICIENCY INDUCED TRANSCRIPTION FACTOR1 (FIT), BRUTUS (BTS), BASIC HELIX-LOOP-HELIX38 (bHLH38), OLIGOPEPTIDE TRANSPORTER3 (OPT3), PHOSPHOENOLPYRUVATE CARBOXYLASE1 (PPC1), and ALCOHOL DEHYDROGENASE1 (ADH1). In M. truncatula, the closest orthologs to AtAHA2 and AtIRT1 in terms of sequence similarity were not responsive to iron. However, a P-type ATPase with 80.4% similarity to AtAHA2, which we named MtAHA2 (Medtr2g036650), was highly induced in M. truncatula roots. Further, a transporter of the ZIP family with high similarity to AtIRT1 at the protein level (Medtr4g083570, 58% identity), which we refer to as MtIRT1, was highly up-regulated in iron-deficient plants. Interestingly, in both species, orthologs of the Zn2+ exporter PLANT CADMIUM RESISTANCE2 (PCR2) were up-regulated, indicative of a conserved Zn detoxification route in addition to sequestration of Zn into the vacuole via METAL TOLERANCE PROTEIN2 (MPA2; Song et al., 2010; Yang et al., 2010). CORONATINE INSENSITIVE1 SUPPRESSOR, coding for an enzyme from the RbfI synthase complex, was up-regulated in M. truncatula and down-regulated in Arabidopsis. Also, in M. truncatula roots, genes involved in the first committed step in RbfI biosynthesis, the synthesis of 2,5-diamino-6-ribosylaminono-4(3H)-pyrimidione 5′-P and 3,4-dihydroxy-2-butanoate-4-P (MtGTPcII; Medtr2g009270), the generation of 6,7-dimethyl-8-(1D-ribityl)lumazine (MtDMRLS; contig_50382_2), and the final step of the pathway, the synthesis of RbfI (contig_57647_1), were strongly induced by iron deficiency (Supplemental Fig. S1). On the contrary, several genes encoding enzymes in the phenylpropanoid pathway were up-regulated in Arabidopsis but down-regulated in M. truncatula. In particular, the abundance of transcripts encoding proteins mediating two steps of the phenylpropanoid pathway, the conversion of caffeoyl-CoA to feruloyl-CoA (At4g34050), and the conversion of 6′hydroxy-feruloyl-CoA from feruloyl-CoA to the dioxygenase AtF6′H1; At3g13610) were significantly increased. Furthermore, the level of transcripts from genes involved in the Met cycle was strongly increased in iron-deficient Arabidopsis roots and decreased in M. truncatula (Supplemental Fig. S1).

Combining Coexpression and Promoter Analysis Predicts Coregulation of Iron Uptake and Secondary Metabolism

To decipher putatively coregulated components in the response to iron deficiency that are common in both species, we generated a hybrid coexpression network comprised of differentially expressed Arabidopsis and M. truncatula genes in which genes were represented by nodes and coexpression relationships by edges between nodes. A flow scheme of this procedure is shown in Figure 2. We first generated individual networks of differentially expressed genes based on pairwise comparison of their coexpression relationships with a Pearson coefficient > 0.75 using the MACCU toolbox (Lin et al., 2011; http://maccu.sourceforge.net/) for each species, and resulting networks had 1,130 and 1,418 nodes for Arabidopsis and M. truncatula, respectively. To merge the two coexpression networks, M. truncatula gene identifiers were substituted by their Arabidopsis orthologs when possible (748 genes). M. truncatula genes without Arabidopsis orthologs were kept in the network with their M. truncatula identifiers. This approach yielded a hybrid network that was slightly smaller than the sum of the two individual networks due to some overlap of nodes that were present in both networks. To reduce the complexity of this hybrid network, we focused on the most relevant components in this expression context by selecting four guide genes with critical functions in iron acquisition, FIT, FRO2, IRT1, and AHA2. Such a guide gene-based approach has been used previously to identify functionally related genes that present common regulatory patterns (Lisso et al., 2005; Persson et al., 2005). For this approach, we selected the first and second degree nodes connected to the four guide genes in the hybrid/ortholog network and obtained a working network with 404 nodes (Supplemental Table S2).

The combination of coexpression networks with information on DNA sequence motifs can increase the accuracy in the prediction of functional modules within the networks (Ma and Bohnert, 2007; Vandepoele et al., 2009; Heyndrickx and Vandepoele, 2012). For this refining approach, we attempted to identify common promoter motifs in genes from the working network. We assumed that the opposite regulation of the phenylpropanoid and RbfI pathways in the two species are likely to be dictated by cis-regulatory elements in the promoters of the genes involved in these pathways. Therefore, we used promoter sequences from genes encoding proteins that mediate the phenylpropanoid pathway in Arabidopsis and genes from the RbfI pathway in M. truncatula as input and searched for common consensus sequences using the MEME Suite toolbox and a threshold of P < 1E-5 (Bailey et al., 2009; http://meme.nbcr.net/meme/). We searched specifically for motifs that were over-represented in orthologs of one species but absent or largely less abundant in the other species. As negative controls, we used homologs that were not responsive to the iron regime (the full gene list is provided in Supplemental Table S3). It should be noted that for several M. truncatula genes, only very short promoter regions are available, which hampered the motif analysis for this subset of genes. This strategy identified a conserved putative cis-regulatory motif with the sequence hhbhAAACCAAv. To combine this sequence information with the coexpression data, we selected
for the final network nodes that were either connected by a stringent Pearson correlation coefficient (>0.90), contained the hhbhAAACCACAv motif, or had been identified as target of FIT (Fig. 2). This approach yielded a final 127-node network that, besides the guide genes (FIT, FRO2, IRT1, and AHA2), contained the iron transporter AtIREG2, the transcription factor bHLH38, the S-adenosylmethionine synthetase AtSAM1, and other genes with known functions in iron homeostasis, such as AtMTPA2 (Fig. 3). This final network also contained four genes from the phenylpropanoid biosynthesis pathway (AtF6’H1, AtHCT, At4CL2, and AtREF3), a putative phenolic efflux carrier, AtPDR9, and two genes from the Rbfl pathway, including MtGTPcII, which acts in the first step of Rbfl synthesis (Fig. 3). For MtDMRLS, which mediates the key step in Rbfl biosynthesis, no coexpression data are available, but the gene showed the strongest up-regulation in terms of reads per kb per million reads of all differentially expressed genes in *M. truncatula*. Interestingly, two genes encoding Hippocampus Abundant Transcript-Like proteins from the major facilitator transporter superfamily (Medtr1g092870 and Medtr1g092880) showed up-regulation and tight coexpression with MtGTPcII (Fig. 3). Furthermore, a 2-oxygenase family gene with 49% homology to F6’H1 at protein level (Medtr2g069300) also showed high up-regulation and tight coexpression with MtGTPcII (Fig. 3). All these three genes were also closely coexpressed with bHLH38 and FIT and are plausible candidates for the production and secretion of Rbfl derivatives. Notably, several uncharacterized protein kinases were found within this cluster, indicative of a sophisticated posttranslational regulation of cellular iron homeostasis in both species. Taken together, these data suggest that Rbfl synthesis in *M. truncatula* and the phenylpropanoid pathway in Arabidopsis are tightly associated with core responses to iron deficiency and constitute an integral part of the iron uptake machinery.

A subset of peroxidase genes linked by a common hub for both species, At1g05260/Medtr2g088770, were down-regulated in the cluster, indicating that the control of reactive oxygen species is important for the regulation of the iron deficiency response. In *M. truncatula*, we also observed a tightly correlated small subcluster of down-regulated genes related to N metabolism, such as nitrate reductase (At1g37130/Medtr5g059820) and Gln synthase (Medtr2g021250).

**The Biological Relevance of Excreted Compounds Depends on the Bioavailability of the Iron Source**

To address the biological relevance of the observed metabolic alterations, we analyzed, using media with different iron sources, the growth of mutants in which either the phenylpropanoid synthesis pathway or the

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*Figure 2. Flow scheme for the construction of an ortholog coexpression network comprising differentially expressed genes in roots of *M. truncatula* and Arabidopsis. See text for a detailed description. [See online article for color version of this figure.]*
secretion of the product(s) of this pathway was compromised. We selected AtF6’H1, catalyzing the last enzymatic step in the phenylpropanoid pathway, and AtPDR9, a putative transporter of phenolic compounds. Wild-type plants and the homozygous mutant lines f6’9h1-1 and pdr9-2 were grown on media containing available iron [avFe; 10 μM Fe(III)-EDTA, pH 5.5] or nonavailable iron [navFe; 10 μM FeCl3, pH 7.0]. Growth parameters and the expression of iron deficiency marker genes were assessed after 6 d. In order to rule out a possible influence of pH on plant growth, an additional control treatment was carried out with 10 μM Fe(III)-EDTA at pH 7.0. None of the genotypes showed an obvious phenotype (Supplemental Fig. S2).

Growing plants on media with available iron produced no visible differences between the genotypes (Fig. 4, A–C). However, dramatic differences were observed in the navFe treatment, where both mutants showed severe growth reduction and leaf chlorosis (Fig. 4, D–F). Similarly, no pronounced differences in leaf fresh weight between the genotypes under investigation were observed for the avFe treatments, while growth on media with navFe caused a 25% to 40% reduction in shoot fresh weight of both mutants relative to the wild type (Fig. 4G). Also, chlorophyll concentration did not differ significantly between the genotypes in avFe conditions, whereas growth on media with navFe caused a 60% to 80% decrease in chlorophyll concentration in f6’9h1-1 and pdr9-2 plants but no significant changes in the wild type (Fig. 4H). In both mutants, total iron content and leaf FER1 transcript levels were significantly lower than in the wild type when grown on navFe media, indicative of a lower iron status of the leaves in these genotypes (Fig. 4, I and J). The expression of AtF6’H1 and AtPDR9 was massively increased during growth under navFe conditions; no transcript was detected in the corresponding mutants (Fig. 4, K and L). Together, these results are indicative of a disturbed iron homeostasis of f6’9h1-1 and pdr9-2 mutant plants when grown with navFe.

Due to the fluorescent properties of phenolic compounds, their production and secretion can be easily monitored. In the wild type and when iron was available, fluorescence was weak and localized around the roots (Fig. 5A). Fluorescence was much reduced after taking plants off the growth medium, indicating that most fluorescent phenolic compounds were contained within the root. Some fluorescence was also apparent in f6’9h1-1 and pdr9-2 plants (Fig. 5A). When plants

Figure 3. Coexpression network of differentially expressed genes in Arabidopsis and M. truncatula as affected by iron deficiency. The network was constructed with the MACCU toolbox following the procedure shown in Figure 2. Square boxes and red edges indicate genes and coexpression relationships from the M. truncatula data set, and diamond-shaped boxes and blue edges denote genes and coexpression relationships derived from the Arabidopsis data set. Oval boxes refer to genes that were common in both networks. Yellow and blue boxes indicate up- and down-regulated genes, respectively. Gray boxes denote genes that were common in both data sets but oppositely regulated in M. truncatula and Arabidopsis. Green circles indicate the presence of the hhbhAAACCAA motif in the promoter, and yellow circles denote regulation by FIT.
were grown on media with navFe, the fluorescence signal in wild-type plants was strongly increased (more than 8-fold; Figure 5B), both within the root system and in the growth media after removing the plants (Fig. 5A). Conversely, in the f6'h1-1 mutant, the fluorescence was largely reduced relative to wild-type plants under the same conditions, both in the roots and the media (75%–95% decrease, respectively; Figure 5B). In pdr9-2 mutants, a strong fluorescence was detected in the roots (Fig. 5A), but after plants had
been removed, little fluorescence was observed in the growth media (70% decrease; Figure 5B). When calculating the percentage of fluorescence that remain in the agar after removal of the plants, for wild-type plants, 94% of the fluorescence remained in the media (Fig. 5C), but only 44% residual fluorescence was observed for pdr9-2 plants (Fig. 5C). This is indicative of a reduced efflux of fluorescent phenolic compounds in the pdr9-2 mutant.

For several flavin-producing species, including *M. truncatula*, it has been shown that high pH inhibits the exudation of flavin compounds (Susín et al., 1993; Rodríguez-Celma et al., 2011a). In *M. truncatula*, growing plants on media with high pH decreased the exudation of the major flavin compound (7-hydroxy-riboflavin) by 60% (Rodríguez-Celma et al., 2011b). Growing *M. truncatula* plants on iron-free media at pH 7.7 for 6 d caused a marked decrease in fresh weight of leaves, roots, and stems when compared with plants with sufficient iron. Decreases were significantly higher (*P* < 0.05) than those observed for plants grown on iron-free media at pH 5.5 (Fig. 6A). Importantly, the leaf iron concentration in plants grown in the −Fe/pH7.7 treatment was dramatically lower (75% and 60%) when compared with those of iron-sufficient and iron-deficient (−Fe, pH 5.5) plants, respectively (Fig. 6B). Both iron deficiency treatments caused visible leaf chlorosis (Fig. 6, C–E), but more severe symptoms were observed in the −Fe/pH7.7 treatment, where necrotic spots appeared (Fig. 6D). As expected, the media in which plants grew on iron-free media at pH 5.5 became yellow due to the secretion of flavins (Fig. 6F), whereas in the −Fe/pH7.7 treatment, no yellow color was observed (data not shown). Thus, decreasing the efflux of flavins greatly diminished the uptake of iron from iron pools that were otherwise available to the plant.

To further test the physiological significance of excreted flavins and phenolics, we grew both mutant genotypes (*f6′*h1-1 and *pdr9-2*) alone or in mixed populations alongside wild-type seedlings of Arabidopsis or *M. truncatula* under navFe growth conditions. If flavins and phenolic compounds excreted by wild-type plants were to improve iron mobilization, the increased bioavailability of iron is expected to rescue the mutant phenotype under these conditions. As shown in Figure 7, growth, chlorophyll, and iron content increased significantly for both mutant genotypes when grown alongside wild-type plants.

**DISCUSSION**

**Products of the Phenylpropanoid and Ribfl Biosynthesis Pathways Are Robustly Up-Regulated under Iron-Deficient Conditions**

In Arabidopsis, transcriptomic and proteomic approaches revealed a strong and robust up-regulation of the general phenylpropanoid pathway. For example, *AtF6′*H1 (*At3g13610*) was reported as being significantly up-regulated in four different transcriptomic studies (Colangelo and Guerinot, 2004; Dinneny et al., 2008; García et al., 2010; Yang et al., 2010), not including this article. iTRAQ analysis revealed a 5.8-fold accumulation of this enzyme at the protein level (Lan et al., 2011). The *AtF6′*H1-mediated step was the most strongly regulated reaction in the phenylpropanoid pathway, indicating that preventing feruloyl-CoA
entering lignin or suberin synthesis (via conversion into coniferaldehyde) under iron deficiency conditions is a key step to redirect the flow of carbon toward the production of phenolics. We previously showed that induction of the phenylpropanoid biosynthesis pathway results in increased levels of scopoletin in Arabidopsis roots upon iron starvation (Lan et al., 2011). It should be noted that, while it appears clear that feruloyl-CoA is hydroxylated in iron-deficient plants, we have no evidence that scopoletin is the compound that is excreted and is physiologically active. The fact that scopoletin may not be a good chelator of Fe(III) would lead to other possible explanations. In roots of M. truncatula, enzymes of the Rbfl synthesis pathway were robustly induced. Up-regulation of Rbfl biosynthesis in response to iron deficiency was associated with the production and exudation of Rbfl derivatives in M. truncatula (Rodríguez-Celma et al., 2011b). Proteomic studies revealed that GTfClII and DMRLS are de novo synthesized in roots of iron-deficient plants (Rodríguez-Celma et al., 2011a). In sugar beet, DMRLS was massively produced under iron-deficient conditions at the protein level, outcompeting most other iron deficiency-induced proteins in terms of abundance in the root protein profiles (Rellán-Alvarez et al., 2010). This study revealed that in M. truncatula, MtDMRLS and MtGTPcII were the most and second most strongly induced genes in terms of increase of transcript abundance, whereas no up-regulation of these genes was observed in Arabidopsis. It thus appears that different secondary metabolic pathways are induced in these species in response to iron deficiency, both of which are grossly up-regulated at the transcript (this study; Rodríguez-Celma et al., 2011a), protein, and metabolite level (Lan et al., 2011; Rodríguez-Celma et al., 2011b). It can further be stated that, in terms of quantity, these metabolic responses are big players in the reprogramming of the transcriptome and proteome in response to iron deficiency, as judged by analyses at the transcript, protein, and metabolite levels.

Induction of the Phenylpropanoid and Rbfl Biosynthesis Pathways Is Phylogenetically Separated in Arabidopsis and M. truncatula

In M. truncatula, genes encoding enzymes that mediate the biosynthesis of phenylpropanoids were either

Figure 6. Effects of pH-regulated Rbfl secretion on iron concentrations and growth in M. truncatula. A, Fresh weight of leaves, stems, and roots. B, Leaf iron concentration. C to E, Plants grown on +Fe (45 μM Fe(III)-EDTA, pH 5.5; C), iron-deplete media (0 μM Fe, pH 5.5; E), or iron-deplete media adjusted to pH 7.7 (0 μM Fe, pH 7.7; D). F, Growth media of iron-deficient plants after 6 d of growth, showing yellowing of the nutrient solution caused by Rbfl and Rbfl derivatives secretion. Measurements were performed after 6 d of treatment. Data represent means of five replicates, and error bars denote SE. Statistical differences were assessed by Duncan test (P < 0.05).

Figure 7. Partial rescue of the f6 h1-1 (A) and pdr9-2 (B) phenotype by growth alongside Arabidopsis or M. truncatula plants. Percentages of total chlorophyll (Chl), iron content, and leaf fresh weight (FW) of mutant plants grown alone or in mixed population with wild-type (col-0) or M. truncatula (Mt) plants when compared with col-0 under navFe conditions. Significant differences (asterisks) were assessed by t test (P < 0.05).
unchanged or down-regulated upon iron deficiency. Vice versa, the synthesis of Rbf1 was not activated in iron-deficient Arabidopsis roots (Supplemental Fig. S1). Ectopic expression of the Arabidopsis transcription factors AtbHLH38 and AtbHLH39 caused increased iron deficiency independent Rbf1 synthesis in tobacco (*Nicotiana tabacum*; Vorwieger et al., 2007), suggesting that Rbf1 synthesis is under the control of FIT-bHLH38/bHLH39 heterodimers that regulate a large subset of iron responsive genes, including *FRO2*, *IR1*, and *AHA2* (Colangelo and Guerinot, 2004; Jakoby et al., 2004; Yuan et al., 2008). Interestingly, overexpression of AtbHLH38 or AtbHLH39 in Arabidopsis or in hairy roots of tomato (*Solanum lycopersicum*) did not result in increased Rbf1 production, indicating species-specific regulation of this pathway (Vorwieger et al., 2007). While the differential regulation of these pathways is most likely explained by the presence of different cis-element in the promoters of the coding genes, this does not provide a physiological explanation for the mutually exclusive induction of the pathways. The ability to produce Rbf1 appears to be genetically defined but can vary across species and cultivars within one genus (Welkie, 2000). The most plausible explanation is that the characteristics of the interactions of Rbf1 derivatives and phenolics with iron may differ and evolutionary pressure for one or the other solution derived from the adaptation to a certain set of soil conditions. However, this assumption needs to be experimentally addressed.

Production of Phenylpropanoids and Flavins Is Tightly Linked to Core Genes of the Iron Acquisition Machinery

Coexpression analysis in combination with information on putative cis-regulatory elements and known transregulation revealed that genes involved in the phenylpropanoid pathway are closely associated with genes encoding proteins that mediate iron acquisition (*FIT*, *FRO2*, *IRT1*, and *AHA2*) and thus appear to constitute an integral part of the reduction-based iron uptake mechanism. F6H is converted into 6-hydroxy feruloyl-CoA (F6H) in a pathway that includes eight enzymes that are encoded by nine genes (Supplemental Fig. S1). Promoters of five genes in this pathway (corresponding to five different steps in the pathway) contain the hhbhAAACCAAv motif (At2g37040, At3g53260, At2g30490, At5g48930, and At3g13610). Three of these steps are regulated by AtFIT (Colangelo and Guerinot, 2004). The conversion of caffeoyl-CoA to feruloyl-CoA is dependent on S-adenosyl- L-Met. One out of three genes encoding S-adenosyl-L-Met synthetases, *SAM1*, is highly up-regulated upon iron deficiency and contained the hhbhAAACCAAv consensus sequence. The last enzymatically catalyzed step of the phenylpropanoid pathway, the conversion of feruloyl-CoA to F6H, is mediated by an iron-containing protein, AtF6H1 (At3g13610). Iron from the plastids, probably released from ferritin and transported into the cytoplasm via AtIREG2, supports AtF6H1 synthesis under iron-limiting conditions. Phenolic compounds, such as scopoletin and/or related phenylpropanoids, are

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**Figure 8.** Mechanisms and regulation of the reduction-based iron acquisition mechanism. The transcription factors FIT and bHLH38/39 regulate proton extrusion, Fe(III) reduction, and uptake of Fe(II). In addition, FIT and bHLH38/39 control the mutually exclusive induction of the phenylpropanoid pathway in Arabidopsis and flavin synthesis in *M. truncatula*, leading to the production of putatively iron-binding compounds (IBCs) that are excreted via specific transporters in the plasma membrane. The consensus sequence hhbhAAACCAAv (indicated by green circles) is likely to be involved in the regulation of the pathways. Iron-binding compounds chelate Fe(III) from non-soluble ferric hydroxides. The resulting chelates are split by reduction via FRO2, and the released ferrous iron is taken up via IRT1. Yellow circles indicate genes that are regulated by AtFIT.
presumably excreted by the AtFIT-regulated, plasma membrane-bound transporter AtPDR9, increasing the solubility of iron pools with low bioavailability in the apoplast or in the rhizosphere. Also, another important component for the mobilization of iron, the proton-ATPase AtAHA2, is tightly coregulated with the genes shown in Figure 3. AtAHA2 is AtFIT regulated, strongly induced by iron deficiency, and responsible for iron deficiency-induced rhizosphere acidification (Santi and Schmidt, 2009; Ivanov et al., 2012). Less information is available regarding the potential coregulation of Rbf synthesis genes with core genes of the iron acquisition machinery in *M. truncatula*. Coexpression analysis showed that, similar to Arabidopsis, expression of the genes encoding enzymes that mediate Rbf synthesis is tightly linked to the three core components of reduction-based iron acquisition, rhizosphere acidification via MtAHA2 (Medtr2g036650), reduction of Fe(III) by MtFRO2 (Medtr7g038510), and uptake of Fe(II) by MtIRT1 (Medtr4g083570). Unfortunately, the lack of sequence information does not allow a final conclusion, but it is tempting to suggest that the hbbhAAACCAAAv motif is controlling the different regulation of the phenylpropanoid pathway between the two species. In any case, it can be stated that the production of species-specific iron deficiency-elicted compounds (phenolics or flavins) is an integral part of the Strategy I type iron uptake mechanisms as first formulated by Römheld and Marschner in 1983.

Secretion of Phenolics and Flavin Compounds Is Essential for the Acquisition of Iron Pools with Low Bioavailability

The role of root exudates in iron uptake has been debated for two decades. Several possibilities, ranging from allelopathic functions, providing cofactors for the activity of enzymes, or modification of the rhizosphere biome have been proposed (Dakora and Phillips, 2002; Cesco et al., 2010). Our data support the hypothesis that Rbf derivatives and flavins function as iron-binding compounds in the utilization of otherwise unavailable iron pools, constituting a key component of the reduction-based strategy of iron acquisition (Fig. 8). For phenolics, experimental evidence for such a function in red clover was recently provided (Jin et al., 2007). Our results support this assumption and provide genetic evidence that compromising the AtF6-H1-mediated step in the phenylpropanoid biosynthesis results in poor performance on media with high amounts of nonavailable iron. We further show that AtPDR9 is critical in this process, most likely by mediating the secretion of phenolic substances. In addition, our data suggest that these compounds are of particular importance when iron is present in fair amounts but not readily available, a situation that occurs in most aerobic soil systems. In *M. truncatula*, hampering the iron deficiency-induced exudation of flavins by increasing the external pH lead to a dramatic reduction in leaf iron concentration, suggesting a role of flavins in the iron acquisition mechanism. Most importantly, we showed that growth of mutants defective in the production or secretion of phenolics can be partially rescued by growing mutant seedlings in close vicinity of either Arabidopsis wild-type or *M. truncatula* seedlings on media with nonavailable iron. This shows that both secreted flavins and phenolics are beneficial for iron acquisition. It can be further assumed that the importance of this metabolic adaptation is easily overlooked under laboratory conditions, where only two iron availability extreme conditions are usually applied: iron is either supplied in highly available chelated forms (i.e. 40 μM Fe(III)-EDTA) or is not supplied at all, further impeding any iron remobilization from the media by using the Fe(II) chelator ferrozine. It can be assumed that exudation of iron-binding compounds may contribute significantly to the iron efficiency of a species or cultivar under natural conditions, where some iron is usually present although its availability is compromised or restricted by pH or other factors.

**MATERIALS AND METHODS**

**Plant Growth Conditions**

Arabidopsis (*Arabidopsis thaliana*) plants were grown in a growth chamber on an agar-based medium as described by Estelle and Somerville (1987). Seeds of the accession Columbia-0 and *fl*/*fl*-1 mutant seeds (SALK_132418C) were obtained from the Arabidopsis Biological Resource Center (Ohio State University). Both mutants were characterized previously (Ito and Gray, 2006; Kai et al., 2008). Seeds were surface-sterilized by immersing them in 5% (v/v) NaOCl for 5 min and 70% (v/v) ethanol for 7 min, followed by four rinses in sterile water. Seeds were placed onto petri dishes and kept for 1 d at 4°C in the dark, before the plates were transferred to a growth chamber and grown at 21°C under continuous illumination (80 μmol m⁻² s⁻¹; Phillips TL lamps). The medium was composed of (in mM) KNO₃ (5), MgSO₄ (2), Ca(NO₃)₂ (2), and KH₂PO₄ (2.5) and (in μM) H₂BO₃ (70), MnCl₂ (14), ZnSO₄ (1), CuSO₄ (0.5), NaCl (10), Na₂MoO₄ (0.2), and Fe-EDTA (40), as well as 4.7 mM MES, supplemented with Suc (43 mM) and solidified with 0.4% Gelrite pure (Kelco). The pH was adjusted to 5.5. For RNA-seq analysis, plants were precultivated for 10 d in complete media and then transferred to fresh agar medium either with 40 μM Fe(II)-EDTA (+Fe plants) or without iron and with 100 μM 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine sulfonate (ferrozine; –Fe plants) to trap residual iron. Plants were grown for 3 d on iron-free medium before analysis. Quantitative PCR analysis of plants was performed after 7 d of precultivation to fresh agar medium with the composition described above, either with 10 μM Fe(II)-EDTA (-Fe/Fe) or with 10 μM FeCl₃, and pH 7.0 (naFe). In the latter treatment, 4.7 mM MOPS buffer was used instead of MES to adjust the pH. Plants were grown for 7 d on the different media before analysis.

Medicago truncatula ‘Jemalong’ plants were grown hydroponically in a controlled environment chamber as described previously (Rodríguez-Celma et al., 2011a). Seeds were imbibed overnight in distilled water and germinated on filter paper for 3 d in darkness at 100% relative humidity. Seedlings were grown for an additional 2-week period in one-half-strength Hoagland nutrient solution (pH 5.5) with 45 μM Fe(II)-EDTA. Plants were then transferred to 10-liter plastic containers (six plants per container) containing one-half-strength Hoagland nutrient solution, and treatments were imposed. Control (iron-sufficient; +Fe) plants were grown with 45 μM Fe(II)-EDTA (pH 5.5), and iron-deficient plants were grown with no added iron (0 μM iron; –Fe) and CaCO₃ to trap residual iron (pH 7.7; –Fe). Plants were grown for 6 d on iron treatments before analysis.

To evaluate the physiological significance of the excreted flavins and phenolics, *fl*/*fl*-1 and *pdr*9-2 plants were transferred after 7 d of precultivation to fresh naFe media (as described above) and grown in mixed populations alongside Arabidopsis plants (four wild-type plants surrounding one mutant plant) or *M. truncatula* (four mutant plants surrounding one *M. truncatula* seedling).
RNA-Seq

For RNA-seq, total RNA was extracted from the root apex of both plant species using the RNeasy plant mini kit (Qiagen), following the manufacturer’s instructions. For analysis, equal amounts of total RNA were collected and cDNA libraries for sequencing were prepared from total RNA following the manufacturer’s protocol (Illumina). The cDNA libraries were subsequently enriched by PCR amplification. The resulting cDNA libraries were subjected to sequencing on a single lane of an Illumina Genome Analyser II. RNA-seq and data collection were done following the protocol of Mortazavi et al. (2008). The length of the cDNA library was maintained from 250 to 300 bp with a 5'-adapter of 20 bp and a 3'-adapter of 33 bp at both ends. Eventually, the fragment length range of the cDNA was 200 to 250 bp.

Quantitative Reverse Transcription-PCR

Total RNA was isolated using the RNeasy mini kit (Qiagen) and treated with DNase using the TURBO DNA-free kit (Ambion) as indicated by the manufacturer. cDNA was synthesized using DNA-free RNA with oligo-dT primer and Superscript II reverse transcriptase (Invitrogen). After incubation at 50°C for 1 h and subsequently at 70°C for 15 min, 1 μL of RNase H was added and incubated for 20 min at 37°C. The cDNA was used as a PCR template in a 20-μL reaction system using the SYBR Green PCR Master Mix (Applied Biosystems) with programs recommended by the manufacturer in an ABI Prism 7500 sequence detection system (Applied Biosystems). Three independent replicates were performed for each sample. The ΔΔCT (cycle threshold) method was used to determine the relative amount of gene expression (Livak and Schmittgen, 2001), with the expression of elongation factor 1 used as an internal control. A list of all primers used in this study is provided in Supplemental Table S4.

Detection of Phenolic Compounds

Production and/or secretion of phenolic compounds into the media was monitored using fluorescence in a BioSpectrum 600 imaging system (UVP). Pictures were taken before and after removing the plants from the agar using 365 nm as excitation wavelength, SYBR Gold 485 to 655 nm as emission filter, and 9 s as exposure time. Image analysis was performed using Adobe Photoshop CS4 software. For quantification of fluorescent compounds, zenithal view pictures were used: Equal areas were measured before and after removal of the plant from the media for each individual plant, and pixel average intensity (p.a.i.) was used as relative fluorescence quantification. For background correction, fluorescence in an equal area of an unused agar plate was measured. Statistical differences were assessed by applying the Duncan test (P < 0.05, n = 5).

Uptake of Insoluble Iron Compounds

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