Iron Uptake and Transport in Plants: The Good, the Bad, and the Ionome

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Contents

1. Introduction 4553
2. Fe Uptake 4553
2.1. Reduction-Based Strategy 4554
2.1.1. Toxic Metals and Fe Deficiency 4554
2.1.2. Sequestration and Buffering of Metal Influx 4555
2.1.3. Uptake of Apoplastic Fe 4556
2.2. Chelation-Based Strategy 4556
2.2.1. Yellow-Stripe 1 4556
2.2.2. Chelation and Toxic Metals 4556
2.3. Combination of Reduction and Chelation Strategies 4557
3. Long-Distance Fe Transport 4557
3.1. Xylem 4558
3.2. Phloem 4558
3.2.1. NA and YSLs 4558
3.2.2. Nicotianamine 4558
3.2.3. NA Levels and Fe Localization 4559
3.2.4. NA and Ni Tolerance 4559
3.2.5. YSLs and Long-Distance Fe Transport in *Arabidopsis* 4559
3.2.6. OsIRT1 4559
3.2.7. ITP 4559
3.3. Control of Long-Distance Fe Transport in Barley 4560
4. Fe and Seeds 4560
4.1. Loading of Fe 4560
4.1.1. NA, YSL1, and YSL3 4560
4.1.2. OPT3 4561
4.2. Storage of Fe 4561
4.2.1. VIT1 4561
4.2.2. NRAMP3 and NRAMP4 4561
4.2.3. FER2 4562
4.3. Fe Bioavailability for Humans 4562
5. Intracellular Fe 4563
5.1. Plastids 4563
5.1.1. FRO7 4563
5.1.2. PIC1 4563
5.1.3. Ferritin 4563
5.2. Mitochondria 4563
5.2.1. Ferritin and Frataxin 4563
5.2.2. ATM3 4564
5.3. Vacuole 4564
5.3.1. NA and the Vacuole 4564
5.3.2. VIT1, NRAMP3, and NRAMP4 4564
6. Conclusions and Future Directions 4564
7. Acknowledgments 4565
8. References 4565

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1. Introduction

Fe is essential for plant growth. At the same time, Fe is highly reactive and toxic via the Fenton reaction. Consequently, plants tightly control Fe homeostasis and react to Fe deficiency as well as Fe overload. The ability of plants to respond to Fe availability ultimately affects human nutrition, both in terms of crop yield and the Fe concentration of edible tissues. Thus, elucidating the mechanisms of Fe uptake and transport is essential for the breeding of crops that are more nutrient rich and more tolerant of Fe-limited soils.

This review covers Fe transport and homeostasis in plants, focusing on the research published in the past five years. Because Fe transporters often have a broad range of substrates, we also examine the relationship between Fe and the toxic metals that often accompany Fe uptake, namely, Cd, Co, and Ni. We begin by discussing Fe uptake into the root, then long-distance transport to the shoot, and finally, the loading of Fe into seeds. And, because Fe is essential to the metabolism of the mitochondria and chloroplast, we also look at the recent discoveries in Fe transport and homeostasis at the intracellular level. We do not cover the regulation of these transporters because this topic has been recently reviewed.[1]

2. Fe Uptake

Plants mainly acquire Fe from the rhizosphere. Although Fe is one of the most abundant metals in the earth's crust, its availability to plant roots is very low. Fe availability is dictated by the soil redox potential and pH. In soils that are aerobic or of higher pH, Fe is readily oxidized, and is predominately in the form of insoluble ferric oxides. At lower pH, the ferric Fe is freed from the oxide and becomes more available for uptake by roots. Because 30% of the world's cropland is too alkaline for optimal plant growth,[2] and some staple crops, like rice, are especially susceptible to Fe deficiency,[3] much research has focused on how plants cope with Fe limitation.

The responses to Fe deficiency include changes in root morphology[2] and up-regulation of genes involved in Fe uptake.[4,5] In fact, in *Arabidopsis thaliana,* up to 85% of the genes expressed in particular regions of the root are differentially regulated by Fe.[4] This transcriptome analysis was made possible by the isolation, via fluorescence activated cell sorting analysis, of cells from specific root layers that were expressing GFP under the control of cell-specific

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promoters.[6] The transcript levels within each layer were then measured via microarray analysis. This allows detection of differential expression profiles among specific cell types that cannot be seen when the root as a whole is examined. Large transcriptional differences between layers in response to Fe deficiency were identified, indicating layer-specific roles (Figure 1). The expression of genes related to metal transport and chelation was increased in the epidermis, while genes related to root hair morphogenesis were down-regulated; in the stele, genes associated with signaling and stress responses were up-regulated. These results suggest that sensing of Fe levels and control of the Fe deficiency response occurs in the vasculature, while regulation of Fe levels in the root is facilitated by modulating uptake in the epidermis.

When these Fe deficiency-induced changes were compared with the response to salt stress, it was found that the vast majority of the transcriptome is altered by environmental stress and that these changes are most dramatic in the root epidermis. Interestingly, there is also a small set of genes unaffected by either stress; this core may define the essential features of each cell type, and mediate the appropriate transcriptional responses to environmental stresses. Of the changes in the epidermis, two specific strategies of Fe uptake have been identified in plants. Nongraminaceous plants reduce Fe^{3+} via a membrane-bound reductase to make it accessible for uptake by a Fe^{2+} transporter, while grasses secrete phytosiderophores (PSs) that readily bind Fe^{3+}, and the Fe−PS complexes are then transported back into the roots.

2.1. Reduction-Based Strategy

Components of the reduction strategy have been described in many nongraminaceous species,[7−10] but it is best characterized in *Arabidopsis* (Figure 2). In response to Fe deficiency, protons are released into the rhizosphere, by AHA H^+-ATPases expressed in the epidermis.[11,12] This lowers the soil pH, making Fe more soluble. While AHA1, AHA2, and AHA7 are all up-regulated in the root epidermis in response to Fe deficiency,[4,5] AHA2 is the primary root H^+-ATPase in the Fe deficiency response.[13] The expression level of *AHA2* is highest among the three, and only the loss of AHA2 was found to reduce rhizosphere acidification during Fe deficiency.[13]

The NADPH-dependent ferric chelate reductase, AtFRO2, then reduces Fe^{3+} to Fe^{2+}. Electrons are transferred from $NADH^+$ across four heme groups to Fe in the rhizosphere.[14] This appears to be the rate-limiting step in Fe uptake in *Arabidopsis*.[15] In fact, the transgenic overexpression of ferric chelate reductases in the roots of rice, tobacco, and soybeans has been successful in increasing tolerance to Fe-limiting conditions.[16−18]

Once reduced, Fe(II) can then be transported into the root epidermal cells by the divalent metal transporter AtIRT1.[19−21] AtIRT1 also transports Zn, Mn, Cd, Co,[22,23] and Ni.[24] Additional root epidermal transporters for these metals have not yet been identified; but in the *irt1-1* loss of function mutant, shoot accumulation of Fe, Mn, Zn, and Co decreases significantly, and the plants become Cd tolerant,[20,25] suggesting that AtIRT1 is a primary transporter for these metals under Fe deficiency. A similarly broad range of metals was found to be transported by the tomato orthologs LeIRT1 and LeIRT2, which complement yeast mutants defective in the uptake of Fe, Zn, Mn, and Cu.[7] Thus, the Fe deficiency response also leads to the uptake of metals other than Fe, all of which are potentially toxic.

2.1.1. Toxic Metals and Fe Deficiency

The presence of Cd and Co have been shown to exacerbate Fe deficiency. Cd interferes with Fe movement from root to

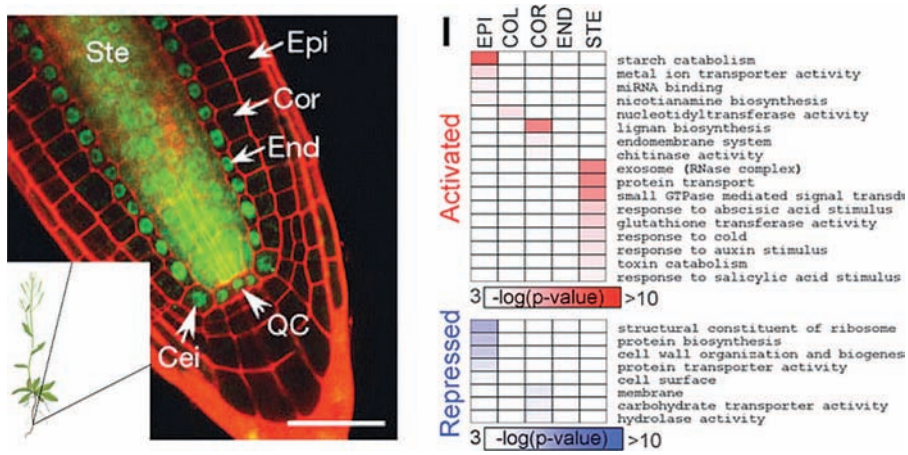

Figure 1. Transcriptional changes in response to Fe deficiency in specific root layers. (A) Root layers marked by propidium iodide staining of the cell wall (red) and expression of GFP in the stele and endodermis. Epi = epidermis; Cor = cortex; End = endodermis; Ste = stele; QC = quiescent center; Cei = cortex/endodermis initial.[153] (B) Enriched Gene-Ontology categories: miRNA, microRNA; RNase, ribonuclease; GTPase, guanosine triphosphatase.[4] Reprinted with permission, copyright 2001 Nature Publishing Group (ref 153), 2008 The American Association for the Advancement of Science (ref 4).

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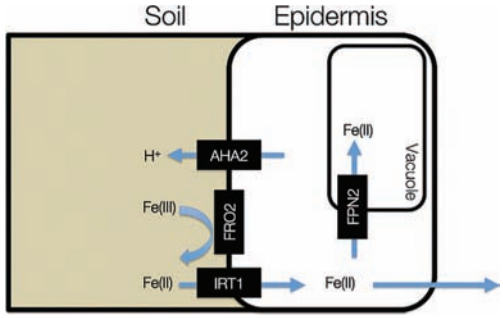

Figure 2. Fe uptake from soil, reduction strategy. In response to Fe deficiency in nongraminaceous species, protons are exuded into the rhizosphere, most likely by the AHA2 H^+-ATPase. The ferric chelate reductase FRO2 is expressed, reducing Fe(III) to Fe(II), which can then be transported into the root epidermis by the divalent metal transporter IRT1. Within the epidermis, the divalent metal effluxer FPN2 is expressed during Fe deficiency on the vacuolar membrane and may serve to buffer Fe uptake by sequestering excess free Fe in the vacuole. The Fe may be bound by phytate or NA in the vacuole. Fe presumably moves out of the epidermis via the plasmodesmata.

shoot; Cd treatment of *Brassica napus* produces a dramatic increase in Fe accumulation in the roots, while the shoots become Fe starved.[26] The level of Fe in the xylem and phloem saps also decreased significantly, while the level of Cd increased. This suggests that the presence of Cd impairs Fe access to the phloem, rather than uptake, at least in *B. napus*. A similar phenotype was seen in mung bean seedlings treated with Co: Fe uptake increased, but Fe was unable to move from the root to the shoot.[27] Additionally, the Fe deficiency response in *Arabidopsis* is up-regulated in Co-treated plants (Morrissey and Guerinot, unpublished data).

The uptake of Cd during Fe deficiency is of special interest, because Cd is considered one of the most toxic crop contaminants. Cd has the opposite bioavailability profile compared with Fe: in aerobic conditions where Fe is oxidized and insoluble, Cd becomes more soluble.[28] Thus, the soil conditions that trigger IRT1 expression also enhance Cd availability for uptake by IRT1. Because ingestion of plants is the primary route of Cd exposure for nonsmokers,[29] efforts have been made to understand and modify the selectivity of IRT1. Expression of mutagenized IRT1 in yeast found that amino acid substitutions in the first third of the protein, especially the first extracellular loop, could modulate selectivity of Fe, Zn, Mn, and Cd.[30] Mutations that destroyed IRT1 function were almost exclusively found in the two α-helices that compose the fourth and fifth transmembrane domains; it is believed that this region forms a metal binding pocket that facilitates ion movement across the membrane. Ultimately, this demonstrates that the broad substrate range of Fe transporters can be adjusted, producing variants optimized to biofortify crops while excluding toxic contaminants.

2.1.2. Sequestration and Buffering of Metal Influx

The influx of toxic metals via IRT1 is counteracted in part by the expression of the metal effluxer FPN2 (IREG2) during Fe deficiency. FPN2 is localized to the vacuolar membrane[24] in the two outermost root layers of *Arabidopsis* and appears to sequester metals in the vacuole. When expressed in yeast, FPN2 confers tolerance to Ni[24] and Co (Morrissey and Guerinot, unpublished data). The Fe-regulated expression pattern and root localization of *FPN2* suggests that it serves as an adaptation to the influx of Ni and Co during Fe deficiency; accordingly, the loss of FPN2 results in increased sensitivity to Ni and Co (Morrissey and Guerinot, unpublished data). A similar role has been described for MTP3, which is also Fe-regulated, localizes to the vacuolar membrane, and is thought to sequester Zn in the vacuole during Fe deficiency.[31]

Fe itself is highly reactive and potentially toxic. It is unclear which ligands bind Fe after transport, but buffering Fe uptake is clearly important. Indeed, a phenotype long attributed to phosphorus deficiency was found to be caused by Fe toxicity.[32] Inhibition of root elongation in the *Arabidopsis* ecotype Col-0 was known to occur during phosphorus limitation and was believed to be caused by a phosphorus-sensing regulatory pathway. Instead, it was recently shown that root growth is restored during phosphorus deficiency by simply removing Fe from the growth medium. The inhibition was actually caused by the toxic effects of Fe that was likely no longer complexed with phosphate, greatly increasing its bioavailability. The influx of Fe via IRT1 may be buffered in the outer root layers by the expression of FPN2, which transports Fe (unpublished data), presumably

sequestering excess Fe in the vacuole. A similar buffering role has been proposed for IRT2, which transports Fe and Zn[33] and has been localized to vesicles in the epidermis.[34]

2.1.3. Uptake of Apoplastic Fe

Another aspect of the Fe deficiency response in nongraminaceous plants is the secretion of phenolic compounds into the rhizosphere[2] and the uptake of apoplastic Fe. It has been observed that as much as 75% of Fe in the roots is attached to the apoplast,[35] because the negatively charged carboxyl groups of the cell walls serve as a cation sink.[2] This pool decreases when a plant becomes Fe deficient, suggesting mobilization into the symplast.[36] How this Fe is taken up is unclear, but it was recently found that phenolics exuded by the root in response to Fe deficiency facilitate the utilization of apoplastic Fe, and the recovery from Fe deficiency.[37] Phenolics secreted by red clover roots were shown to efficiently strip Fe from purified cell walls. To determine whether this is an essential component of the Fe deficiency response, phenolics were filtered from the liquid growth medium by constant recirculation through a resin column. Under normal growth conditions, the level of apoplastic Fe was found to decrease in response to Fe limitation, and although initially chlorotic, the leaves began to regreen. The filtering of phenolics, however, resulted in no decrease in apoplastic Fe, while the Fe concentration in the shoot became lower. This produced plants that were much more chlorotic and could not recover from Fe deficiency. Ferric chelate reductase activity and proton extrusion also increased, but this alone was not able to counteract the severe chlorosis. Thus, phenolic-mediated mobilization of apoplastic Fe is an integral part of the Fe deficiency response (at least in red clover), although it is unclear how phenolics facilitate uptake. Perhaps phenolics mediate extraction of Fe from the negatively charged cell walls, allowing transport into the root symplast. Whether an Fe−phenolic complex is directly transported into the root is unclear, because potential Fe chelate transporters have not yet been characterized in the root epidermis of nongrasses. A candidate would be the Fe−nicotianamine (NA) transporter, AtYSL3, which is up-regulated in the root epidermis under Fe deficiency,[4] although it has not been tested for Fe−phenolic transport.

2.2. Chelation-Based Strategy

Grasses depend on the uptake of Fe chelated by soluble siderophores with a high affinity for Fe^{3+}.[2] In response to Fe deficiency, mugineic acid (MA) family PSs are synthesized from L-methionine and released from the root epidermis (Figure 3), perhaps via anionic channels or vesicles.[38] In barley, the genes required for sulfur uptake, methionine synthesis, and PS synthesis are dramatically up-regulated in the first 24 h of Fe deficiency.[39] In rice, expression of the *OsIRO2* transcription factor increases dramatically over the course of the first 5 days of Fe starvation and is believed to activate the expression of genes related to PS synthesis and Fe uptake.[40]

The resulting Fe(III)−PS complexes are readily transported into the root epidermis via a high-affinity uptake system.[41] The chelation strategy is less sensitive to pH than the reduction strategy, and there is a strong correlation between the volume of PS released and resistance to Fe limiting soils. For instance, barley, which is adapted to alkaline soils, releases a much greater volume of PSs than most rice species,[39] which are adapted for growing in anaerobic soils where Fe is more soluble. Indeed, in *Oryza sativa* var. *japonica*, which grows poorly on calcareous soils, the overexpression of enzymes in the barley PS synthesis pathway greatly increased PS secretion.[3] This resulted in a 4-fold increase in grain yield by rice grown on Fe-limited soil.

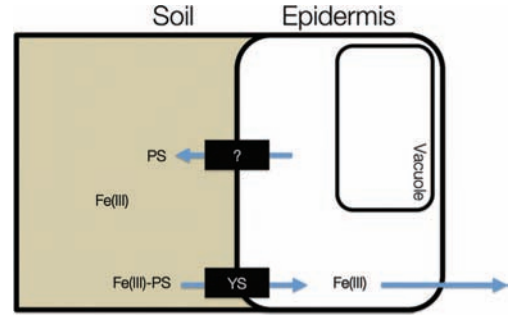

Figure 3. Fe uptake from soil, chelation strategy. In response to Fe deficiency, PSs are synthesized and secreted into the rhizosphere. The PS readily chelate Fe^{3+}, and the Fe(III)−PS complex is transported into the root by members of the YS/YSL family (YS1 in maize and barley, and OsYSL15 in rice).

2.2.1. Yellow-Stripe 1

The most well-characterized Fe−PS transporter is the maize oligopeptide transporter (OPT) family member, ZmYS1. ZmYS1 is expressed in roots in response to Fe deficiency, and its loss results in decreased Fe uptake, and a constitutive Fe deficiency response; in the leaf, the decrease in Fe-containing proteins impairs chlorophyll synthesis, resulting in a yellowing between the veins (interveinal chlorosis).[42,43] The transport of Fe−PS by ZmYS1 appears to be well-adapted to high pH solutions, the type of environment that is Fe limiting for plants. While reduction of Fe^{3+} becomes more difficult with increasing soil pH due to the pH optimum of the reductase, the expression of ZmYS1 in oocytes showed that transport of Fe−PS is still efficient at very high pH.[44]

2.2.2. Chelation and Toxic Metals

The maize PS deoxymugineic acid (DMA) also readily chelates other metals, and ZmYS1 has been shown to transport PS complexed with Zn, Cu, and Ni at the same rate as Fe−PS.[44] ZmYS1 also transported Ni, Fe(II), and Fe(III) complexed with the PS precursor NA. Thus, like IRT1 in nongraminaceous species, ZmYS1 also serves as gateway for a broad range of metals, including those toxic to plants and humans. Interestingly, HvYS1, which is 95% similar to ZmYS1, only transports Fe−PS.[45] Domain swapping between the two transporters showed that the extracellular loop between the sixth and seventh transmembrane regions provided the selectivity.[46] When the loops were synthesized *in vitro*, the HvYS1 peptide formed an α helix in solution, while the ZmYS1 peptide remained flexible, suggesting that this structural difference dictates substrate specificity.

Maize DMA also appears to bind Cd in soil, and while Cd disrupts Fe homeostasis in maize, the Cd−PS complex is not readily transported by ZmYS1.[47] However, the presence of Cd in growth media up-regulates the Fe deficiency response in maize and results in reduced Fe levels in the xylem sap. At the same time, the level of Cd uptake in maize was similar in wild-type and *ys1* mutants, suggesting

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Cd primarily enters the roots through another transporter,[47] perhaps a Ca transporter or channel or a divalent metal transporter similar to IRT1. An IRT1 ortholog was recently identified in barley, and heterologous expression in yeast indicates that it can transport Fe, Mn, Zn, and Cd.[48] Like AtIRT1, HvIRT1 is up-regulated in response to Fe deficiency, but the tissue-specific localization has yet to be determined. Thus, it is premature to say whether barley transports free Fe into the root epidermis like nongrasses. HvIRT1 is also up-regulated under Mn deficiency and higher expression of HvIRT1 correlated with increased Mn uptake by a Mn efficient genotype of barley.[48]

2.3. Combination of Reduction and Chelation Strategies

Another graminaceous species, rice, combines components of the reduction strategy seen in nongraminaceous plants with Fe−PS uptake. Of the 18 yellow-stripe like (*YSL*) genes in rice, OsYSL15 is the primary transporter responsible for uptake of Fe−PS from the rhizosphere.[49,50] OsYSL15 is up-regulated in response to Fe deficiency and is expressed on the plasma membrane in the root epidermis, in addition to the stele, flowers, and developing seeds. Two *osysl15* insertional mutants exhibited chlorotic phenotypes under Fe deficiency and had reduced Fe concentrations in their shoots, roots, and seeds.[50] Reducing OsYSL15 expression with RNAi resulted in severe germination defects, indicating an important role in Fe homeostasis, although these could relate more to Fe loading of seeds than Fe−PS uptake by roots.[49]

But, as mentioned above, rice produces much less PS than maize and barley, making it less tolerant of calcareous soils. Rice compensates by expressing the divalent metal transporters OsIRT1 and OsIRT2 in the root epidermis in response to Fe deficiency. Both are similar to AtIRT1 and transport Fe when expressed in yeast.[51] In fact, in rice mutants without the ability to synthesize PSs, these Fe(II) transporters were found to be dramatically up-regulated in the roots: 30-fold for OsIRT1 and 64-fold for OsIRT2.[52] This compensated in terms of Fe uptake in waterlogged soil where Fe^{2+} is readily available; surprisingly, the mutant plants even accumulated more Fe in roots and shoots than wild-type under these conditions. In aerobic soils (where Fe is limiting) and in hydroponic solution where only Fe^{3+} was supplied, these mutant plants died; thus, the reduction strategy alone is inadequate in Fe^{2+}-limited conditions.

The elevated expression of OsIRT1 and OsIRT2 in rice mutants lacking PSs also resulted in increased concentrations of Zn, Cu, Mn, and Cd in the shoot,[52] while ectopic expression of *OsIRT1* increased Fe, Zn, and Cd accumulation.[53] This suggests these transporters have a similar range of substrates as AtIRT1. When expressed in yeast, both OsIRT1 and OsIRT2 transport Cd, and Fe limitation in rice increases Cd uptake and translocation,[28] as in *Arabidopsis*.

Despite its use of Fe^{2+} transporters, rice roots have very low ferric chelate reductase activity.[16] This is likely because many rice varieties are adapted to the anaerobic paddy environment where Fe^{2+} is readily available. To recreate the reductase strategy system found in nongraminaceous plants, rice was transformed with a ferric chelate reductase.[16] The gene encoding the yeast ferric chelate reductase FRE1, which has optimal activity in acidic conditions, was mutagenized and selected for elevated activity in high pH environments. A common mutation in the alleles tolerant of high pH was a substitution of methionine with arginine at position 312,

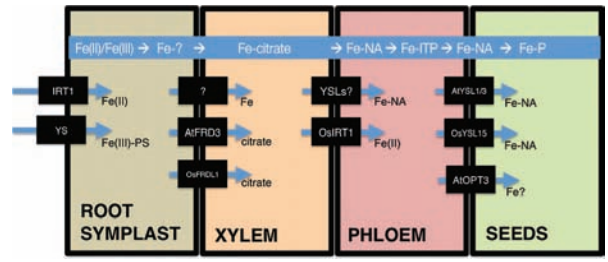

Figure 4. Fe chelation and long-distance Fe transport. Once transported into the root epidermal cells, Fe is almost certainly chelated, although it is unclear by what. It is also unclear, which transporter loads Fe into the xylem, but once in the xylem, Fe is known to be bound by citrate. Citrate itself is transported into the xylem via FRD3. YSLs in rice may transport Fe into the phloem, where it is likely bound by nicotianamine (NA). It has been proposed that NA may serve as a shuttle between the YSL transporters and an iron transport protein (ITP). NA is an essential part of long distance movement to the seeds, although it is unclear in what form the Fe is held, once it is loaded into the seeds. The *ysl1*, *ysl3*, and *opt3* mutants all have decreased seed Fe content, suggesting that they load Fe into the seed.

near one of the four heme-coordinating sites. It would be interesting to determine the structural and functional significance of this change. The resulting ferric chelate reductase coding sequence was fused with the *OsIRT1* promoter to ensure that expression of the modified gene was Fe regulated and that it would be expressed in the same tissue as the Fe^{2+} transporter. This resulted in plants that thrived on high pH soil compared with wild-type plants and produced 7.9 times greater grain yield. Interestingly, the concentration of shoot Fe increased only slightly, while the seed levels did not increase at all. This indicates a tight regulation of Fe homeostasis in rice and that uptake is immediately down-regulated once the necessary amount of Fe has been taken up.[16] This also demonstrates that components of the reduction strategy can be incorporated into grass species to augment Fe uptake, improving crops.

Similarly, incorporating the components of the chelation strategy could increase Fe uptake in nongrass crops, although this has not yet been successfully demonstrated. All plants synthesize the PS precursor NA, and constitutive overexpression of the PS synthesis enzyme nicotianamine aminotransferase (NAAT) in tobacco has been shown to consume NA, leading to intervenial chlorosis and sterility.[54] This suggests that introduction of PS synthesis into nongrasses is feasible, but we still do not understand how PSs are secreted from roots, so this may represent another step that will have to be engineered.

3. Long-Distance Fe Transport

After entering the epidermis, Fe is likely bound by unknown chelators or chaperones (Figure 4), due to its potential reactivity. Fe moves symplastically through the interconnected cytoplasm of the root, perhaps diffusing along the concentration gradient.[2] At the pericycle, Fe is effluxed into the xylem and moves toward the shoot through the transpiration stream. Although Cu chaperones have been identified in many organisms,[55] including plants,[56] the existence of a cytosolic Fe chaperone in plants is unproven. Interestingly, the first cytosolic Fe chaperone was recently identified in humans: PCBP1, a ubiquitously expressed RNA binding protein that also facilitates Fe loading of ferritin.[57] When human ferritin is expressed in yeast, it loads very little

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Fe, indicating the requirement for a chaperone; when PCBP1 is coexpressed, the ferritin fills with Fe. PCBP1 is also found complexed to ferritin *in vivo* and is able to bind Fe *in vitro*; additionally, the knockdown of *PCBP1* increases cytosolic Fe levels in cultured cells. Thus, PCBP1 likely delivers cytosolic Fe to ferritin, facilitating Fe loading. In the *Arabidopsis* genome, the genes most similar to HsPCBP1 have been characterized for their role in binding viral RNA (BTR1) and regulation of flower development (HEN4 and FLK). However, PCBP1 was also first characterized as an RNA binding protein,[58] and its Fe function was only identified in 2008.

In the vasculature, Fe is likely chelated to prevent precipitation. The chelators believed to bind Fe have properties appropriate for their respective environments: citrate readily binds Fe at the xylem pH of 5.5, while NA prevents precipitation of Fe at the pH of phloem sap, 7.5.[59] The exchange of Fe from citrate to NA has been predicted to occur at pH 5.5.[60]

3.1. Xylem

When Fe enters the xylem, it is believed to complex with citrate.[59] In *Arabidopsis*, citrate is effluxed into the xylem via FRD3 (Figure 4), which is expressed in the root vasculature.[61] FRD3 is a member of the multidrug and toxic compound extrusion (MATE) family, of which several other members also efflux citrate to mitigate aluminum toxicity;[62−64] indeed, overexpression of FRD3−GFP increases aluminum tolerance in *Arabidopsis*.[65] While *FRD3* mRNA is detected under Fe sufficiency, it is up-regulated 2-fold in response to Fe deficiency. The loss of FRD3 results in severe chlorosis and a constitutive Fe deficiency response.[66] Xylem exudate collected from the top of *frd3* mutant roots contained nearly 50% less translocated Fe, while Perls staining showed significant Fe^{3+} accumulation in the root vasculature.[65] The shoots of *frd3* plants have been shown to accumulate slightly less Fe than those of wild-type ones.[61,65,67] Without citrate, Fe does not efficiently move through the xylem and is not utilized by the shoot; instead it likely precipitates on the apoplast walls. Accordingly, adding citrate to the growth media regreened the plants, abolished Fe^{3+} accumulation in the root vasculature, and reduced the Fe deficiency response to wild-type levels.[65] Despite the severe phenotype, there is only a 40% decrease in citrate in the xylem, suggesting a role for other citrate effluxers.

The constitutive Fe deficiency response of the *frd3* mutant results in increased IRT1 expression and Fe uptake. But when plants increase IRT1 expression, the uptake of Zn, Mn, Co, and Cd via IRT1 also goes up. In the shoot, growth is tied to the availability of Fe; consequently, the concentration of Fe remains relatively constant under limitation, because growth is retarded. The shoot concentrations of Zn, Mn, Co, and Cd, however, keep increasing with increasing IRT1 expression. At the same time, Mo concentrations decrease, as the acidification of the rhizosphere reduces its availability in soil. This unique pattern in Fe-deficient plants was further substantiated by analyzing the shoot metal profiles of over 70 000 *Arabidopsis* plants grown with different levels of Fe supplementation in the Purdue Ionomics Information Management System (PiiMS).[68] It has been described as an ionomic signature for Fe deficiency[69] and could be utilized as a biomarker to identify Fe-deficient plants

The orthologue of FRD3 was recently identified in rice. While not Fe regulated, OsFRDL1 was found to transport citrate when expressed in *Xenopus* oocytes, and the loss of *OsFRDL1* results in chlorotic plants with Fe precipitation in the xylem.[70] And, like *atfrd3*, the *osfrdl1* loss of function insertion mutant has increased *OsIRT1* expression and accumulates more Zn and Mn in the shoot. Similarly, the elevated expression of *OsIRT1* and *OsIRT2* in MA-free rice mutants also resulted in increased concentrations of Zn, Cu, Mn, and Cd in the shoot.[52] This suggests that the *Arabidopsis* Fe deficiency signature could be adapted to rice, because the substrate specificity of uptake and translocation to the shoot does not diverge between these two species or perhaps even nongrasses to grasses. Interestingly, the loss of OsFRDL1 reduced the concentration of Fe^{3+} in the xylem sap but not Fe^{2+}.[70] This suggests that there is an additional chelator besides citrate involved in Fe movement in the xylem.

3.2. Phloem

3.2.1. NA and YSLs

The *Arabidopsis* orthologs of ZmYS1 and HvYS1 do not transport Fe−PS from soil but play a significant role in the distribution of Fe, most likely via the phloem. The eight *Arabidopsis* yellow stripe like (YSL) transporters are proposed to transport Fe chelated by the PS precursor NA in and out of the phloem (Figure 4).[59] The expression pattern of the rice YSLs also suggest a role in the long-distance transport of Fe complexes, including delivery to the seeds.[49] OsYSL15 and OsYSL2 are both up-regulated in response to Fe deficiency and may coordinate long-distance Fe transport from root to shoot to seed via the phloem: OsYSL15 in the root vasculature, flower, and developing seed and OsYSL2 in the phloem companion cells of the shoot.[49,71] Interestingly, expression in oocytes showed that OsYSL2 transports Fe−NA but not Fe−PS,[71] while OsYSL15 transports Fe−PS but not Fe−NA.[49] OsYSL18, like OsYSL15, also transports Fe−PS but does not appear to be involved in uptake from the rhizosphere. Rather, based on its expression pattern, it may be involved in DMA-mediated Fe distribution in reproductive organs, lamina joints, and phloem cells at the base of the sheath.[72]

3.2.2. Nicotianamine

NA is a nonproteogenic amino acid ubiquitous in higher plants, synthesized by the condensation of three molecules of *S*-adenosylmethionine in a reaction catalyzed by nicotianamine synthase (NAS). NA complexes with Fe^{2+} and Fe^{3+}; it has a higher affinity for Fe^{3+} but forms a more stable complex with Fe^{2+}.[73] NA also readily binds Cu^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, and Mn^{2+}, in decreasing order of affinity.[59]

The immunolabeling of NA in tomato root and shoot sections showed that NA increases within cells in response to increasing Fe levels.[74] This is in contrast to barley, where *NAS1* expression in roots is up-regulated by Fe deficiency,[75] because NA is used as a precursor to MA. Similarly, all three rice *NAS* genes were up-regulated in the roots in response to Fe deficiency, especially in the vasculature.[76] However, while barley roots accumulate (and secrete) higher levels of MA, rice accumulates much more NA in its roots, under both Fe sufficiency and Fe deficiency. This suggests that the NAS expression in rice is used to produce NA predominately for long-distance Fe transport. NA and MA levels were also very high in Fe-deficient rice leaves, while only trace

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amounts were detected in barley.[75] This further indicates a more significant role for NA in long-distance Fe transport in rice compared with that in barley and also a possible role for MA in Fe translocation. The low levels of NA in barley raises the question of what chelators barley uses for long-distance Fe transport. It is interesting that there is such a divergence between graminaceous species and that rice appears to utilize components found in nongraminaceous species for both Fe uptake and long-distance transport.

In *Arabidopsis*, there are four *NAS* genes. During Fe deficiency, *NAS2* and *NAS4* were up-regulated in the root,[77] suggesting a role in Fe translocation to the shoot. *NAS3* expression increased 4-fold after the transition from vegetative to reproductive growth, suggesting NA also mediates Fe movement to the flowers. Despite the varied patterns and Fe regulation, all the single mutants had wild-type NA levels, indicating functional redundancy, presumably because NA is mobile. In fact, interveinal chlorosis and sterility were observed only when the quadruple mutant was created.[77]

3.2.3. NA Levels and Fe Localization

NA levels have a significant affect on metal homeostasis. The overexpression of NAS in tobacco and *Arabidopsis* increases NA levels, resulting in the increased accumulation of Fe, Zn, Mn, and Ni in the shoots.[54,78,79] It is unclear whether these changes are the result of greater root to shoot translocation facilitated by NA or increased metal uptake in the roots driven by the creation of new Fe sinks in the shoots. Similarly, increasing NA levels in rice and *Arabidopsis* alters the localization of Fe. Rice plants with disrupted PS synthesis accumulated up to 43 times more NA than wild-type plants in Fe-starved roots; this dramatic change in NA increased long-distance Fe transport, resulting in seeds accumulating significantly more Fe than wild-type ones.[52] The increased movement of Fe−NA to the seeds most likely involved OsYSL2, which was significantly up-regulated. In *Arabidopsis*, an NAS overexpressing line accumulated 100 times as much NA as wild type, resulting in decreased root Fe, and constitutive IRT1 and FRO2 expression.[80] These plants accumulated significantly more Fe in both the roots and shoots when exposed to Fe-deficient conditions yet remained chlorotic compared with wild-type plants. This indicates that ubiquitous NA increases Fe translocation but impairs the efficient utilization of Fe in the shoot.

Conversely, the loss of NA, either by loss of NAS function in tomato and *Arabidopsis* or by the depletion of NA by the overexpression of the NA-consuming NAAT, leads to symptoms of Fe deficiency like interveinal chlorosis, reduced growth, and sterility.[54,77,81] When NA was depleted in tobacco by NAAT overexpression, Fe accumulated only in the veins of the leaf; the addition of exogenous NA, however, resulted in Fe movement throughout the entire leaf.[54] This suggests that NA is essential for Fe mobilization from the vasculature into the interveinal tissues. Based on what is known about Fe−NA transport in *Arabidopsis*, the YSLs likely play a role in this mobilization.[59]

3.2.4. NA and Ni Tolerance

NA also plays a significant role in Ni tolerance and localization. In *Arabidopsis*, exposure to Ni induces the expression of all four *NAS* genes,[79] and overexpression of NAS confers resistance to Ni.[79,82] Conversely, the NAS quadruple knock-down mutant was found to be highly sensitive to Ni.[77] In the Ni hyperaccumulator *Thlaspi caerulescens*, the high expression level of *NAS* genes in the shoots (relative to *Arabidopsis thaliana*) appears to be a key component of Ni tolerance and long-distance transport.[83] When treated with Ni, NAS expression was only detected in the shoots, yet NA began accumulating in the roots. At the same time, Ni−NA was detected in the xylem, and Ni rapidly accumulated in the shoots. This suggests that in *T. caerulescens*, NA is translocated from the shoot to the root to bind Ni, and the Ni−NA is then translocated back, at least in part, via the xylem sap. Additionally, three TcYSL family members have much higher expression than their *Arabidopsis* orthologs, and although not regulated by Ni, TcYSL3 has been demonstrated to readily transport Ni−NA when expressed in yeast.[84] Thus, there may be a sharing of transporters and translocation pathways by Ni−NA and Fe−NA.

3.2.5. YSLs and Long-Distance Fe Transport in Arabidopsis

Of the eight members of the *Arabidopsis* YSL family, three members, YSL1, YSL2, and YSL3, have been characterized. All but YSL3 transport Fe−NA when expressed in yeast, and all were found to be expressed in a broad range of tissues, especially the vasculature.[41,85−88] It is often proposed that the YSLs serve to translocate Fe from the xylem into the phloem so that it can move to young, growing tissues. The YSLs are also believed to load Fe from senescent leaves for long-distance transport to the flower for loading into the developing seed. Curie et al.[59] have recently reviewed the YSLs.

Of particular interest is the *ysl1 ysl3* double mutant, which had lower Fe levels in both leaves and seeds.[88] Flower and seed set were especially affected, which is discussed in the seed section (section 4.1). The double mutant also displayed what was described as interveinal chlorosis, somewhat similar to the chlorosis seen in the NA-free tomato mutant *chloronerva*.[81] Despite the chlorosis, the Fe deficiency response was not altered,[88] unlike the NA-free tomato mutant. It is interesting that Fe starvation in the interveinal areas of the leaf is not enough to trigger the Fe deficiency response. This suggests a tissue-specific component of Fe sensing in *Arabidopsis*, in which the veins may be more important than the interveinal tissues. A similar role was proposed for the root vasculature, based on the stele-specific up-regulation of signaling genes during Fe deficiency.[4]

3.2.6. OsIRT1

In rice, *OsIRT1−GUS* expression was detected in the phloem of roots and shoots.[51] Expression was up-regulated in response to Fe deficiency, especially in companion cells. It is proposed that OsIRT1 transports Fe(II) into the phloem, where it is then chelated by NA. This role does not appear to apply to AtIRT1, since its expression within the root has only been detected in the epidermis.[20]

3.2.7. ITP

In addition to NA, an Fe binding protein has been identified in the phloem sap of 7 day old castor bean shoots.[89] The iron transport protein, or ITP, is a dehydrin, expressed in the shoots of both seedlings and adult plants. When radiolabeled Fe was applied to the cotyledons, nearly all was recovered in the phloem sap associated with the 17

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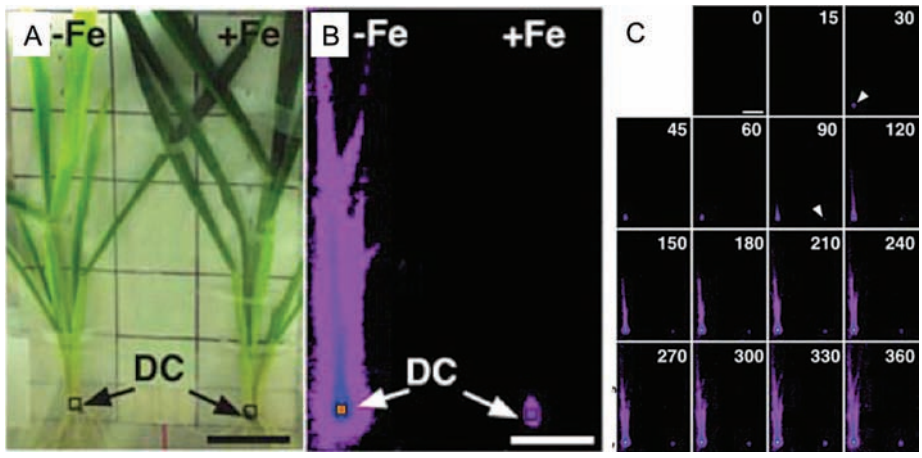

Figure 5. Realtime ^{52}Fe movement in barley shoots: (A) gross image of Fe-deficient (left) and Fe-sufficient (right) barley analyzed using PETIS (the same frame was used for panels B and C); (B) PETIS images of ^{52}Fe accumulation after 6 h; (C) time course of radioactivity accumulation analyzed using PETIS. The images are shown at 15 and 30 min intervals (0−60 and 60−360 min, respectively). Data were scored every 3 min. Arrowheads indicate the first detection of DC (discrimination center) (left arrowhead −Fe; right arrowhead +Fe). This figure is reproduced from ref 90 Creative Commons: Japanese Society of Plant Physiologists and *Plant and Cell Physiology*.

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kD ITP protein, indicating that Fe moves quickly to the phloem and nearly all is bound by ITP. The purified ITP protein was found to preferentially bind Fe^{3+} but not Fe^{2+}. Unfortunately, obtaining large amounts of phloem sap from plant model organisms is difficult, and ITP remains reported only in castor beans. The most similar genes in *Arabidopsis* have annotations related to stress, and several are highly upregulated in response to Fe deficiency in the root (BTI1, BTI2, At1g54410, At2g44060), although none are specific to the stele.[4] But, working under the assumption that an ITP exists in other plant species, it has been proposed that NA serves as a shuttle, facilitating Fe movement in and out of the phloem (via the YSLs), while the actual movement of Fe within the phloem occurs via ITP.

3.3. Control of Long-Distance Fe Transport in Barley

In barley, an Fe discrimination center (DC) in the basal shoot was identified by monitoring the dynamics of Fe uptake and translocation with a positron emitting tracer imaging system (PETIS).[90] PETIS allows the nondestructive visualization of metal movement in live plants, in real time. In both Fe-sufficient and Fe-deficient barley plants, ^{52}Fe was found to first accumulate at a central location in the basal part of the shoot (Figure 5), yet the Fe was only translocated to the leaves in the Fe-deficient plants, suggesting that this region regulates Fe distribution in barley.[90] They also found that damaging the phloem impaired Fe movement to young leaves but not old leaves. This provides more evidence that young leaves receive Fe primarily from the phloem, while older leaves receive Fe from the xylem. PETIS has also been used to visualize and quantify the uptake and translocation of radiolabeled ^{52}Fe and ^{62}Zn in rice plants.[16,91]

4. Fe and Seeds

Fe moves to the seeds, most likely via the phloem, because the flow of the xylem is driven by transpiration and seeds do not transpire.[92] Developing seeds receive Fe from the roots and from senescent leaves. The level of remobilization from shoot to seed varies by species: rice transports only 4% of shoot Fe to the seeds,[93] while wheat transports 77% of shoot Fe to the seeds.[94] The timing and regulation of senescence has been shown to have a significant effect on Fe accumulation in the seeds. In wheat, the knockdown of multiple NAM transcription factors with RNAi was found to delay senescence by over three weeks, and to decrease seed Fe by over 30%.[95] How developmental changes, photosystem deconstruction, and Fe remobilization interact is still unclear. It should be noted that crop breeding has often selected for improved grain maturation time but ignored nutrient accumulation in the grain as a desirable trait. Consequently, many staple crops are agronomically productive but have low levels of nutrients like Fe in the seed.

Cereal seeds provide more than 50% of the world's energy intake[96] and are a large part of the diet in many developing countries. Because the plant-based diet offers relatively low amounts of bioavailable Fe, large portions of the developing world suffer from Fe deficiency, including over 60% of all children in Africa and Southeast Asia.[97] In response to this, research has focused on understanding how nutrients are transported to seeds and how this can be increased. Overexpression of Fe-related genes, however, often creates sinks in the leaves rather than the seed.[98] This shows the importance of determining how Fe levels are sensed at the tissue and intracellular level and how this ultimately affects Fe allocation to the seed.

4.1. Loading of Fe

4.1.1. NA, YSL1, and YSL3

Fe−NA is essential for flower and seed development. The loss or depletion of NA results in deformed flowers and sterility, as well as significant decreases in floral Fe accumulation.[54,81] This fits well with the observed NAS expression pattern in tobacco, with highest expression being seen in flowers, especially in anthers and pollen.[54] Interestingly, the grafting of NA-depleted tobacco shoots onto NAS overexpressing shoots restored Fe mobilization in leaves and flower development but could not completely rescue the impaired seed set.[54] Thus, it would appear that the Fe−NA requirement for normal seed development is especially high. The *Arabidopsis* NAS quadruple knockdown mutant also becomes chlorotic when reproductive growth begins and accumulates significantly more Fe in the leaves during flowering (+216%).[77] At the same time, the

level of seed Fe only decreased 46%, while IRT1 expression in the flower increased, suggesting compensation. When a second quadruple mutant with NA synthesis completely abolished was created, the result was sterility. It should also be noted that IRT1 expression in the flower is also exclusively in the anther,[20] indicating a role for both Fe−NA and Fe(II) in flower development.

Thus, because Fe−NA is critical in seed development, the YSLs play an important, if not essential, role in Fe−NA delivery to the developing seed. In *Arabidopsis*, YSL1 expression was found in and around leaf veins, especially in senescent leaves, in addition to expression in the flower, pollen, young siliques, and embryo.[87] This suggests a role in Fe loading from senescent leaves for transport to developing seed. Indeed, the seeds of the *ysl1* loss of function mutant lines contained 30−65% less Fe and germinated more slowly on Fe-deficient medium. Watering plants with exogenous Fe could not restore Fe accumulation in the seeds, indicating that YSL1 plays a role in seed loading that cannot be compensated by other transporters or chelators. The expression pattern of YSL3 is somewhat similar to that of YSL1: in the vasculature of shoots and in pollen and anthers.[88] When the two loss of function mutants were crossed, the resulting double mutant was chlorotic, and most flowers did not produce siliques. The few resulting seeds were small and irregular and 80% less likely to germinate than wild-type seeds. These phenotypes are similar to the floral deformity and sterility seen in plants lacking NA,[54,81] indicating that seed development requires not just the availability of NA but also specific Fe−NA transporters.

4.1.2. OPT3

OPT3, a member of the family that includes ZmYS1 and the AtYSLs, plays an essential role in Fe loading of the seed. OPT3 is expressed in pollen, the silique vasculature, and the developing embryo; additionally, expression in the root and shoot vasculature is up-regulated in response to Fe deficiency.[99,100] Unlike the *ysl* mutants, the *opt3* null mutant is embryo lethal, indicating an essential role for AtOPT3 in seed development. An *opt3* knock down line, *opt3-2*, allowed embryo formation in seeds, but these accumulated significantly less Fe.[101] The *opt3-2* plants also exhibit constitutive expression of genes involved in the root Fe deficiency response, regardless of exogenous Fe supply. This leads to the accumulation of very high levels of Fe in leaves, resulting in brown necrotic spots, especially during the seed-filling stage. The substrate of OPT3 is unknown, but its phenotypes and relation to the YSLs suggests it likely transports chelated Fe or an Fe chelator. There are eight other members of the *Arabidopsis* OPT subfamily, and many are expressed in the vasculature and reproductive organs; none, however, have reported phenotypes, most likely due to functional redundancy.[100]

4.2. Storage of Fe

In *Arabidopsis*, it has been observed that developing seeds store Mn and Zn complexed with phytate in the vacuoles of the embryo and endosperm, and transiently in the ER.[102] The storage state of Fe in *Arabidopsis* seeds was unknown, although it was long assumed to be stored in ferritin in the plastid. This was based on earlier experiments in legumes that found as much as 90% of Fe in ferritin.[103] Recent work in *Arabidopsis* has found that there is very little ferritin in seeds,[104] raising the possibility that in *Arabidopsis* most seed Fe is bound by phytate or some other chelator in the vacuole.

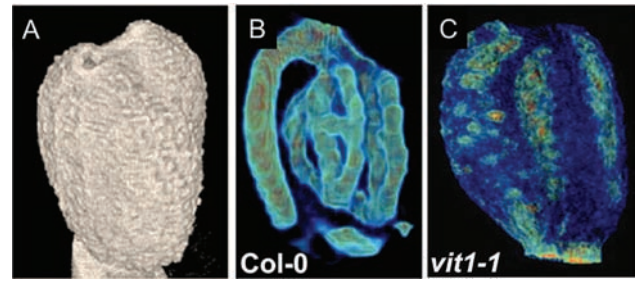

Figure 6. Loss of the VIT1 transporter changes Fe distribution within the seed: (A) three-dimensional rendering of total X-ray absorption of a wild-type *Arabidopsis* seed; (B, C) three-dimensional rendering of Fe Kα X-ray fluorescence in Col-0 and *vit1-1*, respectively, with both seeds identically oriented. Reprinted with permission from ref 105. Copyright 2006 The American Association for the Advancement of Science.

4.2.1. VIT1

In *Arabidopsis*, VIT1 transports Fe^{2+} into the vacuole, and is expressed in the vasculature, especially during embryo and seed development.[105] While the loss and overexpression of VIT1 does not affect total Fe levels in seeds, Fe is severely mislocalized in the loss of function mutant. Visualization of Fe distribution by synchrotron X-ray fluorescence microtomography[106] showed Fe concentrated in provascular strands of the embryo in wild-type seeds, while in the *vit1* mutant, Fe was not associated with the vascular system, but rather was seen throughout the hypocotyl and radicle and was concentrated in a layer of cells just inside the abaxial epidermis of the cotyledons (Figure 6).[105] This mislocalization of Fe resulted in decreased seedling viability on Fe-limited soil. Thus, vacuolar Fe loading via VIT1 is essential for proper Fe distribution in the embryo, which in turn determines seedling viability under low Fe conditions. The Fe stored in the vacuoles of the vasculature may be in the Fe^{3+} form, since Perls staining of Fe in embryos[101] strongly resembles the vascular localization of Fe demonstrated by SXRF.[105]

4.2.2. NRAMP3 and NRAMP4

In *Arabidopsis*, NRAMP3 and NRAMP4 also localize to the vacuoles in the vasculature but transport Fe out of the vacuole. Like *vit1*, the *nramp3 nramp4* double mutant seeds contain the same level of Fe as wild-type but produce seedlings that grow poorly on Fe-limited soil.[107] Visualization of wild-type seeds by electron microscopy showed what were likely Fe−phytate globoids in the vacuole, which disappeared as germination progressed. But, in the double *nramp3 nramp4* mutant, the globules remained during germination, indicating that Fe was not being mobilized from the vacuole, causing the germination defects seen on Fe-limited media. Because the Fe uptake transporter IRT1 is not expressed until the third day of germination, the first two days of growth rely on mobilization of vacuolar Fe stores via NRAMP3 and NRAMP4, hence the germination phenotypes of *vit1* and *nramp3 nramp4* mutants. Interestingly, this also demonstrated that the primary storage form of Fe in *Arabidopsis* seeds is not ferritin, as had been assumed based on earlier work in legume seeds. Instead, the vacuolar globoids, which are ions complexed with phytate,[108] appear

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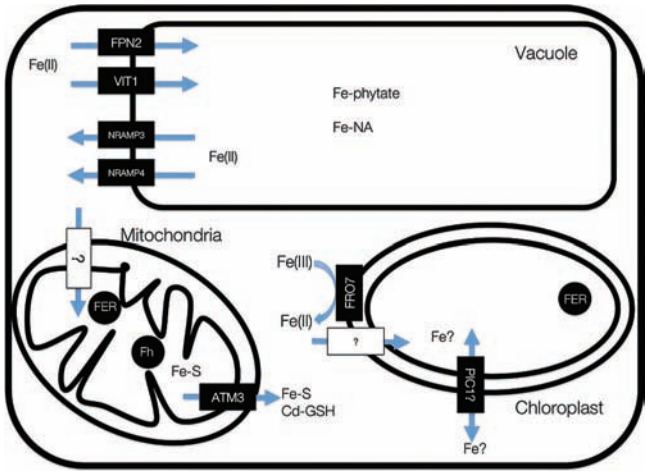

Figure 7. Intracellular Fe transport and sequestration. Fe is transported into the vacuole by FPN2 and VIT1 (although they are expressed in different tissues). Within the vacuole, Fe is known to be complexed with phytate and NA. NRAMP3 and NRAMP4 transport Fe out of the vacuole, most notably during Fe deficiency and germination. In the mitochondria, Fe is sequestered by ferritin and frataxin (FH), most likely to minimize oxidative stress. FH also plays a role in Fe−S cluster assembly or repair. ATM3 is believed to transport Fe−S clusters out of the matrix. To enter the chloroplast, Fe(III) is reduced by FRO7, and then taken up by an Fe(II) transporter, possibly the inner membrane-localized PIC1. Within the chloroplast, Fe is sequestered in ferritin.

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to be a primary Fe storage form in *Arabidopsis* seeds. It would thus be interesting to determine the Fe storage form in the reduced phytate *Arabidopsis* mutant.[109] Perhaps ferritin levels increase to compensate or another chelator (e.g., NA) is able to bind Fe in the vacuole.

Thus, both loading of Fe into the seed vacuole via VIT1 and its release during germination via NRAMP3 and NRAMP4 are essential for seed viability under Fe limited conditions and implicate the vacuole as an integral component of Fe storage in seeds.

4.2.3. FER2

In addition to vacuoles, Fe is found in the plastids bound with ferritin. *Arabidopsis* has four ferritin genes, of which only *FER2* is expressed in developing seeds, and during germination.[110] Accordingly, *FER2* is the only ferritin upregulated in response to the plant hormone abscisic acid (ABA). The *fer2* loss of function mutant does not affect Fe accumulation or seed viability under normal conditions; in fact, ferritin was estimated to account for only 5% of total seed Fe.[104] When the other three ferritin genes were knocked out, the flowers accumulated more Fe and were highly sensitive to Fe supplementation. This resulted in deformed, less functional flowers and increased oxidative stress. Thus, ferritin likely serves more as an Fe buffer, sequestering free Fe to prevent oxidative stress.

4.3. Fe Bioavailability for Humans

Large amounts of the antinutrient phytate accumulate in the seeds of many staple crops, including in the maize embryo and the aleurone cells of wheat, rice, and barley.[111] Phytate is composed of a phosphorylated *myo*-inositol ring and strongly chelates metal cations, including Fe, Zn, and Mn;[112] these salts accumulate as globules in the vacuole, as mentioned above. Because phytate represents around 1−2% of the dry weight of cereal seeds,[113] this poses a serious impediment to dietary Fe uptake. In the developing world, the prevalence of phytate in the plant-based diet is believed to contribute to the high rate of Fe deficiency and anemia.[114] In fact, high fiber diets have been shown to induce Fe deficiency in healthy women,[115] because phytate is presumably binding Fe from other foods in the intestine, making it unavailable for uptake. Conversely, Fe stored within ferritin is believed to be safe and have high bioavailability.[116] Thus, several strategies have been employed to reduce the amount of phytate in seeds, while increasing the amount of ferritin.

One obvious approach is to disrupt phytate biosynthesis. Early attempts to reduce phytic acid across the whole plant successfully reduced accumulation in seeds, but these plants often germinated poorly and were more susceptible to stress.[117] Recently in *Arabidopsis*, the disruption of the inositol polyphosphate kinases required for the later steps of phytate synthesis, AtIPK1 and AtIPK2, was found to produce seeds with 93% less phytate.[109] While these mutations did not affect seed yield or germination, the loss of the phytate precursors did alter phosphate sensing. The authors noted that this could be overcome by using promoters specific for the seeds. Accordingly, low phytate maize and soybean seeds were generated by the seed-specific silencing of an ABC transporter.[118] Although it is unclear to which membrane the transporter localizes or even what it transports, its loss prevents phytate from accumulating in the seed without compromising seed viability. How the reduction of phytate in seeds affects Fe homeostasis has not been examined, but it would be interesting to look at the interplay between vacuolar and plastid Fe pools in these mutants.

A second approach is to overexpress ferritin in seeds. Although the mechanism of dietary ferritin uptake in the human gut is unknown, it is believed that the Fe complexed in ferritin is readily absorbed and a highly accessible source of Fe.[116] Consequently, ferritin has been viewed as a means of increasing bioavailable Fe in staple crops. Indeed, the overexpression of soybean and bean ferritins in rice seed resulted in 2−3-fold increases in seed Fe content;[119−121] rats fed ferritin overexpressing rice recovered from Fe deficiency, indicating that the Fe is bioavailable.[122] Of course, overexpression of ferritin does have consequences for the plant. Overexpression of soybean ferritin in tobacco resulted in a constitutive Fe deficiency response, causing greater Fe uptake and accumulation but also a 2-fold increase in Cd when grown on contaminated soil; at the same time, the increase in sequestered Fe produced improved resistance to oxidative stress.[98,123] Overexpression of alfalfa ferritin in tobacco produced an increased resistance to Fe overload, oxidative stress, and pathogen invasion.[124] Ferritin overexpression with more powerful promoters produced the same fold increase in Fe as transgenics with weaker promoter constructs, suggesting further increasing Fe accumulation is limited by Fe uptake and transport and not by ferritin levels.[125]

Finally, a combination of the two approaches has been undertaken. Transgenic maize plants were generated to ectopically express *Aspergillus* phytase and soybean ferritin in the endosperm.[126] This increased total Fe content in seeds by 20−70% and resulted in the degradation of nearly all endogenous phytate. When paste from the resulting seeds was fed to cultured human cells, Fe uptake was significantly

higher compared with those fed wild-type seed paste. Thus, attempts to increase bioavailable Fe in seeds are becoming more successful.

5. Intracellular Fe

5.1. Plastids

It is believed that chloroplasts hold nearly 90% of the Fe within a leaf.[127] Indeed, Fe is required for photosynthesis, heme biosynthesis, and Fe−S cluster assembly, all of which take place in the chloroplast, yet very little is known about how Fe is transported in and out of this organelle. Transporters likely serve as the gateway for Fe (Figure 7), regulating its levels within the plastid in response to cellular demand outside the plastid.

5.1.1. FRO7

The expression of the ferric reductase FRO7 on the chloroplast membrane indicates that some Fe is trafficked to the chloroplast in the ferric form and must be reduced to enter the chloroplast.[128] Previous experiments with purified pea chloroplasts showed that Fe was likely transported across the inner envelope in the ferrous state.[129,130] Accordingly, the chloroplasts of the *fro7* mutant contain 33% less Fe, resulting in decreased photosynthetic efficiency and fewer healthy photosystems.[128] Additionally, the FRO7-facilitated import of ferrous Fe into the plastid is essential for seedling growth under Fe-limited conditions. While the *nramp3 nramp4* double mutant grows poorly when germinated on Fe-limited soil,[107] the *fro7* loss of function mutant dies. Thus, both Fe mobilization from the vacuole and Fe import into the plastid are essential for seedling development when Fe is limiting. This implies that the plastid Fe pool in seeds is insubstantial, which correlates with the low levels of ferritin found in *Arabidopsis* seed.[104] This further implicates the vacuole, rather than the plastid, as the primary site for Fe storage in seeds and subsequent site for mobilization during germination.

5.1.2. PIC1

PIC1 (Tic21) was originally identified as a chloroplast translocon component, because it immunoprecipitates with the major components of the Toc and Tic translocon.[131] However, it has also been proposed that developmental defects seen in the loss of function mutant are related to impaired Fe homeostasis within the chloroplast, rather than protein translocation. PIC1 localizes to the inner envelope of the chloroplast[131,132] and is essential for Fe homeostasis within the plastid and plant as a whole.[132] The heterologous expression of the plastid-localized transporter in yeast suggested that PIC1 transports Fe and Cu across the membrane. Although overall Fe levels in the leaf do not change in the *pic1* mutant, the plants are dwarfed and chlorotic, with impaired chloroplast development. These plastids were also found to have elevated levels of ferritin and lacked thylakoids; this suggests that Fe was no longer being utilized properly in the plastid and instead accumulates in ferritin. This mislocalization of Fe also changes the expression of nonplastid, Fe-regulated genes in the shoot cells, and the expression of the root Fe uptake transporter *IRT1* was repressed. This indicates that the chloroplast is integral to the Fe sensing mechanism, because the Fe status of the chloroplast affects the Fe homeostasis of the entire cell, in addition to the expression of Fe deficiency response genes in the root.

5.1.3. Ferritin

In *Arabidopsis*, FER1, FER2, and FER3 are predicted to localize to the plastid; FER4 is predicted to localize to the mitochondria, or be dual targeted to both organelles.[110] The roles of the ferritin paralogs is differentiated by localization and regulation: *FER2* is only expressed in the seeds, while the other three ferritins are expressed in the shoots and flowers, in addition to *FER1* expression in the roots.[110] Additionally, the expression of the three nonseed ferritins increases in response to high Fe levels, whereas the seed FER2 is expressed in response to the plant hormone ABA.[110] Ferritins appear to buffer Fe levels and sequester excess free Fe to prevent oxidative stress.[104] When the three genes encoding nonseed ferritins were knocked out, the triple mutant showed a shift in Fe accumulation from stem to flower when supplemented with Fe, resulting in increased oxidative stress and deformed flowers. This supports the hypothesis that chloroplasts are an important Fe sink and that ferritins may sequester some Fe in the leaf plastids. This prevents excess Fe movement to the flower, although it is unclear whether this is by physically sequestering Fe in the shoot or whether the Fe status of the plastid regulates long-distance Fe transport to the flower. The triple mutant also showed no decrease in photosynthesis,[104] indicating that ferritins are not essential for chloroplast development or function. Instead, the ferritins prevent excess free Fe from accumulating in the flower, where it causes damage.

5.2. Mitochondria

Plant mitochondria require Fe for respiration, heme biosynthesis, and the synthesis of Fe−S clusters,[133] but the combination of electrons and free Fe is highly toxic. Thus, proper Fe homeostasis in the mitochondria is vital, and both transporters and Fe sequestering proteins have been found to be essential for mitochondria function (Figure 7). Flower development, especially microsporogenesis, is highly dependent on energy from the mitochondria.[134] Maintaining mitochondrial Fe levels is thus of high importance, because Fe deficiency produces deformed mitochondria in rice pollen and reduces seed yield.[135] Appropriately, many Fe-related genes are highly expressed in the anthers (the portion of the male organ of the flower containing pollen), such as *NtNAS*, *AtOPT3*, *AtYSL1*, *AtYSL3*, and *AtIRT1*.[20,54,88,100]

5.2.1. Ferritin and Frataxin

Recently, the Fe-binding proteins ferritin and frataxin have been localized to the mitochondria in several organisms,[136−138] including *Arabidopsis*.[139] They appear to play a very important role in metal homeostasis not only in the mitochondria, but also in the whole cell.

Very little research has been done on mitochondrial ferritins in plants, other than confirming their presence in purified mitochondria from pea and *Arabidopsis*.[139] Based on its putative transit peptide, AtFER4 is the most likely to localize to the mitochondria, although it may also target to the chloroplast (Aramemnon Plant Membrane Database). The *fer4* loss of function mutant does not have a phenotype, perhaps because one or more of its paralogs are also targeted

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to the mitochondria or frataxin is able to compensate. More likely, FER4 is not essential for mitochondria function under normal conditions. While it is expressed in response to Fe overload, it is down-regulated in response to oxidative stress.[110] Like the mitochondrial ferritin in humans and fruit flies,[136,137] FER4 appears to play an important role in the mitochondria-rich reproductive organs, because *FER4* expression was highest in the flowers and floral stalk. It would be interesting to see the effects of FER4 overexpression in plants, because the overexpression of mitochondrial ferritin in human cells has been linked to cytosolic Fe depletion.[140,141]

Like FER4, frataxin is expressed in the mitochondria of the flowers,[142] in addition to the developing embryo.[143] Unlike mitochondrial ferritin, frataxin is not Fe regulated, and its loss is embryo lethal.[143,144] Frataxin is essential to growth because it has functions beyond mitochondrial Fe sequestration. In addition to sequestering Fe, frataxin is believed to serve as a chaperone, mediating Fe delivery to the Fe−S cluster assembly scaffold.[145] The knock-down of frataxin in *Arabidopsis* is not lethal but results in increased ROS and decreased vegetative growth and seed set.[144] The knock-down line also accumulates more Fe in the root, and the expression of *FER1* and *FER4* is increased, presumably to prevent oxidative stress.[146] Interestingly, Fe−S cluster-related genes in the mitochondria are up-regulated in the mutant; however, the resultant Fe−S-containing proteins (like aconitase) have reduced activity.[144] This indicates that *Arabidopsis* frataxin is essential for functional Fe−S clusters and that even decreased expression of frataxin has serious phenotypic consequences in terms of sequestration of free Fe and Fe−S cluster assembly.

5.2.2. ATM3

In *Arabidopsis*, the only identified mitochondrial Fe transporters are the ATMs, half-molecule ABC proteins that are orthologs of ScATM1. ScATM1 localizes to the mitochondrial inner membrane and is believed to efflux Fe−S clusters from the matrix.[147] The *Arabidopsis* ATMs were first identified by the chlorotic, dwarf phenotype of the *atm3* loss of function mutant (or *sta1*).[148] Like Δ*atm1* yeast, mitochondria of these plants accumulated more nonheme, nonprotein Fe than those of wild-type plants, resulting in increased oxidative stress. When expressed in yeast, the *Arabidopsis* ATMs localized to the mitochondria, but only AtATM3 was able to rescue the Δ*atm1* yeast.[148,149] These results suggest that ATM3, if not the other ATMs, could perform a similar function of Fe−S cluster export from the *Arabidopsis* mitochondria.

ATM3 also appears to play a role in Cd detoxification in *Arabidopsis*.[150] ATM3 is up-regulated in roots treated with Cd or Pb, and the *atm3* dwarfs were more sensitive to Cd than wild-type plants, while ATM3 overexpression enhanced Cd resistance. Because ATM3 is closely related to the fission yeast SpHMT1, a vacuolar phytochelatin−Cd transporter, it has been suggested that ATM3 may also function to export chelated Cd complexes out the mitochondria, in addition to its role in transporting Fe−S clusters. Plants overexpressing ATM3 also accumulate more Cd in roots and shoot, but the concentration of Fe and other metals were not reported. It would be very interesting to investigate how the constitutive export of Fe−S clusters from the mitochondria affects the Fe deficiency response. The increase in shoot Cd suggests that IRT1 expression may be up-regulated.

5.3. Vacuole

As described earlier, the vacuole serves as the most important Fe store in *Arabidopsis* seeds. The vacuole also plays a role in the roots and shoots, storing and releasing Fe (Figure 7) in response to changes in cytosolic Fe levels. Interestingly, it was recently demonstrated that phosphorus availability controls the subcellular localization of Fe in *Arabidopsis* leaves.[151] Using X-probe microscopy, leaf sections from plants grown under phosphorus sufficiency showed Fe and phosphate in globules in the vacuole. Interestingly, these vacuolar Fe globules were only seen in the cells surrounding the vasculature and not in other tissue layers. However, under phosphorus deficiency, the localization of Fe shifts to the chloroplast and *FER1* expression increases, suggesting that ferritin is now binding Fe.

5.3.1. NA and the Vacuole

NA is found in the vacuole[74] and is likely chelated to Fe there. If Fe−NA complexes are indeed transported in and out of the vacuole, YSL4 and YSL6 are candidates, because they were found in the vacuole proteome of *Arabidopsis* suspension cells.[152] In the shoots of tomatoes and peas, NA was found primarily in the cytoplasm during Fe deficiency and Fe sufficiency but was shown to concentrate in the cytoplasm and vacuole during Fe overload.[74] It has been proposed that NA functions as an Fe(II) scavenger, protecting the cell from oxidative stress,[73,74] and this excess Fe−NA may then be sequestered in the vacuole.

When NA was depleted in tobacco, electron microscopy of chlorotic leaf sections showed the appearance of electron dense globules in the vacuole.[54] Although these were not analyzed, similar vacuolar globules were identified as containing Fe and phosphate.[151] Thus, it is worth speculating whether the depletion of NA shifts Fe sequestration within the vacuole into phytate complexes.

5.3.2. VIT1, NRAMP3, and NRAMP4

AtNRAMP3 and AtNRAMP4 are both expressed on the vacuolar membrane in the vasculature of the roots and shoots in response to Fe deficiency. VIT1 most likely loads Fe into the vacuole,[105] and like NRAMP3 and NRAMP4, VIT1 is expressed in the vasculature. Thus, the role of VIT1 may be filling the vacuole with Fe, which is then released into the cells of the vasculature during Fe deficiency by NRAMP3 and NRAMP4.

6. Conclusions and Future Directions

Much progress has been made in the past few years in studying Fe homeostasis in *Arabidopsis*, especially in clarifying ferritin function and in identifying the vacuole's role in Fe storage and mobilization during seed set and germination. Additionally, the recent large-scale characterization of transcriptional changes in specific root layers of *Arabidopsis* has proven an invaluable resource, increasing the resolution of our understanding of the Fe deficiency response. Similarly, the deposition of ICP-MS data from thousands of mutant *Arabidopsis* lines into the PiiMS database[68] has allowed the identification of the Fe-deficiency signature and will likely yield more unexpected discoveries in the future. Some of the discoveries in *Arabidopsis* can be generalized to all plants, but as studies of rice and other grasses have shown, there are species-specific aspects of Fe

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metabolism as well. It must also be noted that many aspects of Fe homeostasis and transport in plants remain unclear. Foremost, a mechanism of Fe sensing has not been discovered in plants. Additionally, it is still unknown what chelates Fe once it is transported into the root epidermis, and very little is known about transport in and out of the mitochondria and chloroplast. Current research aims to answer these questions.

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