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Transporters Contributing to Iron Trafficking in Plants

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ABSTRACT This review will discuss recent progress in understanding the many roles of transporters in the whole-plant physiological processes that maintain iron (Fe) homeostasis. These processes include uptake from the soil via roots, control of transport from roots to above-ground parts of the plant, unloading of Fe from the xylem in above-ground parts, loading of Fe into mitochondria and plastids, transport of Fe to reproductive parts of the plant, and Fe mobilization during seed germination. In addition, we will discuss the mechanisms that plants use to cope with an apparently unintended consequence of Fe acquisition: the uptake of toxic heavy metals via Fe transporters. Rapid progress has been made in understanding the transport processes involved in each of these areas in the last 5 years and this review will focus on this recent progress. We will also highlight the key questions regarding transport steps that remain to be elucidated.

Key words: Nutrient and metal transport; transporters; gene regulation; iron; nicotianamine; phytosiderophores.

INTRODUCTION

Iron (Fe) is one of the most important and most problematic of all the micronutrients used by living organisms. The importance of Fe stems from its role as an essential cofactor for many cellular redox reactions involved in photosynthesis, respiration, and many other reactions. The problematic nature of Fe stems from two of its distinct chemical properties. Fe is highly reactive and, if over-accumulated, can cause cellular damage (Winterbourn, 1995). Fe is also only sparingly soluble in aqueous solution, particularly in well-aerated environments where production of ferric hydroxides and other Fe salts limits Fe solubility (Marschner, 1995). As a response to these key properties of Fe, plants have evolved multifaceted Fe homeostatic mechanisms that regulate Fe acquisition from the environment and the movement of Fe between organelles, cells, tissues, and organs. Transporter proteins feature prominently as mediators of Fe homeostatic control (Table 1).

The ability to acquire adequate amounts of Fe can limit plant growth and productivity. Most soils contain large amounts of Fe, but most of the Fe is present as hydroxides and other insoluble complexes. This sparing solubility of Fe limits the availability of this nutrient to plants, especially in alkaline soils where Fe solubility is particularly low (Marschner, 1995). While deficiencies of other important plant nutrients like nitrogen, phosphorus, and potassium can be readily treated with fertilizers, Fe supplementation is difficult to achieve, since it is the availability and not the abundance of Fe that must be addressed. Application of Fe in the form of iron-chelates, which are relatively costly, can improve Fe availability, even in alkaline soils.

While Fe deficiency in plants is a problem only for some crops and soils, Fe deficiency in humans is a global health issue. Fe deficiency is the most prevalent nutritional deficiency for humankind, affecting 1.62 billion people, or about 25% of the world's population (McLean et al., 2009). The main symptom of Fe deficiency in humans is anemia, which causes major health problems during pregnancy, leads to impaired physical and cognitive development of children, and reduces the work productivity of adults through acute fatigue. A promising and sustainable means of improving Fe nutrition, especially among subsistence farmers, is biofortification: the engineering of staple crops to accumulate additional nutrients in edible parts (Hirschi, 2009). An understanding of the biochemical and molecular mechanisms underlying plant nutrient transport and storage is necessary before safe and effective biofortification strategies can be implemented.

PRIMARY ACQUISITION OF Fe FROM SOIL TO ROOT

Plants use two distinct strategies to overcome the poor bioavailability of Fe and achieve primary Fe uptake. Both of these

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Table 1. Transporters Discussed in this Review.

Transporter	Apparent function	Reference
AtATM3	Export of Fe–S from mitochondria	(Bernard et al., 2009; Chen et al., 2007)
AtFPN1	Iron efflux across plasma membrane; loading of Fe into xylem	(Morrissey et al., 2009)
AtFPN2 (AtIREG2)	Influx of transition metals into vacuole; sequestration of toxic metals during Fe deficiency	(Morrissey et al., 2009; Schaaf et al., 2006)
AtFRD3	Efflux of citrate; loading of citrate into xylem sap	(Durrett et al., 2007; Green and Rogers, 2004; Rogers and Guerinot, 2002)
AtIRT1	Fe(II) influx; primary iron uptake from soil	(Eide et al., 1996; Henrique et al., 2002; Varotto et al., 2002; Vert et al., 2002)
AtIRT2	Fe influx into cortical vesicles	(Vert et al., 2009)
AtMTP3, AtMTP1	Vacuolar import of Zn; Zn exclusion from shoots	(Arrivault et al., 2006; Desbrosses-Fonrouge et al., 2005)
AtNAP14	Iron influx into plastids	(Shimoni-Shor et al., 2010)
AtNRAMP3, AtNRAMP4	Vacuolar efflux of iron; required for normal germination	(Lanquar et al., 2005; Thomine et al., 2003)
PIC1	Fe influx to chloroplasts	(Duy et al., 2007)
VIT1	Vacuolar influx of iron; required for cell-specific Fe localization in *Arabidopsis* embryos	(Kim et al., 2006; Roschzttardtz et al., 2009)
ZmYS1, HvYS1, and OsYSL15	Influx of Fe(III)–PS complexes; primary iron uptake from soil	(Curie et al., 2001; Inoue et al., 2009; Lee et al., 2009a; Murata et al., 2006; Roberts et al., 2004; Schaaf et al., 2004)
AtYSL1, AtYSL2, and AtYSL3	Influx of Fe(II)–NA complexes; remobilization of transition metals during senescence and seed set; iron uptake from xylem	(Chu et al., 2010; DiDonato et al., 2004; Waters et al., 2006)
OsYSL2	Influx of metal–NA complexes; translocation of Fe from root to shoot; loading of Fe in seeds	(Ishimaru et al., 2010; Koike et al., 2004)
OsYSL18	Influx of Fe(III)–PS complexes; facilitates xylem-to-phloem transfer of Fe	(Aoyama et al., 2009)

strategies have been reviewed in detail recently (Walker and Connolly, 2008; Jeong and Guerinot, 2009; Morrissey and Guerinot, 2009) and so will be described here only briefly. In 'Strategy I', an 'acidification/reduction' mechanism is used to enhance Fe solubility prior to uptake. Plants secrete protons into the rhizosphere to lower the pH of the soil and thus increase the solubility of Fe(III). Reduction of the Fe(III) by root ferric chelate reductase further enhances Fe solubility, since Fe(II) is more soluble than Fe(III). Reduction also prepares the Fe for uptake by Iron Regulated Transporter1 (IRT1)-type ferrous transporters, which move Fe(II) across the root epidermal plasma membrane. All three components of Strategy I (proton pumping, ferric chelate reductase gene expression and enzyme activity, and *IRT1* expression) increase markedly when plants are grown in Fe-deficient conditions. Strategy I is used by most types of plants, including the model plant *Arabidopsis thaliana*, facilitating identification of key Strategy I genes: Ferric Reductase Oxidase (*FRO2*), which encodes root ferric chelate reductase (Robinson et al., 1999); and *AtIRT1*, which encodes the ferrous Fe transporter (Eide et al., 1996). A new wrinkle in the Strategy I uptake model has been uncovered recently during work on the *Arabidopsis* IRT2 protein, which, like IRT1, belongs to the Zinc-regulated transporter/Iron-regulated transporter (ZRT/IRT1)-related Protein (ZIP) family of metal transporters. Like *IRT1*, *IRT2* is up-regulated under Fe deficiency and is capable of transporting Fe in yeast functional complementation assays. However, *IRT2* cannot complement the growth defect of *irt1-1* mutants (which are severely chlorotic) and is localized not in the plasma membrane, but in vesicles within root epidermal cells (Vert et al., 2009). Although several explanations for IRT2 function have been proposed, a particularly intriguing one is that Fe-regulated expression of *IRT2* constitutes a 'proactive adaptation' (MacDiarmid et al., 2003) in which IRT2 functions to sequester excess Fe that will be taken up through strongly increased IRT1 activity (Vert et al., 2009). Although more work is needed to confirm this hypothesis, the existence of two closely related and identically regulated Fe(II) transporters with distinct sub-cellular localizations highlights the complex mechanisms that plants have evolved to control Fe uptake.

Certain plants—namely the grasses, which include most of the world's staple grains—have evolved a distinct mechanism to acquire Fe from the soil, known as Strategy II. This strategy is best described as a 'chelation' strategy, similar to that used by many bacteria and fungi (Miethke and Marahiel, 2007), and may have arisen as an adaptation to alkaline soils where acidification of the rhizosphere is difficult to achieve (Ma and Nomoto, 1996). Strong Fe chelators called phytosiderophores (PS) are synthesized by the plant and secreted into the rhizosphere, where they bind Fe(III) (Figure 1; Tagaki, 1976; Tagaki et al., 1984, 1988). The Fe(III)–PS complex is then taken up into root cells via transporters specific for the complex (Romheld and

Marschner, 1986; von Wiren et al., 1994). Phytosiderophores are chemically quite distinct from bacterial and fungal siderophores, and belong to a class of compounds called mugineic acids (MAs; Ma and Nomoto, 1996). The biosynthetic pathway of MAs, starting from a condensation of three S-adenosyl-methionine molecules to form the precursor nicotianamine (NA), is well established (Mori and Nishizawa, 1987; Kawai et al., 1988; Shojima et al., 1990; Ma et al., 1995; Takahashi et al., 1999; Kobayashi et al., 2001).

The Fe(III)-phytosiderophore uptake transporter is called Yellow Stripe1 (YS1), after the mutant phenotype of yellow striped leaves that results from disruption of the gene encoding this transporter in maize (von Wiren et al., 1994; Curie et al., 2001). YS1 proteins are distantly related to the Oligopeptide Transporter (OPT) family of transporters (Yen et al., 2001) and have been identified in several grass species (Curie et al., 2001; Yen et al., 2001; Murata et al., 2006; Inoue et al., 2009; Lee et al., 2009a). YS1 is a proton-coupled symporter of Fe(III)–PS complexes (Schaaf et al., 2004). The transport activity and specificity of YS1 transporters from maize (ZmYS1), barley (HvYS1), and rice (OsYSL15) have been examined using yeast growth complementation and radioactive uptake assays and two electrode voltage clamp experiments in *Xenopus leavis* oocytes (Curie et al., 2001; Roberts et al., 2004; Schaaf et al., 2004; Murata et al., 2006; Inoue et al., 2009; Lee et al., 2009a). The HvYS1 transporter specifically transports Fe(III)–PS and, moreover, this specificity has been attributed to the propensity of a highly variable loop between the sixth and seventh transmembrane domains of the protein to form a coiled domain (Harada et al., 2007). In contrast, the ZmYS1 and OsYSL15 transporters appear to have broader transport capacities, with significant currents produced by application of Ni(II)–, Zn(II)–, and Cu(II)–PS to *Xenopus* oocytes heterologously expressing these transporters (Schaaf et al., 2004; Inoue et al., 2009). The transport differences observed in heterologous expression systems are supported by *in planta* differences in metal–PS uptake by roots of maize and barley. In barley, uptake of Fe(III)–PS was not inhibited by addition of equimolar quantities of pre-formed Cu(II), Zn(II), or Co(III)–PS complexes

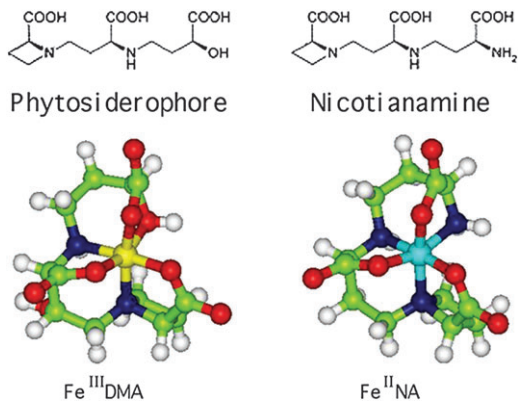

Figure 1. The Structures and Predicted Complex Conformations of a Phytosiderophore, 2′-Deoxymugineic Acid (DMA) and Nicotianamine (NA).

(Ma and Nomoto, 1993), validating the specificity for Fe(III)–PS that was observed in *Xenopus* oocytes. In maize, Zn(II)–PS uptake by the *ys1* mutant, which lacks a functional YS1 gene, is partially compromised, suggesting that YS1 transports Zn(II)–PS *in planta* (von Wiren et al., 1996).

In contrast to the details established for PS biosynthesis and Fe–PS uptake, the molecular details of PS secretion have not yet been characterized. Typically, PS are secreted according to a diurnal cycle, with release occurring after sunrise (Walter et al., 1995; Reichman and Parker, 2007; Nagasaka et al., 2009). Large numbers of vesicles have been observed in barley roots just prior to the daily release of PS, suggesting that PS are secreted by exocytosis (Nishizawa and Mori, 1987; Sakaguchi et al., 1999; Negishi et al., 2002). Furthermore, microarray analysis of barley roots indicated that expression of genes associated with polar vesicle transport increases in the early morning (Negishi et al., 2002). The anion channel blockers anthracene-9-carboxylic acid and phenylglyoxal were shown to inhibit PS secretion by barley roots (Sakaguchi et al., 1999), indicating that anion channels are involved in loading PS to secretory vesicles. Alternatively, anion channels in the plasma membrane could be responsible for PS release. A classically known mutation in maize called *yellow stripe3* (*ys3*) renders plants unable to secrete PS, even though PS are synthesized in normal amounts (Basso et al., 1994). Elucidation of the molecular mechanism of PS release will be an important final step in the full characterization of the Strategy II Fe-uptake mechanism.

FROM ROOT TO SHOOT (XYLEM TRANSPORT)

Following the initial uptake of Fe into root cells, it must be transported to the above-ground portions of the plant via the xylem. The idea that Fe is complexed with citrate in the xylem has been well accepted for many years. There is a strong correlation between the levels of citrate and the levels of Fe in xylem sap (Brown and Tiffin, 1965) and Fe and citrate from xylem sap co-migrate in paper electrophoresis (Tiffin, 1966, 1970). When plants are Fe-deficient, the amount of organic acids (citrate, malate, and succinate) in the xylem increases (Brown, 1966; Lopez-Millan et al., 2000). Theoretical calculations that take into account the known metal chelators (organic acids and amino acids including nicotianamine) and pH present in xylem sap suggest that citrate will be the major complexor of Fe in the xylem (von Wiren et al., 1999; Rellan-Alvarez et al., 2008). Recently, high-pressure liquid chromatography (HPLC) coupled to electrospray time-of-flight mass spectrometry (HPLC–ESI-TOF-MS) and inductively coupled plasma mass spectrometry (HPLC–ICP-MS) were used to identify the naturally occurring Fe–citrate complex in xylem sap of tomatoes as oxo-bridged tri-Fe(III), tri-citrate (Fe_3Cit_3) (Rellan-Alvarez et al., 2010).

The transporters that are responsible for loading Fe and citrate into the xylem have been revealed recently. Citrate appears to be moved into the xylem at least in part via the Multidrug And Toxic compound Extrusion (MATE) family transporter FRD3 (Ferric

Chelate Reductase Defective3; a mutation that was responsible for the phenotype of *man1* (*manganese accumulator1*); Delhaize, 1996; Rogers and Guerinot, 2002; Durrett et al., 2007). The FRD3 protein has strong sequence similarity to two MATE transporters that confer Al resistance in barley and sorghum through efflux of citrate from the root into the soil (Furukawa et al., 2007; Magalhaes et al., 2007). Xylem sap from *frd3* mutant plants contains about 40% less citrate and 49% less Fe than wild-type (WT) plants. The *FRD3* gene is expressed only in pericycle and cells surrounding the vascular tissue and, in *frd3* mutant plants, Fe accumulates adjacent to the xylem in the same cells as those in which *FRD3* is normally expressed, as if it is blocked from entering the vasculature (Green and Rogers, 2004). If high levels of exogenous citrate are provided in the growth medium, Fe accumulation in the root vasculature is alleviated and plants become phenotypically normal (Durrett et al., 2007). When heterologously expressed in *Xenopus* oocytes, FRD3 mediates efflux of citrate, indicating that the block in Fe loading to the xylem sap and accumulation of Fe within the pericycle of roots are caused by lack of citrate in the xylem. Thus, citrate appears to be required in xylem for loading of Fe to occur (Durrett et al., 2007). An apparent FRD3 ortholog, called FRD3-like1 (FRDL1), has been identified in rice and transposon knockout *frdl1* plants exhibit similar phenotypes to *frd3 Arabidopsis*, including reduced citrate and Fe in the xylem (Yokosho et al., 2009). Thus, in spite of having very different primary uptake systems for Fe, grasses and non-grasses appear to share the mechanism that allows translocation of Fe from root to shoot.

The complex phenotypes of *frd3* mutant plants reveal that citrate, in addition to complexing with Fe in xylem sap, may also affect Fe distribution at a local level within leaves. In *frd3* mutants, Fe is translocated to the shoots, presumably via residual xylem transport, and Fe levels in shoots become abnormally high (approximately twofold higher than in WT plants). In spite of this high Fe, all three Strategy I Fe-deficiency responses in *frd3* mutants are activated, suggesting that these plants are experiencing Fe deficiency (Rogers and Guerinot, 2002). WT shoots grafted to *frd3* roots become chlorotic, implying that it is failure to load citrate into the xylem in roots that leads to symptoms of Fe deficiency in shoots. Fe localization in *frd3* leaves has been examined closely by protoplasting to distinguish between intracellular Fe and apoplastic Fe, revealing that intracellular Fe is lower than normal in the mutant, suggesting that normal xylem citrate levels are necessary to allow proper post-xylem Fe distribution into cells (Green and Rogers, 2004).

Iron, too, must be effluxed to the xylem for long-distance transport and a candidate for the protein that accomplishes this step has been identified recently in work describing the *ferroportin* (*FPN*) family of genes in *Arabidopsis* (Morrissey et al., 2009). Mammalian ferroportin, also called Metal Transporter Protein1 (MTP1) and Iron REGulated protein1 (IREG1), is an effluxer that mediates Fe release from macrophages and hepatocytes (Muckenthaler et al., 2008). The three *Arabidopsis FPN* genes have distinct sub-cellular localizations, with FPN1/IREG1 found in the plasma membrane (Morrissey et al., 2009), FPN2/IREG2 in the vacuolar membrane (Schaaf et al., 2006; Morrissey et al., 2009), and FPN3/MAR1/RTS3/IREG3 on the chloroplast envelope (Conte et al., 2009). Transport activity of FPN1 proved difficult to determine, as it does not localize correctly to the plasma membrane in yeast, and so cannot be used in functional complementation assays. The closely related FPN2, however, transports nickel (Ni; Schaaf et al., 2006), cobalt (Co), and Fe (Morrissey et al., 2009) from the cytoplasm to the vacuole. *FPN1* is expressed in the stele of the root, at the root–shoot junctions of seedlings, and in the leaf veins, indicating a role in efflux into the apoplast and/or xylem. Plants with *fpn1* mutations (Morrissey et al., 2009) are chlorotic relative to WT plants, likely indicating Fe deficiency. Root-to-shoot translocation of Co, which is also a substrate for FPN1 efflux, is strongly reduced in *fpn1* mutant plants, again suggesting that FPN1 is responsible for loading metals into the xylem. The levels found in *fpn1* mutant shoots were not significantly different from WT plants, suggesting that FPN1 efflux is not the only means by which Fe is loaded into the xylem for root-to-shoot transport. Identification of additional mechanisms controlling xylem loading of Fe remains an important goal for full understanding of plant Fe homeostasis.

UNLOADING OF XYLEM IN LEAVES

Once the Fe arrives in the leaves, it must once again be taken up into cells, this time from the apoplastic space. We have already seen that citrate is needed to allow efficient movement of Fe from xylem into cells from the *Arabidopsis frd3* citrate efflux mutant, which accumulates Fe in extracellular spaces of the leaves (Green and Rogers, 2004), but the exact role of citrate in xylem unloading remains unclear. In *Arabidopsis*, members of the Yellow Stripe-Like (YSL) family of Fe(II)–nicotianamine transporters have been proposed as key mediators of Fe uptake from xylem.

Nicotianamine (NA; Figure 1) is the direct biochemical precursor to PS and, as such, is structurally quite similar. Consistent with this similarity of structure, NA, like PS, is a strong complexor of various transition metals, particularly Fe(II) (Anderegg and Ripperger, 1989) and Fe(III) (von Wiren et al., 1999), as well as copper (Cu), Ni, Co, manganese (Mn), and zinc (Zn) (Anderegg and Ripperger, 1989). NA is present in shoots and roots at concentrations ranging between 20 and 500 nmol g^{-1} fresh weight (Stephan et al., 1990) and is present in both xylem and phloem (Schmidke and Stephan, 1995; Pich and Scholz, 1996), suggesting that it is a major chelator of metals throughout the plant.

The YSL proteins of *Arabidopsis* were identified based on their strong, full-length sequence similarity to the maize Fe–PS transporter, YS1 (Curie et al., 2001). YSL genes are found in diverse plant species, including both monocots and dicots, gymnosperms, lycopods, ferns, and mosses. Thus, the YSL family is broadly distributed throughout higher plants. YSL proteins are able to use metal–nicotianamine complexes as transport substrates (DiDonato et al., 2004; Koike et al., 2004; Roberts et al., 2004; Schaaf et al., 2004; Le Jean et al., 2005; Murata et al., 2006; Gendre et al., 2007; Harada et al., 2007). Much of

what we know about NA function in plants comes from studies of a mutant of tomato called *chloronerva* (*chln*) in which the function of the single gene encoding *nicotianamine synthase* (*NAS*) is disrupted (Herbik et al., 1999; Higuchi et al., 1999; Ling et al., 1999). A complex phenotype results from this complete lack of NA synthesis. The *chln* plants exhibit interveinal chlorosis in young leaves and constitutively activate their root Fe-uptake systems (Stephan and Grun, 1989; Pich and Scholz, 1991), indicating that they have inadequate Fe. Surprisingly, though, mature leaves of *chln* mutants contain excess Fe (Scholz et al., 1985; Becker et al., 1992). Thus, in the absence of NA, Fe transport to young tissues appears to be impaired, indicating a role for NA in phloem transport of Fe. By contrast, excess Fe is transported to older tissues, presumably via xylem, indicating a possible problem with signaling of shoot Fe status to the root.

Evidence for the idea that YSLs are involved in xylem unloading in *Arabidopsis* comes from a combination of the expression patterns of three *YSL*s (*AtYSL1*, *AtYSL2*, and *AtYSL3*), together with the phenotype of the *ysl1ysl3* double mutant (DiDonato et al., 2004; Waters et al., 2006). Expression of *AtYSL1*, *AtYSL2*, and *AtYSL3* is down-regulated by Fe limitation, showing a pattern that is opposite to that of genes that are part of the Fe-uptake machinery in plants (e.g. *IRT1*, *FRO2*, and *YS1*). This suggests that these YSLs are not involved in Fe acquisition, but instead participate in a distinct aspect of Fe homeostasis. *AtYSL1*, *AtYSL2*, and *AtYSL3* are expressed most strongly in leaves in vascular parenchyma cells closely associated with xylem (DiDonato et al., 2004; Waters et al., 2006). When *AtYSL2* and *AtYSL3* genes were fused to GFP and stably expressed in *Arabidopsis* under their native promoters, green fluorescence was strongest in elongated cells within the vasculature, apparently xylem parenchyma, and was consistent with localization to the plasma membrane (DiDonato et al., 2004; Waters et al., 2006). Strikingly, fluorescence of AtYSL2 and AtYSL3 was only rarely observed at the apical or basal ends of cells, but was instead found almost exclusively on the lateral plasma membranes (DiDonato et al., 2004; Waters et al., 2006). This pattern is consistent with a role for these proteins in the lateral movement of metals within veins. A double mutant that lacks both *ysl1* and *ysl3* gene expression displays strong interveinal chlorosis. Chlorosis can be reversed by application of Fe-EDDHA (iron ethylenediamine-di(*o*-hydroxyphenylacetic) acid) solution to the soil, indicating that this phenotype is caused by a lack of Fe. Leaves of plants grown on standard tissue culture medium show decreased Fe levels, while the levels of other metal ions are normal. Soil-grown plants show perturbations in the levels of Cu, Mn, and Zn as well as Fe (Waters et al., 2006).

We have proposed that the predominant physiological role for AtYSL1, AtYSL2, and AtYSL3 is to take up Fe that has arrived in tissues via xylem transport, thus moving it from apoplast to symplast (DiDonato et al., 2004; Waters et al., 2006). This role is consistent with older studies of the *chln* mutant suggesting that in the absence of NA, movement of Fe into the lamina of the leaf is greatly slowed, and likely occurs through symplastic movement rather than uptake from apoplast (Stephan and Scholz, 1993). In this model, negative regulation of these *YSL*s during Fe starvation allows the plant to restrict exit of metals from the xylem in mature leaves. By restricting uptake in mature tissues, Fe would continue to be translocated apically towards younger leaves higher on the plant. Thus, down-regulation of these *YSL*s would represent a mechanism to enhance long-distance Fe translocation by preventing Fe sequestration in more basal parts of the plant. The strong chlorosis of the *ysl1ysl3* double mutant would result from the plants' inability to efficiently take up Fe from the apoplast following arrival via the xylem.

Integrating the roles of NA and citrate in xylem unloading will be an important next step in forming a complete understanding of the mechanisms used for xylem unloading. If all or most of the Fe arriving in leaves via xylem is bound to citrate as expected (Rellan-Alvarez et al., 2010), then ligand exchange to NA must occur before Fe–NA complexes would be available for transport by YSLs (Curie et al., 2009). Theoretically, such ligand exchange in the relatively acidic apoplast is not expected to be favorable (von Wiren et al., 1999; Rellan-Alvarez et al., 2008). However, it is possible that additional biochemical transformations of Fe and/or citrate could occur at sites of xylem unloading. For example, it has been suggested that Fe(III) must be reduced prior to uptake into leaf mesophyll cells and Fe–citrate is a favorable substrate for leaf ferric chelate reductase (Brüggemann et al., 1993). Reduction at the cell surface could impact the favorability of ligand exchange to NA and promote Fe–NA formation and uptake. Interestingly, Fe(II)–NA but not Fe(III)–NA complexes seem to be readily transported by YSL proteins previously shown to transport metal–NA complexes (DiDonato et al., 2004; Koike et al., 2004; Roberts et al., 2004; Gendre et al., 2007; Chu et al., 2010). In grasses, the requirement for ligand exchange from citrate may not involve a reduction step, since these species use PS (as opposed to NA) throughout their bodies. The rice OsYSL18 transporter, which is expressed only in internal plant tissues, specifically transports Fe(III)–PS complexes, and may facilitate xylem-to-phloem transfer of Fe within the plant vascular system (Aoyama et al., 2009). The question of how ligand exchange from citrate to PS might occur in grass species remains open.

TRANSLOCATION OF Fe DURING REPRODUCTION

The importance of NA during plant reproduction has been recognized for many years. The *chln* mutant fails to produce either flowers or fruits (Stephan and Scholz, 1993). Likewise, tobacco plants engineered to overexpress the enzyme Nicotianamine Amino Transferase (NAAT), which converts NA into a non-functional intermediate in phytosiderophore synthesis, display an array of reproductive abnormalities (Takahashi et al., 2003). The severe reproductive defects exhibited by NA-less plants are likely brought about by compromised phloem transport of Fe (and possibly other transition metals) when NA is absent. The stoichiometry of transition metals and NA

in phloem sap is ~1:1, which has led to the idea that transition metals are bound to NA in the phloem (Schmidke and Stephan, 1995). However, later studies using dialysis of the phloem sap demonstrated that Fe is likely to be bound by a higher-molecular-weight ligand—possibly a peptide (Schmidke et al., 1999). One candidate for such a peptide ligand is the Iron Transport Protein (ITP), which is a member of the Late Embryogenesis Abundant (LEA) family of proteins (Kruger et al., 2002). This has led to the alternative hypothesis that Fe (and other transition metals) could be loaded into the phloem as Fe–NA complex and then exchanged to a larger ligand during transport in the phloem sap. Strong evidence for the involvement of NA in the movement of Fe to seeds comes from studies in which the *NAS* gene is overexpressed, either by activation tagging of the native *OsNAS3* gene (Lee et al., 2009b) or by expression of *NAS* from a constitutive promoter (Wirth et al., 2009). In both cases, high levels of NA promoted higher levels of Fe in seeds.

In rice, OsYSL2 has hallmarks of a transporter that functions in moving Fe–NA complexes into phloem (Koike et al., 2004). OsYSL2 is capable of transporting Fe(II)–NA and Mn(II)–NA in two electrode voltage clamping experiments in *Xenopus* oocytes and is expressed in the phloem companion cells of both roots and leaves. OsYSL2 expression increases dramatically during periods of Fe deficiency, suggesting a role in the Fe-uptake process. This role cannot be in the primary acquisition of Fe from the soil, since expression is confined to the vascular cylinder and does not occur in epidermis and cortex, where Fe uptake from the soil would occur. While OsYSL2 expression in Fe-replete roots is confined to phloem-associated cells, OsYSL2 is broadly expressed throughout the central cylinder during Fe deficiency (Koike et al., 2004). When a hairpin construct was used to knock down OsYSL2 expression, plants (called OsYSL2i) exhibited an accumulation of Fe in the roots and a decline in the amount of Fe in shoots, which was attributed to a translocation defect from roots to shoots (Ishimaru et al., 2010). Phloem loading by OsYSL2 does not explain the observed root-to-shoot translocation defect, since root-to-shoot Fe translocation is expected to occur via xylem. Potentially, the expanded expression of *OsYSL2* observed in Fe-deficient roots suggests an additional role for OsYSL2, such as in translocation of Fe across the root or in loading of the xylem. Importantly, Fe translocation from roots to panicle (i.e. the rice inflorescence) was also compromised in OsYSL2i plants and grains of OsYSL2i plants had strongly decreased levels of Fe relative to WT plants (Ishimaru et al., 2010). While lowered Fe accumulation in grains of OsYSL2i plants could be the result of impaired phloem loading in leaves, it could also result from a more local effect, since OsYSL2 is normally expressed both in developing ovaries and in the endosperm and embryo of developing seeds (Koike et al., 2004). The combination of localization and knock-down phenotype strongly suggests that OsYSL2 plays an important role in the loading of Fe into rice seeds. High ectopic expression of *OsYSL2* using the rice *Sucrose Transporter1* (*OsSUT1*) promoter, which is expected to result in expression in both leaf phloem and developing endosperm, caused marked increases in Fe levels of the grain, again illustrating that YSL transport of Fe–NA complexes can contribute to Fe accumulation in seeds (Ishimaru et al., 2010).

YSLs have also been implicated in metal accumulation in *Arabidopsis* seeds. In addition to the vegetative defects mentioned above, the *ysl1ysl3* double mutant has multiple defects in reproduction: double mutant flowers produce few functional pollen grains and the seeds that these plants do manage to produce are often small and contain embryos arrested at various immature stages, which often fail to germinate (Waters et al., 2006). Seeds produced by *ysl1ysl3* plants have abnormally low Fe, Zn, and Cu content. Strikingly, though, expression of YSL1 and YSL3 in flowers does not overlap and YSL3 is not expressed in fruits and developing seeds at all (Waters et al., 2006), making these reproductive phenotypes puzzling. A likely explanation was provided by the observation that expression of *AtYSL1* and *AtYSL3* increased markedly during leaf senescence and *ysl1ysl3* double mutants plants failed to mobilize Zn and Cu from senescing leaves. No defect in Fe mobilization from *ysl1ysl3* leaves was observed. These results suggested that failure to accumulate metals in the seeds resulted, at least in part, from a failure to remobilize metals out of senescing leaves and into developing flowers and seeds (Waters et al., 2006). This model is consistent with the long-held idea that metals mobilized from vegetative structures account for a significant fraction of the metal content in seeds (Hocking and Pate, 1977, 1978).

Chu et al. (2010) reasoned that if YSL1 and YSL3 are necessary for remobilization of stored Fe from senescing leaves into developing seeds, *ysl1ysl3* inflorescences grafted onto wild-type (WT) rosettes should be fully rescued so that seed and pollen development would be normal and the metal levels and germination efficiency of the seeds would be restored. As expected, seed set (which is a proxy for pollen function, since non-functional pollen results in failure of seed set) and seed weight measurements indicated that YSL1 and YSL3 function is required in leaves for normal pollen and seed development (Chu et al., 2010). Surprisingly, though, metal levels in the seeds remained low after grafting (Chu et al., 2010). This indicates that YSL1 and YSL3 function are required in the grafted portion of the plants, that is, in the inflorescence stems, the flowers themselves, or the developing fruits and seeds. The finding that these YSL transporters are involved in mobilization of metals from leaves and the surprising finding that these mobilized metals are not necessarily accumulated in developing seeds are important clues to the molecular processes involved in metal accumulation in seeds. Since *YSL1* and *YSL3* are only weakly expressed in developing fruits and seeds, it seems likely that YSL activity in flowers, stems, and/or cauline leaves is necessary to achieve correct loading of metals into developing seeds, but the route of metal loading is complex. Waters and Grusak have shown that silique hulls are a likely source of metals that are being loaded into seeds and that, in *ysl1ysl3* plants, Fe and other transition metals are not efficiently translocated trough hulls (Waters and Grusak, 2008).

This finding is consistent with the results of classic experiments performed by Grusak, who demonstrated that Fe applied to the pod wall of peas was rapidly re-translocated into developing seeds (Grusak, 1994). Together, these studies suggest that Fe is translocated repeatedly from organ to organ prior to seed loading: from leaf to developing inflorescence, from inflorescence parts like stems or cauline leaves into hulls, and, finally, from hulls to developing seeds.

TRANSPORT OF Fe IN SEEDS

Knowledge about Fe movement into seeds is essential for biofortification strategies. Although NA and YSLs are implicated in directing metals into the seeds, the identity of the specific transport steps that occur within developing seeds and adjacent maternal tissues remains obscure. Recent work has, however, demonstrated how Fe is stored within the embryo. As mentioned previously, Fe is highly reactive and must either be bound to storage proteins or sequestered in vacuoles to prevent it from causing cellular damage. The ubiquitous intracellular protein ferritin is the primary storage form for Fe in animal cells and this was thought to be the case in *Arabidopsis* as well, until evidence emerged that ferritin's major role is in Fe detoxification rather than storage of Fe (Ravet et al., 2009). Little was known about sub-cellular compartmentation of Fe in seeds until the Vacuolar Iron Transporter, VIT1, was uncovered in *Arabidopsis* (Kim et al., 2006). VIT1 showed 62% amino acid similarity to the yeast CCC1 (Cross-Complements Ca(II) phenotype of *csg1*) Fe–Mn transporter, which is responsible for transport of these metals into vacuoles. Both *Arabidopsis* VIT1 and LeVIT1 from tomato were able to complement the yeast mutant *ccc1*, which is sensitive to high levels of Fe due to an inability to sequester Fe into vacuoles (Li et al., 2001). Fe and Mn content was greatly increased in isolated vacuoles of *ccc1* yeast expressing VIT1 (Kim et al., 2006). Sub-cellular localization experiments indicated unequivocal vacuolar localization for VIT1 in both yeast and plant cells. Synchrotron x-ray fluorescence microtomography of WT *Arabidopsis* seed revealed that Fe is predominantly localized in the pro-vascular strands of the embryonic hypocotyl, radicle and cotyledon. In *vit1-1* seeds, this distinct localization pattern was lost and Fe assumed a more diffuse pattern of localization at the abaxial sides of the cotyledons—the same location as that at which Mn is located in the embryo.

The application of a Perls reagent with diaminobenzidine hydrogen peroxide (Perls/DAB) staining protocol to histological sections has allowed direct visualization of sub-cellular Fe stores in plant tissues (Roschzttardtz et al., 2009). In stage VI wild-type embryos, the main pool of Fe was found in vacuoles of cells surrounding the pro-vascular system. The *vit1-1* mutation led to a redistribution of Fe to a single sub-epidermal cell layer in the cotyledon, again, the same cell layer as that which accumulates Mn. In the hypocotyl/radicle, Fe was restricted to the two layers of cortex cells. Interestingly, however, Fe appeared to remain in vacuoles, which suggests the existence of other vacuolar Fe transport systems.

The vacuole is clearly an important Fe storage compartment, especially in germinating seeds, where it acts as an initial source of metals before uptake from the external environment is possible. Storage of Fe in plant cell vacuoles creates a pool that is essential for proper seed germination, since *vit1-1* seedlings fail to germinate under Fe-deficient soil conditions (Kim et al., 2006). Members of the Natural Resistance Associated Macrophage Protein (NRAMP) family of transporters have key roles in mobilizing vacuolar stores of Fe during germination. The NRAMP transporters are a family of integral membrane proteins found in bacteria, fungi, animals, and plants (Cellier et al., 1995; Williams et al., 2000; Cellier et al., 2001). In *Arabidopsis*, both *NRAMP3* and *NRAMP4* are up-regulated under Fe deficiency and both localize to the vacuole (Thomine et al., 2000, 2003; Lanquar et al., 2005), indicating a possible role in metal remobilization from this compartment. Young *nramp3nramp4* double mutant seedlings exhibited a transient pale cotyledon phenotype that was ameliorated by day 10 after sowing. The double mutants were hypersensitive to low-Fe conditions—a phenotype that could be rescued by expression of either *NRAMP3* or *NRAMP4* alone. The use of transmission electron microscopy (TEM) along with Fe electron spectroscopic imaging (ESI) uncovered Fe in globoids of *nramp3nramp4* cotyledon vacuoles 2 d after germination, while Fe was missing from wild-type globoids at this stage (Lanquar et al., 2005). These data provide strong evidence that NRAMP3 and NRAMP4 are involved in remobilization of Fe from vacuoles during germination. However, the relatively mild phenotype of *nramp3nramp4* double mutants could indicate that other transporters are also involved in vacuolar Fe remobilization, but have yet to be uncovered.

CHLOROPLASTIC AND MITOCHONDRIAL Fe TRANSPORTERS

Not only does Fe have to be transported into and exported from vacuoles; it also must have a means of entry and exit from other organelles. Chloroplasts and mitochondria have large Fe requirements to carry out the various metabolic processes that occur within. However, transporters involved in plant organellar Fe transport have only recently begun to be uncovered. In mitochondria, Fe is required for synthesis of iron–sulfur (Fe–S) clusters and for proper function of the respiratory electron transport chain. Exposure to Fe deficiency caused a decrease in oxygen consumption rate in the roots of cucumber plants and the observed decrease in activities of respiratory complexes was proportional to the number of Fe atoms in each complex (Vigani et al., 2009). Additionally, TEM revealed that mitochondria undergo ultrastructural changes under Fe deficiency. Thus, mitochondria have an essential requirement for Fe, but little is known about how Fe is transported into and out of this organelle.

There are three transporters in *Arabidopsis* (AtATM1, 2, and 3) that are homologous to the yeast mitochondrial ATP binding cassette transporter1 (ScATM1), which is implicated in export of a component required for Fe–S cluster assembly and

maturation in the cytosol (Chen et al., 2007). AtATM–GFP fusions co-localize with MitoTracker Red in yeast, placing each of these transporters in the mitochondrial membrane. Of the three *Arabidopsis* proteins, AtATM3 bears the closest functional resemblance to ScATM1 and is able to complement the yeast *atm1* petite phenotype, suppressing the high-affinity Fe uptake associated with loss of chromosomal ScATM1, reducing intramitochondrial Fe hyperaccumulation, and restoring mitochondrial respiration function (Chen et al., 2007). Thus, AtATM3 likely transports the same substrate as ScATM1. AtATM1 only weakly suppresses the *atm1* phenotype and thus may have a distinct function. AtATM2 is toxic when expressed in the yeast system and, thus, its function has not yet been fully characterized (Chen et al., 2007).

In non-plant eukaryotes, disruption of *ATM1* leads to mitochondrial Fe accumulation (Kispal et al., 1997) and a phenotype of cytosolic Fe deficiency (Schueck et al., 2001), indicating that ATM1 plays an important role in overall cellular Fe homeostasis. Although loss of *Arabidopsis AtATM3* resulted in severe growth defects and chlorosis under normal conditions, expression patterns of *IRT1* and *ferritin* in *atm3-1* mutants indicated a reasonably normal response to external Fe levels, although low levels of IRT1 protein were detected at 10 and 20 μM Fe, and ferritin levels were twofold lower (at 40 μM Fe) in *atm3-1* roots compared to WT (Bernard et al., 2009). Additionally, *atm3-1* mitochondria did not contain elevated levels of Fe, nor was foliar application of iron able to rescue the growth defects and chlorosis of *atm3-1* seedlings. Interestingly however, AtATM3 was required for the activities of cytosolic, but not plastid or mitochondrial Fe–S enzymes. Taken together, these results indicate that AtATM3 is important for Fe–S cluster assembly in the cytosol; however, its role in mitochondrial Fe homeostasis remains unclear. For example, it is possible that plants are able to down-regulate mitochondrial Fe import systems when Fe export mechanisms are lost, which could explain Fe homeostasis phenotypes described by Bernard et al. (2009). Clearly, identification of additional transporters, such as those that import Fe to mitochondria, and more information about the transported substrates of ATM proteins is needed to understand mitochondrial Fe homeostasis in plants.

Chloroplasts are the major plant Fe sink, as they require Fe to carry out several metabolic processes such as photosynthetic electron transport, chlorophyll biosynthesis, Fe–S cluster assembly, and heme biosynthesis. Plastids contain ~80% of the Fe found in leaf cells (Shikanai et al., 2003) and the reactive oxygen species (ROS) produced by the photosynthetic electron transport chain may react with Fe, thereby causing oxidative damage. Thus, as in other cellular compartments, levels of Fe in the chloroplast must be tightly regulated. Unfortunately, we still know very little about how Fe is transported into and out of the chloroplast.

The *Arabidopsis* FERRIC REDUCTASE OXIDASE7 (FRO7) localizes to chloroplasts and, in *fro7* loss-of-function mutants, chloroplasts contained 33% less Fe than WT (Jeong and Guerinot, 2009). The presence of a ferric reductase on the chloroplast envelope indicates that one pathway for Fe entry into chloroplasts may involve a transporter that moves only the reduced form of Fe, Fe(II). ZIP family transporters (e.g. IRT1) can transport divalent transition metals, but, as of yet, no ZIP transporters have been found on the chloroplast envelope (Jeong and Guerinot, 2009).

A screen for metal transporters in plastids uncovered Permease In Chloroplasts 1 (PIC1), which is targeted to the inner envelope and has homology with cyanobacterial permease-like proteins (Duy et al., 2007). *pic1* mutants displayed dwarfism, severe chlorosis, and grew only heterotrophically. Ultrastructural analysis of mutants revealed severely impaired chloroplast development and an increase in ferritin clusters. Additionally, proteins associated with photosynthesis and carbon fixation were absent, while genes involved in Fe–S cluster biogenesis were down-regulated in *pic1* mutants. The ferritin genes *FER1* and *FER4* were up-regulated, with a corresponding increase in protein. Expression of *PIC1* restores growth to the yeast mutant *fet3fet4*, which has defects in Fe uptake, suggesting that it is able to transport Fe. Interestingly, Fe content was unchanged in *pic1* mutants, but Cu levels increased significantly. PIC1 was able to complement the copper +uptake-defective yeast mutant *ctr1*, suggesting that it can also transport Cu. *pic1* mutants also could not be rescued by excess Fe and PIC1 is not transcriptionally regulated by Fe. Thus, the exact role of PIC1 *in planta* may not be completely understood. PIC1 has been described previously as a component of the chloroplast inner-envelope translocon, which may implicate it in protein transport (Teng et al., 2006). Further research is required to fully elucidate the role of PIC1 in chloroplast Fe transport and homeostasis.

Recently, two putative plastid transporters were uncovered in *Arabidopsis* that displayed similarity to the cyanobacterial FutC Fe transporter (Shimoni-Shor et al., 2010). Mutants in both *non-intrinsic ABC transporter protein* (*atnap11* and *atnap14*) genes displayed severe growth defects; however, significant changes in transition metal homeostasis were detected only in *nap14* mutants. These mutants had 18-, 9-, 8-, and 1.5-fold increases in shoot Fe, Mo, Cu, and Zn, respectively. The increased metal content was accompanied by defects in chloroplast ultrastructure and down-regulation of genes involved in Fe homeostasis (FRO7, FER3, FER4, and PIC1). Thus, AtNAP14 may be part of a chloroplast transporter complex or, alternatively, could be acting as a plastid Fe sensor that monitors Fe status and controls Fe flux from roots to leaves (Shimoni-Shor et al., 2010).

TRANSPORTERS INVOLVED IN VACUOLAR SEQUESTRATION OF POTENTIALLY TOXIC METALS DURING Fe DEFICIENCY

IRT1, which is highly up-regulated under Fe deficiency, has a clear function as the primary uptake transporter for Fe into *Arabidopsis* roots (Henrique et al., 2002; Varotto et al., 2002; Vert et al., 2002). In addition to transport of Fe, IRT1 also has the capacity to transport cadmium (Cd), Co, Ni, Zn, and Mn. This ability is thought to lead to the 'ionomic signature' of Fe deficiency in

which levels of Fe are held constant through homeostatic adjustments by the plant, but levels of Mn, Co, Zn, and Cd are elevated, apparently by inadvertent uptake of these metals by IRT1 (Baxter et al., 2008). With multiple metals being imported into the cell as plants respond to Fe deficiency, one can easily see that the cell cytoplasm may become flooded with undesirable toxic metals. One of the ways in which plants protect themselves is to move these metals into the vacuole. Fe-regulated transporters from at least two families perform this function.

Arabidopsis MTP3 (Metal Tolerance Protein3) belongs to the cation diffusion facilitator (CDF) family, members of which are implicated in the transport of transition metal ions such as Zn, Mn, and Fe. MTP proteins are thought to function as exporters, utilizing energy from proton antiport (Kramer, 2005). While NRAMPs and ZIPs appear to have a broad substrate range, MTP transporters appear to be highly specific for zinc (Kramer, 2005). Zn is an essential cofactor for many transcription factors and enzymes, but can cause toxicity when it displaces other ions such as Fe, Mn, Ca, and Mg from their binding sites. Expression of MTP3 increases during periods of Fe deficiency, but the function of this transporter is not to transport Fe. Instead, MTP3, which is localized to the vacuoles of root epidermal cells, transports Zn (Arrivault et al., 2006). When *mtp3* mutant plants are grown under Fe deficiency, they accumulate excess Zn in above-ground tissues. This appears to be due to failure of MTP3 to sequester Zn in the vacuole in the roots, which would ordinarily prevent it from being moved into the vasculature and thus into shoots.

As mentioned earlier, one of the *Arabidopsis* ferroportin transporters, AtFPN2, is localized to the vacuole (Schaaf et al., 2006; Morrissey et al., 2009). In an early study, AtFPN2 (then referred to as IREG2) was unable to complement Fe-related defects in several yeast mutants with perturbed Fe homeostasis, but instead mediated elevated tolerance to Ni in yeast strains lacking the vacuolar cobalt effluxer, Cobalt Tolerance1 (COT1) (Schaaf et al., 2006). In *Arabidopsis*, *AtIREG2* overexpression lines were Ni-tolerant, and *ireg2* mutants accumulated less nickel in roots compared to WT. Interestingly, a clear Ni-sensitive phenotype for *ireg2* mutants was observed only under Fe deficiency, implying that an important function of AtIREG2 is to protect against Ni over-accumulation during Fe deficiency (Schaaf et al., 2006). More recently, FPN2/IREG2 has been revealed as a major quantitative trait locus (QTL) for cobalt accumulation in *Arabidopsis* (Morrissey et al., 2009). Plants with a naturally occurring frame shift mutation in FPN2 have high levels of Co in their shoots. Since FPN2 is capable of transporting Co into vacuoles when heterologously expressed in yeast (Morrissey et al., 2009), FPN2 is again implicated in vacuolar sequestration of toxic metals (Ni and Co) in Fe-deficient roots to prevent translocation of these metals into shoots.

CONCLUSIONS

Due to the concerted efforts of many laboratories, there has been considerable progress recently towards the elucidation of the roles of transport proteins in plant Fe homeostasis. We now know the identities of many transporters responsible for uptake of Fe from soil, transport from roots to shoots, unloading of xylem in above-ground parts, transport to reproductive parts of the plant, mobilization during seed germination, and Fe loading and unloading from vacuoles. However, many unanswered questions still remain. Despite the essential role of Fe in respiration and photosynthesis, a mitochondrial Fe importer has yet to be identified and chloroplast Fe import processes are not yet well defined. Fe transporters such as ZmYS1 and OsYSL15 have the ability to move other metals in addition to Fe; conversely, HvYS1 is highly specific for Fe(III)–MA. The factors that determine the metal substrate specificities of the YS and YSL transporters remain unknown and this will be a fascinating avenue to explore. Sub-cellular localization data are also missing for certain YSLs, namely AtYSL4 and AtYSL6, which were identified in a tonoplast proteomic study. Tonoplast localization for these transporters remains to be experimentally verified and would be an exciting development in the field, as all YSLs characterized thus far localize to the plasma membrane. Additionally, because YSL transporters are known to move Fe–NA conjugates, the presence of AtYSL4 and AtYSL6 on the tonoplast membrane would indicate a role for NA in the sub-cellular environment, in addition to its role in the vasculature. On the whole-plant level, the identification of additional mechanisms controlling xylem loading of Fe remains an important goal, as does an understanding of the roles of NA and citrate in xylem unloading. Finally, an understanding of the molecular mechanism of PS release from roots will be an essential final step in the full characterization of the Strategy II Fe-uptake mechanism used by agronomically important grass species.

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