UNDERSTANDING ARABIDOPSIS ION HOMEOSTASIS IN THE POST-GENOMIC ERA: ASSIGNING FUNCTION TO TWO PROTEINS INVOLVED IN IRON METABOLISM

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by
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The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

UNDERSTANDING ARABIDOPSIS ION HOMEOSTASIS
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Presented by Timothy P. Durrett

A candidate for the degree of Doctor of Philosophy

And hereby certify that in their opinion it is worthy of acceptance.

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ABSTRACT

Two different projects studying aspects of iron deficiency in the model plant *Arabidopsis thaliana* are detailed here. The first project describes the isolation and characterization of the Arabidopsis *frd4-1* and *frd4-2* mutants that do not induce Fe(III) chelate reductase activity in their roots in response to iron deficiency. Map-based cloning revealed that the *frd4* mutations reside in *cpFtsY*, which encodes a component of one of the pathways responsible for the insertion of proteins into the thylakoid membranes of the chloroplast. A number of different hypotheses were tested in an attempt to explain how defects in *cpFtsY* could affect the expression of root Fe(III) chelate reductase activity. The second project involves the further characterization of the protein FRD3, which was previously shown to be important for the efficient translocation of iron from roots to the shoots. Xylem exudate from *frd3* plants contains significantly less citrate and iron than the exudate from wild type plants. Additionally, supplementation of growth media with citrate rescues the *frd3* phenotypes. The ectopic expression of FRD3-GFP results in enhanced tolerance to aluminum in Arabidopsis roots, a hallmark of organic acid exudation. Consistent with this result, the transgenic lines ectopically expressing FRD3-GFP exude higher levels of citrate from their roots. Finally, heterologous studies in *Xenopus laevis* oocytes reveal that FRD3 mediates the transport of citrate. These results confirm previous reports that iron moves through the xylem as a ferric-citrate complex

and strongly support the hypothesis that FRD3 effluxes into the root vasculature the citrate necessary for the formation of this complex.

CHAPTER 1

INTRODUCTION

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Iron is an essential micronutrient for almost all living organisms. Iron's ability to adopt two different ionic states makes it useful for electron transfer. Therefore, many enzymes that catalyze redox reactions possess an iron cofactor (usually in the form of iron-sulfur clusters or heme); similarly many of the proteins present in the electron transport chains of respiration or photosynthesis contain iron. In addition to these two essential pathways, iron containing proteins in plants are involved in other vital processes. For example, the conversion of Mg-protoporphyrin IX to protochlorophyllide, a key step in chlorophyll synthesis, requires an iron containing enzyme, CHL27 in Arabidopsis (Tottey *et al.*, 2003). Additionally, the nitrogenase enzyme complex involved in nitrogen fixation in legumes contains iron in various subunits. For these and other processes, iron is essential for the day to day functioning of plants.

On the other hand though, excess iron can be toxic. Through the Fenton reaction, iron can catalyze the formation of highly reactive hydroxyl radicals, which damage cellular contents such as DNA, proteins and lipids. Consequently plants, like all other organisms, very carefully regulate their uptake and storage of iron.

Despite being the fourth most abundant element in the earth's crust, iron's bioavailability remains limited. Due to an oxidizing atmosphere, most iron exists in the soil as Fe(III) oxides which are barely soluble at most soil pH values. Plants therefore have evolved two different uptake strategies by which they can acquire iron. Dicotyledenous plants and all the non-graminaceous monocots utilize what has been termed Strategy I Iron Uptake; the grasses utilize Strategy II Iron Uptake.

Strategy II Iron Uptake

The grasses use an efficient chelation strategy to obtain iron from the soil: when iron deficient, these plants secrete compounds known as phytosiderophores (PS) into the rhizosphere. There, because of their high affinity for Fe^{3+} ions, the secreted phytosiderophores form complexes with available iron. These Fe^{3+}-PS complexes are then taken up by a specific transporter into the root epidermal cells.

The synthesis of PS has been extensively studied and most of the enzymes involved have been identified in various species (reviewed in Mori, 1999). The initial reaction in this pathway involves the conversion of SAM to nicotianamine (NA) by nicotianamine synthase (NAS) (Herbik *et al.*, 1999; Higuchi *et al.*, 1999; Mizuno *et al.*, 2003). Additional enzymes, unique to the grasses, then modify NA to produce various phytosiderophores. Interestingly, despite the fact that they do not secrete phytosiderophores, dicotyledonous plants also possess NAS and synthesize NA (Ling *et al.*, 1999; Suzuki *et al.*, 1999). As will be discussed later, this production of NA is believed to play a role in the long distance translocation of iron and other metals throughout both Strategy I and Strategy II plants.

The mechanism of PS secretion still remains unknown. PS are probably secreted as monovalent anions via anion channels, along with the symport of K^+ (Sakaguchi *et al.*, 1999). This secretion follows a distinct diurnal pattern, with peak secretion just after sunrise (Takagi *et al.*, 1984). Particular vesicles in the roots of iron deficient barley increase in size and number immediately prior to PS secretion and become shrunken after secretion, suggesting that these vesicles are the site of PS synthesis (Nishizawa and Mori, 1987). Microarray studies in barley have identified a number of genes induced under

iron deficiency with diurnal regulation. Interestingly, some of these genes have previously been shown to be involved in polar vesicle transport in other species, suggesting that vesicular trafficking is important for PS secretion (Negishi *et al.*, 2002). Further molecular insights into PS release will come from the cloning of the maize *yellow stripe3* (*ys3*) mutant, which has long been recognized as being defective in the secretion of PS.

The cloning of the maize *YELLOW STRIPE1* (*YS1*) gene through *Ac*-transposon tagging revealed the Fe^{3+}-PS uptake transporter (Curie *et al.*, 2001). *YS1* mRNA levels increase under iron deficiency in both roots and shoots, as would be expected from a gene involved in iron uptake. Expression of YS1 in the yeast *fet3fet4* iron uptake mutant restores growth on media supplemented with Fe(III)-PS, suggesting that YS1 transports these complexes. More detailed biochemical studies have demonstrated that YS1 is a proton-coupled transporter capable of transporting a broad-range of different metal-PS complexes (Schaaf *et al.*, 2004).

Orthologues of YS1, termed Yellow-Stripe Like (YSL) have been found in other species, including dicotyledonous plants (DiDonato *et al.*, 2004; Koike *et al.*, 2004). As with the case of the nicotianamine synthase genes, it is thought that the YSLs are important for the systemic translocation of iron and other metals in all plants, even those that do not rely on PS for iron uptake.

Strategy I Iron Uptake

All dicots and non-graminaceous monocots coordinately upregulate three biochemical activities in their roots in response to iron deficiency: they acidify the rhizosphere, reduce Fe(III) to Fe(II) and transport Fe(II) (Marschner, 1995).

The acidification of the rhizosphere is brought about by the release of protons from the root. In Arabidopsis, the proteins responsible for this activity have yet to be definitively identified, but are thought to be members of the AHA H^+-ATPase family (Palmgren, 2001). This lowering of the pH in the rhizosphere increases the solubility of Fe(III) chelates, thereby facilitating iron uptake.

In order for iron to be transported into the plant, it must first be reduced by a Fe(III) chelate reductase. In Arabidopsis, the *FRO2* gene encodes this plasma membrane localized enzyme. *FRO2* was originally isolated using a PCR-based strategy that took advantage of conserved cofactor binding sites in other known membrane-bound reductases such as the yeast Fe(III) chelate reductase FRE1 and the human phagocytic NADPH-oxidase component $gp91^{phox}$ (Robinson *et al.*, 1999). The human NADPH oxidase, responsible for the generation of reactive oxygen species (ROS) in phagocytes, is a flavocytochrome b_{558} comprised of the transmembrane proteins $gp91^{phox}$ and $p22^{phox}$ (Segal *et al.*, 1992). Additional cytosolic proteins including $p47^{phox}$, $p67^{phox}$ and rac2 are also necessary for activity (Abo *et al.*, 1992). In activated neutrophil cells, both $p47^{phox}$ and $p67^{phox}$ translocate to the plasma membrane where they bind the cytochrome complex (Clark *et al.*, 1990). rac2 is a small GTP-binding protein and appears to play a role in the regulation of the NADPH oxidase (Abo *et al.*, 1991; Knaus *et al.*, 1991).

Eight respiratory burst oxidase homologs (rboh) of gp91^{phox} have been identified in Arabidopsis. At least two of these proteins, AtrbohD and AtrbohF, are required to generate ROS in response to pathogen attack upon the plant (Torres *et al.*, 2002; Torres *et al.*, 1998). A rac homolog also seems to be involved in the generation of this oxidative burst in rice (Kawasaki *et al.*, 1999). To date, no orthologs of p47^{phox} and p67^{phox} have been recognized in the Arabidopsis genome.

FRO2 possesses little sequence similarity to *FRE1* and gp91^{phox} with the exception of conserved regions responsible for the binding of two heme groups, FAD and NADPH (Robinson *et al.*, 1999). Instead, *FRO2* bears more similarity to other members of the FRO family in Arabidopsis, which encode Fe(III) reductases active in other parts of the plant (Bauer *et al.*, 2004; Mukherjee *et al.*, 2005; Wu *et al.*, 2005). FRO2 is also predicted to have eight transmembrane domains in contrast to the six present in Fre1p and gp91^{phox}. Interestingly though, the C-terminal domain of FRO2 shows 50% sequence similarity (10% identity) to p22^{phox}. This, coupled with the fact that p22^{phox} contains two transmembrane domains, suggests that FRO2 might encode the function of both gp91^{phox} and p22^{phox}. FRO2's role as the important Fe(III) reductase involved in iron acquisition was revealed when it was able to restore iron-deficiency-inducible root Fe(III) chelate reductase activity in *frd1* mutants (Robinson *et al.*, 1999). An earlier study had isolated these Fe(III) chelate reductase deficient mutants, which failed to induce root Fe(III) chelate reductase activity during iron limitation (Yi and Guerinot, 1996). When the *FRO2* gene was sequenced non-synonymous mutations were discovered in *frd1-1* and *frd1-3*, further confirming the role of FRO2 (Robinson *et al.*, 1999). Additionally, *FRO2* expression is upregulated in roots under conditions of iron deficiency, consistent with its

involvement in iron uptake. Later studies demonstrated that in addition to this transcription regulation, *FRO2* is subject to post-transcriptional regulation: in Arabidopsis plants constitutively expressing *FRO2*, Fe(III) chelate reductase activity is increased only when the plants are exposed to iron deficiency (Connolly *et al.*, 2003). The exact nature of this regulation has yet to be determined.

IRT1 (iron regulated transporter 1) functions as the main iron uptake transporter in Arabidopsis. IRT1 was isolated as a cDNA capable of complementing the yeast *fet3 fet4* double mutant which is defective in the uptake of iron (Eide *et al.*, 1996). Further yeast expression studies demonstrated that IRT1 could transport other divalent metal irons such as Zn^{2+} and Mn^{2+} (Eide *et al.*, 1996; Korshunova *et al.*, 1999). *In planta* studies revealed that *IRT1* is transcriptionally upregulated in the roots of iron deficient plants (Eide *et al.*, 1996), mainly in the epidermal cell layer consistent with its role in iron acquisition from the soil (Vert *et al.*, 2002). IRT1 expression has also been detected in flowers; however, as this expression is not regulated by the iron status of the plant, it is thought that IRT1's role in the flower is more of a developmental than a nutritional one (Vert *et al.*, 2002). Ablation of *IRT1* by T-DNA insertions produces extremely chlorotic plants with severe growth defects when grown on soil (Henriques *et al.*, 2002; Varotto *et al.*, 2002; Vert *et al.*, 2002). These phenotypes can all be rescued by supplementing the growth medium of the *irt1* mutants with high concentrations of iron. *irt1* mutants accumulate less iron in their leaves compared to wild type plants (Henriques *et al.*, 2002; Vert *et al.*, 2002). Iron is required for the photosynthetic machinery in plants and the *irt1* mutants possess considerably lower chlorophyll levels, show a dramatic reduction in

thylakoid stacking and exhibit reduced electron flow from PSII (Henriques *et al.*, 2002; Varotto *et al.*, 2002).

IRT2 is the most similar paralog of IRT1, possessing 69% identity and 82% similarity at the amino acid level to IRT1 (Vert *et al.*, 2001). Like IRT1, IRT2 is capable of rescuing the yeast *fet3 fet4* mutant, suggesting that IRT2 is also transports iron. Additionally, transcription of *IRT2* is increased in the roots of iron deficient plants, but this induction is several-fold lower than that for *IRT1*. Despite these similarities, additional evidence suggests that IRT2 does not play a major role in iron acquisition in Arabidopsis. Firstly, histochemical analysis of IRT2-GUS plants revealed expression of IRT2 in the epidermal and cortex layers of iron deficient roots. Additionally, T-DNA insertions in *IRT2* fail to produce any phenotype. Also, over expressing *IRT2* in *irt1* mutants fails to rescue the dramatic iron uptake defective phenotype of these plants (Varotto *et al.*, 2002). These results suggest that IRT1 functions as the major iron uptake transporter under iron deficient conditions and that IRT2 must possess a different function. IRT2 might differ in its intracellular localization or in its substrate specificity or affinity compared to IRT1. Yeast expression studies provide support for the latter idea: IRT2 appears more efficient at transporting Zn than IRT1, and unlike IRT1 does not transport Mn or Cd (Vert *et al.*, 2001).

Based on available genetic sequences, it was quickly realised that many other homologs of IRT1 existed in different species. This large family of metal transporters was given the name ZIP for ZRT, IRT-like Protein. The two most similar genes to *IRT1* in yeast, *ZRT1* and *ZRT2*, encode the high and low affinity zinc uptake transporters, respectively (Zhao and Eide, 1996a, 1996b). Neither of these proteins appears to

transport iron. Similarly, with the exception of *IRT2*, none of the other Arabidopsis ZIPs tested to date transports iron when expressed in yeast (Grotz *et al.*, 1998). Instead, like the yeast ZIPs, these plant transporters probably function in the uptake and transport of zinc. Indeed, it is interesting to note that all the plant ZIPs from Arabidopsis, tomato and pea that are capable of transporting iron tend to cluster together in amino acid alignments (Guerinot, 2000).

Like FRO2, IRT1 is subject to post-transcriptional regulation. Transgenic Arabidopsis lines constitutively expressing *IRT1* under the control of the 35S promoter only accumulate IRT1 protein in the roots under conditions of iron deficiency (Connolly *et al.*, 2002). While the exact nature of this post-transcriptional regulation still is not known, it is tempting to speculate that IRT1 might be regulated similarly to other members of the ZIP family. For example, the yeast zinc transporter ZRT1 is regulated at the post-translation level by ubiquitination, removal from the plasma membrane by endocytosis and subsequent degradation in the vacuole under conditions of high zinc (Gitan and Eide, 2000). In this regard it has been observed that IRT1 possesses two lysine residues in a cytoplasmic variable loop that are necessary for this post-translational regulation (Erin Connolly, unpublished data).

Regulation of Iron Uptake

Very little is known about how iron deficiency in plants triggers an increase in their uptake strategies. The existence of shoot derived signal controlling uptake in the roots has been demonstrated based on studies involving two different pea mutants, *brz* and *dgl*, that constitutively express their Strategy I iron uptake responses. Mutant shoots

grafted onto wild-type roots resulted in constitutive Fe(III) chelate reductase expression whereas wild-type shoots grafted onto mutant roots restored normal regulation of iron uptake (Grusak and Pezeshgi, 1996). The genotype of the shoot determines the phenotype of the roots, suggesting that the shoots generate a transmissible signal that leads to increased iron uptake in the roots. As most iron in plants is found in leaf chloroplasts (Terry and Abadia, 1986), the derivation of this systemic signal from the shoots is not surprising. However, the molecular nature of this signal still remains unknown.

More recent work has suggested the existence of additional, root-derived signals that also control iron uptake. Split root experiments in Arabidopsis and other species have demonstrated that Fe(III) reductase activity is higher in the roots supplied with iron (Schikora and Schmidt, 2001; Vert *et al.*, 2003) implying that the systemic signal generated by iron deficient shoots is further modulated by a local, root derived signal controlled by the root iron pool. Physiological adaptations to iron deficiency, such as the formation of root hairs in Arabidopsis and tomato or the development of specialized transfer cells in some species such as pea, also appear to be governed by the local concentration of iron in the roots, providing additional evidence for the existence of root derived signals that also control iron acquisition (Schikora and Schmidt, 2001).

Recent work has identified transcriptional regulators controlling the expression of genes involved in Strategy I iron acquisition. The tomato *fer* mutant cannot upregulate its root iron deficiency responses; consequently mutant plants become extremely chlorotic and ultimately die unless rescued with high levels of iron supplementation

(Brown and Ambler, 1974; Brown *et al.*, 1971). LeIRT1 and LeNramp1 are not expressed in the *fer* mutant (Bereczky *et al.*, 2003). The cloning of this tomato mutant revealed that *FER* encodes a basic helix-loop-helix (bHLH) transcription factor (Ling *et al.*, 2002). *FER* expression and protein accumulation is restricted to cell types associated with iron uptake or translocation, most notably the epidermis and cells surrounding the vasculature and is reduced upon exposure to high levels of iron (Brumbarova and Bauer, 2005; Ling *et al.*, 2002).

Two groups simultaneously characterized the Arabidopsis ortholog, hence its designation with two different names: FRU (FER-like regulator of iron uptake) (Jakoby *et al.*, 2004) and FIT1 (Fe-deficiency Induced Transcription Factor 1) (Colangelo and Guerinot, 2004). Like *FER*, *FRU/FIT1* is expressed in the root epidermis, as well as the endodermis and vascular cylinder, and this expression is upregulated under conditions of iron deficiency. T-DNA insertion mutants of *FRU/FIT1* are extremely chlorotic and die unless supplemented with high levels of iron. In these mutants, *FRO2* expression and subsequent Fe(III) chelate reductase activity is eliminated under conditions of iron deficiency. *IRT1* mRNA levels remain unaffected, yet interestingly IRT1 protein is absent under these conditions, suggesting the presence of an additional FRU/FIT1-dependent factor that is required for IRT1 translation or stability (Colangelo and Guerinot, 2004). Consistent with their inability to activate the appropriate Strategy I iron deficiency responses, these mutants contain lower levels of iron compared to wild-type plants, explaining their chlorotic appearance. These mutant phenotypes are extremely similar to that of the tomato *fer* mutant. In addition to controlling the Strategy I uptake

response, FRU/FIT1 appears to play a wider role in iron homeostasis, controlling 72 of 179 iron-regulated genes in Arabidopsis (Colangelo and Guerinot, 2004).

As expression of both *FER* and *FRU/FIT1* is regulated by the iron status of the plant, these transcription factors themselves are unlikely to function as the master regulator of iron homeostasis within the plant. Instead, some other upstream factor(s) probably are present that are capable of sensing the iron status of the plant and then regulating downstream components such as FER and FRU/FIT1, as well as expression of *IRT1*.

Systemic Translocation of Iron

Most iron acquired by plants ultimately ends up in leaf chloroplasts. For this final localization to be achieved, iron ions have to be moved radially through the root to the vasculature, either by passing through the plasma membranes of cells in different radial layers, by moving symplastically through plasmodesmata or by moving extracellularly in the apoplastic space. However, even if iron does move this latter way, it still has to cross the Casparian strip, a layer of cells in the endodermis containing suberin in their cell walls. As suberin is hydrophobic, it acts as a barrier to ion and water movement. Thus, even in the case of apoplastic transport, iron must still be transported across the plasma membrane of the endodermis. After being loaded into the xylem vessels, iron (in a chelated form) is translocated aerially to the shoots, where it has to be unloaded out of the vasculature and into leaf cells. Once in leaf cells, most iron must finally be transported into chloroplasts. During this entire process, iron ions therefore have to cross several

different membrane barriers. Consequently, plants possess an elaborate systemic transport system to move iron taken up by root epidermal cells to leaf cells.

As outlined earlier, the sequencing of the Arabidopsis genome revealed that FRO2 was part of the eight member FRO gene family. It was hypothesized that the other FROs encoded Fe(III) chelate reductases required for the reduction necessary prior to transport of iron across other membranes within the plant. Studies in other species had provided a precedent for this. For example, earlier work had demonstrated that the reduction of Fe^{3+} to Fe^{2+} was necessary for the iron uptake in leaf mesophyll cells of *Vigna unguiculata* (Brüggemann *et al.*, 1993). *PsFRO1*, thought to encode the main root Fe(III) chelate reductase involved in iron acquisition in pea, is also expressed in the root cortex and vasculature, as well as in leaves and nodules (Waters *et al.*, 2002). Characterization of individual FRO family members from Arabidopsis has indeed suggested that this hypothesis is correct. Expression of the *FRO* genes in yeast confirmed that they encoded Fe(III) chelate reductases (Wu *et al.*, 2005). Interestingly in these experiments, FRO2 possessed the highest Fe(III) chelate reductase activity, consistent with its role as the major reductase involved in iron uptake from the soil. Detailed analysis of the localization of gene expression has also confirmed that the FRO genes are expressed in different tissues throughout the plant (Bauer *et al.*, 2004; Mukherjee *et al.*, 2005; Wu *et al.*, 2005). *FRO3* expression has been detected in roots, as well as in shoots, flowers and cotyledons. Staining of FRO3-GUS transgenic plants revealed expression in the tips of lateral roots and in the vascular cylinder (Mukherjee *et al.*, 2005). This latter localization suggests that FRO3 might play a role in the loading of iron into the xylem or phloem for transport throughout the plant. *FRO6* is expressed

mainly in the shoots, but also in flowers, siliques and cotyledons (Feng *et al.*, 2006; Mukherjee *et al.*, 2005; Wu *et al.*, 2005). The expression of *FRO6* is increased by the light, consisted with the discovery of several Light Regulated Elements present in this gene's promoter (Feng *et al.*, 2006). This light regulation of *FRO6* is consistent with previous work showing that the iron reductase activity of *Vigna unguiculata* mesophyll cells increased in the presence of light (Brüggemann *et al.*, 1993). *FRO7* possesses an expression profile similar to that of *FRO6.* Interestingly, FRO7 might possess a N-terminal signal sequence targeting it to the thylakoid pathway (Mukherjee *et al.*, 2005). It has been demonstrated that Fe(II) is transported across the chloroplast inner membrane (Shingles *et al.*, 2002) and it is tempting to speculate that FRO7 might encode the Fe(III) reductase involved in this transport.

In addition to having to cross multiple membrane barriers, iron also has to be translocated aerially from the roots to the shoots. As citrate is present in xylem fluid and is capable of forming extremely stable, water-soluble complexes with Fe(III), iron is most probably transported from the roots to the shoots through the xylem in the form of iron-citrate complexes. In fact, mathematical models predict that iron is almost exclusively bound to citrate in xylem fluid. For example, 99.5% of the total iron in soybean exudate is calculated to be chelated by citrate (White *et al.*, 1981). These results are supported by earlier experimental evidence. It has long been recognized that the levels of various organic acids increase in the roots and shoots of both Strategy I and Strategy II plants when iron deficient (reviewed in Abadia *et al.*, 2002). Striking parallels exist between the levels of iron and citrate in the xylem exudates from iron deficient soybean plants

exposed to different levels of iron deficiency (Brown and Tiffin, 1965). Analysis of xylem exudate from a number of different species revealed that iron and citrate comigrated during paper electrophoresis (Tiffin, 1966, 1970). Studies of sugar beets have also demonstrated that the xylem concentration of various organic acids, including citrate, malate and succinate, all increase under iron deficiency (Lopez-Millan *et al.*, 2000). However, the mechanism by which citrate is loaded into the xylem remains unknown.

The FRD3 protein is likely to play a role in this long distance transport of iron. *frd3* mutant plants constitutively expresses their iron uptake responses, resulting in an overaccumulation of iron in their roots and shoots (Rogers and Guerinot, 2002). Despite this overaccumulation, it has been demonstrated that the leaf cells are actually iron deficient, suggesting that iron is being mislocalised (Green and Rogers, 2004; Rogers and Guerinot, 2002). *FRD3* encodes a member of the Multi-drug and Toxin Efflux (MATE) family. Several members of this family have been shown to transport small, organic compounds (Li *et al.*, 2002; Morita *et al.*, 2000; Otsuka *et al.*, 2005). Given its localization to the stele cells surrounding the vasculature in the roots (Green and Rogers, 2004), FRD3 probably functions to transport a small, organic iron chelator into the vasculature that is necessary for the correct localization of iron throughout the plant. Chapter 3 will present work suggesting that FRD3 loads citrate into the root xylem.

The non-proteinaceous amino acid nicotianamine (NA) has also long been implicated in the distribution of iron in plants. As discussed previously, NA is a precursor in the synthesis of phytosiderophores secreted by Strategy II plants to obtain

iron from the soil. However, NA is present in all higher plant species and as it is also capable of chelating both Fe^{2+} and Fe^{3+} (von Wirén *et al.*, 1999), NA likely plays a role in iron homeostasis in Strategy I plants as well. This relevance of NA to iron homeostasis in Strategy I plants is underscored by the phenotype of the tomato mutant *chloronerva*. These mutant plants constitutively express their iron uptake responses and consequently over accumulate iron in their leaves. The leaves however, exhibit interveinal chlorosis, a phenotype typically associated with iron deficiency (reviewed in Scholz *et al.*, 1992). Interestingly, *chloronerva* plants also possess altered copper homeostasis, implicating NA in the distribution of this micronutrient (Pich *et al.*, 1994). Biochemical studies revealed that *chloronerva* was lacking NA and that the phenotype could be rescued by the exogenous application of NA (Budesinsky *et al.*, 1980). These results were later confirmed at the genetic level with the cloning of the *chloronerva* gene, which encoded a nicotianamine synthase (NAS) (Ling *et al.*, 1999). Arabidopsis possesses 4 NAS paralogues with probable redundant function (Suzuki *et al.*, 1999), explaining the lack of *chloronerva*-like Arabidopsis mutants.

Based on the results of a number of studies, NA is thought to play a role in the long distance transport of iron in plants, specifically in the unloading of iron from xylem vessels and in the transfer of iron from the xylem to the phloem for redistribution throughout the plant. Consistent with this, studies in rice have demonstrated that expression of at least three NAS genes is regulated by iron status, and that this expression is confined to cells involved in the long-distance transport of iron (Inoue *et al.*, 2003). Similar work in maize and Arabidopsis has confirmed that NAS genes in these species

are also regulated by iron availability ((Mizuno *et al.*, 2003), Erin Connolly, personal communication).

As discussed previously, Strategy I plants also possess homologs of the maize *Yellow Stripe 1* (YS1) gene necessary for the uptake of iron-phytosiderophore complexes. Because Strategy I plants do not synthesize phytosiderophores, it was hypothesized that the members of this Yellow Stripe Like (YSL) family transport NA, which is similar in structure to the phytosiderophores derived from it. Heterologous expression of Arabidopsis YSL2 in yeast demonstrated that it is capable of transporting Fe^{2+}-NA and Cu-NA complexes (DiDonato *et al.*, 2004). Consistent with the role of NA in unloading iron from xylem vessels, both *AtYSL1* and *AtYSL2* are expressed in leaf veins (DiDonato *et al.*, 2004; Le Jean *et al.*, 2005). YSL2-GFP fusion protein almost exclusively localizes to the lateral plasma membrane, and rarely to the apical or basal membranes, of xylem parenchyma cells in roots and shoots, implying that YSL2 transports Fe-NA complexes laterally within veins (DiDonato *et al.*, 2004). AtYSL1 also appears to play a role in loading seeds with iron and NA: seed of *Atysl1* mutants contained less iron and NA than wildtype seed (Le Jean *et al.*, 2005).

Intracellular Iron Transport

Very little is known about intracellular iron transport and homeostasis in plants. Members of many gene families are probably involved, based on predicted organelle localization. For example, FRO7 might be involved in transporting iron into the chloroplast (Mukherjee *et al.*, 2005). To date however, the vacuole is the only cellular compartment for which iron transporters have been definitively identified.

NRAMP proteins from different species have been shown to transport several different metal cations, including iron (Chen *et al.*, 1999; Goswami *et al.*, 2001; Gunshin *et al.*, 1997). Arabidopsis possesses six members of this metal transporter family. The expression of at least three of them, *AtNRAMP1*, *AtNRAMP3* and *AtNRAMP4*, is regulated by the iron status of the plant, suggesting a role in iron homeostasis. Functional studies have indeed demonstrated that AtNRAMP1, AtNRAMP3 and AtNRAMP4 are all capable of transporting Fe and Mn (Curie *et al.*, 2000; Thomine *et al.*, 2000). AtNRAMP3 and AtNRAMP4 have both been localized to the vacuole, where they are believed to export metal ions into the cytosol (Lanquar *et al.*, 2005; Thomine *et al.*, 2003). Both genes are expressed early in seedling development suggesting that they function to mobilize iron from the vacuole during germination. Not surprisingly, *atnramp3 atnramp4* double mutant seed germinates slowly under low iron conditions (Lanquar *et al.*, 2005). Interestingly, AtNRAMP3 and AtNRAMP4 are expressed in the stele of roots and in the vasculature of leaves and stems (Lanquar *et al.*, 2005; Thomine *et al.*, 2003), arguing for a possible role in the long distance transport of metals. However, at this point in time, little else is known about this potential function.

Recent work has also identified the plant vacuolar iron importer as VIT1, the Arabidopsis ortholog of the yeast CCC1 protein, responsible for the import of iron and manganese into the yeast vacuole. Interestingly, *atvit1* seed germinates slowly under low iron conditions, similar to the phenotype of *atnramp3 atnramp4* double mutant seed (Mary Lou Guerinot, personal communication).

Iron Storage

Plants, like many other organisms, use iron storage proteins known as ferritins to sequester excess iron for later use. Ferritins therefore function both as iron detoxifiers and as iron stores. Despite a surprisingly high variation in sequence between different species, all ferritins possess a highly conserved structure, consisting of a 24-mer complex that is capable of storing up to 4500 iron atoms in its central, hollow core (reviewed in Harrison and Arosio, 1996). Animal ferritins are comprised of two different subunit proteins (termed H and L) whereas plant and bacterial ferritins consist entirely of the H subunit. Additionally, animal ferritins localize to the cytoplasm whereas plant ferritins are found only in chloroplasts. The Arabidopsis genome contains four ferritin homologues that appear to play different roles based on developmental and tissue specific expression patterns (Petit *et al.*, 2001a). *AtFer1* and *AtFer3* expression is upregulated in roots and shoots in response to high iron supply, suggesting these two genes encode the primary ferritins involved in iron storage in vegetative tissue. Based on its expression mainly in older siliques and in dry seeds, *AtFer2* is believed to play a role in seed iron storage. *AtFer4* expression appears restricted to flowering tissues.

Plant ferritins appear to be regulated transcriptionally, as opposed to animal ferritins that are regulated translationally. A 15-bp Iron Dependent Regulatory Sequence (IDRS) has been identified in the promoters of the orthologous maize *ZmFer1* and *AtFer1* genes that is necessary for the transcriptional repression of these genes under low iron supply (Petit *et al.*, 2001b). Most probably, a yet to be identified protein binds this IDRS sequence under iron deficient conditions to inhibit transcription. The IDRS appears to only be important for iron regulation; mutations in its sequence have no effect

on the tissue and developmental-stage specific expression of *AtFer1*, suggesting that other cis elements must control these latter aspects of regulation (Tarantino *et al.*, 2003).

Practical Implications and Future Directions

The practical implications of a better understanding of iron homeostasis in plants are vast. Iron Deficiency Anemia (IDA) has been recognized by the World Health Organisation as the world's leading nutritional disorder, with up to one third of the total population affected (World Health Organization, 2006). IDA is especially prevalent in developing countries where poorer populations depend mainly on plants, a poor source of bioavailable iron, for their nutritional needs. Direct attempts to increase the amount of iron in plants through fertilization are for the most part impractical as any applied iron is rapidly oxidized to its sparingly soluble Fe(III) form. Another, more viable alternative, would be to breed or genetically engineer food crops to increase their uptake and storage of iron. However, a better understanding of the complex mechanisms involved in uptake, transport and storage of iron is needed before this goal can be achieved.

A key process is the systemic distribution of iron throughout the plant. Knowing how plants distribute iron to sink tissues such as the leaves and seed will be important to increase the iron content of most plant foods. Yet, not much is known about how iron is moved from the root epidermal cells to the xylem for translocation to the shoots after uptake by IRT1. Details concerning the loading of iron into the xylem are beginning to emerge, with FRO3 implicated in reducing iron prior to its transfer into the xylem and FRD3 effluxing citrate, the primary xylem iron chelator, into the xylem. However, the identity of the iron transporter itself still remains unknown. Arabidopsis possesses three

ferroportin-like genes, which are similar to animal ferroportins capable of iron efflux from cells. One of these genes, *FPT2*, is expressed in the roots under iron deficiency, making it a likely candidate for effluxing iron into the root vasculature (Mary Lou Guerinot, personal communication). Members of the YSL, NRAMP and ZIP families have also all been hypothesized as playing a role in the loading and unloading of iron into and out of the vasculature. While heterologous expression studies have demonstrated the transport capabilities of some of these proteins, analysis of mutants has failed to reveal any major deficiencies in iron transport. Redundancy within these conserved gene families is probably responsible for this lack of a phenotype. The isolation of the appropriate double and triple mutants hopefully will help elucidate the physiologically relevant functions of these genes.

Another important area concerns the immediate effects of iron deficiency in plants. The mechanism by which iron deficiency is detected remains unknown. As most iron in plants is found in chloroplasts, it would not be too surprising if these organelles were involved in the generation of an iron deficiency signal. Some remodeling of the photosynthetic machinery occurs in order to maximize the use of available iron (Moseley *et al.*, 2002; Naumann *et al.*, 2005). It is therefore tempting to speculate that the reorganization of chloroplast proteins in response to iron deficiency might also result in the generation of this signal.

Lastly, the identity of this long distance iron deficiency signal also remains elusive; its identification will not only increase our knowledge of plant iron homeostasis, but also of plant systemic signaling. Answering all of these questions remains a

challenging and exciting task. Hopefully the results of such work will lead to the development of agronomically important crops with higher levels of bioavailable iron.

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CHAPTER 2

ARABIDOPSIS *cpFtsY* MUTANTS EXHIBIT PLEIOTROPIC DEFECTS INCLUDING AN INABILITY TO INCREASE IRON-DEFICIENCY INDUCIBLE ROOT FE(III) CHELATE REDUCTASE ACTIVITY.[*]

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Abstract

All plants, except for the grasses, must reduce Fe(III) to Fe(II) in order to acquire iron. In Arabidopsis, the enzyme responsible for this reductase activity in the roots is encoded by *FRO2*. Two Arabidopsis mutants, *frd4-1* and *frd4-2*, were isolated in a screen for plants that do not induce Fe(III) chelate reductase activity in their roots in response to iron deficiency. *frd4* mutant plants are chlorotic and grow slower than wild type Col-0 plants. Additionally, *frd4* chloroplasts are smaller in size and possess dramatically fewer thylakoid membranes and grana stacks when compared to wild type chloroplasts. *frd4* mutant plants express both *FRO2* and *IRT1* mRNA normally in their roots under iron deficiency, arguing against any defects in systemic iron deficiency signaling. Further, transgenic *frd4* plants accumulate FRO2-dHA fusion protein under iron deficient conditions, suggesting that the *frd4* mutation acts post-translationally in reducing Fe(III) chelate reductase activity. FRO2-dHA appears to localize to the plasma membrane of root epidermal cells in both Col-0 and *frd4-1* transgenic plants when grown under iron deficient conditions. Map-based cloning revealed that the *frd4* mutations reside in *cpFtsY*, which encodes a component of one of the pathways responsible for the insertion of proteins into the thylakoid membranes of the chloroplast. The presence of *cpFtsY* mRNA and protein in the roots of wild type plants suggests additional roles for this protein, besides its known function in targeting proteins to the thylakoid membrane in chloroplasts.

Introduction

Iron is an essential element in plants, required by many enzymes including those that participate in vital functions such as respiration and photosynthesis. Yet despite being the fourth most abundant element in the earth's crust, iron is not readily available for uptake by plants because it exists in the soil primarily as insoluble Fe(III) hydroxides. Plants therefore possess different approaches to extract iron from their environment. One such strategy, utilized by all dicots and non-graminaceous monocots, involves the coordinated upregulation of three biochemical activities in the roots of iron deficient plants. Protons are released from the root; in Arabidopsis this release is probably mediated by members of the AHA H^+-ATPase family (Palmgren, 2001). The consequent acidification of the rhizosphere increases the solubility of iron present. A root Fe(III) chelate reductase, encoded by *FRO2*, reduces Fe(III) to Fe(II) (Robinson *et al.*, 1999). Lastly an Fe(II) transporter, IRT1, mediates the uptake of iron into the plant root (Eide *et al.*, 1996; Vert *et al.*, 2002).

Iron, however, becomes toxic if present in large quantities. Both Fe(II) and Fe(III) can catalyze the generation of damaging free radicals (Halliwell and Gutteridge, 1992). Plants therefore tightly regulate their iron uptake mechanisms in order to maintain optimal levels of this essential yet potentially harmfully element. For example, both *IRT1* and *FRO2* are only expressed in Arabidopsis roots under iron deficient conditions (Eide *et al.*, 1996; Robinson *et al.*, 1999) and the transcript levels of both genes are rapidly turned over after exposure to iron-sufficient conditions (Connolly *et al.*, 2003; Connolly *et al.*, 2002). Additionally, both *IRT1* and *FRO2* are subject to post-

transcriptional regulation: transgenic plants that constitutively express *IRT1* only accumulate IRT1 protein in the roots under iron deficient conditions (Connolly *et al.*, 2002). Likewise, in Arabidopsis plants constitutively expressing *FRO2*, Fe(III) chelate reductase activity is increased only when the plants are exposed to iron deficiency (Connolly *et al.*, 2003). These multiple levels of control demonstrate the importance of careful regulation of iron uptake in plants. Not much is known about the mechanisms controlling iron uptake, except that they appear to depend on the amalgamation of two distinct signals. One signal is endogenous to the roots and depends on local iron concentrations (Schikora and Schmidt, 2001). The other signal controls systemic iron uptake and originates in the shoot (Grusak and Pezeshgi, 1996). Given that most iron in the plant is found in chloroplasts, (Terry and Abadia, 1986) the derivation of this latter, systemic signal from the shoots is not surprising. Little is known however, about how iron deficiency is detected in the leaves or how this signal is transported to the roots.

This study describes the isolation of an Arabidopsis mutant *frd4*, that is unable to increase Fe(III) chelate reductase activity under iron deficient conditions. Interestingly, these mutants contain lesions in *cpFtsY*, a gene encoding the FtsY ortholog in the chloroplast Signal Recognition Particle (cpSRP) pathway. The cpSRP pathway is one of at least four protein transport mechanisms responsible for the insertion of proteins into the thylakoid membranes of the chloroplast (reviewed in Keegstra and Cline, 1999). Briefly, in this pathway the stromal proteins cpSRP43 and cpSRP54 bind to the target protein to form a transit complex which is then targeted to the thylakoid membrane by the cpSRP receptor, cpFtsY (Tu *et al.*, 1999). A thylakoid integral membrane protein ALB3 and the hydrolysis of GTP are then required for the subsequent insertion of the target

protein (Moore *et al.*, 2000). cpFtsY is similar to both prokaryotic FtsY orthologs and to the SRα subunits of eukaryotic endoplasmic reticulum SRP receptors. Biochemical studies of cpFtsY have suggested that it localizes to the stromal side of the thylakoid membrane and that it associates with other components of the cpSRP pathway (Kogata *et al.*, 1999), (Moore *et al.*, 2003).

Results

Isolation and initial characterization of frd4 mutants

As aberrations in iron homeostasis are known to result in chlorosis in Arabidopsis (Henriques *et al.*, 2002; Rogers and Guerinot, 2002; Varotto *et al.*, 2002; Vert *et al.*, 2002) approximately 40 chlorotic mutants available from the ABRC and the NASC were screened for their ability to upregulate root Fe(III) chelate reductase activity after growth under iron deficient conditions. A similar screening strategy, applied to EMS mutagenized populations, previously identified two mutants, *frd1* and *frd3*, with alterations in Fe(III) chelate reductase activity or its regulation (Rogers and Guerinot, 2002; Yi and Guerinot, 1996). Two chlorotic lines, CS3171 and CS3173 failed to induce Fe(III) chelate reductase activity under iron-deficient conditions. These mutant lines were subsequently backcrossed to wild type Col-0. In both cases, the F_1 progeny did not possess the mutant phenotype, whereas the F_2 progeny segregated in a 3 wild type: 1 mutant ratio (data not shown), demonstrating that in each line the mutant phenotype is caused by a recessive mutation in a single nuclear-encoded gene. The F_1 progeny resulting from a cross between the two mutant lines also failed to upregulate Fe(III) chelate reductase activity (data not shown). This failure to complement the mutant

phenotype implies that the mutations in each line affect the same gene. Therefore CS3171 and CS3173 were named *frd4-1* and *frd4-2*, respectively, for ferric chelate reductase defective. Quantification revealed that Fe(III) chelate reductase activity in *frd4-1* and *frd4-2* mutants was reduced 10-fold and 6-fold respectively compared to wild type Col-0 plants under iron deficiency (Figure 2.1) The Fe(III) chelate reductase activity of the mutants under iron sufficient conditions was reproducibly slightly higher than that of wild type seedlings. This may be due to a general stress response in the *frd4* mutants. The *frd4* mutants are similar to *frd1-1* mutants that contain a mutation in the *FRO2* gene encoding the Fe(III) chelate reductase enzyme (Robinson *et al.*, 1999; Yi and Guerinot, 1996) because both lack iron-deficiency inducible ferric chelate reductase activity. However, crossing *frd4-1* and *frd1-1* resulted in F_1 plants with wild type levels of Fe(III) chelate reductase activity (data not shown), demonstrating that the phenotype in *frd4* is not caused by a mutation in *FRO2*. Additionally, the *frd4* mutants are extremely chlorotic in appearance compared to both wild type and *frd1-1* plants (Figure 2.2) Further, *frd4* mutants grow considerably slower than wild type and *frd1-1* plants, particularly on soil. The average shoot weight of a 14 day old *frd4-1* seedling is approximately half that of a 14 day old wild-type seedling (4.0 ± 0.4 mg as compared to 7.2 ± 0.4 mg). These additional phenotypes cosegregated with the Fe(III) chelate reductase defective phenotype in the back-crossed F_2 population (data not shown), implying that the same mutation is responsible for these multiple phenotypes. The pleiotropic nature of the *frd4* mutations suggest that they affect the function of other proteins in addition to the Fe(III) chelate reductase enzyme FRO2.

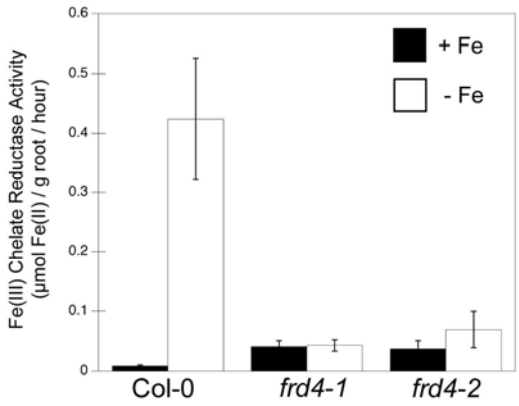

Figure 2.1. *frd4* mutants lack iron deficiency inducible root Fe(III) chelate reductase activity.

Iron deficiency inducible root Fe(III) chelate reductase activity was measured in iron sufficient and deficient wild type Col-0, *frd4-1* and *frd4*-2 seedlings. Values shown represent the mean (± SD) derived from three replicates.

Figure 2.2 Chlorotic and dwarf phenotype of the *frd4-1* mutant.

Wild type, *frd1-1* and *frd4-1* plants were grown axenically on B5 medium for two weeks. *frd4-1* and *frd4-2* plants are visually indistinguishable.

Chloroplast ultrastructure of frd4 *mutants*

Chlorophyll levels were measured to quantify the chlorosis present in the *frd4* mutants. Consistent with their appearance, chlorophyll levels in *frd4-1* (220.8 ± 6.26 µg Chl/g FW) and *frd4-2* (232.7 ± 13.61 µg Chl/g FW) were dramatically reduced to 19.8 and 20.8% of wild type levels (1117.8 ± 70.87 µg Chl/g FW), respectively.

To determine whether the mutant chloroplasts also contained structural defects, the ultrastructure of *frd4* chloroplasts was examined using transmission electron microscopy. Chloroplasts from the leaves of wild type plants had a characteristic lens-like shape and possessed a well developed thylakoid membrane system with numerous grana stacks (Figure 2.3a, b). In contrast, the chloroplasts in *frd4-1* mutant plants were smaller and more distended in shape, with a large reduction in thylakoid membrane content and little evidence of grana stacking (Figure 2.3d, e). Also, leaf cells from *frd4-1* mutant plants were slightly smaller than wild-type leaf cells and contained fewer chloroplasts per cell (6.4 ± 0.7 versus 8.2 ± 1.1; $p = 0.0037$). The reduced chloroplast number and thylakoid membrane content of the *frd4* mutants is consistent with their chlorotic appearance and explains their slower growth on soil: presumably these mutants photosynthesize less efficiently and therefore grow slower in the absence of a sugar source such as sucrose.

Because the *frd4* mutants were isolated based on a root phenotype, we also examined plastids from the roots of mutant and wild-type seedlings (Figure 2.3c, f). In contrast to chloroplasts in the leaves, wild type and mutant root plastids do not differ

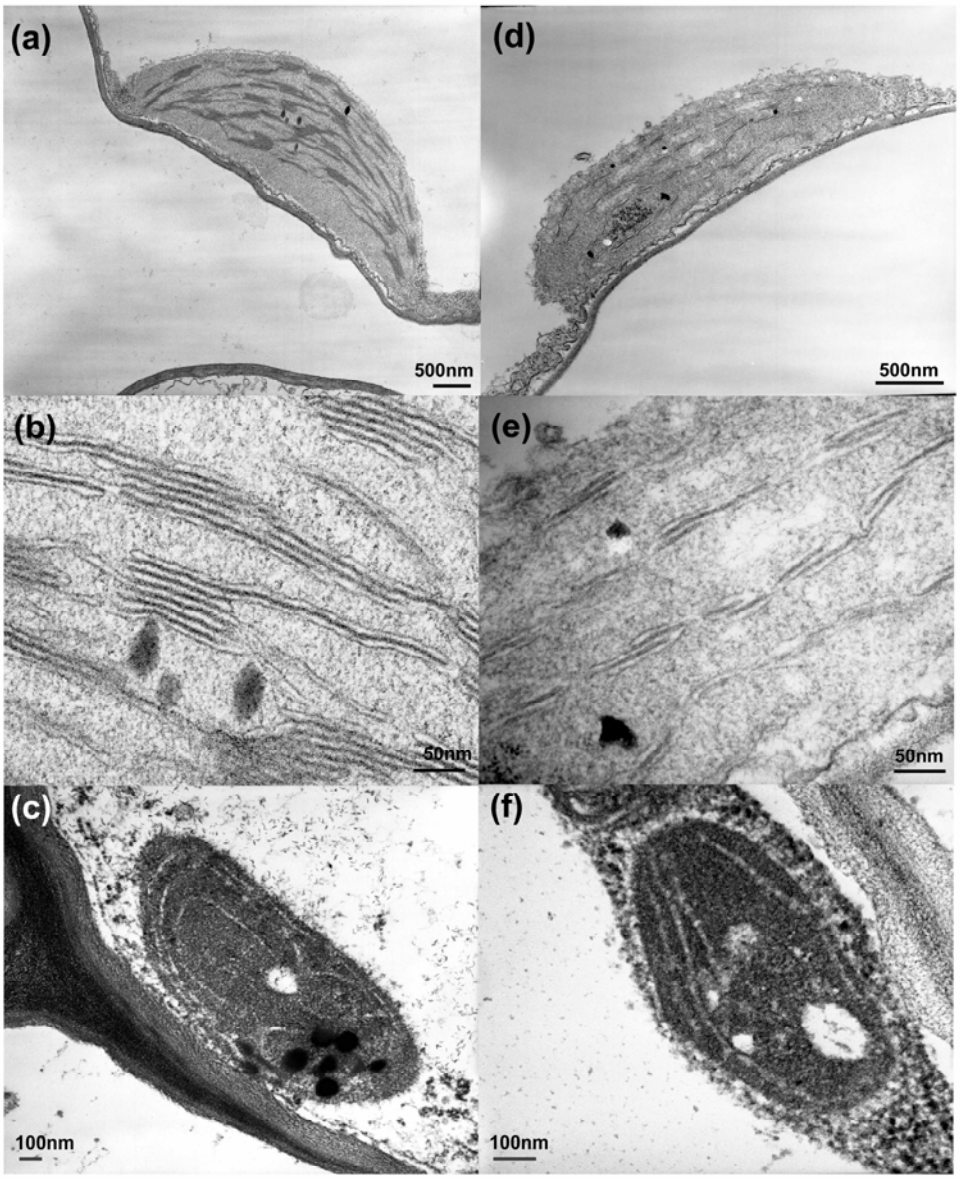

Figure 2.3: *frd4-1* chloroplasts possess fewer thylakoid membranes and grana stacks than wildtype chloroplasts.

(a, b) Transmission Electron Micrographs of Col-0 chloroplasts; (d, e) Transmission Electron Micrographs of *frd4-1* chloroplasts; (c, f) Transmission Electron Micrographs of root plastids from Col-0 (c) and *frd4-1* (f). Bars represent 500nm (a, d), 50nm (b, e) or 100nm (c, f).

significantly in their appearance. The root plastids appear to be some type of immature plastid or proplastid as they contain a rudimentary internal membrane structure. This is not surprising for the roots of seedlings grown in the light on agar plates.

Iron deficiency responses in frd4

To establish whether other components of iron uptake are affected in *frd4,* the expression of *FRO2* and *IRT1* in wild type and mutant roots was examined using RNA blotting. As expected, the levels of both *FRO2* and *IRT1* mRNA were considerably higher in iron deficient wild type roots compared to iron sufficient roots (Figure 2.4a). Similarly, both *FRO2* and *IRT1* expression was upregulated in *frd4-1* and *frd4-2* under conditions of iron deficiency. This level of activation was lower in *frd4-1* (23 % of WT *FRO2* expression) but this difference does not fully account for the dramatic reduction of Fe(III) chelate reductase activity (10% of WT activity). Additionally, *FRO2* expression levels under iron deficiency are similar in *frd4-2* compared to wild type, yet *frd4-2* is indistinguishable phenotypically from *frd4-1*, suggesting that some other abnormality in addition to the low expression of *FRO2* in *frd4-1* must explain the lack of Fe(III) reductase activity present in both of these mutants.

FRO2 protein levels were determined by fusing two HA epitopes to the C-terminus of FRO2 expressed under the control of the *FRO2* promoter. There is no available antibody specific for the FRO2 protein so a tagged construct was necessary. This construct is capable of complementing the *frd1-1* mutant phenotype, suggesting that the FRO2-dHA fusion protein is fully functional (ELC, unpublished data). Transformation of FRO2-dHA into Col-0, *frd4-1* and *frd4-2* did not affect Fe(III) chelate

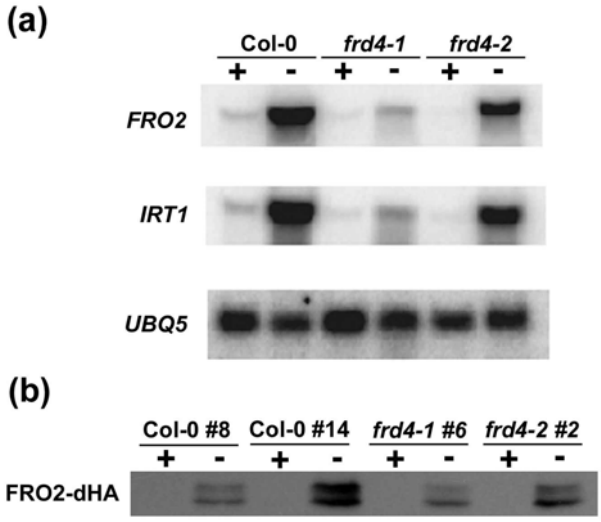

Figure 2.4. Expression of iron deficiency responses in mutant and wild type seedlings.

(a) *FRO2* and *IRT1* expression was upregulated in the roots of seedlings exposed to iron deficiency (-) compared to those with sufficient iron (+). 10 µg of total RNA extracted from the roots of iron sufficient and deficient seedlings was subjected to RNA blot analysis. A ubiquitin probe (*UBQ5*) was used as a loading control.

(b) FRO2-dHA fusion protein accumulates in transgenic Col-0, *frd4-1* and *frd4-2* in response to iron deficiency. Approximately 10 µg of total protein from the roots of iron deficient (-) and iron sufficient (+) T_2 homozygous plants was subjected to Western blot analysis using an anti-HA antibody. A similar gel was stained with Coomassie blue to confirm equal loading (data not shown). Shown are representative samples of at least eight individual transgenic lines.

reductase activity in T_2 homozygous lines compared to untransformed plants (data not shown). FRO2-dHA levels were examined in eight individual transgenic lines for both Col-0 and *frd4-1* transformants, and in four individual transgenic lines for *frd4-2* transformants. Variable amounts of FRO2-dHA were detected in different lines; shown are the most representative lines for each transformation (Figure 2.4b). FRO2-dHA protein accumulates in the roots of iron deficient Col-0 transgenic lines, but not when the plants are grown under conditions of iron sufficiency (Figure 2.4b). FRO2-dHA protein was detectable in both *frd4-1* and *frd4-2* transgenic lines grown under iron deficient conditions at levels comparable with Col-0 transgenic lines (Figure 2.4b). IRT1 protein levels in the mutants were regulated similarly to wild type plants (data not shown).

The *frd4* mutations do not affect the localization of FRO2-dHA. In iron-deficient Col-0 transgenic lines, FRO2-dHA localizes to the plasma membrane of root epidermal cells but is absent from similar iron-sufficient plants (Figures 2.5a-c). This is in good agreement with previous *in situ* hybridization data on *FRO2* (Connolly *et al.*, 2003). Interestingly, cross-sections of iron deficient plants suggest that FRO2-dHA protein concentrates on the outer side of the epidermal cells (Figure 2.5d), exactly where one would predict the enzyme to be located given its role in reducing iron in the rhizosphere. Examination of *frd4-1* lines expressing FRO2-dHA suggests a similar pattern of localization under iron deficient conditions. Strong staining of the plasma membrane and the lack of significant staining in the cytoplasm of *frd4-1* epidermal cells argues against any defects in the trafficking of the protein to the plasma membrane (Figure 2.5e). Again, as in Col-0 lines, stronger staining on the outer edge of the epidermal cells is evident (Figure 2.5f).

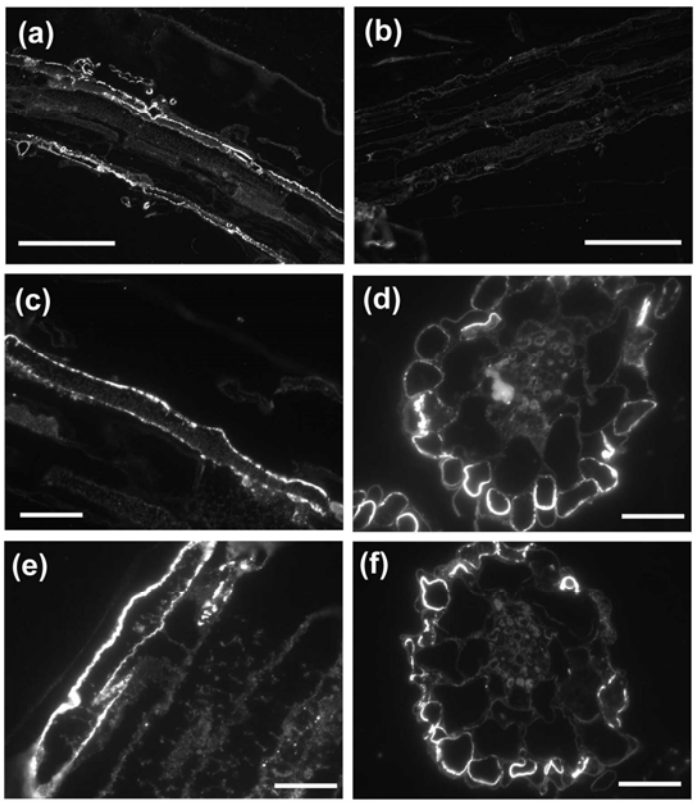

Figure 2.5. FRO2-dHA localizes to the plasma membrane of iron deficient root epidermal cells.

(a, b) Immunolocalization of FRO2-dHA in longitudinal root sections from iron-deficient (a) and iron-sufficient (b) Col-0 T_2 homozygous seedlings. Bars represent 100µm.

(c - f) Close-up views of longitudinal sections (c, e) and cross sections (d, f) from iron-deficient Col-0 (c, d) and *frd4-1* (e, f) T_2 transgenic roots. Bars represent 50µm.

The *FRO2* gene is one of a family of ferric chelate reductases in Arabidopsis. The only other gene with significant expression in the roots is *FRO3* (Mukherjee *et al.*, 2005; Wu *et al.*, 2005). However, promoter-GUS analysis reveals that FRO3 expression is limited to the root vascular bundle (Mukherjee *et al.*, 2005), therefore, *FRO3* expression was not examined in the *frd4-1* and *frd4-2* mutants.

Previous work has demonstrated that organic acid concentrations increase in the roots of iron deficient plants (reviewed in Abadia *et al.*, 2002). Root citrate levels increased by approximately 2.5 fold under iron deficiency in the roots of wild-type, *frd4-1* and *frd4-2*, suggesting these metabolic responses to iron deficiency were not affected in the *frd4* mutants.

The *frd4* mutants accumulated similar levels of iron and other metals compared to wild type plants, as measured by ICP-MS (TPD, Bret Lahner, David Salt, EER, unpublished data). This indicates that the IRT1 protein is fully functional in the *frd4* mutants; plants with alterations in IRT1 protein levels show alterations in their Fe, Mn or Zn levels (Henriques *et al.*, 2002; Rogers and Guerinot, 2002; Varotto *et al.*, 2002; Vert *et al.*, 2002).

Map-based cloning of frd4

To identify the gene affected by the *frd4* mutations, *frd4-1* was crossed to Landsberg *erecta* and mapped using markers designed from the Monsanto Arabidopsis Polymorphism Database (Jander *et al.*, 2002). Approximately 1100 chlorotic F_2 mutant progeny were genotyped to map the *frd4* mutation to an 82-kb interval at the bottom of chromosome II (Figure 2.6a). No predicted open reading frames within this region

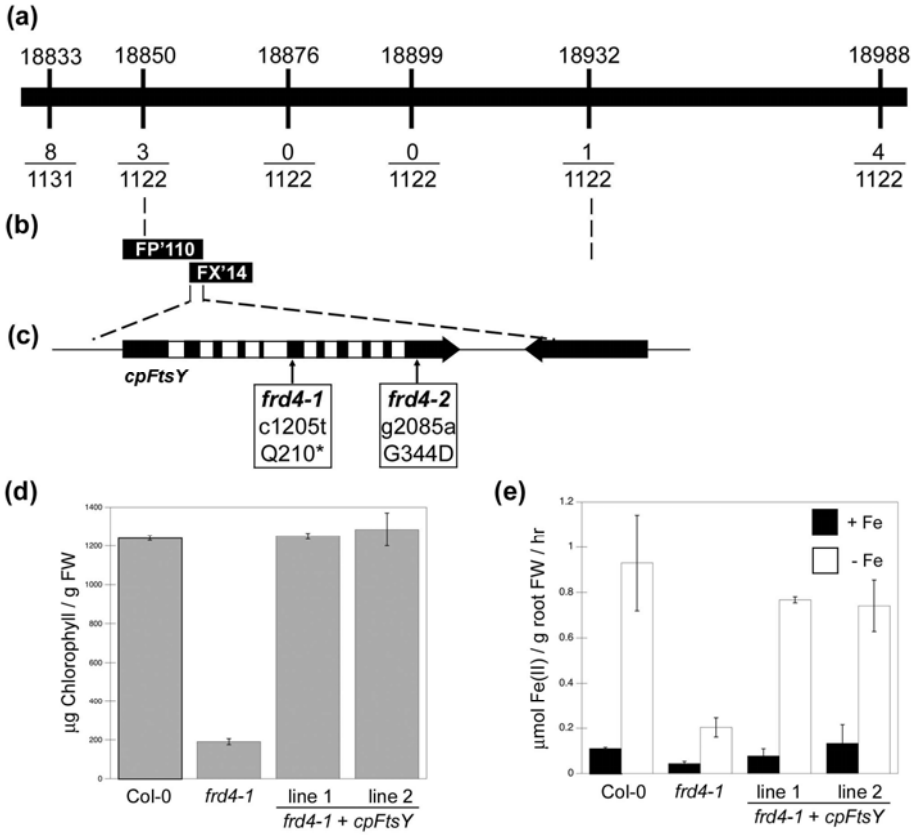

Figure 2.6. The map-based cloning of *frd4*.
(a) The genotyping of approximately 1100 F2 mutant plants mapped the *frd4* mutation to an 82 kb region between the markers 18850 and 18932 at the bottom of chromosome II. The marker names (above the line representing chromosome II) correspond to their position (in kb) along chromosome II. The number of recombinant lines for each marker over the total number of F2 mutant lines genotyped is shown below the line.
(b) Two subclones from the BAC F4I18, FP'110 and FX'14 were able to complement the chlorotic phenotype of the *frd4-1* mutant. Non-complementing subclones are not shown.
(c) The 3.3 kb overlap between these two subclones contains only one complete predicted open-reading frame, *cpFtsY*. The position of the mutations identified in each *frd4* allele is indicated. Black rectangles represent the predicted exons of *cpFtsY*, white rectangles represent the introns.
(d, e) Transformation of *cpFtsY* into *frd4-1* mutants complements both the chlorotic phenotype (d) and the Fe(III) chelate reductase activity phenotype (e). Shown are representative samples of six individual transgenic lines. Values shown represent the mean (± SD) derived from three replicates.

appeared to encode likely candidate genes. Therefore the BAC F4I18, which spans this 82-kb region, was subcloned to create a complementation library. Two such clones, FP110 and FX14, complemented the chlorotic phenotype when transformed into *frd4-1* (Figure 2.6b). The 3.3-kb shared region between these two clones contained only one complete open reading frame annotated as *cpFtsY* (At2g45770). This candidate gene was sequenced from both *frd4-1* and *frd4-2*. Comparisons with the known genomic sequence from Col-0 revealed that *cpFtsY* in *frd4-1* contains a T for C substitution at nucleotide 1205, resulting in a premature stop codon at position 210 in the protein. Likewise, an A for G substitution at nucleotide 2085 of *cpFtsY* in *frd4-2* changes the glycine at position 344 in the protein to an aspartate (Figure 2.6c). A comparison of the cpFtsY protein with homologues from different phylogenetic kingdoms reveals that the glycine in position 344 is highly conserved, even in more divergent proteins (Figure 2.7). This conservation points to the essential nature of glycine 344 for cpFtsY function and may explain why its substitution in the *frd4-2* mutant results in such a dramatic mutant phenotype.

To further confirm that *cpFtsY* was the gene mutated in the *frd4* mutants, the wild type gene under the control of its own promoter was transformed into the *frd4-1* mutant. Transformed T_1 plants resembled wild type plants in appearance, suggesting that wild type *cpFtsY* was able to complement the chlorotic phenotype of the *frd4-1* mutant (data not shown). T_2 lines homozygous for individual insertions of wild type *cpFtsY* possessed chlorophyll levels similar to those in Col-0 (Figure 2.6d). The same lines were able to upregulate their Fe(III) chelate reductase activity under iron deficiency in a manner similar to that of wild type plants (Figure 2.6e), confirming that mutations in *cpFtsY* are responsible for the Fe(III) chelate reductase defective phenotype.

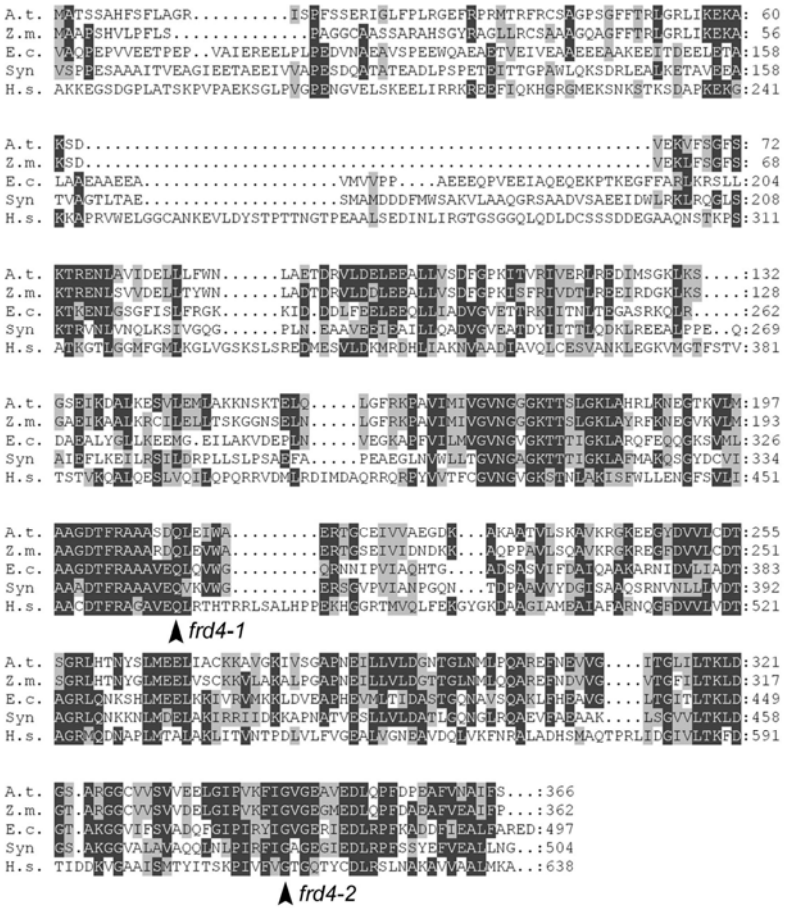

Figure 2.7. Conservation of key amino acid residues in Arabidopsis cpFtsY homologs.

Arabidopsis cpFtsY (At) was aligned with cpFtsY from maize (Zm), the bacterial SRP receptors from *E. coli* (Ec) and *Synechocystis PCC6803* (Syn), and the human SRP receptor alpha subunit (Hs). Residues identical among at least three of the proteins are shaded black. Similar residues are shaded gray. Triangles indicate the location of the mutations in *frd4-1* and *frd4-2*.

Expression of cpFtsY

Because previous studies of cpFtsY had focused only on its role in leaves and chloroplasts (Asakura *et al.*, 2004; Kogata *et al.*, 1999; Tu *et al.*, 1999), and given the root based phenotype of the *frd4* mutants, it was of great interest to determine whether the *cpFtsY* gene is expressed in the roots. Therefore, RNA blot analysis was performed on RNA extracted from the roots and shoots of Arabidopsis seedlings exposed to either iron sufficient or iron deficient conditions. As expected, *cpFtsY* mRNA was present in the shoots of Col-0 seedlings (Figure 2.8a). Further, *cpFtsY* was also expressed in the roots of Col-0 seedlings (Figure 2.8b) at similar levels to the shoots, suggesting that cpFtsY might play a role in the roots. The iron status of the seedlings did not appear to affect the expression of *cpFtsY* in either the shoots or the roots (Figure 2.8a, b).

cpFtsY mRNA levels were considerably lower in both the shoots and roots of *frd4-1* seedlings compared to Col-0 wild type plants (Figure 2.8a, b). This reduction may be a result of nonsense mediated degradation of *cpFtsY* mRNA due to the premature stop codon present in the *cpFtsY* gene in the *frd4-1* mutant. Interestingly in the *frd4-2* mutant, *cpFtsY* expression in the shoots was doubled compared to wild-type plants (Figure 2.8a), possibly pointing to some feedback mechanism controlling gene expression. Expression of *cpFtsY* in *frd4-2* roots was similar to that in wild-type roots (Figure 2.8b).

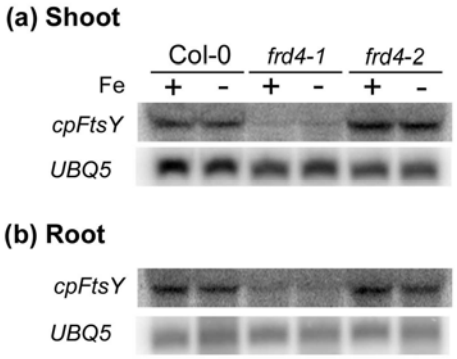

Figure 2.8. Expression of *cpFtsY* in roots and shoots.

10 µg of total RNA extracted from the shoots (a) and roots (b) of iron sufficient (+) and iron deficient (-) wild type and mutant seedlings was subjected to Northern blot analysis to detect *cpFtsY* expression. A ubiquitin probe (*UBQ5*) was used as a loading control

Localization of cpFtsY protein in the roots

To confirm the presence of cpFtsY in the roots and to determine the protein's localization, a cpFtsY-green fluorescent protein (GFP) fusion protein was created and transformed into both wild type and *frd4-1* plants. The cpFtsY-GFP fusion protein was expressed under the control of the *cpFtsY* promoter to reflect endogenous gene expression. This construct was capable of complementing all the *frd4-1* mutant phenotypes, suggesting that the fusion protein is fully functional (data not shown).

Previous work had demonstrated that cpFtsY protein localized to the chloroplasts (Asakura *et al.*, 2004; Kogata *et al.*, 1999; Tu *et al.*, 1999). Consistent with these results, green fluorescence colocalized with chlorophyll auto-fluorescence in the leaves of wild type seedlings expressing cpFtsY-GFP (data not shown). Confocal microscopy of roots revealed GFP fluorescence in all cell types (Figure 2.9a, b). Closer examination of the epidermal layer in the roots showed a punctate pattern of GFP present at the edge of the cells (Figure 2.9c, d). Cells of this type tend to possess very large vacuoles which restrict the cytoplasm to the edge of the cell. Close up images of root hairs also clearly demonstrate the presence of cpFtsY-GFP in a punctate cytoplasmic pattern (Figure 2.9e, f). Given the localization of cpFtsY-GFP to chloroplasts in the leaves, these cytoplasmic structures most likely represent some type of immature proplastid or plastid in the roots.

Detailed examination of the chloroplast protein import pathways in root plastids has not been performed. Chromoplasts from the roots of red bell pepper have been shown to contain functional Sec and Tat chloroplastic protein import pathways but not a functional SRP pathway (Summer and Cline, 1999). Therefore we assayed RNA extracted from roots of wild type or *frd4* seedlings for the presence of three other

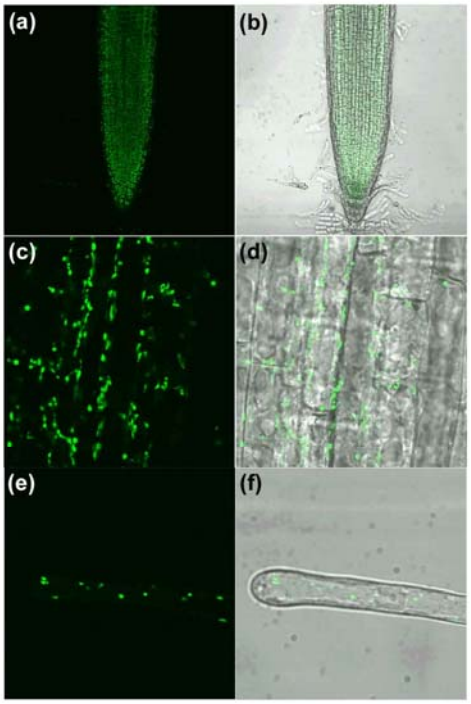

Figure 2.9. cpFtsY localizes to punctate cytoplasmic structures in root cells.

(a, c, e) are confocal fluorescence sections of a wild type root expressing the fusion protein cpFtsY-GFP under the control of the endogenous *cpFtsY* promoter. (b, d, f) are the GFP images from a, c and e respectively, overlaid with the transmission view of the same field.

(a, b) View of an entire root tip showing expression in all tissues.

(c, d) Close up view of the root epidermal layer demonstrating confinement of GFP fluorescence to punctate cytoplasmic organelles.

(e, f) Close up view of a root hair, again demonstrating localization of GFP to punctate cytoplasmic organelles.

components of the SRP pathway: ALB3, cpSRP43, and cpSRP54. All three mRNAs were detectable by RT-PCR (data not shown).

Fe(III) chelate reductase activity of other chlorotic mutants

In order to discount the possibility that the *frd4* mutants are simply too chlorotic and unhealthy to increase Fe(III) chelate reductase activity, the ability of other chlorotic mutants to increase this activity when iron deficient was measured. No obvious correlation between chlorophyll levels and Fe(III) chelate reductase activity under iron deficient conditions was observed (Figure 2.10). Both the *frd4* alleles clustered by themselves in a plot of reductase activity versus chlorophyll content. The *frd4* mutants are clearly separate from the other chlorotic mutants that all possessed higher levels of Fe(III) chelate reductase activity. These results suggest that the inability of the *frd4* mutants to increase Fe(III) chelate reductase activity is not an indirect effect caused by the chlorotic nature of the mutants.

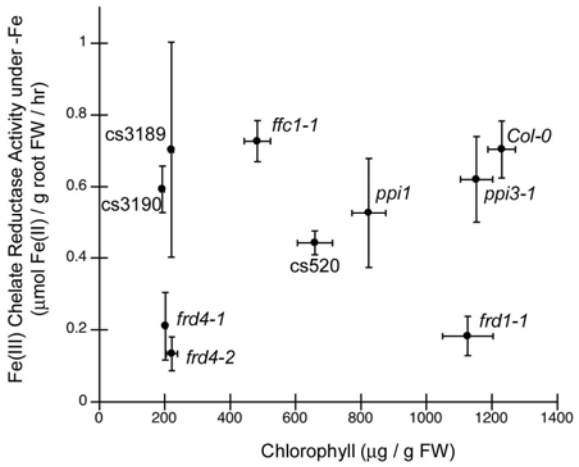

Figure 2.10. Other chlorotic mutants are capable of activating root Fe(III) chelate reductase activity when exposed to iron deficiency.

The iron deficiency inducible root Fe(III) chelate reductase activity of several chlorotic mutants exposed to iron deficiency was plotted against the chlorophyll level of each mutant. Values shown are the means (± SD) derived from at least three replicates.

Discussion

The experiments described above reveal that mutations in the *cpFtsY* gene dramatically reduce the activity of the root iron deficiency inducible Fe(III) chelate reductase (Figure 2.1). The identification of *cpFtsY* is based on the discovery of mutations in the same gene in two different mutant alleles and is confirmed by the fact that a 5.6-kb wild-type genomic fragment containing this gene is capable of complementing all the phenotypes associated with the *frd4* mutants (Figure 2.6). The location of the mutations also explains the chlorotic phenotype of the *frd4* mutants: the cpSRP pathway, which is responsible for insertion of chlorophyll binding proteins into the thylakoid membranes, is obviously affected by mutations in *cpFtsY*. To our knowledge, this is the first example of a chloroplast protein that is involved in the expression of an iron-deficiency response.

This serendipitous identification of an Arabidopsis *cpFtsY* mutant completes the collection of mutations in known components of the cpSRP pathway and should provide an important tool to further elucidate the function of this pathway. The chlorotic phenotype (Figure 2.2) and the dramatic reduction of the thylakoid membrane system (Figure 2.3) in the *frd4* mutants are similar to the phenotypes found in other known mutants of the cpSRP pathway. In this regard however, mutations in *cpFtsY* appear to be more severe than those affecting cpSRP54 and cpSRP43 function. For example, lack of thylakoid membrane organization and reductions in chlorophyll levels are both greater in *frd4* compared to transgenic Arabidopsis plants expressing dominant negative forms of cpSRP54 (Pilgrim *et al.*, 1998), or to *ffc* mutants affected in cpSRP54 function (Amin *et al.*, 1999) or to *chaos* mutants affected in cpSRP43 function (Klimyuk *et al.*, 1999).

Instead, the *frd4* mutants appear very similar to *chaos*/*ffc* double mutants, both in terms of reduced chlorophyll levels and lack of thylakoid membrane organization (Hutin *et al.*, 2002). While possessing more severe phenotypes than mutations affecting cpSRP43 and cpSRP54 function, mutations in *cpFtsY* appear to affect the operation of the cpSRP pathway less than those in *ALB3* which encodes the membrane translocase of this pathway (Moore *et al.*, 2000). For example, *alb3* mutants die at the seedling stage unless supplemented with exogenous sugar and their chlorophyll levels are considerably reduced relative to the *frd4* mutants (Sundberg *et al.*, 1997). Research into this pathway can now be furthered with this isolation of mutations in Arabidopsis *cpFtsY.*

Interestingly, the maize *csr1* mutants which contain *Mu* transposon insertions in *cpFtsY* are seedling lethal (Asakura *et al.*, 2004), whereas both the *frd4* mutant alleles are fully viable. Unlike the *csr1* mutants, the *frd4* mutants are capable of completing a normal life cycle when grown on soil, albeit more slowly and with less fecundicity compared to wild-type plants. The *frd4-1* mutant allele is almost certainly a complete null: in addition to reduced *cpFtsY* mRNA levels (Figure 2.8), the presence of a premature stop codon means that any cpFtsY protein present would lack the conserved GTP binding domains necessary for function (Kogata *et al.*, 1999). This survival of the *frd4* mutants over their maize counterparts could be explained by two different hypotheses: (1) that other thylakoid membrane targeting pathways are capable of compensating for the elimination of the cpSRP pathway in Arabidopsis, or (2) that in maize the cpSRP pathway is essential for some other function besides targeting proteins to the thylakoid membrane. The first hypothesis has been discounted by studies of Arabidopsis mutants defective for both cpSRP43 and cpSRP54 (Hutin *et al.*, 2002).

Additionally, chloroplasts in the *csr1* mutants appear less disrupted than those found in the *frd4* mutants. Thus the survival of *frd4* mutants is not the result of them possessing more functional chloroplasts due to the existence of alternate targeting pathways. Instead, the preliminary evidence presented here favors the second hypothesis and points to another role for cpFtsY in maize besides targeting proteins to the thylakoid membrane.

Despite being unable to increase the activity of Fe(III) chelate reductase, an enzyme involved in iron acquisition in plants, the *frd4* mutants were capable of accumulating equivalent amounts of iron compared to wild-type plants on a dry-weight basis (data not shown). However, previous work has suggested that the long term accumulation of iron in Arabidopsis is more dependent upon the activity of the IRT1 iron uptake transporter than the reduction of Fe(III) chelates to Fe(II). Loss of function mutants of *IRT1* are lethal (Henriques *et al.*, 2002; Varotto *et al.*, 2002; Vert *et al.*, 2002) whereas *frd1* mutants containing lesions in *FRO2* are reasonably healthy (Robinson *et al.*, 1999; Yi and Guerinot, 1996). Additionally, the iron levels in *frd1* plants grown on soil or under iron deficient conditions do not vary significantly compared to those in wild-type plants (Yi and Guerinot, 1996). Therefore root Fe(III) chelate reductase activity is not essential for the long term accumulation of iron in Arabidopsis. Consequently it is not too surprising that the iron levels in *frd4* mutants are similar to those in wild-type plants.

The presence of *cpFtsY* mRNA in the roots (Figure 2.8b) further suggests a function for the protein in parts of the plant that do not contain mature chloroplasts. Expression of cpFtsY-GFP fusion protein under the control of the endogenous *cpFtsY* promoter results in the localization of cpFtsY-GFP to small cytoplasmic organelles in the

roots (Figure 2.9), further confirming the presence of cpFtsY protein in the roots. These organelles are probably immature plastids based on Figure 2.3 and on previous work localizing cpFtsY to chloroplasts in the leaves (Asakura *et al.*, 2004; Kogata *et al.*, 1999). Several different hypotheses have been considered in an attempt to uncover the role of cpFtsY in inducing root Fe(III) chelate reductase activity under iron deficiency.

One possibility could simply be that these mutants are too chlorotic and unhealthy to activate this enzyme. However, many other chlorotic mutants (including plants containing mutations in other components of the cpSRP pathway) all increase their Fe(III) chelate reductase activity when exposed to iron deficiency (Figure 2.10). Likewise, *irt1-1* mutant plants which are extremely chlorotic, are also capable of inducing Fe(III) chelate reductase activity (Vert *et al.*, 2002). Therefore, the lack of this activity in the *frd4* mutants is likely caused by the absence of functional cpFtsY rather than being an indirect side effect caused by the chlorosis present in these mutants. Additionally, as Fe(III) chelate reductase activity is an energy-intensive process, ATP levels were compared between wild-type and mutant plants. If the *frd4* mutant plants contained lower levels of ATP than the wild type, that might explain their lowered reductase activity. However, ATP levels in the roots of both *frd4* alleles are indistinguishable from wild type (data not shown). These experiments therefore all suggest that the lack of Fe(III) chelate reductase activity in the *frd4* mutant is not an indirect result of lowered energy production caused by decreased photosynthetic capability.

The fact that other cpSRP mutants are capable of increasing root Fe(III) chelate reductase activity under iron deficiency suggests that this phenotype is specific to defects

in cpFtsY and not to problems with the cpSRP protein targeting system as a whole. This finding is similar to results in *E. coli* where FtsY, but not Ffh (the *E. coli* SRP54 homolog) plays in a role in the association of ribosomes to the inner membrane, suggesting that FtsY homologs may possess functions independent of their role in the SRP pathway (Herskovits and Bibi, 2000).

Another potential explanation is that the systemic iron deficiency signaling apparatus is affected by the elimination of cpFtsY function. However, RNA blotting results demonstrate that expression of both *FRO2* and *IRT1* genes is upregulated in the roots of *frd4* mutants after growth under iron deficient conditions (Figure 2.4a). Similarly, FRO2 protein levels increase in *frd4* roots during iron deficiency (Figure 2.4b). Further, the *frd4* mutations do not appear to affect the localization of FRO2-dHA (Figure 2.5). Finally, root citrate levels are increased upon exposure to iron deficiency in the *frd4* mutants. Therefore, *frd4* mutant roots appear to respond appropriately to the systemic iron deficiency signal. This suggests that the mutations in *cpFtsY* are influencing Fe(III) chelate reductase activity after the translation and correct localization of the FRO2 enzyme. The *frd4* mutants represent the first example of a plant where the FRO2 protein levels are not correlated with the root epidermal ferric chelate reductase activity. However, because antibodies specific for FRO2 have been difficult to obtain, this is one of the first times FRO2 protein levels have been examined.

Heme is required for the function of other members of the NADPH oxidase super-family of which FRO2 is a member. Like other NADPH oxidases, FRO2 possesses two conserved heme binding sites (Robinson *et al.,* 1999) suggesting that heme is necessary for FRO2 function. As plastids appear to be the major site of heme synthesis in plants

(Cornah *et al.*, 2002), and given that the chloroplasts in *frd4* mutants are highly atypical (Figure 2.3), yet another possible explanation for the inability of the *frd4* mutants to upregulate Fe(III) chelate reductase activity under iron deficiency could be that their chloroplasts are unable to synthesize enough heme for enzymatic function. However, the levels of heme-containing proteins in both roots and shoots are similar in *frd4* mutants compared to wild-type plants (data not shown) arguing against the possibility that lower heme levels in *frd4* are responsible for the Fe(III) chelate reductase defective phenotype. Likewise, the levels of NADPH and NADH are similar in *frd4-1* compared to Col-0 (data not shown).

One intriguing possibility explaining the lack of Fe(III) chelate reductase activity in the *frd4* mutants is that the proposed post-transcriptional control of FRO2 (Connolly *et al.*, 2003) is affected, either directly or indirectly, in these mutants. Indeed, it is interesting to note that two distinct populations of FRO2-HA protein are detected in the roots of iron deficient wild type plants (Figure 2.4b) suggesting that post-translational modification of the protein is occurring. The *frd4* mutants appear to possess reduced amounts of the larger molecular mass protein species suggesting that they might be unable to implement this modification.

In summary, the *frd4* mutant is chlorotic and contains reduced chloroplast thylakoid membrane content – phenotypes directly attributable to the location of mutations in *cpFtsY*. This serendipitous discovery of Arabidopsis cpFtsY mutants should lead to further knowledge concerning the role of cpFtsY in targeting proteins to the thylakoid membranes. The *frd4* mutant is also unable to activate Fe(III) chelate reductase activity in its roots when exposed to iron deficiency, despite the accumulation of the

FRO2 protein in the plasma membrane of root epidermal cells. Future work will be needed to explain the apparently complex relationship between cpFtsY and the induction of Fe(III) chelate reductase activity. Such study of the *frd4* mutants will hopefully further our understanding of the intricate mechanisms that govern iron uptake in plants.

Experimental Procedures

Arabidopsis lines, growth conditions and biochemical characterization

Chlorotic *Arabidopsis thaliana* mutants were obtained from the Arabidopsis Biological Resource Center (ABRC) at The Ohio State University. *frd1-1* has been described previously (Robinson *et al.,* 1999), (Yi and Guerinot, 1996). Plants were grown axenically as described previously (Rogers and Guerinot, 2002). Briefly, surface sterilized seeds were plated on Gamborg's B5 medium (Caisson Laboratories, Rexburg, ID) in Petri dishes. Fourteen day old seedlings were then transferred to plates with 50 µM Fe(III) EDTA for iron-sufficient conditions or 300 µM ferrozine for iron-deficient conditions for three days before analysis.

Wild type Col-0, *frd4-1* and *frd4-2* plants were transformed using the floral dip method (Clough and Bent, 1998). Transgenic plants were selected by germinating T1 seedlings on B5 media containing 50 µg mL^{-1} kanamycin or by spraying soil-grown T1 plants three times with 250 mg L^{-1} Basta. Lines homozygous for individual insertions were generated through selection at the T2 and T3 generations. At least eight such individual transgenic lines were analyzed for each transformation.

Fe(III) chelate reductase assays have been described previously (Yi and Guerinot, 1996). Chlorophyll was extracted by soaking approximately 20mg leaf tissue in 1ml methanol, and total chlorophyll levels quantified by measuring the absorbance at 652, 665 and 750 nm (Porra *et al.*, 1989). Heme containing proteins were detected by staining native protein gels with TMBZ (Quinn and Merchant, 1998). Pyridine nucleotides were extracted by grinding 100mg of root tissue in 1ml extraction buffer and then quantified using an enzymatic cycling assay (Wagner and Scott, 1994). ATP was extracted by grinding 1g of root tissue in 12ml chilled 5% trichloroacetic acid (TCA). These extracts were then centrifuged for 6 min at 4 °C and the resulting supernatant diluted 1:1000 with 0.1M Tris-acetate buffer (pH 7.75) (Alferez *et al.*, 2005). ATP levels were measured with a bioluminescence detection kit (Promega, Madison, WI) using a Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, CA). Citrate was extracted by grinding roots in 80% ethanol and incubating at 75 °C for 1 hour. Citrate levels were then determined according to the instructions of a citrate analysis kit (Roche Molecular Biochemicals): NADH consumption was measured in a solution containing 100mM glycylglycine pH 7.9, 0.2mM $ZnCl_2$, 0.56 mM NADH, 12U/ml Malate Dehydrogenase, 24U/ml Lactate Dehydrogenase and 1.6U/ml Citrate Lyase. Enzymes and NADH were obtained from Sigma (St. Louis, MO).

RNA blot analysis

RNA was extracted from root and shoot tissue using the LiCl method (Verwoerd *et al.*, 1989). RNA blotting was performed using standard methods (Ausubel *et al.*, 2004). *IRT1*, *FRO2* and *UBQ5* probes were made as described previously (Eide *et al.*, 1996),

(Robinson *et al.*, 1999), (Rogers and Ausubel, 1997). The *cpFtsY* probe was made by amplifying the 3' end of the gene with the primers ATGTACCGTCTTTACTGAATCTGAG and ATGAAAACTAATCGAATTATGGATCATGTC. The resulting PCR product was gel-purified and labeled by random priming. RNA blots were visualized and quantified using a Molecular Dynamics Storm 860 PhosporImager (Amersham Biosciences, Piscataway, NJ).

Construction and immunolocalization of FRO2-dHA

The stop codon of the FRO2 cDNA was replaced with a BamHI restriction site, which was then used to insert the dHA tag. The FRO2 promoter was then inserted to control the expression of the FRO2-dHA fusion protein. This entire construct was cloned into 35SpBARN (LeClere and Bartel, 2001) from which the 35S promoter was subsequently excised.

Roots from FRO2-dHA transgenic plants were fixed, embedded in methacrylate and sectioned as previously described (Baskin and Wilson, 1997). Rabbit anti-HA antibody (1:50 dilution in 1x PBS, Sigma) and Alexa Fluor® 488 goat anti-rabbit IgG (1:200 dilution in 1x PBS, Invitrogen) were used to immunolocalize FRO2-dHA protein. Images were obtained on a Leica DMRE microscope using either 20x or 60x objectives.

FRO2-dHA western blotting

Western blotting was performed as previously described (Connolly *et al.*, 2002). Briefly, total protein was extracted by grinding root tissue (2 mL buffer/1g tissue) on ice in

extraction buffer (50mM Tris pH 8.0, 5% glycerol, 4% SDS, 1% polyvinylpolypyrrolidone, 1mM AEBSF Hydrochloride) followed by centrifugation at 4 ºC for 15 min at 14,000g from which the supernatant was recovered. Samples for SDS-PAGE were diluted with an equal volume of 2x sample buffer (125 mM Tris-HCl pH 6.8, 8 M urea, 10% SDS, 10% ß-mercaptoethanol, 0.004% bromophenol blue). Total protein was separated by SDS-PAGE (Laemmli, 1970) and transferred to nitrocellulose membrane by electroblotting (Towbin *et al.*, 1979). Equal protein loading of gel lanes was verified by Coomassie staining of equivalently loaded gels run in parallel (Ausubel *et al.*, 2004). Membranes were incubated in blocking solution (0.25% gelatin, 0.1% bovine serum albumin, 5% nonfat dry milk, 1x PBS) for 3 hr at 37 ºC and then washed twice in 1x PBST for 15 min each. The membranes were then incubated overnight at 4 ºC with rabbit anti-HA antibody (1:1000 dilution in blocking solution; Sigma). The membranes were then washed 4 times for 15 min each in 1x PBST. Next, the membranes were incubated for 1 h with goat anti-rabbit IgG (1:2000 dilution in blocking solution; Sigma) followed by 4 washes for 15 min each in 1x PBST. Chemiluminescence was performed using the SuperSignal West Pico chemiluminescent substrate kit (Pierce).

Electron microscopy

Two-week-old seedlings were fixed with in 2% glutaraldehyde and 2% paraformaldehyde in 0.1M sodium cacodylate pH 7.4. After the primary fixation the sample was rinsed twice with 0.1M sodium cacodylate buffer followed by three rinses with ultra pure water. The samples were then post fixed with a 1% buffered osmium tetroxide, followed by 4 rinses with ultra pure water. Samples were dehydrated with

acetone and then infiltrated with Epon-Spurr's Resin and polymerized for 24hr at 60 ºC. 75nm ultra thin sections were cut for each sample using a Leica Ultracut UCT Ultramicrotome. Samples were viewed in the JEOL 1200-EX Transmission Electron Microscope.

Mapping and complementation of frd4

CAPS and SSLP markers were designed based on information obtained from the Monsanto Arabidopsis Polymorphism Database (Jander *et al.*, 2002). A complementation library was constructed by digesting the BAC F4I18 with appropriate restriction enzymes to generate 10 to 15-kb fragments which were subcloned into the binary vector pCAMBIA2200 (www.cambia.org). Clones containing desired fragments were identified with colony PCR using primers specific for each fragment. The clone containing only the *cpFtsY* open reading frame was constructed by excising the 5.6-kb SalI-BamHI fragment from the complementing clone FP'110 and ligating it into the binary vector pCAMBIA2300. DNA sequencing was performed at the University of Missouri DNA Sequencing Core Facility using an Applied Biosystems 377 automated sequencer (Foster City, CA).

cpFtsY-GFP *construction and localization*

To construct cpFtsY-GFP, the *cpFtsY* stop-codon was replaced with a BamHI restriction site using PCR. The *GFP* sequence was obtained by PCR from pCAMBIA1302, with incorporated EcoRI and BamHI ends. The ligation of this *GFP*

sequence after the modified *cpFtsY* gene created an open reading frame encoding GFP minus its first four amino acid residues fused to the C-terminal of cpFtsY.

GFP fluorescence was visualized in living whole-mount roots using a Bio-Rad (Hercules, CA) Radiance 2000 confocal system coupled to an Olympus IX70 inverted microscope at the University of Missouri Molecular Cytology Core facility.

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APPENDIX 1

THE CHARACTERIZATION OF ARABIDOPSIS *cpFtsY* MUTANTS: UNPUBLISHED DATA AND FUTURE DIRECTIONS

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This Appendix contains data arguing against the hypothesis that *frd4* mutants are unable to increase Fe(III) chelate reductase activity because they are too chlorotic and unhealthy to activate this enzyme. This data was either described briefly in the previous manuscript as unpublished data or was mentioned in an accompanying letter to the editors of The Plant Journal. In addition, further lines of investigation are discussed.

Results

The reduced photosynthetic capabilities of the *frd4* mutants might result in lowered plasma membrane potentials, which could explain the reduced activity of the membrane bound Fe(III) chelate reductase. Therefore, the relative plasma membrane potentials of Col-0 wild-type and *frd4-1* mutant seedlings were compared by loading intact roots with the potentiometric dye, $DiBAC_4$. Cells with normal plasma membrane potentials are able to efflux the dye, lowering their fluorescent intensity. Under these conditions, Col-0 and *frd4-1* roots exhibit similar levels of $DiBAC_4$ fluorescence (Figure A1.1), suggesting that plasma membrane potentials are not affected by the *frd4* mutation. Therefore, the lack of Fe(III) chelate reductase activity in the *frd4* mutants is not an indirect effect caused by lower membrane potentials.

As mentioned in the manuscript, ATP levels were measured in wild-type and *frd4* plants to determine whether lower ATP levels might explain the reduced Fe(III) chelate reductase activity. However, ATP levels from both *frd4-1* and *frd4-2* were indistinguishable from Col-0, under both iron-sufficient and iron-deficient conditions (Figure A1.2). These results further confirm that the reduced Fe(III) chelate reductase

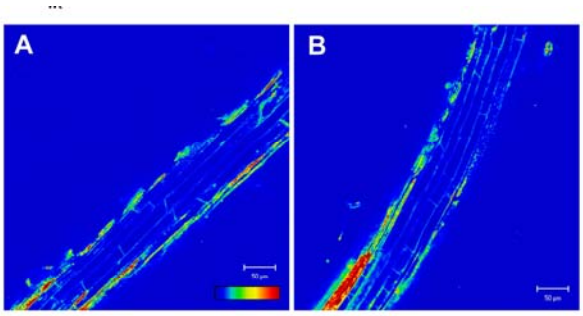

Figure A1.1. *frd4* Mutants Possess Similar Plasma Membrane Potentials to Col-0 Wild-type.

Col-0 wild-type (A) and *frd4-1* mutant (B) roots were preloaded with the anionic dye $DiBAC_4$. Confocal pseudo-color images were taken 30 minutes after removal of the dye. Dye intensity ranges from blue (background) through to deep red (maximum plasma membrane depolarization). Bars, 50µm.

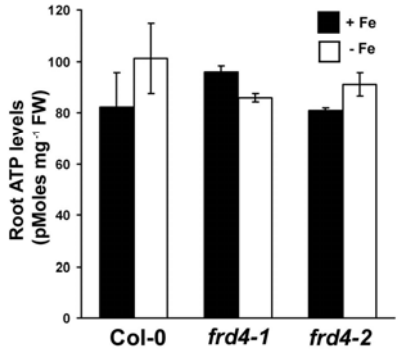

Figure A1.2. *frd4* Mutants Possesses Similar ATP Levels to Col-0 Wild-type Plants. ATP levels were measured in iron-sufficient and iron-deficient wild-type Col-0, *frd4-1* and *frd4-2* seedlings.

activity of the *frd4* mutants is not an indirect result of lowered energy production caused by a reduced ability to photosynthesize efficiently.

Like other members of the NADPH oxidase family, FRO2 obtains its reducing potential from NADPH. Therefore, lowered NADPH levels might explain the lack of Fe(III) chelate reductase activity in the *frd4* mutants. However, levels of NADPH and $NADP^+$ are not dramatically altered in these mutants compared to Col-0 wild-type plants (Figure A1.3), suggesting that these mutants possess sufficient reducing power for Fe(III) chelate reductase activity.

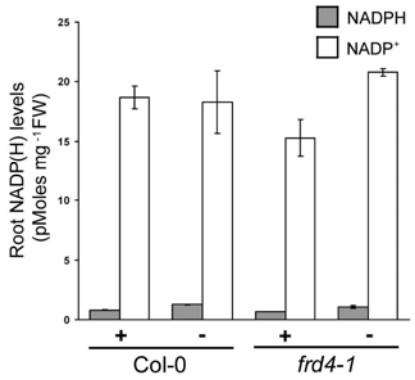

Figure A1.3. *frd4* Mutants Possess Similar Levels of NADPH and NADP$^+$ Compared to Col-0 Wild-type Plants.

NADPH and NADP$^+$ levels from the roots of iron-sufficient (+) and iron-deficient (-) Col-0 and *frd4-1* seedlings.

Future Directions

As noted in Chapter 2, it is interesting to note that two distinct populations of FRO2-dHA protein are present in the roots of iron-deficient wild-type plants, suggesting that FRO2 undergoes some sort of post-translational modification. These modifications might be important for the proposed post-transcriptional regulation of FRO2 (Connolly *et al.*, 2003). Interestingly, the *frd4* mutants appear to possess reduced amounts of the larger molecular mass protein species suggesting that the mutants might be unable to implement this modification, possibly explaining the *frd4* phenotype should this modification be required for Fe(III) chelate reductase activity. Therefore, future experiments should focus on trying to uncover the nature of this post-translational modification.

The phosphorylation of proteins has long been recognized as a mechanism to regulate protein activity. It is somewhat unlikely that a single phosphorylation event could lead to the observed size difference between the bands. However, the phosphorylation state of FRO2 could in theory be easily determined by seeing whether incubating a protein extract with a phosphatase results in the elimination of the higher molecular mass protein species. A similar approach, using PNGase-F, could be used to investigate whether FRO2 was glycosylated.

Modifications such as acylation or prenylation control the membrane localization of target proteins. As the plasma membrane localization of FRO2 appears unaffected by the *frd4* mutations, it is unlikely that the protein is lacking these modifications.

FRO2 belongs to the same NADPH oxidase family as the human NADPH oxidase $gp91^{phox}$, which is responsible for the generation of reaction oxygen species (ROS) in

phagocytes. Additional proteins, $p47^{phox}$, $p67^{phox}$ and rac2, are necessary for the activation of $gp91^{phox}$ (Abo *et al.*, 1992). These proteins are translocated to the plasma membrane in order to activate ROS production (Clark *et al.*, 1990). Assuming that FRO2 requires a similar mechanism for its activation, another explanation for the lack of Fe(III) chelate reductase activity could be that this mechanism is somehow defective in the *frd4* mutants. A necessary first step in exploring this possibility will be to identify the Arabidopsis $p47^{phox}$, $p67^{phox}$ and rac2 orthologs. However, as no orthologs are immediately recognizable in the Arabidopsis genome, these proteins will have to be identified based on their association with FRO2. $p47^{phox}$ phosphorylation appears to be necessary for it translocation and the subsequent assembly of the NADPH oxidase (reviewed in Bokoch and Knaus, 2003). Therefore, a split ubiquitin yeast two hybrid screen using FRO2 as bait will probably not pull out their Arabidopsis orthologs as translated prey proteins will not possess the required phosphorylated residues. A strategy with a higher chance of success, albeit it more technically challenging, would be to immunoprecipitate FRO2-dHA from extracts of iron-deficient roots. Hopefully, associated proteins will coprecipitate; these could then be identified through standard proteomic procedures. If necessary, cross-linking strategies will be pursued to preserve protein-protein interactions. Candidates obtained through this procedure will be confirmed by determining whether these proteins move to the plasma membrane and associate with FRO2 under conditions of iron deficiency. Such translocation could be detected using either immunolocalization (which would require the development of specific antibodies) or by expressing YFP-fusions of the candidate proteins. This latter approach has the additional advantage in that the same construct could also be used to

confirm association with FRO2-CFP using FRET. The translocation of these proteins could also then be studied in the *frd4* mutants to determine whether their movement is affected.

Admittedly, these proposed experiments are risky and will involve a lot of work to further investigate the *frd4* mutant. On the other hand, the identification of proteins that activate FRO2 will greatly increase our understanding of the regulation of iron uptake in plants. The scope of this project therefore extends beyond studying *frd4*. Additional characterization of these proteins will be of great interest. For example, T-DNA insertion mutants for the genes encoding these proteins can be isolated to determine whether eliminating protein function inhibits activation of Fe(III) chelate reductase activity under iron deficient conditions.

Experimental Procedures

Biochemical analyses

NADPH was extracted by grinding 100mg of root tissue in 1ml extraction buffer and then quantified using an enzymatic cycling assay (Wagner and Scott, 1994). ATP was extracted by grinding 1g of root tissue in 12ml chilled 5% trichloroacetic acid (TCA). These extracts were then centrifuged for 6 min at 4 °C and the resulting supernatant diluted 1:1000 with 0.1M Tris-acetate buffer (pH 7.75) (Alferez *et al.*, 2005). ATP levels were measured with a bioluminescence detection kit (Promega, Madison, WI) using a Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, CA).

Evaluation of plasma membrane potentials

Six day old Arabidopsis roots were loaded with $DiBAC_4$ as previously described (Zhang *et al.*, 1998). Briefly, the intact roots were incubated in the dark at 4 °C for 2 hours in a solution containing 0.2mM $CaCl_2$, 50mM sorbitol and 20µM $DiBAC_4$ (Molecular Probes, Eugene, OR). The roots were washed by incubating in 0.2mM $CaCl_2$ and 50mM sorbitol for 1.5 hours at 20 °C. Fluorescence images of plasma membrane potential were recorded with a Zeiss LSM 510 Meta NLO 2-Photon (120E) system at the University of Missouri Molecular Cytology Core facility.

Acknowledgements

I am extremely grateful to Mayandi Sivaguru for all his assistance, including providing the $DiBAC_4$ probe, when examining the plasma membrane potentials. I would also like to thank Doug Randall for providing the reagents for the NADPH assays.

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CHAPTER 3

THE FRD3-MEDIATED EFFLUX OF CITRATE INTO THE ROOT VASCULATURE IS NECESSARY FOR IRON TRANSLOCATION

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Abstract

Iron, despite being an essential micronutrient, becomes toxic if present at high levels. As a result, plants possess carefully regulated mechanisms to acquire iron from the soil. The *frd3* mutant of *Arabidopsis thaliana* is chlorotic and exhibits constitutive expression of its iron uptake responses. Consequently, *frd3* mutants over accumulate iron; yet paradoxically, the *frd3* phenotypes are due to a reduction in the amount of iron present inside *frd3* leaf cells. The FRD3 protein belongs to the multidrug and toxin efflux (MATE) family, members of which are known to export low-molecular weight organic molecules. We therefore hypothesize that FRD3 loads an iron chelator into the xylem necessary for the correct distribution of iron throughout the plant. One such potential chelator is citrate. Xylem exudate from *frd3* plants contains significantly less citrate and iron than the exudate from wild type plants. Additionally, supplementation of growth media with citrate rescues the *frd3* phenotypes. The ectopic expression of FRD3-GFP results in enhanced tolerance to aluminum in Arabidopsis roots, a hallmark of organic acid exudation. Consistent with this result, approximately three times more citrate was detected in root exudate from plants ectopically expressing FRD3-GFP. Finally, heterologous studies in *Xenopus laevis* oocytes reveal that FRD3 mediates the transport of citrate. These results all strongly support the hypothesis that FRD3 effluxes citrate into the root vasculature, a process important for the translocation of iron to the leaves, as well as confirming previous reports suggesting that iron moves through the xylem as a ferric-citrate complex. Our results provide additional answers to long-standing questions about iron chelation in the vasculature and organic acid transport.

Introduction

Plants, like most other organisms, require iron for essential everyday processes. Iron's usefulness is primarily derived from its ability to adopt two different ionic states; consequently iron is present in many enzymes that catalyze redox reactions or are involved in electron transfer. Iron is abundant in most soils, yet exists mostly as Fe(III) hydroxides, which are sparingly soluble at neutral pH. Plants use two different strategies in order to extract iron under these conditions. One approach, called Strategy I and utilized by non-graminaceous species, involves the coordinate upregulation of three biochemical activities in the roots of iron deficient plants (Marschner, 1995). The rhizosphere is acidified by the release of protons from the roots, increasing the solubility of iron present. A membrane-bound Fe(III) reductase, encoded by *FRO2* in Arabidopsis, reduces Fe(III) to Fe(II) (Robinson *et al.*, 1999). Once in its reduced form, iron can then be transported into the root epidermal cells via the IRT1 transporter (Eide *et al.*, 1996; Vert *et al.*, 2002). While the components of iron uptake into the roots have for the most part been identified, very little is known about how iron is translocated from the roots to the shoots, where most iron is ultimately localized.

The Arabidopsis *frd3* (*ferric reductase defective 3*) mutants are chlorotic and constitutively express all three of their Strategy I iron uptake responses, even when grown under iron sufficient conditions (Rogers and Guerinot, 2002). Consequently, *frd3* mutant plants over-accumulate iron as well as other metals, such as manganese and zinc, in their roots. The accumulation of these additional metals is probably due to the constitutive expression of IRT1, which has previously been shown to transport other divalent metals such as zinc and manganese (Eide *et al.*, 1996; Korshunova *et al.*, 1999).

Accumulation of iron in *frd3* shoots appears dependent on the plant's growth conditions: when grown on Petri dishes under conditions of high iron availability, *frd3* accumulates leaf iron levels approximately twice that of wild-type (Rogers and Guerinot, 2002). However when less iron is available, for example when grown on potting soil, iron levels in *frd3* leaves are 10% lower than wild-type leaves (Lahner *et al.*, 2003).

Iron localization is dramatically altered in *frd3* mutant plants. Protoplasts isolated from *frd3* leaves contain about one-half the iron levels of wild-type protoplasts, despite the fact that the *frd3* leaves used for the protoplast isolation accumulated twice as much iron as corresponding wild-type leaves (Green and Rogers, 2004). Additionally, *frd3* fails to accumulate the iron storage protein ferritin, even when grown under conditions of iron sufficiency (Rogers and Guerinot, 2002). These lower cellular iron levels in *frd3* plants explain two of the characteristic *frd3* phenotypes. Presumably, *frd3* leaves are chlorotic because they are iron deficient. Further, because they are iron deficient, the *frd3* leaf cells constantly signal the roots to take up more iron, hence the constitutive Strategy I iron uptake response.

FRD3 is a member of the multi-drug and toxin efflux (MATE) family, a group of proteins with 12 to 14 transmembrane domains capable of transporting small, organic compounds (Brown *et al.*, 1999). NorM from *Vibrio parahaemolyrticus*, the first MATE family member identified, is a Na^+/drug antiporter, capable of effluxing antimicrobial agents (Morita *et al.*, 2000; Morita *et al.*, 1998). Only a few of the 56 predicted Arabidopsis MATE proteins have been studied to date; their characterization, however, is consistent with them also transporting small, organic compounds. The FRD3 protein localizes to the stele cells that surround the vasculature in the roots (Green and Rogers,

2004). Based on this localization, the efflux capabilities of other MATE proteins and the *frd3* mutant phenotypes, it was hypothesized that FRD3 transports a small, organic iron-chelator into the xylem that is necessary for the correct localization of iron throughout the plant.

A number of known iron chelators present in plants have been implicated in the systemic distribution of iron and are candidates for transport by FRD3. One such chelator is nicotianamine (NA), a non-proteinaceous amino acid capable of binding both Fe^{3+} and Fe^{2+}, that has long been implicated in the distribution of iron throughout plants (von Wirén *et al.*, 1999). The tomato mutant *chloronerva* is unable to synthesize NA and consequently exhibits aberrations in its regulation of iron homeostasis (Ling *et al.*, 1999). For example, *chloronerva* plants consitutively activate their iron uptake responses, resulting in an increased accumulation of iron in leaves. The mutants also exhibit interveinal chlorosis in their leaves, a symptom of iron deficiency. However, there are a number of reasons why FRD3 is unlikely to transport NA. First, *chloronerva* mutants also exhibit alterations in copper homeostasis (Pich *et al.*, 1994); *frd3* mutants, however, possesses normal levels of copper (Lahner *et al.*, 2003; Rogers and Guerinot, 2002). Additionally, in *chloronerva* plants, both apoplastic and symplastic iron pools are higher compared to wild type plants (Becker *et al.*, 1992; Pich and Scholz, 1991); in contrast, *frd3* plants have symplastic iron levels half that of wild-type plants (Green and Rogers, 2004). These significant differences make it unlikely that the *frd3* phenotype is caused by lack of NA, therefore suggesting that FRD3 does not transport NA.

A more likely candidate for FRD3's substrate is citrate, which is predicted to chelate 99.5% of iron present in xylem exudate (White *et al.*, 1981). Analysis of xylem

exudate from a number of different species reveals that iron and citrate comigrate during paper electrophoresis (Tiffin, 1966, , 1970). Additionally, studies of sugar beets have demonstrated that the xylem concentration of various organic acids, including citrate, malate and succinate, all increase under iron deficiency (Lopez-Millan *et al.*, 2000). While these physiological experiments all demonstrate the importance of citrate in iron translocation, the means by which citrate enters the vasculature has until this point remained unknown.

Here we present data demonstrating that FRD3 loads citrate into the vasculature, a process necessary for the correct localization of iron throughout the plant. First, xylem from *frd3* plants contains less citrate and iron than wild-type xylem. Additionally, growing *frd3* plants on citrate rescues their characteristic mutant phenotypes. The ectopic expression of FRD3 confers tolerance to aluminum, consistent with the efflux of organic acids. Root exudates from these transgenic plants ectopically expressing FRD3 contain increased amounts of citrate. Finally, the expression of FRD3 in *Xenopus laevis* oocytes mediates the transport of citrate.

Results

frd3-1 *xylem exudate contains less citrate than Col-0 wild-type exudate*

In order to identify FRD3's substrate, xylem fluid was collected from wild-type Col-0 and *frd3-1* mutant plants grown to maximize their vegetative growth. Under these conditions, the *frd3-1* phenotype was similar to soil grown plants at the same developmental stage: *frd3-1* plants were slightly more chlorotic than wild-type plants and over accumulated zinc and manganese in their leaves (data not shown). Leaf iron levels were slightly lower in *frd3-1* than in wild-type plants (data not shown), consistent with previous analysis of soil grown *frd3-1* plants (Lahner *et al.*, 2003). Concentrations of iron chelators known to be present in xylem were then measured in the xylem collected from wild-type Col-0 and *frd3-1* plants. One such chelator is citrate, which is predicted to chelate up to 99.5% of the total iron present in soybean xylem exudate (White *et al.*, 1981). Comparison of citrate levels revealed that *frd3-1* xylem contained 40.2% less citrate than wild-type Col-0 xylem (Figure 3.1A). Interestingly, xylem iron levels were also lower in *frd3-1*, only 49.2% of wild-type levels (Figure 3.1B). This correlation between citrate and iron levels in the xylem is consistent with earlier work (Brown and Tiffin, 1965). The levels of two other iron chelators known to be present in xylem, malate and nicotianamine, were also measured. However, the concentration of these two compounds in xylem exudates was below the detectable limits of the assays used (data not shown).

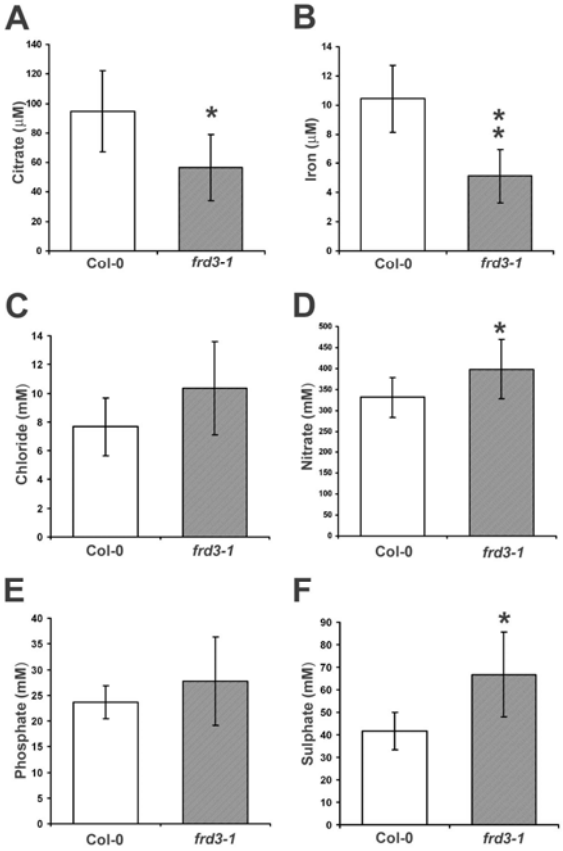

Figure 3.1. Xylem from *frd3-1* Mutants Contains Less Citrate than Xylem from Col-0 Wild-type Plants.

Xylem exudate was collected from Col-0 and *frd3-1* plants and analyzed for citrate (A), iron (B), chloride (C), nitrate (D), phosphate (E) and sulfate (F). Values shown represent the mean (± SD) derived from at least five samples. Student's t-test: $p < 0.05$ ⋆, $p < 0.01$ ⋆⋆.

In order to confirm that the differences in the concentrations of citrate and xylem were not the result of indirect factors, such as variations in the transpiration rate between Col-0 and *frd3-1*, the levels of other anions present in xylem were also measured. No dramatic differences in the concentrations of chloride, nitrate, phosphate and sulfate ions were noted (Figure 3.1C-G), arguing that the lower levels of citrate and iron in *frd3-1* xylem exudate are caused by the elimination of FRD3 function.

Citrate Supplementation Rescues the frd3 *phenotype*

frd3-1 seedlings germinated on plates with citrate added to the growth medium were noticeable larger and greener than those grown on un-supplemented media (Figure 3.2A). This regreening is reflected in the significantly higher chlorophyll levels of *frd3-1* seedlings grown on citrate, which approach those of Col-0 wild-type plants grown under identical conditions (Figure 3.2B). Growth on media supplemented with other organic acids, such as malate and succinate, had no effect on the *frd3*-1 phenotype (data not shown). Other characteristic *frd3* phenotypes were also rescued by growth on citrate-containing media. The *frd3-1* mutant possesses constitutive Fe(III) chelate reductase activity, even when grown under iron-sufficient conditions (Rogers and Guerinot, 2002); however, addition of citrate to the growth medium reduced Fe(III) chelate reductase activity to wild-type levels (Figure 3.2C). Similar results were also obtained with the *frd3-3* mutant allele (data not shown). Iron accumulates in the root vasculature of *frd3-1* seedlings, as indicated by Perls' stain (Green and Rogers, 2004); Figure 3.2D).

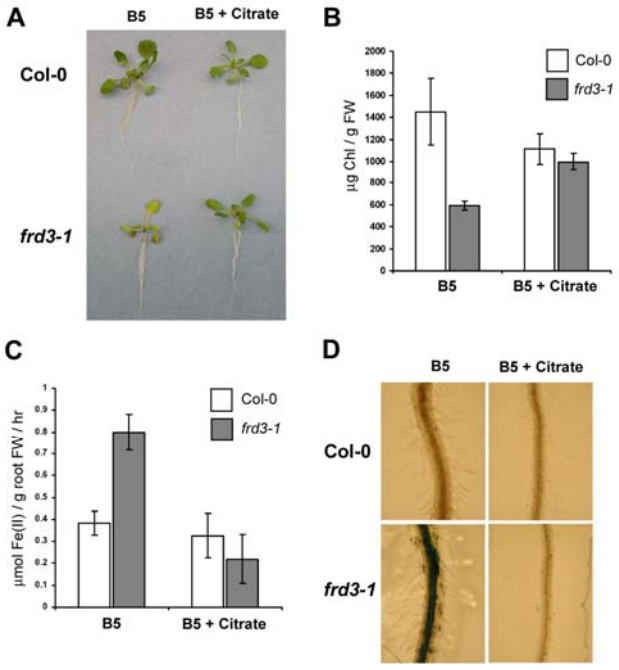

Figure 3.2. Growth on Citrate Rescues the *frd3* Phenotype.

(A) Wild type Col-0 and *frd3-1* seedlings were grown axenically on B5 medium, or B5 medium supplemented with 3 mM sodium citrate. Chlorophyll levels (B) and Fe(III) chelate reductase activity (C) were measured after three weeks.

(D) Perl's Staining of three-week old Col-0 and *frd3-1* seedlings grown in the absence or presence of 3 mM sodium citrate.

Yet when grown on citrate, this over-accumulation of iron all but vanishes from the roots of *frd3-1* mutant plants (Figure 3.2D). The ability of high levels of exogenous citrate to rescue the *frd3* phenotype suggests these mutants are defective in the transport of this iron chelator, furthering the idea that FRD3 effluxes citrate into the root vasculature.

Ectopically Overexpressing FRD3 Confers Increased Tolerance to Aluminum

In order to protect themselves from the toxic effects of aluminum, the roots of a number of different plant species secrete organic acids into the rhizosphere (Delhaize *et al.*, 1993; Miyasaka *et al.*, 1991; Pellet *et al.*, 1995). If FRD3 does efflux citrate, then its ectopic expression might increase the resistance of transgenic plants to aluminum. Therefore, a construct consisting of a FRD3-GFP fusion protein under the control of the 35S promoter was transformed into wild-type Col-0 plants. This 35S::FRD3-GFP construct is capable of complementing *frd3-1* mutant plants, suggesting that the fusion protein is functional (data not shown). T_3 Col-0 lines homozygous for individual insertions were analyzed for aluminum tolerance using a root growth assay. In the absence of aluminum, the root growth of most transgenic lines was similar to that of untransformed plants. However, when exposed to 75 µM and 125 µM aluminum, four of the five transgenic lines ectopically expressing FRD3 possessed an enhanced tolerance to aluminum, as evidenced by better root growth under those conditions (Figure 3.3A). These results are consistent with FRD3 effluxing an organic acid. As other organic acids besides citrate, such as malate or oxaloacetate, can also confer aluminum tolerance to plant roots, root exudates were collected from these transgenic lines and citrate levels

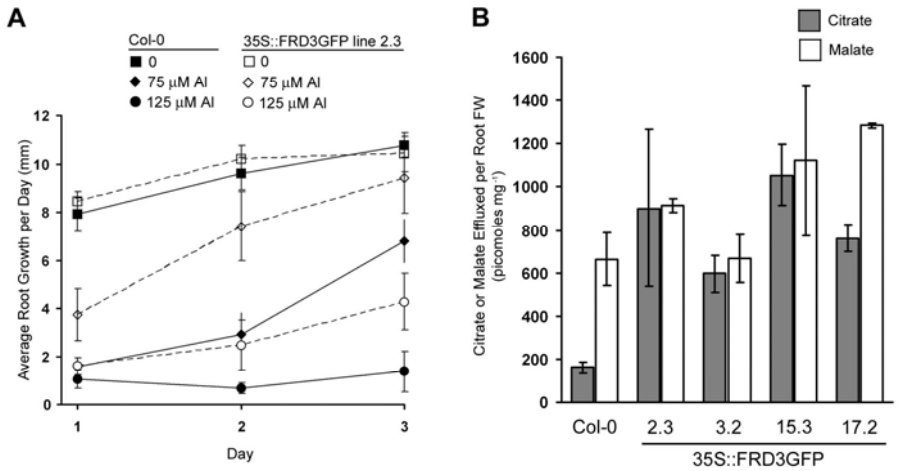

Figure 3.3. Transgenic Plants Ectopically Expressing FRD3GFP Possess Enhanced Tolerance to Aluminum due to an Increased Efflux of Citrate.

(A) Seven day old Col-0 wild type seedlings (solid symbols) and Col-0 transgenic seedlings containing 35S::FRD3GFP (open symbols) were transferred to vertical plates containing growth media supplemented with 0 µM Al^{3+} (squares), 75 µM Al^{3+} (diamonds) or 125 µM Al^{3+} (circles). Root lengths were measured at 24 hour intervals after transfer. Shown are the results from a representative experiment. Values represent the mean (± SD) of at least 5 roots from Col-0 wild-type and a single, representative transgenic line.

(B) Root exudates from fourteen day old wild type Col-0 and transgenic 35S::FRD3GFP seedlings were collected and levels of citrate (solid bars) and malate (open bars) measured. Values represent the mean (± SD) of citrate and malate effluxed normalized to the root mass of the seedlings from a single, representative experiment.

measured. Plants ectopically expressing FRD3-GFP had significantly higher amounts of citrate in their root exudates compared to untransformed control plants (Figure 3.3B). The exudate of malate, however, was not dramatically increased in the transgenic lines compared to wildtype plants (Figure 3.3B).

Expression of FRD3 *in* Xenopus *oocytes Mediates Transport of Citrate.*

To further confirm that FRD3 transports citrate and to investigate the specificity of this transport, the characteristics of FRD3 were examined in oocytes using two electrode voltage clamping. Oocytes injected with *FRD3GFP* cRNA produced inward currents when the bath solution contained citrate, but not malate (Figure 3.4A). These currents were specific for the transport of citrate by FRD3: removal of citrate from the bath solution quickly returned the current to resting levels. Additionally, the magnitude of these currents was dependent on the concentration of citrate. In contrast, uninjected oocytes responded to neither citrate nor malate. Figure 3.4B summarizes the response of a number of oocytes from different batches, demonstrating that the response to citrate was significantly larger in *FRD3GFP*-injected oocytes compared to uninjected oocytes. By convention, these inward currents are indicative of the influx of net positive charge or the efflux of net negative charge.

In order to study the efflux of citrate more directly, oocytes were injected with citrate immediately prior to voltage clamping. In these experiments, uninjected oocytes experienced an outward current (Figure 3.4C). Similar currents were obtained when both uninjected and FRD3GFP oocytes were injected with water or malate immediately before

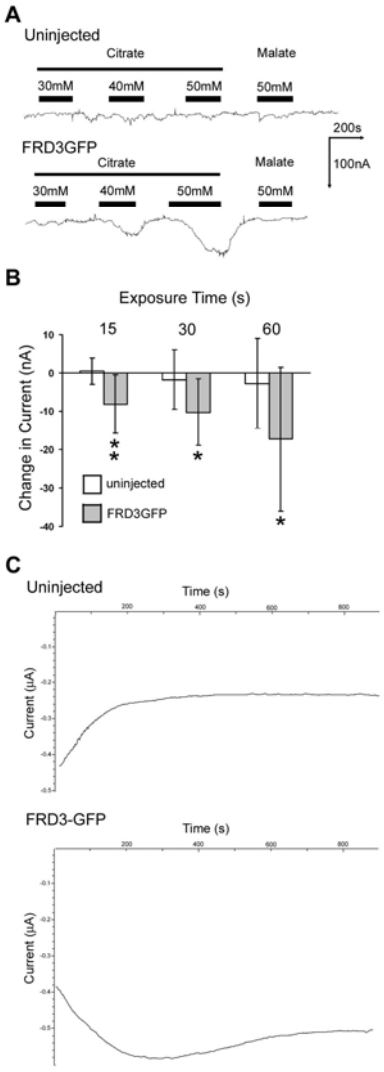

Figure 3.4. Transport of Citrate in Oocytes Expressing FRD3GFP.
(A) Currents produced when uninjected oocytes (top) and oocytes expressing FRD3GFP (bottom) were exposed to citrate and malate. Presence of substrate is indicated by a black bar above the current trace. Oocyte membrane potentials were clamped at -80 mV.
(B) Average currents induced after different times of exposure to 30mM citrate at a membrane potential of -80mV. Values shown represent the means (± SD) for at least 10 oocytes from four different batches of oocytes. Student's t-test: $p < 0.05$ ✶, $p < 0.01$ ✶✶.
(C) Currents produced when uninjected oocytes (top) and oocytes expressing FRD3GFP (bottom) were injected with 23nl 1mM citrate prior to clamping at a membrane potential of -80 mV.

voltage clamping (data not shown). These currents may be the result of the membrane resealing after being disrupted by the citrate injection and by the subsequent penetration for voltage clamping. However, FRD3GFP oocytes experienced a strong inward current when injected with citrate before voltage clamping (Figure 3.4C). The inward currents from both sets of experiments are indicative of the flow of anions out of the oocytes and are therefore consistent with the efflux of citrate by FRD3.

To further study the efflux of citrate from oocytes expressing FRD3GFP, oocytes were loaded with [^{14}C]citrate and the consequent efflux of radioactivity measured. Oocytes expressing FRD3GFP effluxed almost twice as much radioactivity compared to uninjected oocytes (Figure 3.5), confirming that FRD3 can mediate the efflux of citrate.

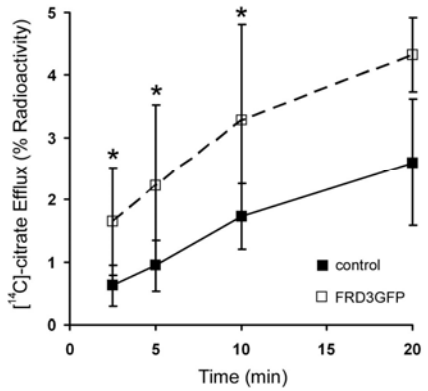

Figure 3.5. Efflux of ^{14}C-Citrate via FRD3GFP

Control oocytes (■) and oocytes expressing FRD3GFP (□) were injected with [^{14}C]citrate and the effluxed radioactivity measured at the indicated time points. Values are expressed as the percentages of the total radioactivity injected into the oocytes and represent the mean (± SD). Student's t-test: $p < 0.05$ ✶.

Discussion

FRD3 was previously hypothesized to transport a low molecular weight compound, most probably an iron chelator, into the xylem (Green and Rogers, 2004). FRD3's substrate is required for the correct localization of iron throughout the plant; in its absence, lower amounts of iron enter the leaf cells, resulting in chlorosis and the constitutive activation of the root iron uptake responses.

Analysis of xylem exudates revealed that *frd3-1* mutants contain less citrate in their xylem compared to Col-0 wild-type plants (Figure 3.1A). Citrate has long been hypothesized to be the main iron chelator in the xylem. These results support that hypothesis and suggest that FRD3 is responsible for the loading of citrate into the xylem to fulfill this function. Interestingly, iron levels in *frd3-1* xylem were also lower than that of wild-type plants (Figure 3.1B). The dramatic accumulation of iron in the *frd3* root vasculature revealed by Perl's Staining(Green and Rogers, 2004), implies that iron is less soluble in the xylem of the mutant plants. This reduced ability to transport iron in the vasculature explains why iron levels are lower in *frd3-1* xylem exudate. The correlation between citrate and iron levels in the xylem is consistent with earlier work that demonstrated striking parallels between the levels of iron and citrate in the xylem exudates from soybean plants grown under different levels of iron deficiency (Brown and Tiffin, 1965). The concomitant lowering of iron and citrate levels in *frd3* xylem provides further evidence that citrate is necessary for the efficient translocation of iron from the roots to the shoots. Based on the phenotype of *frd3* plants grown on plates, which experience intracellular iron deficiency despite an overall overaccumulation of iron in their leaves, citrate also appears to be required for the efficient uptake of iron into leaf

cells. We speculate that in the absence of citrate, leaf apoplastic iron precipitates onto cell walls and is therefore unavailable to the endogenous iron uptake systems. Combined with the data demonstrating that FRD3 transports citrate when expressed in *Xenopus* oocytes (Figures 3.4, 3.5), these results strongly support the hypothesis that FRD3's function *in planta* is to transport citrate into the xylem.

Surprisingly, xylem citrate levels were only reduced by 40.2% in the *frd3-1* mutant. If FRD3 loads citrate into the root vasculature, one would expect xylem citrate levels to be more dramatically reduced in its absence. A number of explanations have been considered to explain this discrepancy. One possibility is that the Ala to Asp change caused by the *frd3-1* mutation does not completely eliminate the function of the FRD3 protein so that it can still transport reduced amounts of citrate. This explanation is somewhat unlikely: for the most part, *frd3-1* mutant plants are indistinguishable from other isolated *frd3* mutant alleles that all result in truncated proteins (Rogers and Guerinot, 2002).

Another probable explanation for the surprisingly high *frd3-1* xylem citrate levels could be the presence of other citrate efflux transporters capable of compensating for the loss of FRD3 function. In a dendrogram of MATE family members, FRD3 falls into a discrete cluster with four other Arabidopsis genes (Rogers and Guerinot, 2002). Based on their sequence similarity, these paralogs are likely candidates to provide this redundant function. However, *in silico* analysis predicts either these genes localize to different intracellular compartments than FRD3, or are not expressed in the roots, suggesting that they are unlikely to partially complement the loss of FRD3 (data not shown). Additionally, T-DNA insertion mutants of the closest FRD3 paralog, FRDL,

possess no apparent phenotype associated with iron homeostasis: unlike *frd3* mutants, *frdl* mutants regulate Fe(III) chelate reductase activity normally, do not show any signs of chlorosis and possess wild-type levels of different metals (data not shown). Likewise, double *frd3-3 frdl* mutants do not possess an enhanced phenotype relative to *frd3-3* mutants (data not shown), further suggesting that FRDL is not redundant to FRD3. Therefore, it is somewhat unlikely that the higher than expected levels of citrate in *frd3*-1 xylem is due to the citrate transport capabilities of FRD3 paralogs.

A more probable explanation derives from the fact that *FRD3* expression increases under iron deficiency (Rogers and Guerinot, 2002). As xylem exudate was collected from plants grown under iron sufficient conditions, it is likely that the lower expression levels of *FRD3* in wild-type plants resulted in less citrate being present in the wild-type xylem. Indeed, it has been demonstrated that xylem citrate levels rise upon exposure to iron deficiency (Brown and Tiffin, 1965; Lopez-Millan *et al.*, 2000). Therefore, wild-type citrate levels might be understated, reducing the apparent effect of the *frd3* mutation. Preliminary attempts to measure citrate levels in the xylem of iron deficient wild-type Col-0 plants revealed no dramatic differences compared to previous results for iron sufficient plants (data not shown). However, limitations with the Rockwool hydroponics system suggest that the plants might not have been suffering from iron deficiency when xylem was collected. Future work will focus on developing a suitable growth system capable of providing the necessary amounts of xylem from iron deficient plants.

There are several possible mechanisms through which citrate could enter the vasculature to rescue the *frd3* phenotype (Figure 3.2). It would be possible for exogenous

citrate to access the root vasculature through the root apical meristem. This portion of the root is not surrounded by the Casparian strip, a layer of cells with cell walls containing suberin, which acts as a barrier to the radial movement of water and ions in the roots. The Casparian strip would also block the entry of exogenous citrate from ultimately entering the root vasculature apoplastically throughout much of the root length. However, as the Casparian strip does not extend all the way to the tip of Arabidopsis roots, this portion of the root could be the entry point for exogenous citrate to enter the root vasculature and thereby rescue the *frd3* mutant phenotypes. It is also possible for citrate to enter through non-FRD3 dependent routes: low affinity transporters could efflux citrate when exposed to these high substrate levels. Alternatively, uncharged citrate could diffuse across membranes.

The ectopic expression of FRD3 in wildtype plants results in an enhanced tolerance to aluminum toxicity (Figure 3.3A), consistent with FRD3 effluxing an organic acid such as citrate: with FRD3 expressed in other cell types beside the stele, these transgenic plants are now capable of effluxing increased amounts of organic acids into the growth media, resulting in their enhanced tolerance to aluminum. Analysis of root exudates from these transgenic lines revealed increased amounts of citrate relative to untransformed control plants (Figure 3.3B), consistent with FRD3 effluxing citrate. On the other hand, exudate malate levels from the transgenic lines were either unchanged or only slightly increased compared to untransformed control plants. These results therefore suggest that the FRD3 mediated efflux of citrate, and not malate, is responsible for the enhanced aluminum tolerance of these plants.

Heterologous expression studies of FRD3 in *Xenopus* oocytes further confirmed FRD3's ability to transport citrate. By convention, the inward currents experienced by oocytes expressing FRD3GFP when exposed to citrate (Figure 3.4A,B) are indicative of either an influx of cations, an efflux of anions, or a combination of both. These results are therefore consistent with an efflux of negatively charged citrate ions. Presumably, due to the high external concentrations used in these experiments, citrate somehow enters the oocytes and is then subsequently effluxed by FRD3. Under these conditions the concentration of citrate molecules that are uncharged and therefore able to cross the plasma membrane of the oocytes is 160 μM, a concentration that is about two-fold higher than found in xylem. Additional experiments involving the direct injection of citrate into oocytes prior to voltage clamping also resulted in inward currents only in those oocytes expressing FRD3GFP. Similar currents were never observed in control oocytes (Figure 3.4C). These inward currents are again consistent with FRD3 mediating the efflux of citrate anions out of the oocytes. Lastly, FRD3's ability to efflux citrate is further confirmed by the fact that oocytes expressing FRD3GFP were capable of effluxing almost twice as much [^{14}C]citrate compared to uninjected oocytes (Figure 3.5).

A number of lines of evidence all suggest that FRD3 specifically transports citrate and not other organic acids. First, transgenic lines ectopically expressing FRD3GFP exhibited an enhanced secretion of citrate but not malate from their roots (Figure 3.3B), suggesting that FRD3 transports citrate preferentially over malate. Additionally, oocytes expressing FRD3GFP experienced inward currents only when exposed to citrate, and not when exposed to comparable levels of malate (Figure 3.4A). Similarly, preliminary experiments involving the direct injection of malate failed to produce inward currents in

oocytes expressing FRD3GFP (data not shown). Consistent with these results, only citrate appears capable of rescuing the *frd3* phenotype (Figure 3.2). The supplementation of media with other organic acids such as malate and succinate failed to restore a wild-type phenotype to *frd3-1* mutant plants (data not shown).

In summary, the results presented in this study all support the hypothesis that FRD3 transports citrate into the root vasculature. Mutations in FRD3 result in lower levels of citrate in the xylem. The ectopic overexpression of FRD3, on the other hand, yields an increase in the efflux of citrate from roots, resulting in an enhanced tolerance to aluminum. Finally, heterologous expression studies in *Xenopus* oocytes further confirm the ability of FRD3 to transport citrate. Transport of citrate via FRD3 into the root vasculature is important for the translocation of iron to the shoots, and subsequent uptake by leaf cells. This role of FRD3 might be especially relevant when considering the so-called "iron chlorosis paradox", a phenomenon where the leaves of plants grown on calcareous soils become chlorotic, despite possessing normal levels of iron in their leaves (Morales *et al.*, 1994; Nikolic and Romheld, 2002). This condition is reminiscent of the phenotype of *frd3* mutants grown on Petri dishes which are chlorotic despite overaccumulating iron in their leaves (Rogers and Guerinot, 2002). Interestingly, plants suffering from the "iron chlorosis paradox" regreen when citrate or other weak acids are applied to their leaves (Álvarez-Fernández *et al.*, 2004; Kosegarten *et al.*, 2001). It is therefore tempting to speculate that there might be a connection between the role of FRD3 in providing citrate and the "iron chlorosis paradox". Plants suffering from this phenomenon might possess lower levels of citrate in their xylem due to lack of FRD3 activity. In this regard, it is interesting to note that soybean and tomato varieties resistant

to iron deficiency contain more citrate and iron in xylem compared to other varieties susceptible to iron deficiency (Brown and Chaney, 1971) The further study of FRD3 orthologs in different crop species could be important in understanding this common agricultural problem.

Future Directions

Biochemical characterization of FRD3

An obvious area for additional study involves the further biochemical characterization of transport by FRD3. This work has been hindered by availability of a suitable system with which to study substrate efflux. Heterologous expression in *Xenopus* oocytes probably remains the most feasible system to obtain the desired biochemical data. Obtaining reliable expression of FRD3 will greatly increase the efficiency with which this data can be obtained. Two different approaches, either singly or in combination, could achieve this goal. In the past, eliminating the Arabidopsis 5' UTR appeared to increase protein expression. Additional manipulation of the 5' UTR could further enhance expression: insertion of a yeast 5' UTR increased expression of Arabidopsis OPT4 in oocytes (Walter Gassmann, personal communication). Therefore, modifying the 5' UTR with this yeast sequence will hopefully increase the amount of FRD3 protein expressed in the oocytes. Another approach could be to alter the codon usage for optimal expression in oocytes. Once these improvements have been made, and if they result in consistent FRD3 expression levels, a number of different experiments could be performed.

Firstly, the affinity of FRD3 for citrate transport could be determined by measuring the efflux of radioactivity after FRD3 expressing oocytes are injected with different concentrations of [^{14}C]citrate. A Lineweaver-Burk plot could then be constructed to determine the K_m and V_{max} for transport.

While the results presented suggest that FRD3 transports citrate and not other organic acids, the [^{14}C]citrate efflux procedure could be adapted to further confirm this

specificity. Other, unlabelled organic acids will be injected along with [^{14}C]citrate and the efflux of radioactivity measured. If FRD3 is capable of transporting other substrates, they will compete for transport with the [^{14}C]citrate, reducing the amount of radioactivity effluxed.

The bacterial MATE protein NorM uses the antiport of sodium ions to energize the efflux of toxins from the cell (Morita *et al.*, 2000). As previous work has suggested that most plant homologs of bacterial Na^+ antiporters utilize protons in their antiport mechanism, it is likely that the FRD3 mediated efflux of citrate is linked to proton uptake. An obvious approach to test this hypothesis will be to determine the effect of the pH of buffer solution on the efflux of [^{14}C]citrate from oocytes expressing FRD3. If FRD3 is a citrate-proton antiporter then the efflux of citrate should be increased when the oocytes are exposed to lower pH buffers. Experiments varying the concentrations of other potential antiport ions could also be performed in a similar manner.

Characterisation of other MATE Proteins

FRD3 is most similar to four other paralogs that cluster together in a dendogram of all the Arabidopsis MATE proteins (Rogers and Guerinot, 2002). As discussed previously, these paralogs are unlikely to be redundant with FRD3. The possibility remains however, that they are capable of transporting organic acids in different tissues of the plant. Interestingly, recent reports have demonstrated that a sorghum MATE protein effluxes malate in order to provide tolerance to aluminum (Kochian *et al.*, 2006), demonstrating that other genes similar to FRD3 are also involved in transporting organic acids. Preliminary investigations into the transport capabilities could be easily achieved

by ectopically overexpressing FRD3 paralogs in both wild-type Col-0 and *frd3* mutant plants. If these proteins are capable of transporting organic acids, their ectopic expression in wild-type plants should result in an enhanced tolerance to aluminum. Root exudates from these transgenic lines could then be analyzed to determine which organic acids were being transported. Additionally, it would be interesting to determine whether the ectopic expression of the FRD3 paralogs could rescue the *frd3* mutant. These results could be particularly enlightening depending on the nature of the organic acid transported, suggesting whether other organic acids could perform the same physiological function of citrate in the translocation of iron from the roots to the shoots.

Investigating the Role of FRD3 in Iron Deficiency Chlorosis

Chlorosis caused by iron deficiency is a major problem for soybean growers in the Midwestern United States, resulting in large yield losses. Occurrence appears to depend on a number of factors such as soil type, weather and crop genotype. Iron deficiency chlorosis is common on calcareous soils that contain high levels of calcium carbonate and that possess a high pH (Chen and Barak, 1982). Soybean cultivars differ in their resistance to iron deficiency chlorosis on calcareous soils; however, the genetics of this resistance appear complex. Different genetic studies have identified a large number of non-overlapping QTLs linked to resistance to iron deficiency chlorosis (Diers *et al.*, 1992; Lin *et al.*, 1997; Lin *et al.*, 2000). Most interestingly however, earlier physiological studies have demonstrated that soybean cultivars resistant to iron deficiency chlorosis contain more citrate and iron in their xylem exudate compared to susceptible cultivars (Brown and Chaney, 1971). Given the role of FRD3 in effluxing

citrate into the vasculature, it would be interesting to see whether orthologs of FRD3 might function in providing resistance to iron deficiency chlorosis.

First, the soybean FRD3 ortholog will have to be isolated. A number of soybean ESTs similar to *AtFRD3* have been identified. RACE techniques will be used to obtain any missing 5' and 3' sequences. Analysis of expression patterns could be used to determine which sequence is the *AtFRD3* ortholog. Presumably, the ortholog will only be expressed in roots, and specifically in the pericycle. One potential problem is that given the large number of MATE genes present in plant species, probes could recognize multiple, similar transcripts. This situation could be exacerbated given large amount of gene duplication present in the soybean genome. Therefore, probes will need to be designed carefully to ensure specificity. In this regard, as 3' UTRs are often highly variable, these sequences will be initially used to design these specific probes. The identity of orthologs would be further confirmed by looking for functional complementation after transformation into *frd3* mutant plants.

Once *GmFRD3* has been identified, a number of experiments can be performed to determine its influence on resistance to iron deficiency chlorosis. For example, it would be particularly interesting to determine whether the lower xylem citrate levels in susceptible soybean cultivars were caused by reduced GmFRD3 function in these plants relative to the resistant cultivars. For example, expression levels of the gene might be higher in the resistant cultivars. Should this be the case, comparison of promoter regions might reveal important regulatory motifs. Alternatively, genetic variation between the two cultivars also might result in a GmFRD3 protein from the resistant cultivar that is

more efficient in transporting citrate. Such differences in protein function could be dissected biochemically using heterologous expression in *Xenopus* oocytes.

Another experimental approach would be to overexpress either *AtFRD3* or *GmFRD3* in susceptible cultivars to determine whether this could result in enhanced tolerance to iron deficiency chlorosis. Should it succeed, this approach has obvious agronomic and economic implications for crop development.

Experimental Procedures

Arabidopsis Lines and Growth Conditions

The mutants *frd3-1* and *frd3-3* have been described previously (Rogers and Guerinot, 2002). Surface sterilized seeds were plated on Gamborg's B5 medium (Caisson Laboratories, Rexburg, ID) in Petri dishes. Where indicated, organic acids were added to the growth medium prior to autoclaving in the form of their conjugate base sodium salt. Two weeks after germination, seedlings were transferred to fresh plates for another week, after which Fe(III) chelate reductase activity and chlorophyll levels were measured.

Fe(III) chelate reductase assays have been described previously (Yi and Guerinot, 1996) Chlorophyll was extracted by soaking approximately 20mg leaf tissue in 1ml methanol, and total chlorophyll levels quantified by measuring the absorbance at 652, 665 and 750 nm (Porra *et al.*, 1989). Perl's Stain for Fe(II) was performed using established protocols for Arabidopsis roots (Green and Rogers, 2004).

Xylem Collection and Analysis

Wild type Col-0 and *frd3-1* mutant seed were grown axenically on B5 medium for two weeks after which they were transferred to Rockwool (Worms Way, Bloomington, IN) soaked in 0.25X Gamborg's B5 medium without sucrose. Seedlings were grown under a 8h day, 16h night light cycle and were watered once a week with 0.25X Gamborg's B5 medium. After seven weeks, the shoots were excised from the roots at the hypocotyl with a sharp razor blade. To reduce cellular contamination, the first drop of

xylem exuded from the decapitated roots was always discarded. Xylem was collected for no more than two hours after the shoots were initially removed.

Xylem citrate levels were determined according to the instructions of a citrate analysis kit (Roche Molecular Biochemicals): NADH consumption was measured in a solution containing 100mM glycylglycine pH 7.9, 0.2mM $ZnCl_2$, 0.56 mM NADH, 12U/ml Malate Dehydrogenase, 24U/ml Lactate Dehydrogenase and 1.6U/ml Citrate Lyase. Malate levels were measured by measuring the production of NADH in a solution containing 50mM 2-amino-2-methylpropanol pH 9.9, 40mM glutamate, 2mM NAD^+, 3.5U/ml Malate Dehydrogenase and 0.9U/ml Aspartate Transaminase. Enzymes and NADH were obtained from Sigma (St. Louis, MO).

Anion quantification of xylem was performed as described previously (Dionex Corporation, 2004). Briefly, samples were analyzed using a DX-500 BioLC system (Dionex, Sunnyvale, CA) with an IonPac AS11 Analytical Column and a 0.5 to 38mM NaOH gradient at a flow rate of 2.0ml/min. Gradient parameters were 0.5mM NaOH for 0 to 2.5 min, 0.5 to 5mM NaOH for 2.5 to 6 min and 5 to 38mM NaOH for 6 to 18 min. Anions were detected with an ED40 Electrochemical Detector.

Iron levels were measured by adding the xylem to a solution of 0.3M ascorbate and 1mM ferrozine and incubating in the dark overnight. Iron concentrations were then determined by comparing absorption readings at 562nm with a standard curve.

Aluminum Tolerance

To obtain 35S::FRD3GFP, a full-length FRD3 cDNA was cloned into the yeast expression vector pFL61 (Minet *et al.*, 1992). Homologous recombination was then used

to replace the C-terminal of FRD3 with sequence corresponding to the C-terminal fused to GFP. The inserted sequence was confirmed with sequencing. FRD3GFP was then cloned into 35SpBARN under the control of the 35S promoter (LeClere and Bartel, 2001). Wild-type Col-0, *frd3-1* and *frd3-3* plants were transformed with 35S.FRD3.GFP using the floral dip method (Clough and Bent, 1998). Transgenic plants were selected by spraying T_1 seedlings three times with 250 mg l^{-1} Basta. Lines homozygous for individual insertions were generated by analysis of segregation ratios at the T_2 and T_3 generations.

Seedlings were grown on vertical plates in the absence of Al^{3+} for seven days, after which they were transferred to fresh vertical plates containing different concentrations of $AlCl_3$. The medium used in these vertical plates, and its supplementation with $AlCl_3$, has been described previously (Sivaguru *et al.*, 2003). Root growth was measured by marking root lengths every 24 hours. The Petri plates were then digitally scanned and root growth accurately measured using NIH image (developed at the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/)

In order to measure citrate efflux, fourteen day old seedlings grown on B5 media were carefully washed and their roots placed in 3 mL water for 24 hours. This root-bathing solution was lyophilized and resuspended in 150 µL water. Citrate and malate levels were measured using the enzyme based assay described previously.

Two Electrode Voltage Clamping and [^{14}C]Citrate Efflux Experiments

A full length *FRD3* cDNA was subcloned into the oocyte expression vector pOO2 (Ludewig *et al.*, 2002). The FRD3 N-terminal was then replaced with sequence

containing the N-terminal fused to GFP. To enhance expression, the long plant 5' UTR was subsequently removed to obtain pOO2.FRD3GFPshort. For oocyte expression, pOO2.FRD3GFPshort was linearized with *MluI* and capped cRNA transcribed *in vitro* using the SP6 mMessage mMachine kit (Ambion, Austin, TX). The isolation of oocytes from *X. laevis* frogs has been described previously (Osawa *et al.*, 2006). Oocytes were injected with 23ng of *FRD3GFP* cRNA using a Nanoject II injector (Drummond Scientific, Broomall, PA) and then incubated in ND96 Ringers solution at 17 °C as described previously (Osawa *et al.*, 2006).

Oocytes were voltage clamped 4 days after cRNA injection in a bath solution containing 5mM MES-Tris pH 5.0, 1mM $MgCl_2$ and 1.8mM $CaCl_2$, with osmolality adjusted to 240-260 mosmol kg^{-1} with D-sorbitol. Voltage clamping was controlled and currents were recorded with a TEV-200A amplifier (Dagan, Minneapolis, MN) and pCLAMP 6.0 software (Axon Instruments, Union City, CA). To study citrate efflux directly, oocytes were injected with 23nl 1mM citrate using glass micropipettes. The injected oocytes were allowed to recover for 1 min before clamping at a membrane potential of -80mV.

For [^{14}C]citrate efflux, six oocytes (either uninjected or previously injected with *FRD3GFP* cRNA) were injected with 23nl 2mM [^{14}C]citrate (4.6 nCi/oocyte) in ND96 buffer using fine-tipped glass micropipettes. The oocytes were incubated for 1 min in ice-cold ND96 buffer and then transferred into 750 µl ND96 at room temperature. At indicated time points, 650 µl of the buffer was removed for measuring radioactivity and replaced with fresh buffer. At the end of each experiment, the oocytes were dissolved in 10% SDS buffer. Radioactivity of the efflux buffer at the various time points and

remaining in the oocytes was counted using liquid scintillation. [^{14}C]citrate effluxed was expressed as a percentage of total [^{14}C]citrate injected.

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