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Characterization of the Role of FRO6 in Metal Homeostasis in Arabidopsis Thaliana

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Characterization of the Role of *FRO6* in Metal Homeostasis in *Arabidopsis* thaliana

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DEDICATION

This thesis is dedicated to my wonderful family:

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ABSTRACT

Iron deficiency is the most common human nutritional disorder in the world today, affecting approximately 2 billon people worldwide, almost a third of the world's population. For most of the world's population, plant foods serve as the major source of dietary iron. One approach that may contribute towards the solution of iron deficiency is the development of crop plants that contain higher levels of bioavailable iron. This project focuses on *FRO6,* a ferric chelate reductase in *Arabidopsis thaliana* that is thought to reduce apoplastic iron for transport across the plasma membrane in leaf cells. We hypothesize therefore that *FRO6* controls iron content of leaves and provides iron needed for photosynthesis, a vital process in plants. In order to examine the proposed function of *FRO6,* we obtained two FRO6 loss-of-function lines, *fro6-1* and fro6-*2* and compared these lines to the WT to determine the role of FRO6 in iron homeostasis in plants. *fro6-1* contains an insertion in the 8th intron of the gene, while *fro6-2* contains an insertion in the 8th exon of the gene. We demonstrate that *fro6-1* and *fro6-2* are both homozygous for the insertion. Despite this, *fro6-1* and *fro6-2* mutants express *FRO6* transcript, possibly because the insertion does not destabilize the transcript. Leaf disks prepared from *fro6-1* and *fro6-2* show a significant decrease in ferric reductase activity compared to WT leaf disks, indicating a putative role for FRO6 in reduction of apoplastic Fe in leaves. Consistent with the fact that FRO6 is not expressed in roots, *fro6-1* mutant roots did not show a significant decrease in ferric reductase activity compared to the roots of WT plants.

The *fro6-1* plants show a reduced flowering time as compared to WT plants. Taken together, these data indicate that FRO6 is likely responsible for mediating the reduction of ferric iron to ferrous iron at the plasma membrane of leaf cells.

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LIST OF ABBREVIATIONS

AHA ..... ATPase

DMA ..... Deoxymugeinic Acid

DMAS ..... Deoxymugeinic Acid Synthase

FER ..... Ferritin

FRO ..... Ferric Reductase Oxidase

ICP-MS ..... Inductively Coupled Plasma Mass Spectroscopy

IRT ..... Iron Regulated Transporter

MA ..... Mucigenic Acid

NA ..... Nicotianamine

NRAMP ..... Natural Resistance Associated Macrophage Protein

PIC ..... Permease in Chloroplasts

PS ..... Phytosiderophore

SAM ..... S-Adenosyl Methionine

SMF ..... Suppressor of *mif1-1*

VIT ..... Vacuolar Iron Transporter

YSL ..... Yellow Stripe Like

ZIP ..... ZRT/IRT –like Protein

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CHAPTER 1

IRON UPTAKE, TRAFFICKING AND HOMEOSTASIS IN *ARABIDOPSIS thaliana*

For the majority of organisms, iron (Fe) is a necessary micronutrient for maintaining life. Iron is important because it serves as a cofactor in proteins that are involved in integral cellular functions, such as DNA replication and repair, photosynthesis, electron transport, and nitrogen assimilation (Jeong and Guerinot, 2009). Iron's many valuable properties in life arise from the fact that it is a transition metal, meaning it has the ability to change redox state (the accepting and donating of electrons), which makes it an ideal candidate for participation in the electron transport chain of respiration and photosynthesis. Fe associated with proteins is most commonly found as Fe-S clusters or heme groups.

The properties that make iron so vital for life also make it potentially harmful to life. Copious amounts of ferrous iron are harmful, because this form of Fe has the potential to generate reactive oxygen species (ROS), via the Fenton reaction. The Fenton reaction involves ferrous iron Fe^{2+} interacting with H_2O_2 and O_2 within the cell to generate superoxides and/or hydroxyl radicals. These ROS are strong oxidants that can cause serious damage to DNA, proteins, and lipids, and therefore greatly decrease the viability and integrity of the cell (Halliwell, 1992).

Iron is the fourth most abundant element in the earth's crust. But the majority of the iron that is present in the soil is not readily available for use by plants. In particular, in aerobic soils at neutral or alkaline/basic pH, Fe has low bioavailability.

In these soils, Fe forms insoluble oxyhydride polymers that cannot be used by some plants (Jeong and Guerinot, 2009). Alkaline soils account for approximately one third of all agricultural lands in the world, so Fe limitation is a major problem in agricultural settings (Nano and Strathmann, 2006).

According to the World Health Organization, iron deficiency is the most common nutritional disorder in the world today, affecting over approximately 2 billion people (http://www.who.int/nutrition/topics/ida/en/index.html). While plant food serve as the major source of dietary iron for humans and other mammals (Connolly et al., 2002), plants are generally considered poor sources of iron. More iron rich foods include red meat, which is considered a better source of iron versus plants for primarily two reasons. First, plant foods contain less iron than meat. Secondly, not only is there less iron in plants than in meat but the iron that is present is less bioavailable than the iron found in meat (Miret et al., 2003). A diverse plant-based diet or eating meat may be a solution to this problem, but these alternatives are not plausible in some parts of the world, due to lack of affordability or conflict with religious or cultural beliefs (Miret et al., 2003). Since plant-based diets seem to be a significant contributor to this nutritional deficit pandemic, there is great interest in bioengineering plants to have enhanced amounts of bioavailable Fe in the edible portion of the plant. So, now there is much focus on

understanding how plants uptake, transport, and store iron. A clearer understanding of these mechanisms may lead to the development of iron-enriched crops that may be used to combat iron deficiency anemia.

To achieve this goal, we must first understand the mechanisms of plant iron uptake, transport, and storage. Plants have evolved two strategies to increase acquisition of iron from the soil under limiting conditions. Strategy I plants include all non-grasses, such as tomato, pea and *Arabidopsis thaliana*, while Strategy II plants include all the grasses, such as rice, maize, wheat and barley.

Iron Uptake from Soil

Strategy I

When iron is limiting, Strategy I plants employ a three-pronged approach to mediate the uptake of iron by the roots (see Figure 1.1). This system is similar to that of the iron uptake system found in the yeast *Saccharomyces cerevisiae*, which uses a two-step iron uptake strategy, in which Fe^{3+} iron is reduced to Fe^{2+} by FRE1 and then transported into the cell by a high affinity transporter (Askwith, 1994). In Strategy I plants, protons (H^+) are first pumped out into the rhizosphere. This step is crucial, because it serves to acidify the soil and make the ferric Fe^{3+} iron more soluble (Mukherjee et al., 2006). A one unit drop on the pH scale corresponds to 1000-fold increase in Fe solubility (Palmer and Guerinot, 2009). The family of genes thought to be responsible for proton extrusion in the rhizosphere is the AHA (Arabidopsis H+ ATPase) family. *AHA2*, along with *AHA1* and *AHA7* are all up- regulated in iron deficient conditions (Palmer and Guerinot, 2009). However, AHA2 is the most likely candidate for

extruding protons in the Strategy I mechanism (see Figure 1.1) (Santi and Schmidt, 2009; Hindt and Guerinot, 2012).

Next, the solubilized ferric iron is reduced to ferrous Fe^{2+} iron by FRO2, which is the principle root surface ferric chelate reductase (or Ferric Reductase Oxidase) responsible for reduction of rhizosphere iron (see Figure 1.1). FRO2 is the founding member of the FRO family and the first member to be fully characterized. The Arabidopsis *FRO* family includes 8 genes. The *FRO* family of genes was identified due to their similarity to the human respiratory burst NADPH oxidase (gp91phox) and to the yeast ferric chelate reductase (Yi and Guerinot 1996). Additionally, *FRO2* was found to map to same locus as *frd1,* a mutant which lacks ferric reductase activity. *FRO2* was able to functionally complement or rescue the *frd1* mutant phenotype proving that *FRO2* encodes the root surface reductase (Robinson et al., 1999). *FRO2* is expressed primarily in the roots and is localized to the root plasma membrane (Mukherjee et al., 2006).

FRO2 is predicted to contain eight hydrophobic domains that form helices that span across the membrane. Two of the transmembrane helices (4 and 6), are believed to each contain two conserved histidines which are thought to coordinate two heme groups. Also, within FRO2 there is a region that is highly conserved in all flavocytochrome family members. It is thought that FRO2 oxidizes NADPH in the cytoplasm and then transfers the electrons from NADPH through the two heme groups and subsequently across the membrane to reduce Fe^{3+} to form Fe^{2+} (Robinson, 1999 ; Schagerlof 2006). This step is considered to be the rate-limiting step in iron uptake from the soil (Connolly, 2003).

Finally, ferrous iron is transported across the plasma membrane into the cell via IRT1 (iron-regulated transporter 1) (see Figure 1.1) (Connolly et al, 2002; Vert et al. 2002). *IRT1* was discovered using yeast complementation studies (Eide et al., 1996). In addition to Fe, IRT1 can transport zinc, manganese, cobalt, and cadmium (which is toxic at low levels) as well.

Expression of IRT1 is crucial for survival of the plant, as evidenced by IRT1 loss-of-function mutants, which show seedling lethality unless they are provided with excess iron (Vert et al., 2002). Experimentally, it has been shown that after 3 days of growth on iron deficient medium, expression of IRT1 is greatly increased in the plasma membrane of the roots. Additionally it has also been shown that *IRT1* mRNA and protein are quickly degraded and are undetectable just 12 hours after iron is resupplied to plants (Connolly et al. 2002).

Regulation of Strategy I

FRO2 and *IRT1* are both iron deficiency response genes that are controlled by the iron responsive transcription factor FIT (FER-like Iron- deficiency- induced transcription factor). FIT was discovered in *Arabidopsis* based on its sequence similarity to the tomato FER protein and it is a basic helix-loop-helix (bHLH) transcription factor (Ling 2002; Bauer et al, 2007). FIT, like its tomato ortholog, is needed to induce iron-deficiency responses (Colangelo, 2004). Under iron-deficient conditions, there is high induction of FIT in the roots. Similar to *irt1* mutants, *fit* mutants exhibit chlorosis and do not produce seed, unless supplied with exogenous iron (Colangelo and Guerinot, 2004; Vert, 2002). Additionally, *FRO2* transcript and activity (ferric chelate reduction) are not detectable in

the roots of the *fit* mutant (Colangelo and Guerinot, 2004). On the other hand, IRT1 shows post-transcriptional regulation by FIT, because in *fit*, *IRT1* mRNA is present, but there is no accumulation of the IRT1 protein (Colangelo and Guerinot, 2004).

Strategy II

Strategy II is the mechanism by which the grasses acquire iron and it is deemed much more efficient than Strategy I because Strategy II plants can grow on calcareous soils (Römheld, 1987). Strategy II plants respond to iron deficiency by synthesizing phytosiderophores (PS) in the roots, and secreting them out into the rhizosphere. PSs chelate or bind Fe^{3+} with a very high affinity (Chu et al., 2010). These Fe(III)-PS complexes are then transported across the root plasma membrane for use by the plant (see Figure 1.1) (Chu et al., 2010).

Nicotianamine serves as the precursor for all phytosiderophores. Nicotianamine is a non-proteinogenic amino acid. Nicotianamine synthetase synthesizes NA by condensing three molecules of S-adenosyl methionine. Then, nicotianamine aminotransferase converts NA into a 3”-ketoacid. Next DMA synthase (DMAS) reduces the 3”-ketoacid (Dell’mour, 2010) to create mucigenic acid (MA) or phytosiderophores. The two most common muginenic acids are hydroxymugineic acid (HMA) and deoxymugineic acid (DMA) (Dell'mour, 2010).

After the phytosiderophores have been pumped out into the rhizosphere, they chelate ferric iron, and now must be transported back into the plant (see Figure 1.1) (Nozoye, et al., 2011). In 2001, Curie et al identified the transporter needed to transport the Fe(III)-PS complexes through the characterization of the *yellowstripe1 (ys1)* mutant in maize. A wild type copy of *ZmYS1* was transformed into the *fet3fet4* yeast strain,

which shows reduced growth on iron deficient media, but when transformed with WT *ZmYS1* and supplied with Fe(III)-DMA, the transformed mutant yeast strain was able to grow normally. On the other hand, growth was still limited if the media included Fe(III)-citrate as the substrate. So, the YS1 transporter is specifically able to transport the phytosiderophore bound Fe (Fe-DMA) (Curie, 2001). Furthermore, *ZmYS1* was shown to be expressed in both iron deficient roots and shoots, indicating that YS1 may play a role in iron acquisition from the soil and iron transport to the shoots (Roberts, 2004).

In Strategy II plants, the biosynthesis of PS and Fe(III)-PS transporter had been well characterized, but the actual PS effluxor had remained a mystery. However, recent studies have identified this efflux protein (Nozoye et al., 2011). TOM1 of rice and HvTOM1 from barley are both members of the major facilitator family (MFS) and both were shown to function in efflux of PSs (Nozoye et al., 2011). When expressed in Xenopus oocytes, TOM1 and HvTOM1 were both able to efflux 14C-labeled deoxymugineic acid but did not transport 14C-labeled NA, which indicates that TOM1 and HvTOM1 specifically efflux the phytosiderophore DMA (see Figure 1.1).

Iron Trafficking in Plants

The *FROs*- metalloreductases

In Arabidopsis, there are eight *FROs* named *FRO1-8.* Due to their subcellular localization and expression patterns and their sequence similarity to FRO2, other *FROs* are believed to function to reduce Fe in different tissue of plants and in different subcellular compartments. Ferric reductase activity has been reported in leaves of both sunflowers (de la Guardia, MD and Alcantara E., 1996) and *Vigna unguiculata* (Bruggemann et al. 2003), which suggests that Fe is reoxidized after uptake by roots and

that Fe needs to be reduced again before entering leaf cells. Only *FRO2* and *FRO7* have been fully characterized, while *FRO4* and *FRO5* have been partially characterized. FRO7 localizes to the chloroplast (Jeong et al., 2008) and is highly expressed in all green aerial portions of the plant (Mukherjee et al., 2006). Chloroplasts isolated from *fro7* loss-of-function mutants contains 33% less Fe than WT (Jeong and Guerinot, 2009). Further, *fro7* chloroplasts show 75% less ferric chelate reductase activity than WT (Jeong et al., 2008). This indicates that FRO7 is important for providing the large amount of Fe that is needed by the chloroplast.

Recently, two additional *FROs* have been partially characterized. *FRO4* and *FRO5* are expressed in both roots and shoots, and both localize to the PM (Mukherjee et al., 2006; Bernal et al., 2011). Recently, FRO4 and FRO5 have both been shown to be

FRO4 and FRO5 have been shown to be under control of the transcription factor SPL7 (SQUAMOSA Promoter Binding Protein-Like 7), which is the master regulator Cu deficiency responses (Bernal, et al., 2011). The promoters of *FRO4/FRO5* both contain multiple copies of the GTAC core motif, to which SPL7 can bind under Cu deficient conditions (Bernal, et al., 2011). A *fro4fro5* double mutant is unable to reduce Cu at the root surface. Additionally, it was shown that high affinity Cu uptake in the roots first requires reduction of Cu^{2+} to Cu^{1+} by FRO4/FRO5. Taken together, this data indicates that FRO4/FRO5 function redundantly as Cu chelate reductases.

There remain four FROs to be characterized; FRO1, FRO3, FRO6, and FRO8. Of these remaining FROs, FRO3 and FRO8 both localize to mitochondria. *FRO3* is expressed in both the root and shoot tissue of the plant. While FRO8 is expressed mainly

in the shoots (Mukherjee et al., 2006). FRO3 and FRO8 are believed to be responsible for reducing Fe for subsequent uptake by mitochondria.

FRO6 shares a high level of sequence similarity with FRO7 (approximately 95% amino acid sequence similarity) (Wu et al., 2006). FRO6 has been shown to localize to the plasma membrane in protoplasts (Jeong and Guerinot, 2009). *FRO6* is regulated in light-dependent manner, with its promoter containing many light responsive elements (LREs), such as the I-box, GT1, and GATA motif, which indicates that FRO6 may be indirectly involved in photosynthesis, perhaps through delivery of Fe to photosynthetic complexes (Feng et al., 2006). Li et al. expressed the Arabidopsis *FRO6* gene under the control of a 35S promoter in tobacco plants (Li et al., 2010). These transgenic tobacco plants showed increased ferric reductase activity in leaves grown under both iron sufficient and iron deficient conditions compared to WT plants grown in the same conditions (Li et al., 2010). The 35S::FRO6 plants also contained higher levels of Fe^{2+} and chlorophyll, compared to WT plants (Li et al., 2010). Taken together, this data suggests that FRO6 is predicted to be responsible for reducing apoplastic iron for transport across the plasma membrane in leaf cells. The functional characterization of FRO6 is the focus of this thesis, as described in Chap 2.

Additional Transporters of Iron

There are additional known transporters of iron that exist in plants, beside IRT1 and YS1. Arabidopsis IRT2 has high amino acid sequence similarity to the main iron transporter in Strategy I plants, IRT1. IRT2 is also able to alleviate the Fe- limited growth phenotype of the *fet3fet4* yeast strain on iron deficient media, like IRT1. Also like IRT1, IRT2 is expressed only in the roots, under iron deficient conditions. However, unlike

IRT1, IRT2 does not transport manganese or cadmium. So IRT2 is more specific in its transport of metals (Vert, 2002). Additionally, the IRT2 mutant, *irt2,* does not exhibit any signs of iron deficiency. Furthermore, *IRT2* cannot rescue the chlorotic and Fe-deficient growth phenotypes of the *irt1-1* mutant and it is not localized to the plasma membrane like IRT1, but instead to vesicles within root epidermal cells (Vert et al., 2009). There are many ideas about the precise function of IRT2 and one interesting explanation proposes that IRT2 serves as a "proactive adaptation" (MacDiarmid et al. 2003) where IRT2 acts to sequester excess Fe that is produced when IRT1 is induced (Vert et al., 2009). However more work needs to be done to support this idea (Conte and Walker 2011).

There is also another family of iron transporter genes called the NRAMPs (Natural Resistance Associated Macrophage Protein). The yeast *SMF* (Suppressor of *mif1-1*) gene and the mammalian *DMT1* are both NRAMP homologs, and both have been shown to participate in metal uptake. Arabidopsis contains six NRAMP genes, and half of them (*NRAMP1*, *NRAMP3*, and *NRAMP4*) can transport iron when expressed in yeast (Curie et al., 2000). Additionally, like *IRT1* and *IRT2*, some *NRAMP*s are up-regulated during times of iron deficiency (Curie et al, 2000).

Intercellular Transport of Fe in Plants

Once iron has been taken up into root cells, it undergoes lateral movement from the root epidermal cells to the xylem and then is moved to the green above-ground portions of the plant, where it is needed in great quantities for photosynthesis. The process of transporting iron from the roots to shoots is thought to involve organic acids, such as citrate, which bind iron in the xylem and carry it to the shoots (Rellán-Alvarez, 2010). There are also other chelators of iron. For example, NA, which binds both Fe^{2+}

and Fe^{3+}, is present in both Strategy I and Strategy II plants, and these Fe-NA complexes are found in the vasculature of plants (Chu et al., 2010). Iron is transported to the root, flower, seeds, and other parts of the plant via the phloem (Palmer and Guerinot, 2009).

Vascular Transport of Fe

Citrate is thought to be the major binder of iron in the xylem because the pH of xylem favors Fe(III)-citrate complexes (Curie et al., 2009). Additionally, there is evidence that Fe moves from roots to shoots as Fe(III)-citrate (Tiffin, 1966). Analogs of xylem sap show that Fe and citrate co-migrate in paper electrophoresis. Indeed,"Theoretical calculations that take into account the known metal chelators (organic acids and amino acids including nicotianamine) ... suggest that citrate will be the major complexor of Fe in the xylem" (Conte and Walker 2011). Recently, Fe-complexes that occur in nature were identified as oxo-bridged tri-Fe(III), tri-citrate ($FeCit_3$) using HPLC-ICP-MS to analyze the xylem sap of tomatoes (von Wiren et al., 1999; Rellan-Alvarez et al., 2008; Conte and Walker, 2011). The presence of Fe(III)-complexes in xylem exudates suggests that Strategy I plants reoxidize the reduced Fe back to the ferric form once it crosses the plasma membrane of root epidermal cells.

Two mutant lines have provided significant insight into transport of Fe within the xylem. The *chloronerva* mutant, which lacks the single *NAS* gene that encodes nicotianamine synthase and thus lacks NA synthesis, shows elevated levels of citrate in the xylem sap (Pich et al., 2001). The *Arabidopsis frd3* mutant contains less xylem citrate and accumulates Fe at high levels in the root, which indicates that FRD3 is required for the proper transport of Fe across long distances (Rogers and Guerinot 2002). FRD3 *(*ferric reductase defective) is a member of the MATE (Multidrug and toxin efflux)

protein family and is thought to function in the efflux of citrate to the xylem. It is localized to the root pericycle and cells around the vasculature. Thus, FRD3 is thought to release citrate into the root xylem, where it complexes with Fe so that it may be translocated to the aerial portions of the plants (Rogers and Guerinot, 2002). *FRD3* is also strongly expressed in the seed and flower, indicating a possible role in efflux of citrate to the apoplast for transport of iron in the xylem of flowers (Roschzttardtz et al., 2011). In the Arabidopsis *frd3* mutant, iron accumulates in the cell directly next to the vasculature, where FRD3 is localized, suggesting that there is blockage of Fe entering the vasculature (Green and Rogers, 2004).

When FRD3 was expressed in the heterologous *Xenopus oocyte* system, it was able to facilitate efflux of citrate. In rice, there is *FRD3*-like gene *FRDL1,* (FRD3-like1), that has 57% sequence similarity to FRD3 and has an analogous function to FRD3 and similar phenotypes to the *frd3* mutant (Yokosho et al., 2009). Although Strategy I and Strategy II plants have differing Fe uptake mechanisms from the rhizosphere, both types of plants seem to share similar Fe translocation systems (Conte and Walker, 2011).

At this time, the mechanism for loading iron into the xylem is not yet entirely known. However, recently, a likely candidate has been identified and the name of this protein is FPN1 (ferroportin). In Arabidopsis, the ferroportin family includes three genes: FPN1/IREG1 which localizes to the plasma membrane (Morrissey et al., 2009), FPN2/IREG2, which localizes to the vacuolar membrane (Schaaf et al., 2006; Morrissey et al., 2009), and FPN3/MAR1/RTS3/IREG3, which localizes to the chloroplast envelope (Conte et al., 2009). In mammals there are ferroportins called Metal Transporter Protein 1 and Iron Regulated protein 1 (IREG1) that facilitate efflux of Fe from macrophages and

hepatocytes (Muckenthaler et al., 2008; Conte and Walker 2011). The functional activity of FPN1/IREG1 could not be determined, because FPN1 protein does not localize to the plasma membrane in the yeast heterologous system (Schaaf et al., 2006). But the closely related FPN2 can transport Ni, Co, and Fe from the cytoplasm to the vacuole (Morrissey et al., 2009). FPN1 is localized to the "stele of the root, root-shoot junctions of seedlings and leaf veins, indicating a role in efflux into apoplast and or xylem" (Morissey et al., 2009).

Transport of Fe within the Shoot

Once Fe travels to the leaf apoplast via the xylem, it must be transported across the leaf plasma membrane (Conte and Walker, 2011). The importance of citrate in the proper transport of iron in xylem has been demonstrated in the *frd3* mutant, which accumulates iron in the extracellular space, as noted above (Green and Rogers, 2004). However the role that citrate plays in xylem unloading remains a mystery. In Arabidopsis, members of the Yellow Stripe-Like (YSL) family, which transport Fe^{2+} nicotianamine complexes, are thought to be the principal "mediators" of uptake of Fe from the xylem (Conte and Walker, 2011).

Fe is thought to be transported through the phloem bound to nicotianamine (NA). NA is synthesized by both Strategy I and Strategy II plants and is a precursor to the PS found in the Strategy II grasses. Thus NA is structurally similar to PS, so it can chelate Fe^{3+}, along with other metal species including Fe^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, and Zn^{2+} (Chu et al., 2010). NA is found in both shoots and roots (Stephan et al., 1987; Conte and Walker, 2011) and both xylem and phloem (Schmidke and Stephen, 1995; Pich and Scholz, 1996; Conte and Walker, 2011).

Identification of the tomato NA synthesis deficient mutant, *chloronerva,* demonstrated the importance of NA in iron translocation. The *chloronerva* mutant shows severe interveinal chlorosis of young leaves (Stephan and Grun, 1989; Higuchi, 2001). The interveinal chlorosis phenotype indicates that the plant is iron deficient and the mutant shows constitutive induction of the iron deficiency uptake response (i.e. upregulation of *AHA2, FRO2,* and *IRT1*) (Becker et al, 1992). However, the mature leaves surprisingly contain an excess of iron (Becker et al. 1992). So the fact that there is more iron in the mature leaf tissue and less Fe in the younger tissues suggests that NA plays a role in proper distribution of Fe (Conte and Walker, 2010).

As noted above, when Fe is traveling through the phloem it is believed to be chelated or bound by NA (Conte and Walker, 2011). The YSL family of proteins is thought to be responsible for loading and unloading of these Fe-NA complexes. The YSLs are a subfamily of oligopeptide transporters (OPT) (Curie et al, 2009). These genes were identified based on their high sequence similarity to YS1, the PS-Fe(III) transporter in Strategy II plants (Curie et al, 2009). *YSLs* are expressed in a wide variety of plants including gymnosperms, mosses, lycopods, ferns, monocots and dicots (Conte and Walker, 2011). YSLs have been shown to transport various metal-NA chelates as substrates (DiDonato et al., 2004, Koike et al., 2004; Roberts et al., 2004; Schaaf et al., 2004; Lejean et al., 2005; Murata et al., 2006; Gendre et al., 2007; Harada et al., 2007).

YSL1 is a well-characterized member of the YSL family. YSL1 is expressed in the shoot vasculature, siliques, pollen grains and maturing seeds (Lejean et al., 2005). The *ysl1* mutant contains less iron in its seeds and these seeds show a reduced germination rate on iron-deficient medium, which indicates that YSL1 is needed to load

iron into the seed (Waters et al., 2006). Additionally, another member of the YSL family, *YSL3* also localizes to the shoot vasculature and pollen grains (Waters et al., 2006). The double mutant *ysl1*, *ysl3*, contains less Fe, Cu, and Zn in leaves, which indicates roles for YSL1 and YSL3 as metal transporters in leaves (Waters et al., 2006). Expression of *YSL1*, *YSL2*, and *YSL3* is suppressed under iron deficiency. The opposite holds true for the iron uptake genes (*YS1, FRO2* and *IRT1*). Taken together, these data suggest that the YSL family is responsible for maintaining iron homeostasis via intercellular transport of Fe-NA complexes (Conte and Walker, 2011).

Intracellular Iron Transport

Once Fe has reached the appropriate tissue and crossed the plasma membrane, it must be distributed to various subcellular compartments to be used and/or stored. Two major organelles that require large amounts of Fe are the chloroplast and mitochondria (Conte and Walker, 2011). In addition, iron may be stored in the vacuole (Kim et al., 2007).

The Chloroplast

Almost 90% of leaf cell Fe is found in the chloroplast. Much iron is found here, because this is the organenelle where photosynthesis takes place. Many photosynthetic processes, complexes, and proteins require Fe, including the photosynthetic complexes, chlorophyll synthesis, and heme (Kim and Guerinot, 2007). Additionally, Fe is needed as a co-factor in the enzyme Fe-SOD, which prevents oxidative damage by converting ROS to H_20_2, reducing the number of harmful superoxides that are normally formed during the photosynthetic electron transport chain (Kim and Guerinot, 2007).

Ferritin in plants, much like ferritin found in animals, is involved in storage of iron. Plant ferritins can store up to 4500 atoms of Fe and are localized to the plastids (chloroplast) (Hintze and Theil, 2006). In Arabidopsis, there exist four *FER* genes. FER1 is believed to be involved in senescence, because the age-dependent rate of senescence was increased in the *fer1* mutants, due to toxic accumulation of ROS. All four ferritins (FER1-4) localize to the chloroplast and have been shown to function primarily to protect the plant from oxidative damage rather than in storage of Fe (Ravet, et al., 2008).

Recently, transport of Fe into the chloroplast has been elucidated. Transport into the chloroplast involves two main components: FRO7 and PIC1. As noted above, *FRO7* belongs to the FRO family of ferric chelate reducatses. It is highly expressed in all green aerial tissues and is localized to the chloroplast membrane. In Arabidopsis, *fro7* mutants germinate on alkaline soils, but quickly die (Jeong et al., 2008). Chloroplasts isolated from *fro7* plants show 75% less ferric chelate reductase activity than WT. They also have less iron than WT chloroplasts, which indicates that FRO7 plays a major role in acquiring Fe for the chloroplasts (Jeong et al., 2008).

In recent years, the chloroplastic Fe transporter has been identified as PIC1 (Permease in Chloroplast 1). PIC1 is a permease-like protein in cyanobacteria (Duy, et al., 2007). PIC1 was first identified as a part of a "protein-conducting channel" on the inner-envelope of the chloroplast (Teng, Y et al., 2006). However it was shown to be able to complement the yeast iron uptake mutant *fet3fet4* . Additionally, the *pic1* mutant shows phenotypes that are indicative of compromised iron transport. For instance, the *pic1* mutants are severely chlorotic and have higher levels of ferritins (Duy et al., 2007).

It is not known whether or not PIC1 transports Fe^{2+} or Fe^{3+} or some other molecule, and the precise role of PIC1 remains unclear (Jeong and Guerinot, 2009).

Mitochondria

Mitochondria, like chloroplasts, require a high amount of iron (Briat, 2007). As previously mentioned, iron serves as a cofactor in the electron transport chain of respiration. Additionally, Fe-S clusters are constructed in mitochondria, as well as in chloroplasts. FER4 dually localizes to mitochondria and to the chloroplast and is thought to have the same function as the chloroplastic ferritins FER1, FER2, and FER3, which is to store iron safely in a bioavailable form and to therefore protect cells from oxidative stress (Taratino, 2010). In recent years, the transporter responsible for transporting Fe into the mitochondria has been identified. MIT was discovered using through screening of a T-DNA (transfer DNA) library to identify rice T-DNA lines that exhibited iron deficiency symptoms. MIT is a member of the mitochondrial solute carrier family (MSC) and members of this family are known to localize to the inner mitochondrial membrane and also to transport a diverse variety of substrates, including Fe (Kunji and Robinson, 2006). MIT function is essential for survival, as homozygous knockouts for MIT are not viable (Bashir et al., 2011). MIT was able to rescue the growth phenotype of *Δmrs3Δmrs4* yeast, which has defective transport of iron into the mitochondria. The MIT-knockdown, *mit-2*, shows a reduced growth phenotype, although it accumulates excess iron. Moreover, there was a decrease in aconitase activity in both the cytosol and mitochondria, which indicates that Fe-S cluster formation is compromised in *mit-2* plants. Taken together, these results point to MIT as the protein responsible for transporting iron into the mitochondria of rice (Bashir et al., 2011).

Vacuoles

The vacuole is a critical compartment for storage and sequestration of iron within plants (Lanquar et al., 2005; Kim et al., 2006). VIT1 has been shown to transport Fe into vacuole (Kim et al., 2006). Expression of *VIT1* in yeast was able to rescue the yeast *ccc1(Ca^{2+}cross complementer 1)* mutant, because there was an increase in the amount of Fe in the vacuoles, which suggests that VIT1 functions in transporting iron into the vacuole (Kim, et al., 2006). VIT1 is expressed highly during the development of seeds, and *vit1* mutants cannot survive under Fe-limiting conditions, such as alkaline soil (Kim et al, 2006).

Remobilization of Fe from the vacuole is another important part of iron metabolism in plants. Two members of the NRAMP family have been shown to aide with this process. NRAMP3 and NRAMP4 both export Fe from the vacuoles (Kim et al, 2006). Both NRAMP3 and NRAMP4 are up-regulated under iron deficient conditions (Lanquar et al, 2005). Neither *nramp3* nor *nramp4* single mutants show phenotypes, when grown on iron deficient medium. However, the double mutant *nramp3nramp4* does not germinate under iron deficiency (Lanquar et al., 2005). Interestingly, the mutants do not contain less iron that WT seeds, but electron microscopy shows that *nramp3nramp4* plants accumulate Fe in the vacuole, suggesting that NRAMP3 and NRAMP4 play a role in exporting Fe from the vacuole (Jeong and Guerinot, 2009).

A thorough understanding of the mechanisms that regulate metal homeostasis is critical to generating iron-enriched biofortified crops. This goal will be attainable by continual in- depth study and analysis of the ferric reductase oxidases, transporters, chelators and regulatory proteins involved in metal metabolism. In recent years, much

progress has been made to elucidate the molecular mechanisms of Fe homeostasis, but much remains unknown. The focus of this thesis is the role of FRO6 in reduction of Fe by leaf cell.

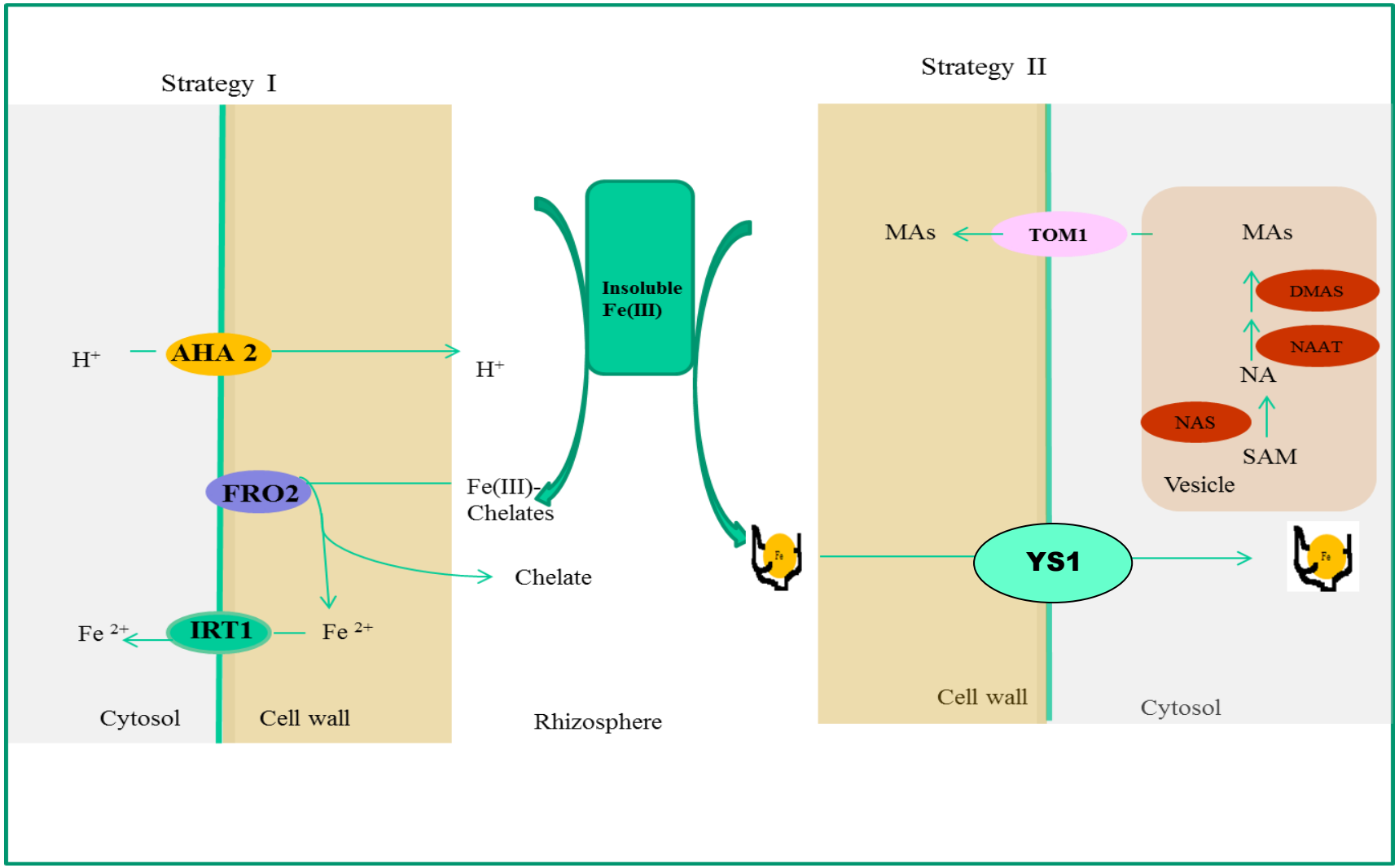

Figure 1.1 Strategy I versus Strategy II Iron Uptake Mechanism. (Adapted from Kobayashi and Nishizawa, 2012). Strategy I plants (all non-graminaceous dicots) use a Reduction Strategy, where Fe is reduced by FRO2, then taken up into the roots by IRT1. Strategy II plants (grasses) use a "Chelation" based strategy, where phytosiderophores are released into the soil and directly chelate the Fe present. The Fe-phytosiderophore complexes are then taken up into the plant.

CHAPTER 2: Characterization of the Role of FRO6 in Metal Homeostasis in *Arabidopsis thaliana*

Introduction

For the majority of organisms, iron (Fe) is essential for life. This is because it acts as a cofactor in many proteins involved in replication and repair of DNA, photosynthesis, and respiration (Jeong and Guerinot, 2009). Although iron is required for many cellular processes, over accumulation of iron generates toxic hydroxyl radicals via the Fenton reaction (Halliwell and Gutteridge, 1992). Even though iron is abundant in the soil, it is not readily available for use by plants in aerobic soils at neutral or basic pH, where it forms insoluble oxyhydroxide polymers (Jeong and Guerinot, 2009). Alkaline soils account for approximately one third of all agricultural lands in the world, so iron deficiency is a major problem that we face in the world today (Nano and Strathmann, 2006). Indeed, plants grown under iron deficient conditions often exhibit yellowing or chlorosis of the leaves (Briat and Lobreaux, 1997) and reduced crop yields (Guerinot and Yi, 1994). According to the World Health Organization, iron deficiency anemia is the most common nutritional disorder in the world today, affecting over approximately 2 billion people. Plants serve as the major source of dietary iron for humans and other mammals (Connolly et al., 2002). So, there is great interest in understanding the mechanisms by which plants uptake and regulate iron.

Plants have evolved two mechanisms to combat Fe limitation (Guerinot and Yi, 1994) (see Figure 1.1).

Strategy I plants include all non-grasses, such as tomato, soybean and the model plant *Arabidopsis thaliana*, while Strategy II plants include all the grasses, including rice, maize and wheat. When iron is limiting, Strategy I, plants employ a three-step process to mediate the uptake of iron in the roots. In the first step, protons are pumped out into the rhizosphere by an ATPase; this step serves to acidify the soil and make the ferric (Fe^{3+}) iron more soluble (Vert, 2002; Eide et al., 2006; Connolly and Walker, 2008). Then, the solubilized ferric iron is reduced to ferrous (Fe^{2+}) by FRO2 (ferric reductase oxidase), which is the principle root ferric chelate reductase and a member of the *FRO* family of genes. Finally, ferrous iron, is transported across the plasma membrane into the cell via IRT1 (iron-regulated transporter 1) (Eide et al., 1996; Vert et al., 2002; Connolly et al, 2002). Strategy II is deemed much more efficient, than Strategy I. Strategy II plants respond to iron deficiency by synthesizing phytosiderophores (PS) in the roots, and secreting them out into the rhizosphere. PSs chelate or bind Fe^{3+} with a very high affinity (Chu et al., 2010). These Fe(III)-PS complexes are then transported across the plasma membrane by the YS1 (yellow stripe 1) iron transporter (Chu et al., 2010) (see Figure 1.1).

In Strategy I plants, IRT1 is the transporter that is responsible for transporting iron and other bivalent metal cations across the plasma membrane. Its expression is crucial for survival of the plant, as the *irt1* loss-of-function line displays seedling lethality (Vert et al., 2002). Experimentally, it has been shown that after 3 days of plants growth on iron deficient medium, expression of *IRT1* is greatly increased. Additionally it has also been shown that *IRT1* mRNA and protein are quickly degraded, after resupply of Fe (Connolly et al. 2002).

Similarly, *FRO2* is also transcriptionally up regulated under iron deficiency (Connolly et al., 2003). FRO2 is the main ferric chelate reductase responsible for converting ferric iron to ferrous iron, a pivotal and rate limiting step for transport of iron across the plasma membrane (Grusak et al., 1999). Plants which lack *FRO2* exhibit severe chlorosis, indicating the importance of this gene in Strategy I plants (Robinson et al., 1999). FRO2 is expressed primarily in root epidermal cells and is localized to the plasma membrane in these cells (Connolly et al, 2003).

The *FROs* constitute a family of genes thought to be responsible for reducing iron and/or copper in various parts of the plant. The Arabidopsis *FRO* family includes 8 genes, named *FRO1-8* (see Table 2.1). When iron is limiting, there is transcriptional induction of some of the *FRO* genes and studies have shown that different *FROs* are expressed and localized in different parts of the plant (Wu et al., 2005; Mukherjee et al., 2006; Jeong et al., 2008; Bernal et al., 2012). Recently, three *FROs* have been functionally characterized. In 2008, Jeong et al. showed that FRO7, which is expressed highly in all green tissue, localizes to the chloroplast and plays a role in acquiring iron in chloroplast. *FRO7* is essential for proper functioning of photosynthesis in young seedlings under iron deficient conditions (Jeong et al., 2008). On the other hand, *FRO4* and *FRO5* are expressed in the shoots and roots and localized to the plasma membrane. Recently, these genes have been shown to be instrumental in maintaining Cu homeostasis, because they reduce Cu for high affinity uptake of Cu by roots. Furthermore, transcription of *FRO4* and *FRO5* is under the control of the transcription factor that controls Cu deficiency responses, *SPL7* (Bernal et al, 2011).

There are 4 *FROs* that have not been characterized yet. *FRO3* and *FRO8* are thought to be localized to the mitochondria (Jeong and Connolly, 2009). *FRO3* is ubiquitously expressed throughout the plants, while *FRO8* is only expressed in senescing leaves (Mukherjee et al., 2006). *FRO3* and *FRO8* are both thought to be involved in the reduction of iron that is needed for use by the mitochondria. *FRO1* is not highly expressed in any tissue tested (Mukherjee, et al., 2006, Wu et al., 2005).

This work focuses on functional characterization of another uncharacterized *FRO*, *FRO6.* FRO6 is localized to the PM and expressed at a high level in all green aerial portions of the plant (Mukherjee et al., 2006; Feng et al., 2008; Jeong et al., 2008) Interestingly, the *FRO6* promoter contains multiple, light responsive elements (LREs), including a GATA motif, the I-box, and GT1, thus *FRO6* is regulated in a light-dependent manner (Feng H et al., 2006). Overexpression of *FRO6* in transgenic tobacco plants resulted in increased ferric reductase activity in the shoots, but not the roots of plants grown on iron sufficient and deficient media, with ferric reductase activity being higher in iron deficient transgenic plants (Li et al., 2010). Additionally, these *35S::FRO6* transgenic plants showed increased levels of chlorophyll and Fe.

As of now, the mechanism for how Fe is taken up across the PM of the root is well understood: 1. AHA2 lowers pH of soil to solubilize Fe, 2. FRO2 reduces the solubilized Fe^{3+} to Fe^{2+}, and 3. IRT1 transports the Fe^{2+} across the root PM, as described above. However, the mechanism for how Fe is taken up across the PM of the shoot is not well understood.

Previous studies support the idea that a ferric reductase is required for Fe uptake by leaf cells. For example, ferric reductase activity has been reported in leaves of both

sunflowers (de la Guardia, MD and Alcantara E., 1996) and *Vigna unguiculata* (Bruggemann et al. 2003). The presence of ferric chelate reductase activity indicates that some percentage of Fe is likely oxidized back to its Fe^{3+} form after entry into the root cells and therefore needs to be reduced again before entering the leaf cells. The actual metalloreductase responsible for providing the Fe needed for photosynthesis has been a mystery for quite a while and the presence of the LREs in the *FRO6* promoter suggests that it may be responsible for providing the Fe needed for photosynthesis (Feng et al., 2008). Moreover, the increased ferric reductase activity and Fe content of *FRO6* overexpressing transgenic tobacco plants provide strong evidence that FRO6 is indeed the actual FRO responsible for providing the iron needed for transport across the leaf PM (Li et al, 2010). Here, I provide additional evidence to support this hypothesis through the analysis of *fro6* loss-of-function lines.

Table 2.1 Arabidopsis FRO Family Summary

	Expression Pattern	Localization	Function
FRO1	Unknown	Unknown	Unknown
FRO2	Roots	PM	Reduction of Fe at root soil interface
FRO3	Roots and shoots	Mitochondria	Postulated reduction of Fe for mitochondria
FRO4	Roots and shoots	PM	Reduction of Cu for high affinity uptake in the roots
FRO5	Roots and shoots	PM	Reduction of Cu for high affinity uptake in the roots
FRO6	Shoots	PM	Postulated reduction of Fe for transport across leaf PM
FRO7	Shoots	Chloroplast	Reduction of Fe for chloroplast
FRO8	Shoots		Postulated reduction of Fe for mitochondria

Materials and Methods

Arabidopsis lines

Wild type Arabidopsis (ecotype Columbia *gl-1*) was used as a control for all experiments. Two *FRO6* loss-of-function mutants *fro6-1* (SALK_085659) and *fro6-2* (*SALK_099597C),* were ordered from the SALK Institute Genomic Analysis Lab (SIGNAL). *fro6-1* has a T-DNA insertion in the 8th intron (see Figure 2.1A), while *fro6-2* has a T-DNA inserted in the 8th exon (see Figure 2.2A).

Genotyping

The *fro6-1* and *fro6-2* lines were backcrossed to the WT Col *gl-1* twice. To confirm that the *fro6-1* and *fro6-2* knockouts are homozygous, plants were genotyped using primers specific to *FRO6* along with primers specific to the T-DNA insertion. The primers used for genotyping are as follows: FRO6KOLP Forward, FRO6KO Reverse, and LBB1 (see Table 2.2). PCR was stopped after 35 cycles.

DNA Extraction

In order to extract DNA, a single leaf was selected from a single plant and ground in an eppendorf tube, using 400 µl of extraction buffer (200 mM Tris, pH 7.5, 250 mM NaCl, 25mM EDTA, and 0.5% SDS). Samples were centrifuged at 13,000 rpm for two minutes. Then, 300 µl of the supernatant was removed and mixed with 300 µl of isopropanol and the samples were centrifuged again at 13,000 rpm for 5 minutes.

Next, the supernatant was discarded and the pellet was washed with 70% ethanol and allowed to dry. The pellet was resuspended in 50 µl of water and used for genotyping assays (Klimyuk et al., 1993).

Transcript Analysis

To verify that the *fro6-1* and *fro6-2* mutants do not contain any FRO6 transcript, semi-quantitative reverse transcriptase-PCR (RT-PCR) was performed. The primers used are specific to the *FRO6* 3´UTR. It was necessary to design primers that correspond to the 3´UTR, because the sequence of *FRO6* is highly homologous to the *FRO7* gene and the greatest degree of variability between the two genes is in the 3´UTR. RNA was extracted from the shoots of wild type Arabidopsis seedlings grown for 14 days on Gamborg's B5 medium (Sigma-Aldrich, St. Louis). cDNA was synthesized from this RNA following the manufacturer's protocol (Superscript First-Strand Synthesis from PCR Kit, Life technologies/Gibro-BRL). Actin cDNA was used as a control when doing semi-quantitative RT-PCR. In RT-PCR, the polymerase chain reaction (PCR) was stopped at particular intervals, the sample was then collected, and PCR was resumed, after each pause. In addition to the WT control, a No RT control was included, to ensure that there was no genomic DNA present in the sample. Moreover, a No cDNA control was used to ensure the absence of contamination of the reagents used to prepare the PCR samples. For transcript analysis of *FRO6*, samples were stopped at the 29th, 32th, and 35^{th} cycles. For actin transcript analysis, the PCR reaction was stopped at the 23^{th}, 26^{th}, and 29^{th} cycles.

Leaf Disc Ferric Reductase Activity

Leaf discs (de la Guardia, 1996) from 3-4 week old seedlings (grown on soil)were submerged in assay solution and were kept in light. Leaves of the same age were selected from 8 different plants. Preparation for leaf discs required the use of the "Taped-Arabidopsis Sandwhich" method in order to peel off the leaf epidermis to expose the PM (Wu et al., 2009). A hole punch was used to cut out the leaf discs, while the leaves are still attached to the tape. Then, the leaves were completely submerged in 300 ul of assay solution. The assay solution used contains 0.1mM Fe(III)-EDTA with 0.3mM Ferrozine. Absorbance was then measured at 562 nm at 5 minutes, 15 minutes, 30 minutes 45 minutes and 60 minutes. Absorbance data was normalized to leaf disc weight. A student's t-Test was conducted to determine statistical significance.

Root Ferric Reductase Activity

Ferric reductase assays were conducted on roots of plants grown on Gamborg's B5 medium (Sigma-Aldrich, St. Louis) for 12-14 days, then transferred to iron sufficient (50µM Fe(III)-EDTA) or iron deficient (300 µM Ferrozine) medium for an additional three days. Roots were submerged in the assay solution containing 0.1mM Fe(III)-EDTA and 0.3mM Ferrozine. Then the plants were placed in the dark and the absorbance was then measured at 562 nm at 0, 20, 40, 60 minutes). The absorbance data was normalized to fresh root weight (Yi and Guerinot, 1996. Ten replicates of each line were used. A student's t-Test was conducted to determine statistical significance.

Flowering time

Flowering time was measured for *fro6-1* and WT Col *g1-1* plants, as a control. Plants were grown in long-day, short-day, constant light, and high light. Flowering time

was measured by counting the total number of leaves when the initial flowering bud appeared (cotyledons were not included). 10 replicates for each line were used. A student's t-Test was conducted to determine statistical significance (Clarke et al., 1995).

Root Growth Assay

A root growth assay was used to measure the effects of iron deficiency on the growth of roots of *fro6-1* mutants. Plants were grown for 5 days on Gamborg's B5 medium (Sigma-Aldrich, St.Louis) supplemented with 2% sucrose, 1mM MES, and 0.6% agar, pH 5.8 (Mukherjee, et al., 2006). Then the 5 day old seedlings were transferred to either iron sufficient (50 µm Fe (III)- EDTA) or iron deficient (300 µM Ferrozine) plates (Yi and Guerinot, 1996). The plates were positioned vertically in the growth chamber. Col *g1-1* plants were used as controls. Measurements were taken every day for six days (Connolly et al., 2002). 12 replicates of each line were used. A student's t-Test was conducted to determine statistical significance.

Chlorophyll Content

Plants were grown on Gamborg's B5 medium (Sigma-Aldrich, St.Louis) for 14 days, and then transferred to either iron sufficient (50µM Fe (III-EDTA) or iron deficient (300 µM Ferrozine) medium for an additional three days. Col *g1-1* plants were used as controls. Chlorophyll content was measured using a protocol adapted from Arnon (1949), in which .1 g of tissue was ground in an eppendorf in 1 ml of 85 % acetone. The homogenate was spun down and the supernatant absorbance was read at 663 and 644 nm. Chlorophyll content was calculated as previously described (Arnon, 1949). Eight replicates of each line were used. A student's t-Test was conducted to determine statistical significance.

Table 2.2 Primer Sequences Primer sequences used for RT-PCR and PCR

PRIMER	SEQUENCE 5'-3'
FRO6KOFORWARD	5'-TTCCGAAAATATATGGGCAGC-3'
FRO6KOREVERSE	5'-CGAGCCTCGCTTGGTACGTC-3'
LBB1	5'-GCGTGGACCGCTTGCTGCAACT-3'
FRO6RTF-3	5'-TGGAAACAGCTATGGTTGATATG-3'
FRO6RTR-3	5'-TGTCCAATGTAGAAACACCAACA-3'
FRO6RTF(HINDIII)	5'-CTTATGGCCATGAATCTCCATA-3'
FRO6RTR(HINDIII)	5'-AGCTTGACAGAAGGATGCAC-3'

RESULTS

Previous work has shown that *FRO6* is highly expressed in all green aerial portions of plants (Mukherjee et al., 2006; Wu et al., 2005; Feng, et al., 2006; Li, et al., 2010) and is localized to the PM (Jeong et al., 2008). In addition, it has been shown that *FRO6* encodes a protein that displays ferric reductase activity using a yeast system (Wu et al., 2005; Jeong et al., 2008). This data has led us to hypothesize that FRO6 is ferric chelate reductase involved in Fe homeostasis, specifically providing reduced Fe for transport across the leaf plasma membrane that is needed for important biological processes such as photosynthesis.

Mutants (*fro6-1* that contains a T-DNA insertion in the 8th intron) (see Figure 2.1A) and (*fro6-2* that contains a T-DNA insertion in the 8th exon) (see Figure 2.2A) of the Arabidopsis *FRO6* gene were obtained from the SALK Institute Genomic Analysis Laboratory collection. The inserts were confirmed using sequence analysis at the University of South Carolina. PCR genotyping was used to identify homozygous mutant plants (see Figure 2.1B and Figure 2.2B).

However, semi-quantitative RT-PCR transcript analysis showed that these mutants express transcript comparable to WT (Col *gl-1*) (see Figure 2.3 B). A No RT control was used to check for the presence of genomic DNA and a no cDNA control was used as well to check for the presence of DNA contamination in the PCR reagents.

In the No RT control there was a band present, indicating some genomic contamination, but this band was much fainter than the others. Next, we checked the

FRO6RTF-3′ and FRO6RTR-3′ primer specificity, because FRO6 and FRO7 share approximately 92% sequence similarity. Of the 23 bases in the FRO6RTF-3′ primer, 15 bases match the corresponding *FRO7* sequence and of the 23 bases in FRO6RTR-3′ primer, 14 bases match the corresponding *FRO7* sequence. FRO6RTF-3′ and FRO6RTR-3′ were used with *FRO7* cDNA and showed that these primers are able to amplify *FRO7* under the PCR conditions used.

Next, we designed primers FRO6RTF (HINDIII) and FRO6RTR (HINDIII) for semi-quantitative RT-PCR to amplify a region of the cDNA that contains a HindIII site that is specific to *FRO6*. Because *FRO6* and *FRO7* share such high sequence similarity, it was important to cut at a site that is present in *FRO6* but not *FRO7*, so that after digestion with HindIII, the actual identity of the transcript can be verified (see Figure 2.4 A). The RT-PCR product was digested with the restriction enzyme HindIII. Two bands of sizes 100 bp and 200 bp correspond to the digested *FRO6* transcript and the undigested 300 bp band corresponds to the *FRO7* transcript (see Figure 2.4 B). When the amplified product was digested with HindIII, it was found that the product corresponded to both *FRO6* and *FRO7*. This result confirmed that the primers amplified both *FRO6* and *FRO7* and also confirmed that the *fro6-1* and *fro6-2* lines express *FRO6* transcript.

In order to determine the function of FRO6 in the leaves, we conducted a ferric chelate reductase assay. There was a significantly less leaf ferric chelate reductase activity in *fro6-1* mutants (see Figure 2.5). Additionally, there was a significantly less leaf ferric chelate reductase activity in *fro6-2* mutants as well (see Figure 2.6). In contrast, root ferric chelate reductase was not affected by loss of *FRO6* (see Figure 2.7).

Since other *FRO* mutant lines display altered flowering time, flowering time of *fro6-1* was measured. *fro3* was used as a negative control, because it has been shown to have a delayed flowering time compared to WT. The *fro6*-1 mutants show a significantly faster flowering time than both WT and *fro3* mutants when grown in high light conditions (see Figure 2.8).

Root growth is used as a typical marker for a plant's functions under conditions of metal stress. Under both Fe sufficient and Fe deficient conditions, there was no significant difference in the root length of the *fro6-1* mutants versus WT (see Figure 2.9). Chlorophyll content is often affected by alterations in Fe homeostasis as Fe is required for chlorophyll synthesis. Additionally, chlorophyll requires Fe to function, so measurement of chlorophyll may indicate whether there is proper reduction and transport and delivery of Fe to the photosynthetic apparatus. Mutants of genes involved in Fe homeostasis have been shown to have compromised chlorophyll content or a chlorotic phenotype (including *fro2, fit, irt1,* and *fro7*). There was no significant difference in chlorophyll content of between WT and *fro6-1*or *fro6-2* mutants (see Figure 2.10).

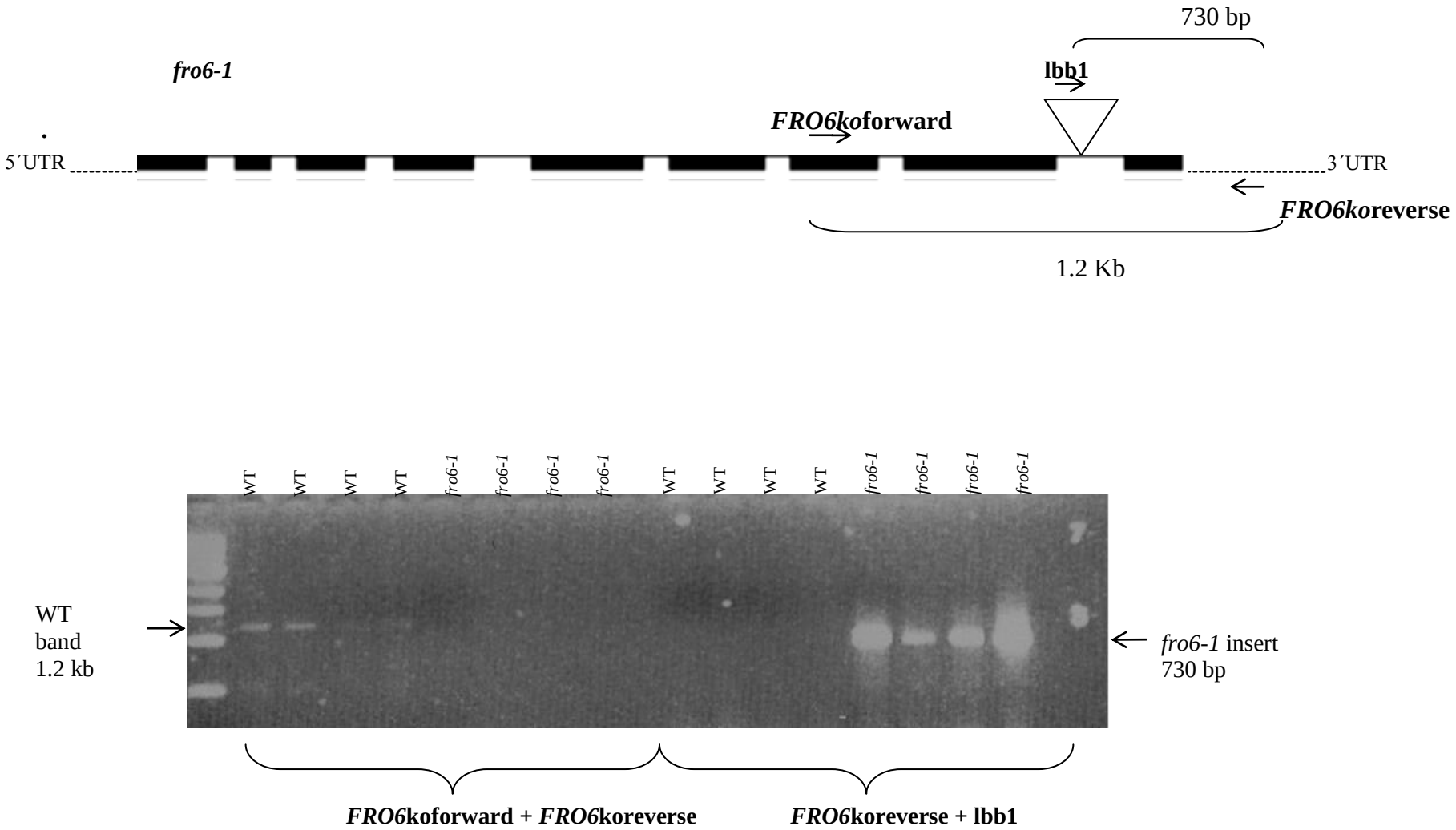

Figure 2.1: Genotyping of *fro6-1* mutants
A. Schematic of *FRO6* gene and T-DNA insertion (triangle) as well as the locations of gene specific primers *FRO6*KOforward and *FRO6*KOreverse, and T-DNA specific primer lbb1 (adapted from Arabidopsis.org) (black boxes = exons, spaces = introns) (adapted from Arabidopsis.org) (black boxes = exons)
B. Agarose gel of PCR reaction using gene specific primers *fro6KO* forward and *fro6KO* reverse and T-DNA specific primer LBB1 and gene specific primer *fro6KO* reverse.

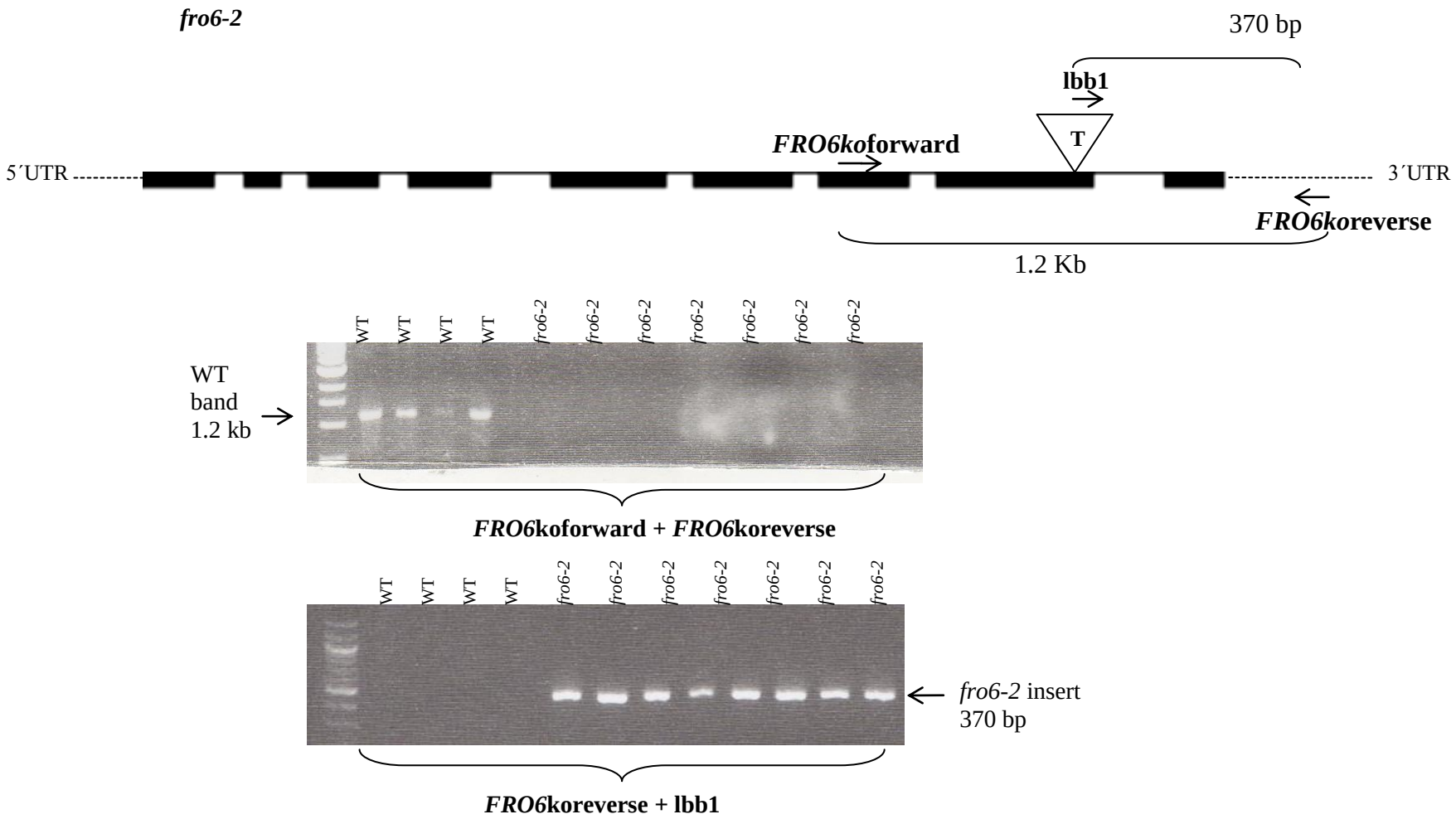

Figure 2.2: Genotyping of *fro6-2* mutants

A. Schematic of *FRO6* gene and T-DNA insertion (triangle) as well as the locations of gene specific primers *FRO6*KOforward and *FRO6*KOreverse, and T-DNA specific primer lbb1 (adapted from Arabidopsis.org) (black boxes = exons, spaces = introns) (adapted from Arabidopsis.org) (black boxes = exons)

B. Agarose gel of PCR reaction using gene specific primers *fro6KO* forward and *fro6KO* reverse and T-DNA specific primer LBB1 and gene

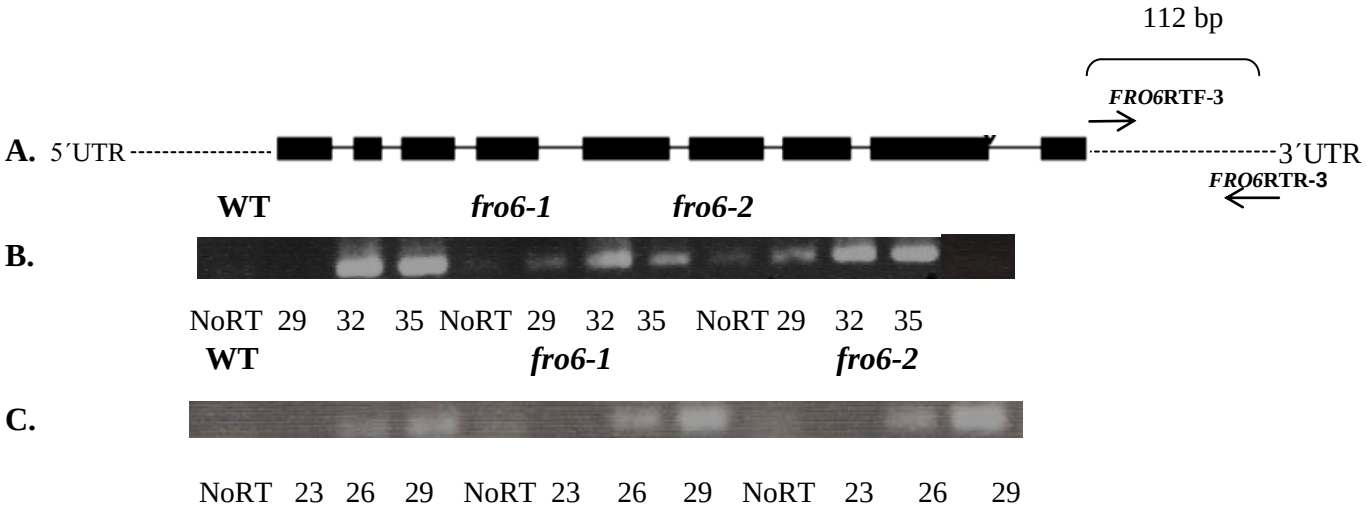

Figure 2.3: Transcript Analysis

A. Schematic Drawing of FRO6 gene (black boxes = exons) (adapted from Arabidopsis.org).
B. RT-PCR transcript analysis of FRO6 transcript in Col *gl-1* and *fro6-1* mutant. Cycles 29, 32, 35for FRO6 transcript
C. RT-PCR transcript analysis of Actin transcript in Col *gl-1* and *fro6-1* mutant. Cycles 23, 26, 29 for actin transcript

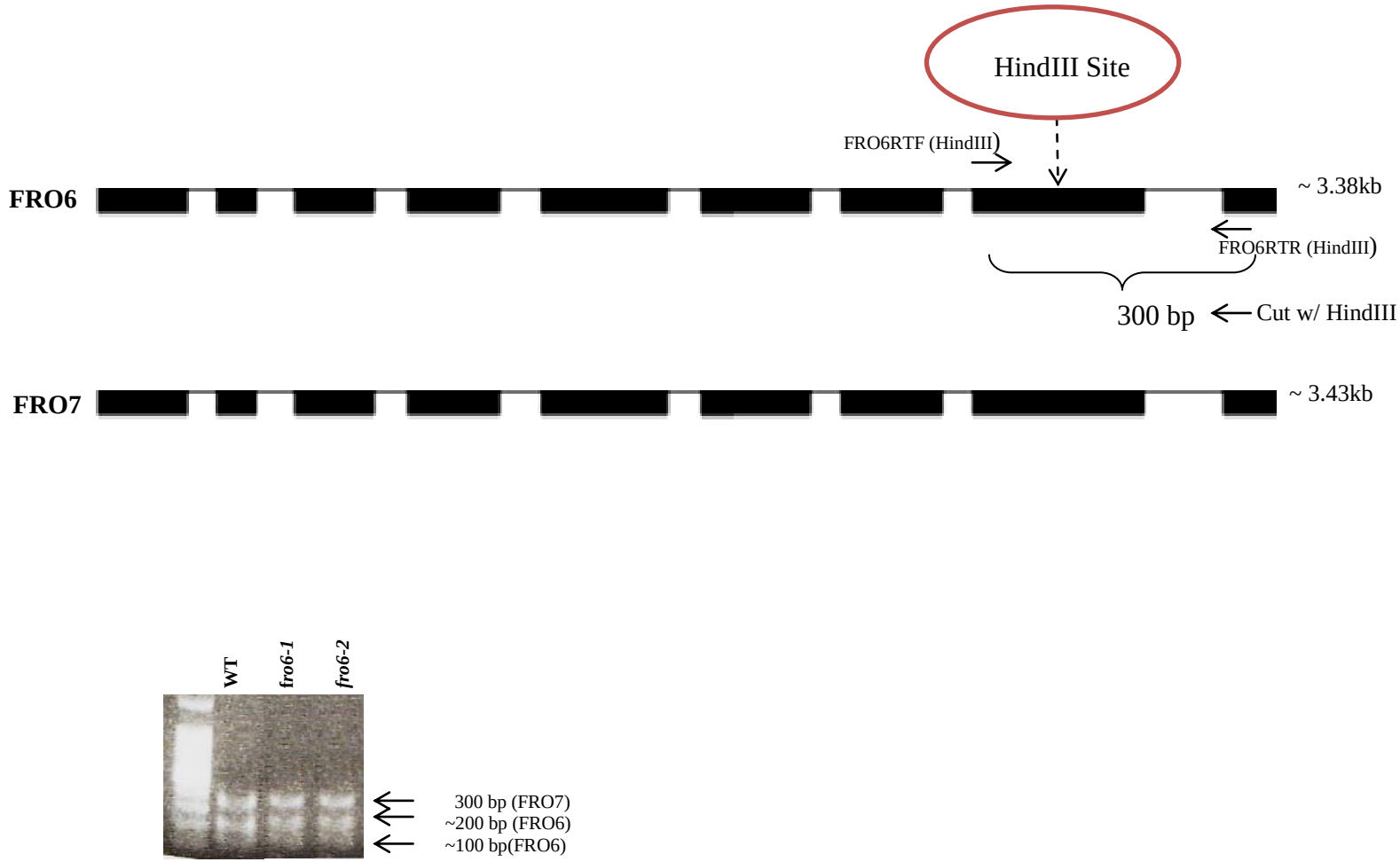

Figure 2.4: Digestion of RT-PCR Product with HindIII
A. Schematic of FRO6RTF (HINDIII) and FRO6RTR (HINDIII) flanking the HindIII site and the predicted band size that FRO6RTF (HindIII) and FRO6RTR (HindIII) amplify of FRO6. (adapated from Arabidopsis.org). (black boxes = exons, spaces between boxes= introns)
B. Digestion of Col-*gl1* and *fro6-1* mutant RT-PCR transcript using HindIII

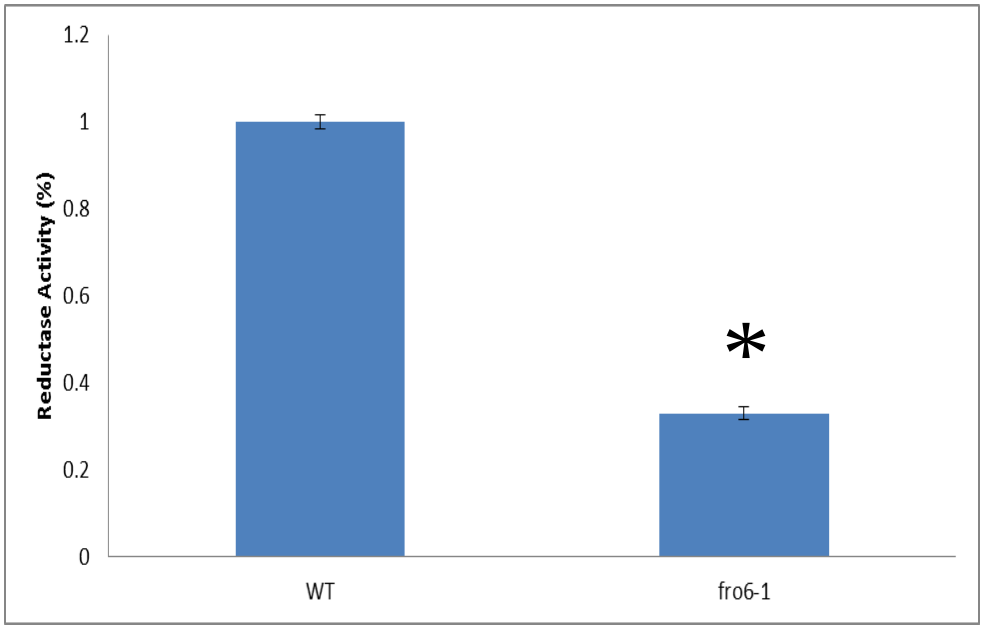

Figure 2.5: Leaf Disks Ferric Reductase Activity of *fro6-1*. Plants were grown on B5 medium for 2 weeks. Then transferred to soil for 2 weeks. Leaf Discs were prepared using the "Tape Sandwich" method and a hole punch. Leaf discs were then submerged in FCR assay solution and the absorbance at 562 nm was measured at 0, 20, 40, 60 minutes. Data was normalized by leaf disc weight. An asterisk indicates that there was a significant difference using a student's t-Test for statistical analysis.

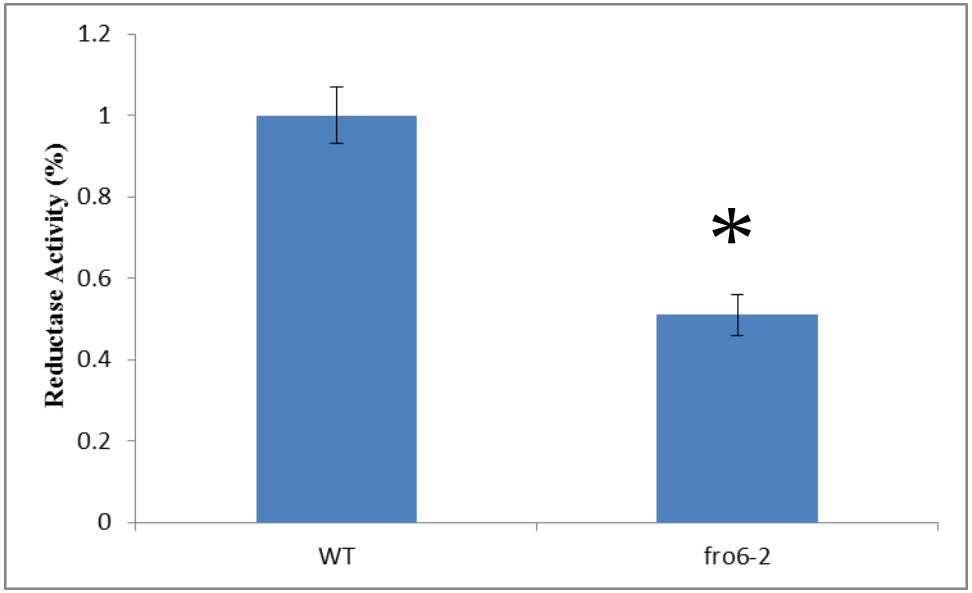

Figure 2.6: Leaf Disks Ferric Reductase Activity of *fro6-2*. Plants were grown on B5 medium for 2 weeks and then transferred to soil for 2 weeks. Leaf Discs were prepared using the "Tape Sandwich" method and a hole punch. Leaf discs were then submerged in FCR assay solution and the absorbance at 562 nm was measured at 0, 20, 40, 60 minutes. Data was normalized by leaf disc weight. An asterisk indicates that there was a significant difference using a student's t-Test for statistical analysis.

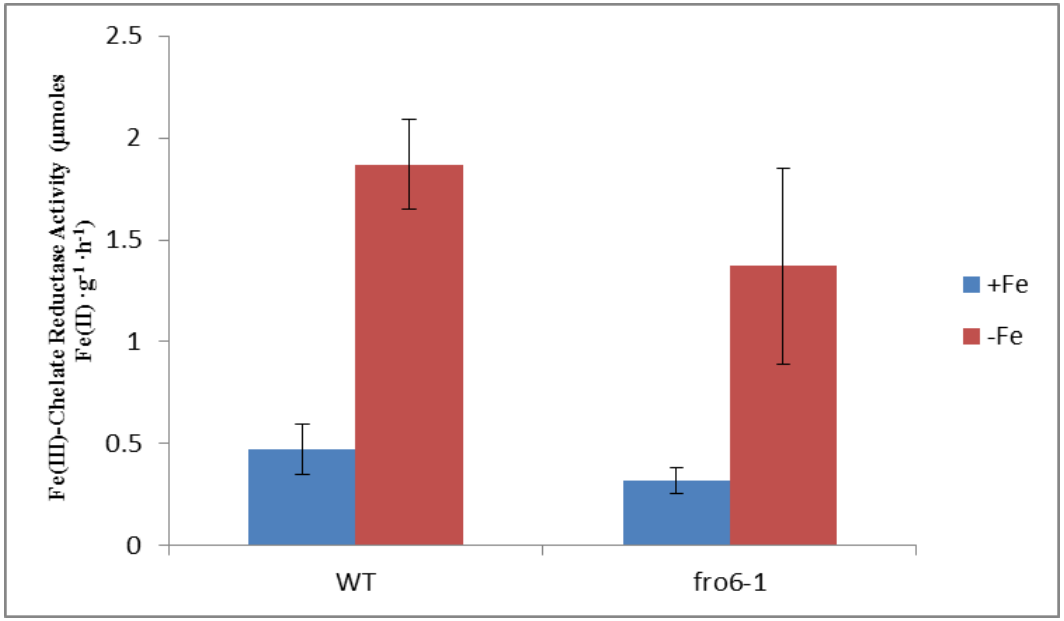

Figure 2.7: Root Ferric Reductase Activity of Col *gl-1* and *fro6-1* grown on B5 medium for two weeks then transferred to Fe deficient and Fe sufficient medium. Bars represent standard error. Roots were then submerged in FCR assay solution and the absorbance at 562 nm was measured at 0, 20, 40, 60 minutes. Data was normalized by leaf disc weight.

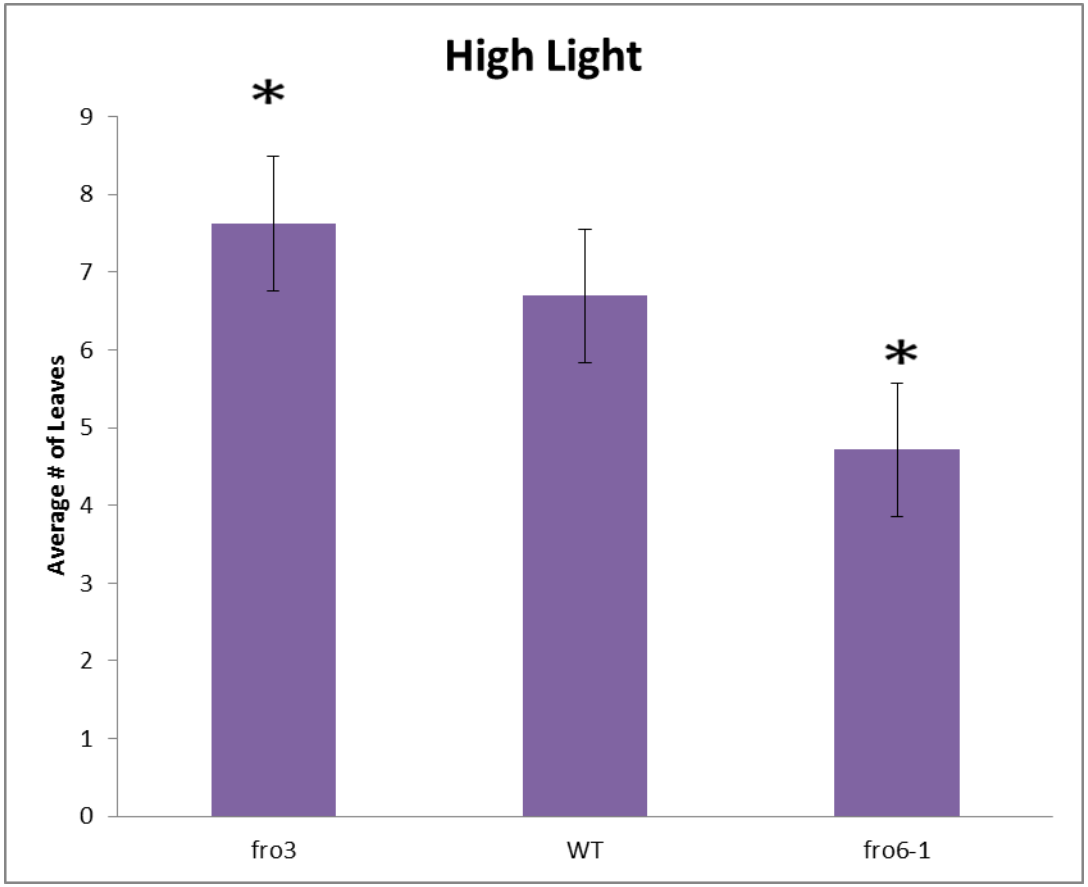

Figure 2.8: Flowering Time. Average number of leaves for each plant at time of bolting. Seeds were grown on soil and leaves were counted at flowering. WT and *fro3* mutants used as controls. Bars represent standard error. An asterisk indicates that there was a significant difference using a student's t-Test for statistical significance.

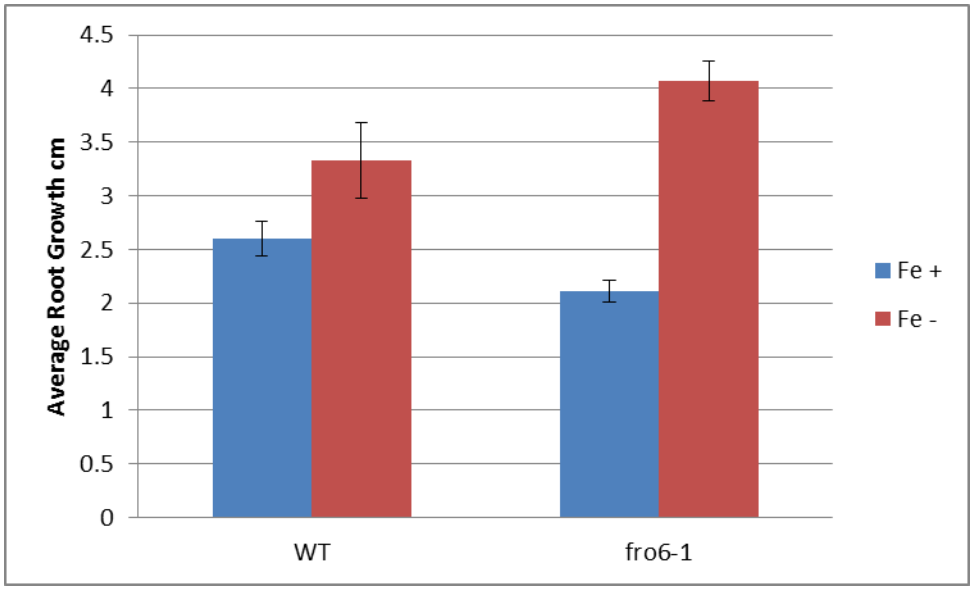

Figure 2.9: Average growth rate of roots grown on +/- Fe medium. Plants were grown on iron sufficient or iron deficient medium and measurements were taken every day for six days and averaged. Bars represent standard error.

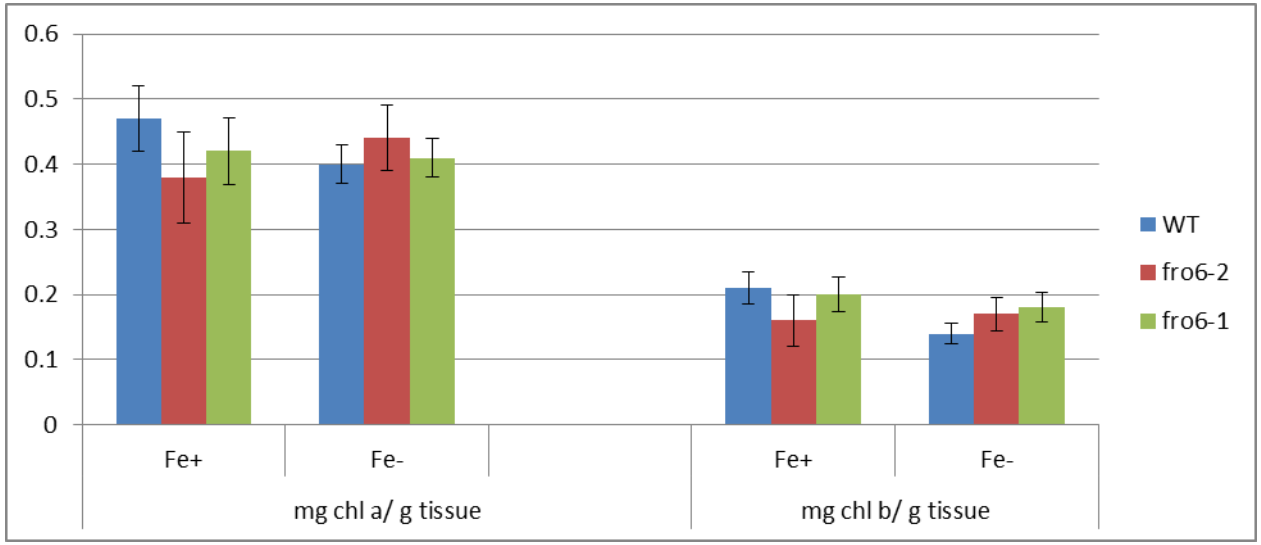

Figure 2.10: A. Chlorophyll Content of Col *gl-1* and *fro6-1* grown on B5 medium for two weeks then transferred to Fe deficient and Fe sufficient medium. Bars represent standard error. An asterisk indicates that there was a significant difference using a student's t-Test for statistical significance.

Discussion

At this time, much is known about the localization and expression pattern of FRO6, but not much is known about the role it may play in iron uptake (Mukherjee et al., 2006; Feng et al., 2006; Jeong et al., 2008). In protoplasts, FRO6 has been shown to localize to the plasma membrane (Jeong et al., 2008). It is regulated in a light-dependent manner, with its promoter containing many light responsive elements (LREs), such as the I-box, GT1, and GATA motifs (Feng et al., 2006). While overexpression of *AtFRO6* in transgenic tobacco plants resulted in a higher rate of ferric chelate reductase activity of leaves grown under iron sufficient and deficient conditions, there was no difference in ferric chelate reductase (FCR) activity of the roots of the transgenic plants compared to WT. Additionally, overexpression of *FRO6* in tobacco plants, resulted in elevated Fe and chlorophyll content (Li et al., 2010). Thus we hypothesized that *FRO6* is responsible for reducing apoplastic iron for transport across the plasma membrane in leaf cells.

The results here indicate that FRO6 is involved in reduction of iron at the leaf PM. Ferric reductase activity is significantly reduced in the *fro6-1* and *fro6-2* mutants as compared to WT, suggesting that FRO6 functions in reduction of iron at the leaf PM (see Figure 2.4 and 2.5). However, despite the fact that the *fro6-1 and fro6-2* mutants are homozygous (see Figures 2.1B and 2.2B), the lines show detectable *FRO6* transcript (see Figure 2.3). It is possible that the transcript that is present in the *fro6-1* and *fro6-2* mutants is aberrant in some way and does not encode a functional protein.

Our results indicate that there is no significant difference between the root ferric chelate reductase activity between *fro6-1* and WT seedlings (see Figure 2.7). This is not surprising, because FRO6 is localized at the PM of leaf cells and is not expressed at all in the root PM. FRO2 is still present and should be able to continue effectively providing the iron that is needed for the whole plant.

We also observed that *fro6*-1 mutants display faster flowering, in high light (see Figure 2.8). *fro3* plants were used as control because they have been shown to flower slower than WT (Mukherjee, unpublished data). The faster flowering phenotype only occurred under high light conditions when plants are under photo-oxidative stress. It is possible that increased apoplastic ferric iron may be participating in the Fenton reaction to produce toxic hydroxyl radicals. It has been shown that under extreme light conditions, plants cannot absorb the excess light during photosynthesis, which leads to a higher rate of ROS production (Golan, et al., 2006).

Lastly, we did not observe a significant difference in the chlorophyll content of either *fro6-1* and *fro6-2* mutants and the WT plants (see Figure 2.10). Many proteins involved in photosynthesis require iron, including chlorophyll, cytochrome oxidase complex, heme, ferrodoxin, and iron-sulfur proteins (Li et al., 2010; Bang et al., 2008). It is possible that the plants maintain chlorophyll synthesis when Fe content is lower, but that some other Fe-containing proteins suffer. Thus, FRO6 may be important for providing Fe only for a certain subset of Fe proteins. This data along with the residual ferric reductase activity in leaf disks suggests that there is another pathway that acts redundantly with FRO6 to provide the iron needed for photosynthesis. There are a number of cytochromes in Arabidopsis that might act redundantly in leaf apoplastic iron

reduction. For example, the AtRBOHs are closely related to the FRO family or another FRO might act redundantly with FRO6 to reduce iron at the PM. The best candidate is FRO5, because it is expressed in the roots and shoots and it is localized to the PM (Wu et al., 2005; Mukherjee et al., 2006).

Recently, FRO6 was shown to be 6.2 fold down regulated in the *chl27-t* mutant (Bang et al., 2008). The CHL27 protein is the membrane bound subunit of the aerobic cyclase in the chlorophyll biosynthesis pathway. It contains a "consensus D/EExxH motif specific to carboxylate-liganded di-iron-binding enzymes"(Bang et al., 2008) and so it is possible that FRO6 is providing the iron needed for this reaction (Moseley et al. 2000; Tottey et al. 2003; Bang et al, 2008). But there was no difference in chlorophyll content of *fro6*-1 mutants compared to WT, which suggests that FRO6 is not responsible for providing the Fe needed for CHL27 synthesis of chlorophyll.

Future directions

In the future, we plan to develop a FRO6 antibody to check FRO6 protein levels. Additionally, for full characterization of FRO6 function, we can complement the *fro6-1* and *fro6-2* mutants with a copy of the *FRO6* gene under the control of the endogenous promoter to check if it rescues the mutant phenotype. Although the *fro6* mutants showed significantly reduced FCR, there is still some activity observed and this residual activity may be due to the activity of FRO3, FRO7 and FRO8, because creation of leaf discs exposes some of the plant's cytoplasm to the assay solution. The residual activity also could indicate that FRO6 acts redundantly with another FRO. The most likely candidate is FRO5, because both FRO6 and FRO5 are localized to the PM and expressed in the shoots. In the Connolly lab, there are presently *fro5fro6* mutants. Since FRO6 is thought

to provide iron for proteins of the photosynthetic apparatus, Blue Native Gel electrophoresis can be done to determine if components of the photosynthetic apparatus, such as PSI (Photosystem I), PSII (Photosystem II), and/or LHCI (Light Harvesting Complex I), are compromised in any way, because they are all large sinks for Fe (Jeong et al., 2008).

In this thesis, I have presented one project that focuses on the reduction of Fe in leaves and characterized a mutant of FRO6, in order to determine its function in maintaining iron homeostasis. Our data indicates that AtFRO6 is responsible for reducing the apoplastic Fe that is needed for the transport across the leaf PM. This study enables a more thorough understanding of how plants maintain Fe equilibrium and thus will aide in the production of iron enriched plants to combat one of the leading issues facing the world today, iron deficiency anemia.

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