FUNCTIONAL CHARACTERIZATION OF FRO4 AND FRO5: TWO MEMBERS OF THE FERRIC CHELATE REDUCTASE FAMILY OF ARABIDOPSIS

by

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DEDICATION

This manuscript is dedicated to two people who have helped me in more ways than imaginable: Rod Kinard and the late Ed Morris. They were always there during the highs, the frustrations, and the tears to offer jokes, words of encouragement, and comfort.

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ABSTRACT

Iron (Fe) is the fourth most abundant element within the earth's crust and is an essential micronutrient for plants and animals. Fe plays key roles in photosynthesis, respiration and chlorophyll biosynthesis in plants and in hemoglobin in animals. Like Fe, copper (Cu) is also an important micronutrient in plants and is needed for photosynthesis and respiration, especially in the important copper-containing protein plastocyanin. Copper also is important in scavenging reactive oxygen species and ethylene perception. The reduction of Fe^{3+} to Fe^{2+} at the root surface of *Arabidopsis thaliana* during times of Fe deficiency has been a well-characterized process; however, reduction of Cu^{2+} to Cu^{1+} at the root surface is less well understood. It is known that a member of the FRO family of Arabidopsis genes, *FRO2*, functions to reduce Fe^{3+} to Fe^{2+} prior to import, but a role for copper reduction in Cu uptake in response to Cu deficiency was not previously known. The work presented in this thesis describes the characterization of two additional members of the FRO family, *FRO4* and *FRO5*, that have been shown to have high amino acid sequence similarity. FRO4 and FRO5 function in the reduction of Cu^{2+} to Cu^{1+} at the root surface. For the characterization of these two genes, we isolated a T-DNA knock-out line of FRO4, *fro4*, which lacks full-length *FRO4* transcript. In addition, we generated and characterized artificial microRNA knockdown lines for FRO5 and for both FRO4 and FRO5 (double knockdown line). Under copper deficiency, *FRO4* and *FRO5* are highly expressed in root and shoot tissue. Loss-of-function mutants show only basal levels of reductase activity under Cu deficiency and grow poorly on Cu deficient

hydroponic media compared to their wild-type counterparts. Taken together, these data support the hypothesis that FRO4 and FRO5 are the principle copper reductases during Cu deficiency in Arabidopsis and function redundantly to reduce Cu^{2+} to Cu^{1+} as part of the high affinity Cu uptake system.

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List of Abbreviations

AHA2 ..... Arabidopsis H+-ATPase2

ATX1 ..... Antioxidant1

bHLH ..... basic Helix-Loop-Helix

CRR1 ..... Copper response regulator1

CCH ..... Copper Chaperone

CCS ..... Copper chaperone for superoxide dismustase

COPT/CTR1 ..... Copper transporter

CuRE ..... Copper Response Element

EIN3 ..... ETHYELENE INSENSITIVE3

EIL1 ..... ETHYELENE INSENSITIVE3-LIKE1

FIT ..... Fer-like iron deficiency inducted transcription factor

FRE ..... Ferric reductase

FRO ..... Ferric reductase oxidase

HMA ..... Heavy Metal ATPase

NA ..... Nicotianamine

NO ..... Nitric Oxide

PAA ..... P-type ATPase of Arabidopsis

PSII ..... Photosystem II

ZIP ..... ZRT/IRT-like Protein

CHAPTER 1

IRON AND COPPER HOMEOSTASIS IN *ARABIDOPSIS THALIANA*

Importance of Iron:

Iron (Fe) is an essential micronutrient required by plants and is used in many cellular processes including photosynthesis, respiration, and nitrogen fixation (Briat and Lobreaux, 1997). Iron is required in chloroplasts where it is used by the photosynthetic complexes, is required for chlorophyll biosynthesis, and is essential for the production of Fe-S clusters (Kobayashi and Nishizawa, 2012). Iron metabolism in plants is an important area of study because iron deficiency anemia is one of the most common human nutritional deficiencies worldwide (http://www.who.int/home-page). In addition, most people acquire their iron from plant sources, so there is significant interest in understanding the molecular basis of iron uptake and accumulation in plants.

Despite the significant iron requirement of plants, it is important to note that too much iron is toxic to plants, leading to the generation of reactive oxygen species (Halliwell and Gutteridge, 1992). More importantly, even though iron has a high abundance in the earth's crust, it is generally present as insoluble oxyhydroxide ferric iron complexes at neutral or basic pH (Grotz and Guerinot, 2006). Approximately 30% of soils worldwide are considered iron deficient and plants grown under conditions of low iron availability tend to have chlorosis of the leaves (Briat and Lobraeux, 1997) and

suffer from reduced crop yield (Guerinot and Yi, 1994). Despite the low bioavailability of iron in soil, plants manage to utilize two strategies to combat iron deficiency and take up the nutrient (Guerinot and Yi, 1994).

Uptake and Transport of Iron:

Plants can be classified as using either Strategy I or Strategy II for iron acquisition. Strategy I plants are all non-grasses, including *Arabidopsis thaliana*, while Strategy II plants are graminaceous monocots (Guerinot and Yi, 1994). When under iron deficient conditions, Strategy I plants engage in a three-step process to take up iron. At the root surface, protons are pumped out into the rhizosphere via an ATPase, likely thought to be AHA2 (Santi and Schmidt, 2009), a member of the *Arabidopsis* H^+-ATPase gene family. This serves to lower the pH of the surrounding rhizosphere and increase iron solubility. Ferric (Fe(III)) iron chelates are then reduced to ferrous (Fe(II)) iron by a plasma membrane ferric chelate reductase; FRO2 (*Ferric Reductase Oxidase 2*) is known to function as the ferric reductase that reduces iron at the root surface (Yi and Guerinot, 1996; Robinson et al, 1999). Finally, Fe^{2+} is transported into the cell across the plasma membrane via IRT1 (*Iron-Regulated Transporter 1*; Eide et al., 1996; Vert et al., 2002).

Strategy II plants respond to iron deficiency by synthesizing phytosiderophores (PS) and then releasing them from the roots into the surrounding rhizosphere; PSs, such as mugineic acid, bind Fe(III) with high affinity (Walker and Connolly, 2008). These Fe(III)-PS complexes are then transported across the root membrane via the YS1 (*Yellow Stripe1)* iron transporter (Curie et al., 2001).

Iron Homeostasis:

Iron uptake and trafficking need to be tightly regulated. Iron is associated with citrate as it is transported through the xylem from the roots to the shoots and with nicotianamine in phloem (Briat et al., 2007). Having these iron complexes helps prevent free iron from causing damage via the generation of hydroxyl radicals and keeps iron from precipitating. In addition, ferritins are present in plastids and mitochondria and serve as iron storage units; data also show that ferritins play a role in protecting a plant against oxidative stress during growth and development (Ravet et al, 2009). It has also been shown that iron can be stored within the vacuole (Kim et al., 2006).

Not much is known about Fe sensing and signaling. However, the expression of *FRO2* and *IRT1* is regulated transcriptionally by the transcription factor, FIT (Colangelo and Guerinot, 2004). Two bHLH transcript factors, AtbHLH38 and AtbHLH39, have been shown to form heterodimers with FIT to control the transcriptional activation of *FRO2* and *IRT1* (Yuan et al., 2008). The recently discovered bHLH transcription factor POPEYE (PYE) also plays a role in roots during iron starvation, and has been shown to function in the stele to regulate the expression of multiple genes involved in Fe homeostasis (Long et al., 2010).

In addition to transcriptional regulation of the iron deficiency response, IRT1 has been shown to be post-translationally regulated (Connolly et al., 2003; Kerkeb et al, 2008; Barberon et al, 2011; Shin et al, 2013). Recently, it was discovered that proper turnover of FIT is required for optimal expression of *IRT1* and *FRO2* during Fe-deficiency. This involves the process of constant degradation of "exhausted" FIT by ubiquitination and proteosomal degradation of the transcription factor on the promoters

of *IRT1* and *FRO2* that is then replaced by newly synthesized FIT protein (Sivitz et al, 2011).

Hormones also play roles in the regulation of plant responses to Fe availability (Hindt and Guerinot, 2012). Two ethylene transcription factors, EIN3 and EIL1, are important in the ethylene pathway; it is theorized that these proteins bind directly to FIT, strengthening the Fe-deficiency response in Arabidopsis (Lingam et al, 2011). Double mutants of *ein3 eil1* show reduced FIT protein abundance and lower expression of *FRO2* and *IRT1* (Lingam et al, 2011). Two additional positive hormone regulators of Fe-deficiency in Arabidopsis are NO and auxin. Auxin is a regulator of plant growth, and under Fe-deficiency, Fe can work to increase lateral root growth through the auxin transporter, AUX1. This serves to increase the surface area of roots to allow for more uptake of Fe from the environment (Giehl et al, 2012). The signaling molecule NO is produced in response to Fe deficiency and is important for stabilization of FIT (Graziano and Lamattina, 2007; Meiser et al, 2011) Hormones that negatively regulate the Fe-deficiency response are cytokinin and jasmonate. When exogenous cytokinin is added to plants, *FIT*, *FRO2*, and *IRT1* expression is down-regulated (Seguela et al, 2008). The effect of exogenous jasmonate is the same (Maurer et al, 2011).

IRT1 and the FRO family:

In Strategy I plants, IRT1 is the major transporter of iron, and other divalent cations such as zinc, manganese, cobalt and cadmium from the soil (Eide et al, 1996; Vert et al, 2002). IRT1 belongs to the ZIP family of metal transporters (Kim and Guerinot, 2007). It is an essential gene as plants that lack IRT1 are inhibited in their ability to take up iron and die early unless they are watered with high levels of a soluble form of iron

(Vert et. al., 2002). During times of iron deficiency, expression of *IRT1* at the root plasma membrane is greatly increased. Peak levels in expression of *IRT1* mRNA occur 3 days after detection of iron deficiency and coincide with noticeable levels of the IRT1 protein (Connolly et. al., 2002). Previous studies have shown that when two-week-old *Arabidopsis* seedlings are placed on media deficient in iron for three days and then transferred to plates with media sufficient in iron, *IRT1* mRNA and protein begin to disappear, and are undetectable after 12 hours. This rapid turnover is thought to help prevent the uptake of too much iron (Connolly et al, 2002).

Like *IRT1*, transcription of *FRO2* is up-regulated during times of iron deficiency, with *FRO2* mRNA levels reaching a peak at three days after the transfer to Fe deficient conditions (Connolly et al., 2003). FRO2 functions to reduce Fe(III)-chelates to Fe(II), which can then be transported across the root plasma membrane. This step of the iron uptake process is thought to be rate limiting (Grusak et al., 1990; Connolly et al., 2003). Mutants deficient in FRO2, known as *ferric reductase defective 1(frd1)*, display enhanced chlorosis (as compared to WT) when iron is limiting, showing just how important FRO2 is to the Strategy I pathway. FRO2 was identified due to its sequence similarity to yeast *FRE1*, and to a subunit of gp91phox (the human respiratory burst NADPH oxidase; Robinson et al, 1999). Fe(III) reductase genes have been characterized in tomato and pea and are expressed in similar locations as FRO2 (Li et al., 2002; Waters et al., 2002; Kim and Guerinot, 2007).

The *Arabidopsis FRO* gene family contains 8 members, named *FRO1-8* (Mukherjee, et al, 2006). A subset of the *FRO* genes are induced in response to Fe deficiency and studies have shown that the FROs are expressed in different locations

within the plant (Wu et al, 2005; Muhkjeree et al, 2006). FRO2 localizes to the root plasma membrane and, as mentioned above, is the major enzyme involved in iron reduction at the root-soil interface during iron deficiency (Robinson et al., 1999). FRO3 and FRO8 are believed to localize to the mitochondria and may have a role in the reduction of iron in that organelle (Jain and Connolly, 2013), while FRO7 has been shown to localize to the chloroplasts and to function in supplying iron to the organelle (Jeong, et al., 2008). FRO4 and FRO5 are targeted to the secretory pathway, and preliminary data show that FRO5 localizes to the plasma membrane (Jeong and Connolly, 2009). Predictions suggest that FRO4 may also localize to the plasma membrane.

Importance of Copper:

Copper (Cu), like Fe, is also an essential micronutrient in plants. Copper is an important cofactor of proteins important for photosynthesis and respiration, scavenging reactive oxygen species, and in ethylene perception (Marschner, 1995; Rodrigues et al., 1999; Pilon et al, 2006). One important and abundant copper-containing protein is plastocyanin (PC), which functions in Photosystem I of photosynthesis (Kiselback et al., 1998; Raven et al., 1999). Copper/Zinc superoxide dismutase (Cu/ZnSOD) is a second important copper-containing protein that functions to scavenge reactive oxygen species (Bowler et al., 1994). Since most copper is located in leaves, when copper is limiting, plants tend to have withered leaves and slowed growth (Hansch and Mendel, 2009). It is important to note that iron and copper homeostasis are intertwined processes. Should copper become limiting, plants can shift from the use of copper-containing proteins to

iron-containing proteins in order to traffic available copper to the exceedingly more important plastocyanin, a topic discussed more below.

Copper Homeostasis in Yeast:

In yeast, before iron and copper can be taken into the cell, the metals must be reduced. *Saccaromyces cerevisiae* FRE1 and FRE2 are metalloreductases involved in the reduction of iron and copper at the cell surface (Martins et al., 1998). Once reduced, copper is transported into the cell via the high and low affinity yeast transporters CTR1 and CTR3 (Freitas et al., 2003). Much like *FRO2* and *IRT1* in *Arabidopsis*, *FRE1, FRE2, CTR1,* and *CTR3* are regulated by metal status. The transcription factor MAC1 is induced under copper deficiency and acts to regulate genes involved in copper homeostasis (Jungmann et al., 1993). MAC1 binds to the copper responsive elements of *FRE1, CTR1,* and *CTR3* to activate gene expression when copper is limiting; MAC1 is inactive when copper is available (Freitas et al., 2003).

Copper Homeostasis in plants:

Copper homeostasis needs to be tightly regulated, as too much copper can be toxic to plants. When copper is limiting, the plant must shift how it utilizes copper, as copper is a cofactor in many components of photosynthesis and respiration (Marschner, 1995). Much research has been done in the green algae *Chlamydomonas reinhardtii* to help understand the response to copper deficiency (Merchant et al., 1991). In *Chlamydomonas* there is a shift from using proteins that contain copper to proteins that contain iron as a cofactor. This shift also exists in *Arabidopsis*, though the proteins differ. Under copper deficiency, *Arabidopsis* shifts from using Cu/Zn superoxide dismutase (Cu/ZnSOD) to using Fe superoxide dismutase (Abdel-Ghany et al., 2005). By doing this, copper can then be directed to plastocyanin, which is a vital component of the

photosynthetic electron transport chain (Yamasaki et al., 2007). Recently, it has been shown that a microRNA plays a role in copper homeostasis in *Arabidopsis* (Yamasaki et al., 2009). MicroRNA *miR398* is induced under copper deficiency and is controlled by the transcription factor, SPL7 (SQUAMOSA promoter binding protein-like7). *miR398* functions by degrading the transcript of two Cu/Zn SODs, CuZn/SOD1 and Cu/ZnSOD2.

SPL7 also regulates the transcription of many copper deficiency response genes in *Arabidopsis* such as *COPT1* and *COPT2*, *FeSOD*, and even *FRO3* (Yamasaki et al, 2009). Mutant *spl7* plants do not up-regulate these genes under copper limitation. Data presented here and in publication also show that *FRO4* and *FRO5* are controlled by SPL7 and are two of the most highly up-regulated genes during Cu deficiency (Bernal et al., 2012).

Uptake and Trafficking of Copper:

Like iron, copper may need to be reduced from Cu^{2+} to Cu^{1+} before the plant can take it up. Some evidence suggests that FRO2 may play a role in the reduction of copper at the root surface, as *frd1* plants are impaired for copper reduction (Robinson et al., 1999). However, *frd1* plants are not copper deficient, so it is possible that plants take up copper as Cu^{2+}, or that other FRO family members function in reduction of Cu.

Reduced copper has been shown to be transported into the plant via the copper transporter, COPT1 (Puig et al., 2007a; Penarrubia et al., 2009). The COPT1 transporter belongs to a family of six copper transporter proteins in *Arabidopsis* (Sancenon et al., 2003). It is possible that plants can also take up the divalent form of copper as members of the ZIP family of transporters, ZIP2 and ZIP4, have been shown to be up-regulated

during copper deficiency (Wintz et al., 2003). However, this hypothesis lacks support, which is discussed more in Chapter II.

Inter- and Intracellular Copper Homeostasis:

Several members of the COPT family of Cu transporters have been characterized to date. COPT2 has been shown to play a role during both Fe and Cu deficiencies by functioning to alleviate some of the stress induced by Fe deficiency (Peria-Garcia et al, 2013). COPT5 has been shown to function as a Cu exporter across the tonoplast under Cu deficient conditions (Garcia-Molina et al, 2011; Klaumann et al, 2011), and finally COPT6 has been shown to be involved in redistribution of Cu in aerial tissue under Cu deficiency (Garcia-Molina, 2013).

Once Cu enters the root as Cu^{1+}, it needs to be loaded into the xylem. It is believed that HMA5 can move Cu into the xylem where it then can bind to NA, although this is still unclear (Andres-Colas et al, 2006; Curie et al, 2009). In which oxidative state Cu is transported into leaf cells is currently unknown, as well as which transporter is involved in the process. It was speculated that FRO6 may be involved in Cu reduction prior to leaf import, however it has been shown that *FRO6* expression is actually reduced under Cu deficiency (Mukherjee et al, 2006). Other data suggests that FRO5 may play a role due to its expression pattern and control by Cu status, but this hypothesis needs to be further tested.

Cu is needed in cells for use by the Cu transporter RAN1 for ethylene perception (Binder et al, 2010), Cu/ZnSODs, cytochrome c oxidase in mitochondria (Carr and Winge, 2003), and plastocyanin. Much work has been done to understand chaperones and

transporters involved in the shuttling and import of Cu into various organelles, as well as transcriptional response to Cu deficiency (Burkhead et al, 2009).

Due to the cellular environment and the fact that Cu is reactive and can lead to oxidative damage, Cu is bound to chaperones that aid in the shuttling of the metal to target proteins. One chaperone characterized in yeast is Atx1 (Pufahl et al, 1997). This protein functions to move Cu to an ATPase located in the golgi. The first Arabidopsis homolog of the yeast *Atx1* gene characterized was *CCH* (Himelblau et al, 1998). *CCH* expression was found to be up-regulated under Cu-deficiency conditions and low under Cu excess (Puig et al, 2007b). The second characterized *Atx1* homolog in Arabidopsis was *ATX1*, which is expressed under Cu excess. Both chaperones have been show to interact with RAN1 and HMA5, suggesting that these two chaperones deliver copper to the machinery for ethylene perception and function in Cu detoxification under high Cu stress (Andres-Colas et al, 2006; Puig et al, 2007b). However, recent data has shown that ATX1 and not CCH is required for tolerance to excess and low Cu conditions (Shin et al, 2012).

The final Cu chaperone is CCS, which is the only plant homolog of the yeast and human Cu chaperone for SODs; CCS functions to provide Cu to the three isoforms of Cu/ZnSOD in Arabidopsis (Chu et al, 2005). The three isoforms of Cu/ZnSOD are localized to the cytosol, chloroplast, and peroxisomes. CCS has been shown to be active in both plastids and the cytosol, and mutants of CCS show reduced activity in all three SODs, demonstrating that CCS is the primary Cu chaperone for these proteins (Chu et al, 2005).

Data has shown that two P-Type ATPases, PAA1 and PAA2, are responsible for the transport of Cu into chloroplasts (Abdel-Ghany et al, 2005). These proteins localize to the chloroplast inner membrane and thylakoid membrane, respectively, and are important for delivery of Cu to plastocyanin and the electron transport chain (Abdel-Ghany et al, 2005; Bernal et al, 2004). *PAA1* and *PAA2* single mutants are not lethal and mutant phenotypes can be rescued by addition of extra Cu, however *paa1paa2* double mutants are embryo lethal, underscoring the importance of efficient transport of Cu to chloroplast (Shikanai et al, 2003; Abdel-Ghany et al, 2005).

Regulation of Copper Homeostasis

It was discovered that the transcription factor Crr1 in *Chlamydomonas* is a regulator of the Cu deficiency response (Kropat et al, 2005). Crr1 binds to a GTAC motif under Cu deficiency and activates transcription of Cu assimilation genes (Quinn et al, 2000). In Arabidopsis, work with *miRNA398* led to the discovery of the Arabidopsis homolog of the Ccr1 transcription factor from *Chlamydomonas*. *miRNA398* is up-regulated under Cu deficiency and binds to transcripts of non-essential Cu proteins, that when lost, causes an attenuated response to Cu status (Yamasaki et al, 2007; Yamasaki et al, 2009). It was initially hypothesized that the *SPL* family of transcription factors could be mediating the response to Cu deficiency given the fact that these proteins recognize the GTAC motif (Birkenbihl et al, 2005). Arabidopsis SPL7, which shares the highest sequence similarity to *Chlamydomonas* Crr1, has been shown to be the master regulator of the Cu deficiency response in Arabidopsis (Yamasaki et al, 2009; Bernal et al, 2012).

Objective:

Arabidopsis *FRO4* and *FRO5* are two *FRO* genes that were previously uncharacterized. Based on data generated previously in our lab, as well as RNA-Seq data

provided by our collaborators, *FRO4* and *FRO5* have been shown to be two of the most highly up-regulated genes under Cu deficiency and most likely function as root surface Cu reductases. My aim with this thesis project was to obtain and characterize mutants of *FRO4* and *FRO5* and to confirm their function *in vivo*.

CHAPTER 2:

FUNCTIONAL CHARACTERIZATION OF FRO4 AND FRO5

INTRODUCTION

Copper is an essential micronutrient in plants, where it is needed as a cofactor in proteins involved in photosynthesis and respiration, in scavenging reactive oxygen species, and in ethylene perception (Marshner, 1995; Rodrigues et al, 1999; Pilon et al, 2006). One of the most important copper containing-proteins in plants is plastocyanin (PC), which functions in Photosystem I of the photosynthetic machinery (Kiselback et al, 1998; Raven et al, 1999). A second important Cu-containing enzyme is Cu/Zn superoxide dismutase, which functions to scavenge reactive oxygen species (Bowler et al, 1994). Finally, Cu has importance in cell wall metabolism and seed coat metabolism, functioning through glycoproteins known as laccases (Turlapati et al, 2011).

Cu is a highly toxic and reactive metal and it has been reported that levels of Cu in plants can range from 2 μg^{-1} g to 5 μg^{-1} g DW depending on the plant species (Epstein and Bloom, 2005). A normal Cu concentration in shoots is considered to be 6$\mu g\ g^{-1}$ DW (Cohu and Pilon, 2007), while deficiency occurs at less than 5$\mu g\ g^{-1}$ and toxicity occurs at greater than 20 μg^{-1} g dry-weight (Marshner, 1995). Cu deficiency in plants leads to chlorosis of leaves, reduced growth and seed set, and impaired photosynthetic efficiency

PS (II) is more susceptible to Cu toxicity than PS(I) as high concentrations of Cu enhance the negative impact of periods of prolonged, high intensity light, which leads to damage to PSII (Aro et al, 1993; Bernal et al, 2004).

Cu uptake is a well-characterized process in the photosynthetic green alga *Chlamydomonas reinhardtii* and in Arabidopsis. Like in plants, plastocyanin is one of the most abundant Cu-containing proteins in *C. reinhardtii*. During Cu deficiency, *C. reinhardtii* induces the expression of cytochrome c_6, which is an Fe-containing protein that can perform the function of plastocyanin (Merchant et al, 1991). This ensures that photosynthetic activity is maintained under Cu deficiency (Quinn and Merchant, 1995). The Cu homeostasis network on *C. reinhardtii* is controlled by the transcription factor Crr1, which targets genes that contain a CuRE motif, such as cytochrome c_6 (Kropat et al, 2005).

The Arabidopsis homolog of *C. reinhardtii* Ccr1 transcription factor is SPL7 and is up-regulated during Cu-deficiency (Yamasaki et al, 2009). This transcription factor serves to activate the transcription of copper assimilation genes, such as the transporters *COPT1* and *COPT2,* as well as the Cu chaperone, *CCH*. In addition, SPL7 controls the activation of several microRNAs, mainly *miR397, miRNA398,* and *miRNA408,*, which serve to down-regulate the translation of non-essential Cu-containing proteins such as Cu/ZnSOD and laccases (Yamasaki et al, 2009; Burkhead et al, 2009).

Previously, it was believed that two ZIP transporters, ZIP2 and ZIP4, might function in the uptake of Cu^{2+} from the root surface. These genes are up-regulated during copper deficiency and are able to rescue the ctr1 yeast mutant which lack a functional Cu transporters (Sancenon et al, 2003). However, there has not been any evidence to suggest

that these two transporters work in the high affinity Cu uptake pathway (Wintz et al, 2003, Puig et al, 2007a, Yamasaki et al, 2009; Burkhead 2009; del Pozo et al, 2010; Bernal et al, 2012). Recent studies using stable isotopes support a reduction-based method for Cu uptake (Jouvin et al, 2012). Stable isotope studies show that Cu^{2+} is reduced to Cu^{1+} prior to import via a cell-surface reductase and Cu^{1+} is the principle form in which Cu is brought into plant during Cu deficiency.

While it was known that COPT1 acts as the root Cu^{1+} transporter in Arabidopsis (Sancenon, et al 2003; Sancenon et al, 2004), it was unknown whether a member of the Arabidopsis FRO family facilitated the reduction of Cu^{2+} to Cu^{1+} for subsequent uptake into roots via COPT1 FRO2 can function as Cu reductase, however, although mutants of FRO2 (*frd1*) fail to induce Cu reductase activity under Fe deficiency, they do not suffer from reduced Cu levels (Robinson et al, 1999). This suggested that other FROs may function to reduce Cu under copper deficiency. Two additional, uncharacterized candidates were *FRO4* and *FRO5*. These two genes lie in tandem on chromosome 5 and share high amino acid similarity. It was previously shown that *FRO5* was induced by Cu deficiency in roots, and both proteins are predicted to localize to the secretory pathway/plasma membrane (Mukherjee et al, 2006). An RNA-Seq study showed that under Cu deficiency, *FRO4* and *FRO5* are two of the most highly up-regulated genes in roots (Bernal et al, 2012). Additionally, *spl7* mutants failed to up-regulate *FRO4* and *FRO5* under Cu deficiency, showing that expression of *FRO4* and *FRO5* is under the control of this transcription factor. Indeed, both *FRO4* and *FRO5* have several repeats of the GTAC motif in their upstream promoter regions, further supporting the hypothesis

that these two genes are under the control of SPL7 since SPL7 is known to bind to the GTAC promoter motif (Bernal et al, 2012).

My thesis project was to characterize mutants of *FRO4* and *FRO5* and study their function *in vivo* in Arabidopsis. My data, combined with the data of our collaborators, shows that FRO4 and FRO5 are part of the high affinity copper uptake system in Arabidopsis and serve as the principle Cu reductases in the roots.

METHODS AND MATERIALS:

Plant lines and growth conditions

Wild type Arabidopsis (ecotype Col *gl-1*), was used as a control in all experiments. A T-DNA mutant of FRO4 (SAIL_H09_159; http://signal.salk.edu/cgi-bin/tdnaexpress) was obtained from the Arabidopsis Biological Resource Center (ABRC; https://abrc.osu.edu) and *fro5* and *fro4fro5* mutants were generated using artificial microRNA technology (Schwab et al, 2006). The T-DNA insertion of *fro4* was mapped to the first exon. For solid media, seeds were surface sterilized with 25% bleach and 0.2% SDS for 15 minutes, then washed several times with autoclaved water. Seeds were then stored at 4°C for two days prior to plating. For normal growth, seeds were plated on Gamborg's B5 medium (Phytotechnology Laboratories, Shawnee Mission) supplemented with 2% surcrose, 1mM MES, and 0.6% agar, pH 5.8. After autoclaving, 1 mL of 1000x Gamborg's Vitamin solution was added (Phytotechnology Laboratories, Shawnee Mission). For hydroponic experiments, plants were grown in half strength liquid Hoagland's media (Bernal et al, 2012) under 11h day/13h night for three weeks, before being shifted to 16h day/8h night to promote flowering.

Isolation of mutant lines:

Homozygous *fro4* (SAIL_159_H09; *Colg-1* background) mutants were selected by growing plants on solid agar media supplemented with 50μM glufosinate ammonium (Basta, Cresent Chemical, Islandia, NY, USA) and by genotyping. A T-DNA primer specific for the left border of the insert (LB1 (5' GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC 3'; Sessions et al, 2002) was used in conjunction with a FRO4 specific reverse primer (5' ATTTGTGCAATGGAGTTGCTC 3') to show the presence of the insert; this primer pair produced a 1kb band. Mutants were then backcrossed to the wild-type twice to insure only a single insertion was present. In order to determine the location of the insert, the 1kb PCR product was gel purified using a PCR purification kit and following the manufacturers instructions (Qiagen, Maryland, USA) Samples were shipped to Engencore (http://selahgenomics.com/genomic-services/) for sequencing using LB1 and the FRO4 specific reverse primer, and the insert was determined to be in the 1st exon, approximately 27 bp downstream of the transcription start site.

Cloning:

Artificial microRNA lines for *FRO5* and *FRO4/FRO5* were made previously by Huijun Yang (Connolly Lab) using the Web MicroRNA Designer (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi; Schwab et al, 2006) The FRO5 amiRNA was constructed using the following primers: FRO5miR-sense, 5'-gaTTATTAGAGAATCGTGCCCCGtctctcttttgtattcc-3'; FRO5miR-antisense, 5'-gaCGGGGCACGATTCTCTAATAAtcaaagagaatcaatga-3'; FRO5miR*sense, 5'-gaCGAGGCACGATTCACTAATATtcacaggtcgtgatatg-3'; FRO5miR*antisense, 5'-gaATATTAGTGAATCGTGCCTCGtctacatatatattcct-3'; primer A, 5'

CTGCAAGGCGATTAAGTTGGGTAAC-3'; and primer B, 5'-GCGGATAACAATTTCACACAGGAAACAG-3'. FRO4/5 amiRNA was constructed using the following primers: FRO4FRO5miR-sense, 5'-gaTAGTATTAGAGAGTCATGCCTtctctcttttgtattcc-3'; FRO4FRO5miR-antisense, 5'-gaAGGCATGACTCTCTAATACTAtcaaagagaatcaatga-3'; FRO4FRO5miR*sense, 5'-gaAGACATGACTCTCAAATACTTtcacaggtcgtgatatg-3'; FRO4FRO5miR*antisense, 5'-gaAAGTATTTGAGAGTCATGTCTtctacatatatattcct-3'; primer A, 5'-CTGCAAGGCGATTAAGTTGGGTAAC-3' and primer B, 5'-GCGGATAACAATTTCACACAGGAAACAG-3'. Further details on the construction of the mutants have been published previously (Bernal et al, 2012)

To generate a 35S-FRO5-YFP-HA construct, the Gateway cloning system was used (Earley et al., 2006). In order to generate cDNA for cloning, plants were grown on standard Gamborg's B5 agar plates for 7 days under constant light prior to RNA isolation. FRO5 cDNA was amplified (from cDNA prepared from RNA isolated from *Col gl-1* roots using oligo dT primers and Superscript First Strand Synthesis kit; Introgen; Carlsbad, CA, USA) using the following primers: FRO5GATEFOR: 5'CACCATGGGGAATATGAGAAGCTTAGTG 3' and FRO5GATEREVNOSTOP 5' CCAGTTGAAACTAATTGCCTCAAAGTG 3', with the forward primer containing the added bases CACC at the 5' end as required for Gateway cloning by recombination and the reverse primer lacking the stop codon. The PCR product was then recombined into the pENTR/D/TOPO vector as per the manufacturers protocol (Invitrogen, Carlsbad, CA, USA). One Shot TOP 10 competent *E. coli* cells were transformed with the resulting pENTR-FRO5 construct. Transformants were selected on 50µg/mL Kanamycin; plasmids

were isolated and the inserts were sequenced. The construct was then recombined into the pEarleyGate101 destination vector using Gateway LR Clonase II Plus (Invitrogen, Carlsbad, CA, USA) to create the 35S-FRO5-YFP-HA plant transformation vector (Earley et al., 2006).

Because *amiFRO5* constructs already conferred resistance to Basta (glufosinate ammonium), and we needed to transform the amiFRO5 line with a FRO5-YFP construct (see results section) a second YFP expression vector was constructed for this study. FRO5-YFP-HA was cloned from the previously generated 35S-FRO5-YFP-HA construct using the following primers: FRO5BglIIFwd 5' GAAGATCTTCACCATGGGGAATATGAGAAGC 3' and FRO5HANheIRev 5' CTAGCTAGCTAGTTAAGCGTAATCTGGAACATC3'. The product was then subcloned into the pCambia1302 vector (http://www.cambia.org/daisy/cambia/home.html, Australia). Following sequencing, the plasmid was transformed into *Agrobacterium tumefaciens* strain GV3101 (Koncz and Schell, 1986). *amiFRO5* and *Col gl-1* plants were then transformed by the floral dip method (Clough and Bent, 1998). Transgenic plants were selected on Gamborg's B5 media supplemented with 50 μg mL^{-1} hygromycin.

Cloning of 35S:*FRO4-GFP* and 35S:*FRO5-YFP*

35S:FRO5-YFP expressing plants were generated prior to my joining the lab by Huijun Yang (Connolly Lab). For 35S:FRO4-GFP, bacteria containing cloned *FRO4* were obtained. The obtained bacteria were grown over night in liquid LB media at 37°C. Plasmids were isolated from bacteria using a Qiagen miniprep kit following manufactures' instructions (Qiagen, Maryland, USA). Full length *FRO4* was then cloned

from the plasmid using the following primers: FRO4GATEFOR: 5' CACCATGGGGAATATGAGAAGCTTAGTG 3' and FRO5GATEREVNOSTOP: 5' TCACCAGTTGAAACTAATTGCCTCAAG 3'. A *FRO5* specific reverse primer was used because the 3' end of *FRO4* and *FRO5* are identical. Cloning and transformant selection proceeded as described above for *35S:FRO5-YFP*, with the exception that *FRO4* was cloned into pEarleyGate103, which expresses GFP (Earley et al, 2006). Confocal microscopy was done at the University of North Carolina, Raleigh with the help and guidance of Dr. Terri Long using a Zeiss LSM 710 confocal microscope. Plants were grown for 4 days on standard Gamborg's B5 media. Before imaging, live root tissue was stained with 10μM propidium iodide for several seconds.

RNA Isolation and Transcript Analysis:

To analyze transcript levels, total RNA was extracted from 100 mg of frozen leaf tissue of two-week old seedlings grown on standard Gamborg's B5 media from *Col gl-1*, *fro4, fro5*, and *fro4fro5* using TRI-Reagent (Sigma, Saint Louis, MO, USA). DNase I (Bio-Rad, Hercules, CA, USA) treatment was conducted using 3.5 μg of RNA at 37°C for 10 minutes. Superscript First-Strand Synthesis System for semi-quantitative RT-PCR (Invitrogen, Carlsbad, CA, USA) was used to synthesize cDNA from 3.5 μg of RNA. For full length *FRO4* and *FRO5* transcripts, PCR was conducted using the *FRO4* full-length (Forward: 5' CACCATGGGAAATATGAGAAGCTTAGTGAAGAC 3'; Reverse: 5' TCACCAGTTGAAACTAATTGCCTCAAG3') primer pair and a *FRO5* full-length primer pair (Forward: 5' CACCATGGGGAATATGAGAAGCTTAGTG 3'; Reverse: 5' TCACCAGTTGAAACTAATTGCCTCAAG 3') . Primers specific for actin (Forward: 5' CCTTTGTTGCTGTTGACTACGA 3'; Reverse: 5' GAACAAGACTTCTGGGCATCT

3') were used for control reactions. PCR reaction mixtures contained 1μg of cDNA when using FRO4 or FRO5 primers, and 1/10th as much cDNA for actin reactions.

Semi-quantitative RT-PCR was used to examine transcript levels and amplification was monitored over the course of the PCR reaction to ensure that amplicon comparisons were made during the exponential phase of amplification. The primers used were: FRO4 RT Forward: 5' GCGTTTTTAGACCTAATCTTCCCACTG 3' and FRO4 RT Reverse: 5'GCGCCATAAGAAAACTACACTGGAA 3'; FRO5 RT Forward: 5' GCATTTTTAGACCTAATCTTCCCTTCA 3' and FRO5 RT Reverse: 5' TGCGCCACAAGAAAATTATGCTTGAC 3'. The PCR was paused and samples removed at the cycle numbers indicated in figures 2.2B, 2.3B, and 2.4B.

Copper Reductase Assay:

Prior to sowing seeds on Cu sufficient or deficient media, glass petri dishes were soaked overnight on 0.1N HCl, followed by four washes to remove as much metal as possible. Copper reductase assays were then performed on plants grown for 23 days on +Cu/-Cu agar medium in glass plates (Becher et al, 2004; Bernal et al, 2012). For measurement of reductase activity, plant roots were submerged in 300 μL of assay solution in a 96-well plate consisting of 0.2mM $CuSO_4$, 0.6mM Na_3Citrate, and 0.4mM BCDS (Sigma-Aldrich) and placed in the dark for 30 minutes. The assay solution absorbance was then measured at 483 nm and activity was standardized to fresh weight of roots (Yi and Guerinot, 1996). Ten plants were used for each genotype for each assay, and activities for two biological replicates were averaged. A student's t-test was used to perform statistical analysis.

Arabidopsis membrane preparation:

Arabidopsis total membranes were isolated from Col *gl-1* and *amiFRO5* plants transformed with the *35S:FRO5-YFP-HA* construct, as previously described (Zhao et al., 2002). Two-week-old seedlings were ground with liquid nitrogen using homogenization buffer (1 mL g^{-1} tissue fresh biomass) containing 30 mM Tris pH 8.5, 150 mM NaCl, 1 mM EDTA, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 2 mM Pefabloc (Roche, Indianapolis, IN, USA) at 4°C. Ground samples were then filtered through Miracloth (Calbiochem, Sand Diego, CA, USA) to remove plant debris. The extract was centrifuged at 8,000xg at 4°C for 15 minutes. The supernatant was recovered and centrifuged at 100,000xg at 4°C for 30 minutes to pellet microsomal membranes. Isolated membranes were resuspended (200 μL g^{-1} fresh biomass of starting tissue) in 10 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, and 2 mM Pefabloc and stored at -80°C.

Western Blot:

Protein concentrations were estimated using the Bradford Assay (BioRad, Hercules, CA, USA). Membrane extracts (15 μg) were diluted with an equal volume of 4X loading buffer and placed at 37°C for 1 h prior to being separated by SDS-PAGE and transferred to a PVDF membrane by electro-blotting using standard protocols (Towbin et al, 1979). FRO5-YFP-HA protein was detected using an anti-HA antibody (Roche Applied Science, Penzberg, Germany). Membranes were incubated with 5% non-fat dairy milk in 1x PBST for one hour prior to incubation with the antibody. Membranes were then washed with 1x PBST buffer 2x for 5 minutes each. For primary antibody incubation, anti-HA was diluted to 1:2500 in 1% non-fat dairy milk in 1x PBST and placed in 4ºC overnight. The membrane was then washed 5x with 1x PBST buffer for 5

minutes each on the following day. A chemiluminescent substrate (Thermo Scientific, Rockford, IL, USA) was used to examine abundance of protein in the dark room. To confirm equal loading, a gel loaded and run in parallel was stained with coomassie for 1 hour and then washed with destaining solution until bands were crisp and viewable.

RESULTS:

Previously, two groups had looked at the expression of all eight members of the FRO family. Wu et al (2005) showed slight expression of *FRO4* in Fe-deficient roots and leaves, and showed some expression of *FRO4* in cotyledons. *FRO5* was shown to be more highly expressed than *FRO4* in both shoots and roots, while also showing expression in flowers. Muhkerjee, et al (2006) only saw *FRO5* expression in roots, which was slightly higher under Fe-deficient conditions, while seeing no expression of *FRO4*. Additionally, Muhkerjee et al examined expression in response to Cu status, and *FRO5* was shown to be up-regulated under Cu-deficiency (2006). It is possible that the differences in expression could be accounted for by different growth conditions used in the two studies.

Our collaborators also examined expression of FRO4 and FRO5 shortly after the discovery that the transcription factor SPL7 regulates the Cu-deficiency response (Yamasaki et al, 2009). Using RNA-Seq, it was found that *FRO4* and *FRO5* are two of the most highly expressed genes under Cu-deficiency in roots, with *FRO5* showing higher expression. Also, it was shown that both genes are under the control of SPL7, due to the fact that expression of *FRO4* and *FRO5* was lost in *spl7* mutants (Bernal et al, 2012).

In parallel, our lab obtained a T-DNA insertion line for *FRO4* and generated artificial microRNA lines for *FRO5* and *FRO4/FRO5* (using a microRNA designed to target both FRO4 and FRO5). This approach was necessary, as a true T-DNA knockout line for *FRO5* did not exist.

Analysis of transcript abundance in *fro4, fro5* and *fro4,fro5*

To determine transcript abundance of *FRO4*, semi-quantitative reverse transcriptase PCR was performed. Primers that amplify the full coding sequence of *FRO4* and *FRO5* were used to test transcript levels in wild type (*Col gl-1*) and *fro4* plants (Figure 2.1A primer pair e and f; Figure 2.2A). I tested *FRO5* transcripts in these plants due to the fact that *FRO4* and *FRO5* lie in tandem on chromosome 5 and previously it has been shown that a T-DNA insertion in *FRO6* can have an effect on transcript abundance of *FRO7*, both of which also lie in tandem on chromosome 5. (unpublished data).

Full-length *FRO4* transcript was undetectable in the *fro4* mutant, while *FRO5* transcript abundance was unaffected by the disruption of *FRO4* (Figure 2.1B). Once a homozygous line was established for *fro4*, I then mapped the insertion point of the T-DNA. Using the LB1 primer and FRO4 internal reverse primer (2.1A; primer "a"), I was able to map the insertion to 27 bp downstream of the transcription start site in the 1st exon, which is marked by the triangle in Figure 2.1A.

Prior to my joining the lab, Huijun Yang began the process of constructing artificial microRNA mutants. The WMD 2 – Web microRNA Designer software was used to design 21bp artificial microRNAs that targeted: 1. just *FRO5* and 2. both *FRO4* and *FRO5* simultaneously. (Figure 2.2A, Figure 2.3A). The microRNAs targeted sequences in the 7th exon of each gene. For *FRO5*, red letters indicate complementary

bases between the microRNA and *FRO5*, while lower case red letters indicate mismatches that would correspond to *FRO4*; blue letters are mismatch bases (Figure 2.2A). For the microRNA targeting both *FRO4* and *FRO5* simultaneously, red letters indicate complementary bases, while blue and black letters are mismatches to *FRO4* and *FRO5* respectively (Figure 2.3A; Bernal et al, 2012).

After isolating several homozygous lines for both microRNA constructs, I examined transcript abundance. First I tested transcript abundance of *FRO4* and *FRO5* in two *amiFRO5* lines (7 and 10), using internal primers (Figure 2.2B, FRO5I-Fand FRO5I-R). Transcript abundance in these two lines was unaffected when compared to the wild type. At first this was confusing; however, recent data has shown that it is possible to achieve translational repression using artificial microRNA constructs as opposed to transcription knockdown (Gu and Kay, 2010).

To test if this phenomenon was occurring in these plants, a *35S:FRO5-YFP-HA* construct was generated and transformed into the wild-type and *amiFRO5* backgrounds. If translational repression was occurring, then we should be able to detect *FRO5* transcript in both wild type and *amiFRO5* plants, but we should not be able to detect any FRO5-YFP-HA fusion protein in the *amiFRO5* background. Using both western blot and confocal microscopy, we confirmed that no FRO5-YFP-HA fusion protein was detectable in the *amiFRO5* background, despite the fact that it was detected in the control (WT) background and that translational repression of FRO5 was indeed occurring in the *amiFRO5* lines (Figure 2.4A; Bernal et al, 2012). Using an anti-HA antibody and membrane preps, I was able to detect a 105kD band that corresponded to a FRO5-YFP-HA fusion protein in wild type plants transformed with the construct, but not in the

amiFRO5 background. This data was also supported by confocal microscopy performed by our collaborators (Bernal et al, 2012). Examining *FRO5* transcripts in these plants showed no detectable differences between the wild type and *amiFRO5* mutants (Figure 2.4B).

Examination of transcript abundance in two independent homozygous mutants for *amiFRO4FRO5* (lines 27 and 48) showed that transcript levels of *FRO4* were below detectable levels, while transcript abundance of *FRO5* was largely unchanged compared to wild-type (Figure 2.3B). Because of the translational repression of FRO5 shown in the *amiFRO5* lines, we assume that FRO5 protein is also repressed in the *amiFRO4FRO5* lines as well.

Phenotypic analysis of mutants

Since data indicated that *FRO4* and *FRO5* are expressed more highly under copper deficiency, and because of the fact that they are under the expression of the SPL7 transcription factor, next we wanted to test copper reductase activity in wild type and all mutants. Plants were grown for three weeks on either Cu-sufficient or Cu-deficient media before a root copper reductase assay was performed. Under Cu-deficient conditions, wild-type plants show high induction of activity, while *fro4* and *fro5* single mutants showed greatly reduced activity, and double mutants showed only basal level activity (Figure 2.5; Bernal et al, 2012).

In addition to copper reductase activity, experiments performed by our collaborators show that short-term high affinity uptake of Cu in single and the double mutants was greatly reduced. Plants were grown on either Cu-sufficient or –deficient media for three weeks and then roots were placed in a solution containing 10nM $CuSO_4$

for ten minutes. A fluorescent dye, known as CS1 (Coppersensor-1), was used to visualize Cu^{1+} in root tissue. Wild-type plants showed large amounts of Cu^{1+} within root cells, while single mutants showed greatly reduced fluorescence due to a decrease in Cu^{1+} uptake and the double mutant showed nearly abolished uptake of Cu^{1+}, further showing the importance of FRO4 and FRO5 to the reduction of Cu^{2+} to Cu^{1+} prior to import into the plant (Bernal et al, 2012).

To examine growth of wild type and mutant plants under both Cu sufficiency and Cu deficiency, I grew plants hydroponically from the seedlings stage until senescence. While all plants looked healthy under copper-sufficient conditions, under copper deficient conditions, single and double mutants of *FRO4* and *FRO5* show stunted growth, and fewer branches. However, seed weight showed no observable difference (Figure 2.7) Despite the dramatic phenotype of mutant plants grown under copper deficiency, when grown on soil, the mutants are mostly similar to wild-type plants, with *amiFRO4FRO5* plants showing only slightly stunted growth (Figure 2.6).

Preliminary confocal data appear to show that FRO4 and FRO5 are localized to the plasma membrane; however, only specks of fluorescence could be seen. (Figure 2.8)

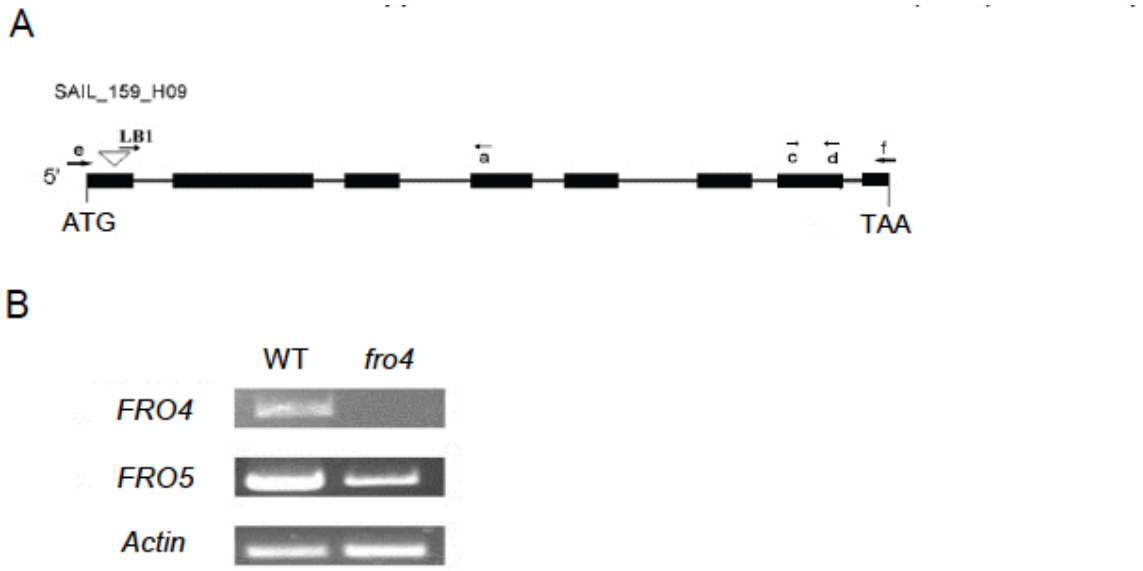

Figure 2.1: Gene map for *FRO4*.
A) A homozygous, single insertion T-DNA mutant of *FRO4* (*fro4*) was obtained from ABRC (SAIL_159_H09). The insertion was mapped to a position downstream of the transcription start site in the 1st exon, marked by the triangle. To genotype for homozygous lines, LB1 and a FRO4 reverse primer, labeled "a" were used. Black boxes represent exons, while lines represent introns. Letters above the gene indicate primers used.
B) Transcript analysis of WT and *fro4*. Full length *FRO4* was tested in WT and fro4 mutants. *fro4* mutants show no detectable full length *FRO4* transcript and *FRO5* levels in the mutant are unaffected. *Actin* is used as a control. (Bernal et al, 2012 © The Plant Cell).

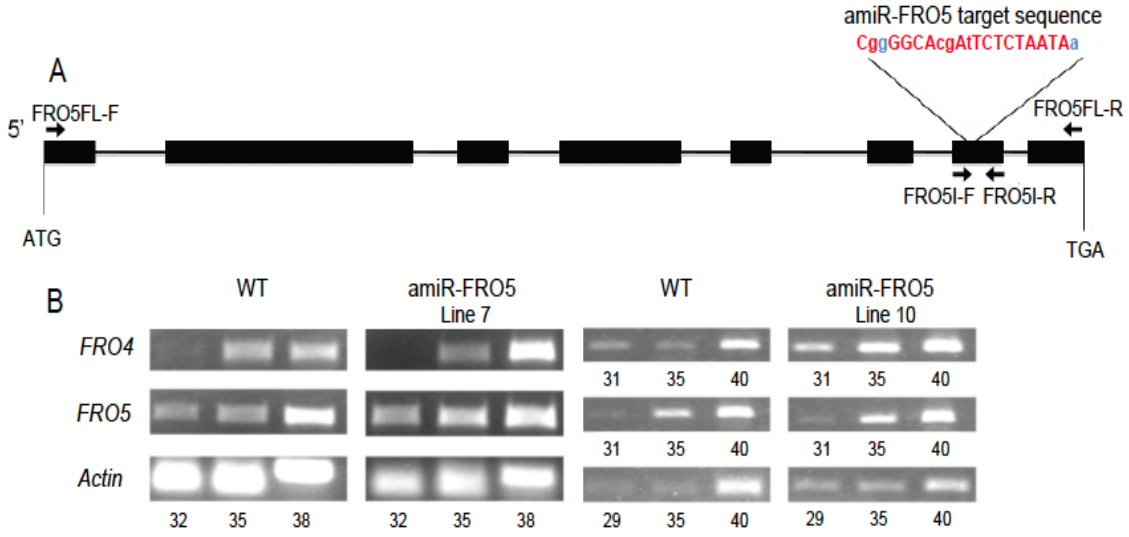

Figure 2.2: Generation of *amiFRO5* lines.

A) Gene map of *FRO5*. An artificial microRNA target sequence was generated to target the 7th exon of *FRO5*. Red letters indicate complementary bases to *FRO5*, while lower cases letters are mismatch bases to *FRO4*. Blue letters indicate mismatch pairs of *FRO5*. FRO5FL-F and FRO5FL-R were primers used to amplify full length *FRO5*. B) Transcript analysis of two independent mutant lines for *amiFRO5*. *FRO4* and *FRO5* levels were tested in WT and mutants. FRO5I-F and FRO5I-R were used to test transcript abundance of *FRO5*; *FRO4* internal primers, labeled "c "and "d"(Figure 2.1B), were used to test *FRO4* transcript abundance. *FRO4* and *FRO5* transcript is unaffected in in both *amiFRO5* lines. *Actin* used as control. (Bernal et al, 2012. © Plant Cell)

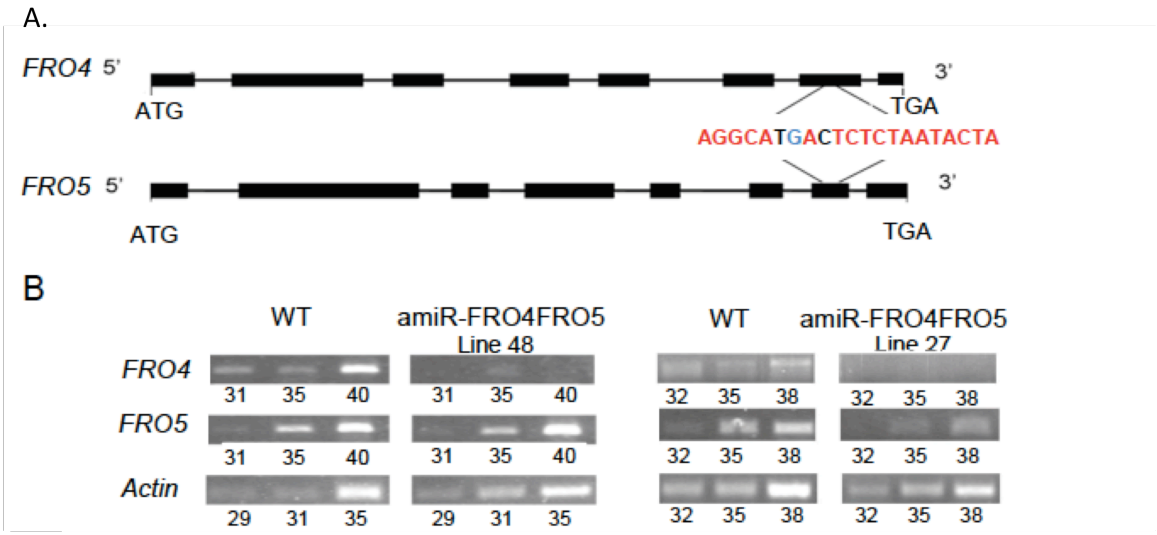

Figure 2.3: Transcript analysis of *fro4fro5* mutants.

A) Gene map of *FRO4* and *FRO5* with artificial microRNA target sequence indicated in the 7th exon. Red letters indicate complementary base pairs to both genes, while blue and black letters represent mismatches to *FRO4* and *FRO5* respectively. Black bars represent exons while lines represent introns.
B) Transcript abundance of *FRO4* and *FRO5* in two independent *amiFRO4FRO5* mutants. *FRO4* levels are greatly reduced in both lines, while *FRO5* levels appear unaffected. *Actin* is used as a control; cycle numbers indicated under each lane.

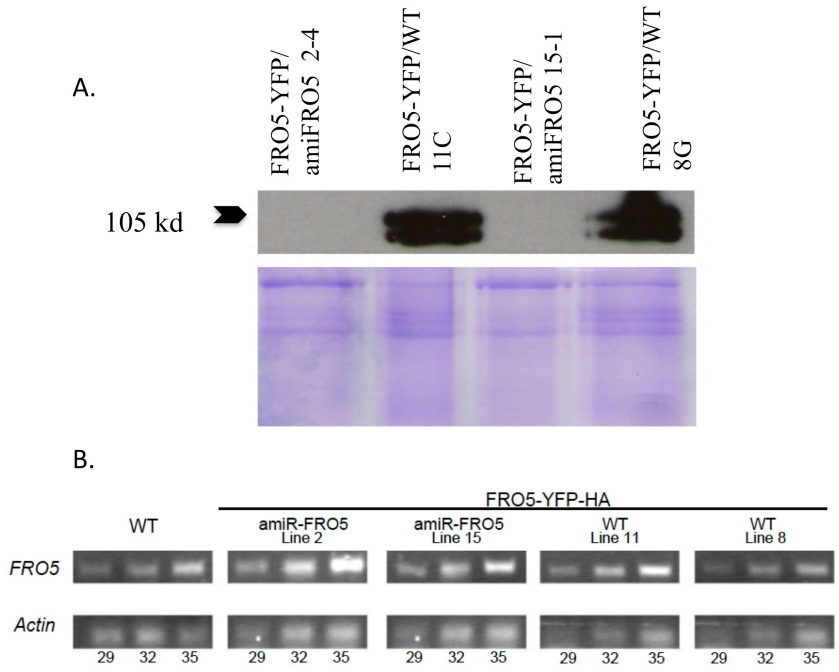

Figure 2.4: Translational repression of FRO5 in *amiFRO5* mutants. A) WT and *amiFRO5* plants were transformed with a *35S::FRO5-YFP-HA* construct. Two independent homozygous lines were obtained for each genotype. *amiFRO5* plants transformed with *35S::FRO5-YFP-HA* showed no protein accumulation when probed with an anti-HA antibody, while WT plants show protein accumulation. B) *FRO5* transcript levels were unaffected in all four lines tested. Actin is used a control. (Bernal et al, 2012. © Plant Cell)

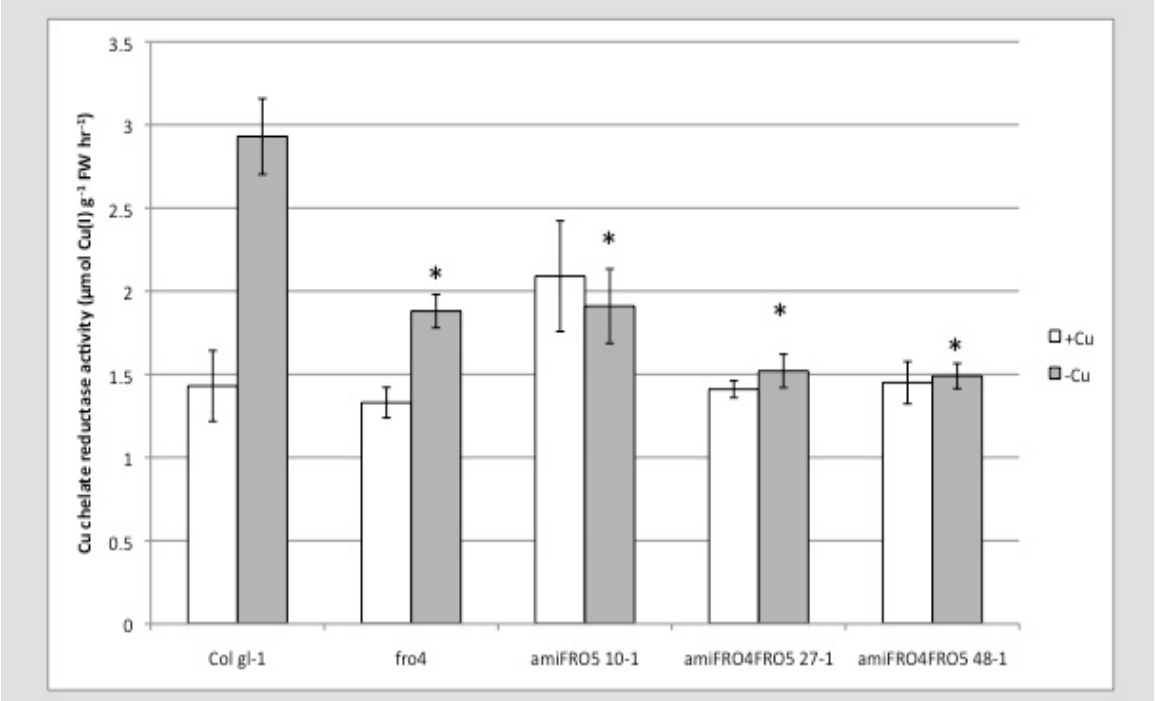

Figure 2.5: Root copper reductase assay.
WT and mutant plants were grown on +Cu or –Cu solid agar media for 3 weeks at 11h day/13h night photoperiod. Roots were completely submerged in copper reductase assay solution and placed in the dark for 30 minutes. The assay solution color change was measured at 483nm and activity was normalized to fresh root weight. Two biological replicates were averaged. Asterisks indicate a significant difference, with $p < 0.05$ using a Student's t-test.

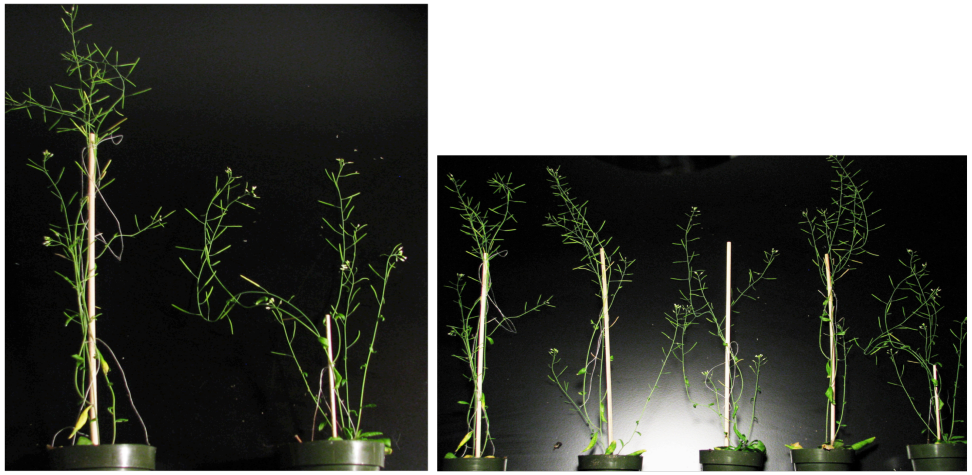

Figure 2.6: Phenotype of soil grown wild-type and mutant plants.
There is no visible difference between wild-type and the single mutants when grown on standard soil in constant light. *fro4fro5* plants show only a slightly stunted growth compared to wild-type, *Col gl-1*.

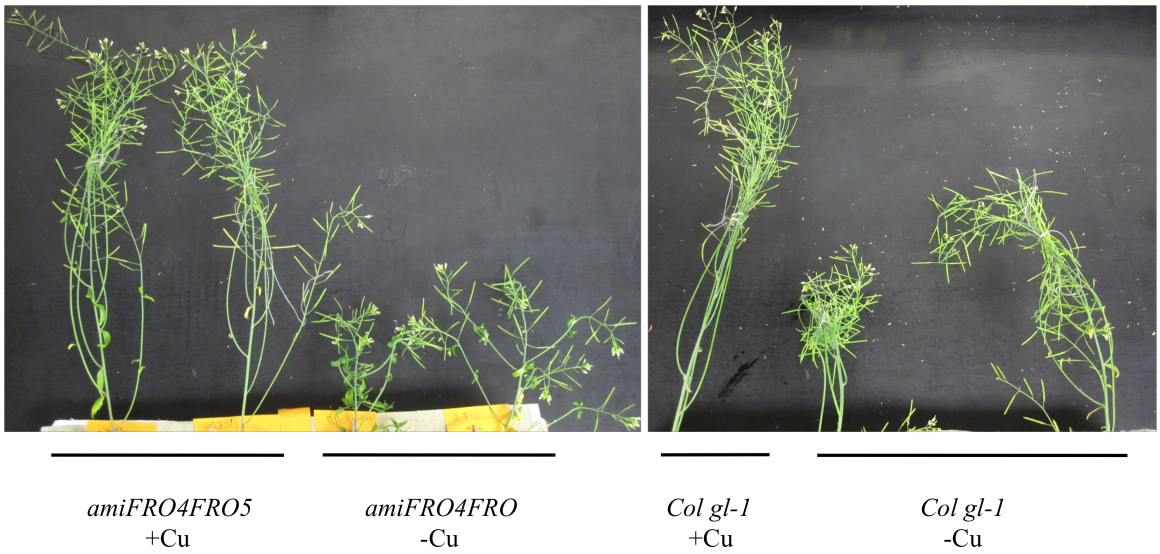

Figure 2.7: Hydroponically grown wild-type and *fro4fro5* mutants. Plants were grown for 3 weeks in a 11h day/13h night photoperiod before being transitioned to a 16h day/8h night photoperiod to promote flowering. *Col gl-1* and *fro4fro5* mutants show no difference in growth under copper sufficient conditions. However, *fro4fro5* mutants show severely stunted growth compared to wild-type.

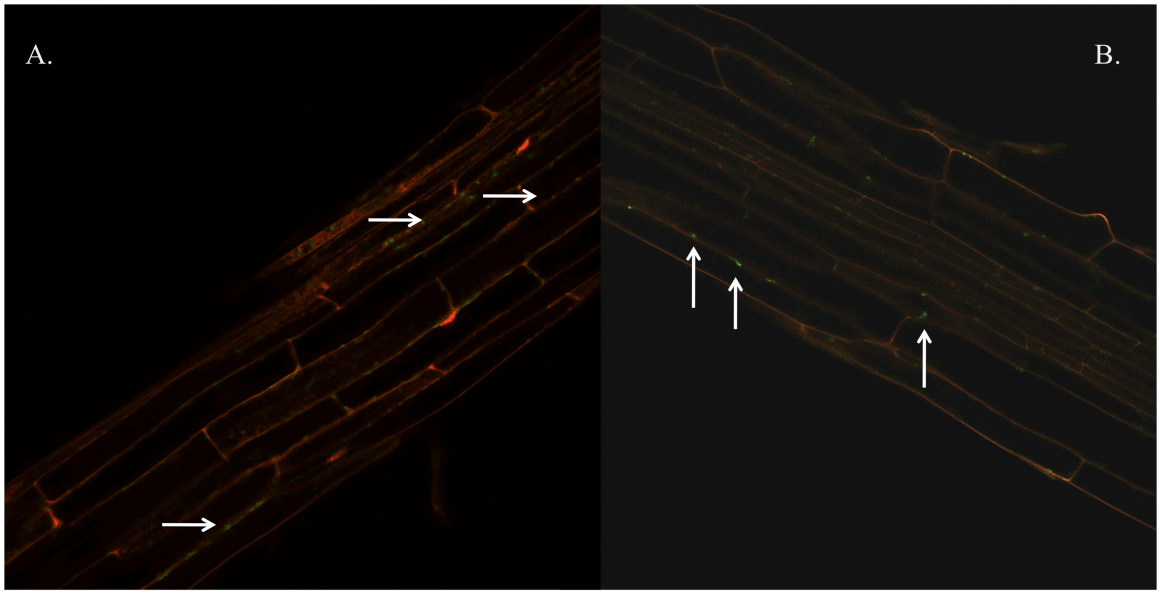

Figure 2.8 Confocal imagery of *35S:FRO4-GFP* and *35S:FRO5-YFP* Arrows indicate areas of possible fluorescence of FRO4-GFP (A) and FRO5-YFP (B) fusion proteins. Cell walls were stained with 10 μM propidium iodide and live root cells were imaged with a Zeiss LSM 710 confocal microscope.

DISCUSSION:

Much is known about other members of the FRO family. FRO2 is the principle Fe reductase at the root surface (Robinson et al, 1999), while FRO7 functions to reduce Fe at the surface of the chloroplast (Jeong et al, 2008). Finally, it is believed that FRO3 and FRO8 function at the surface of the mitochondria to reduce Fe (Jain and Connolly, 2013, Jain et al, 2014).

FRO4 and FRO5 represent two functionally uncharacterized FRO members. Data suggested that *FRO4/FRO5* are expressed in tissue ranging from shoots, to roots, to flowers, while being predicted to localize to the secretory pathway (Wu et al, 2005; Mukherjee et al, 2006). Data also show that, in the case of *FRO5*, expression was induced by Cu deficiency (Mukherjee et al, 2006). A T-DNA mutant of *FRO4* was obtained whose insertion was mapped to the 1st exon. No full-length *FRO4* transcript was detected in these plants, and *FRO5* transcript was unaffected, indicating that any phenotypes observed in this mutant would be the effect of a single gene mutation as opposed to a double mutation. Due to a lack of an insertion knockout in *FRO5*, artificial microRNA knockdown lines were generated for *FRO5* as well as a double knockdown for *FRO4* and *FRO5*. Surprisingly, all *amifro5* lines tested show transcript; however, no protein associated with FRO5 was detected, suggesting translational repression was occurring in these plants (Bernal et al, 2012). It remains to be seen if this phenomenon is occurring in *fro4fro5* mutants, as these plants also show transcript for *FRO5,* but show undetectable levels of *FRO4.*

The biochemical assay to test for reductase activity showed differences between wild type and mutant plants. While *fro4* and *fro5* both showed greatly reduced activity,

fro4fro5 plants showed only basal levels of Cu-deficiency inducible Cu reductase activity. Coupled with the short-term high affinity Cu uptake data (Bernal et al, 2012), it appears that FRO4 and FRO5 are required for the reduction of Cu^{2+} to Cu^{1+} prior to import, and they function redundantly. Both single mutants showed greatly reduced uptake of Cu, while double mutants showed almost no detectible Cu^{1+}. This clearly shows that FRO4 and FRO5, and not an additional FRO, are needed for Cu reduction at the root surface during Cu deficiency (Bernal et al, 2012).

Experimentally it has been shown that FRO5 localizes to the plasma membrane in protoplasts (unpublished data). Stable transgenic lines expressing either a *35S:FRO4-GFP* or a *35S:FRO5-YFP* construct were generated and isolated. Preliminary confocal data I obtained show specks of fluorescence along the periphery of root cells next to cell wall stained with propidium iodide for both constructs. Interestingly, confocal data obtained by our collaborators of *35S:FRO5-YFP-HA* plants show that, under normal growth conditions, FRO5 protein was detected within the cytosol of root cells (Bernal et al, 2012). These results could simply be due to the fact that the protein was under the expression of the constitutively active 35S promoter and only under Cu deficient conditions would the protein localize to the plasma membrane. This remains to be tested experimentally, however.

Data has shown that *FRO4* and *FRO5* show expression in aerial tissue as well as root tissue (Wu et al, 2005; Mukhjeree et al, 2005; Bernal et al, 2012). However, only *FRO5* appears to be under the control of SPL7 in this tissue. This is probably due to the fact that normally, *FRO4* expression is low and shows higher expression only in older leaf tissue. The role these two genes play in leaves is currently unknown. Data has

suggested that FRO6 works to reduce Fe in leaves prior to its import into cells (Feng et al, 2006). Previously in our lab, leaf disc ferric reductase assays were done with *fro6* mutants. While reductase activity was reduced, some residual activity remained (Maynes, 2013). It is possible that this residual activity could be due to the activity of FRO5 also working to reduce Fe in leaves. While *fro5fro6* double mutants have been generated, work still needs to be done to characterize this mutant to test this hypothesis.

The discovery that two Arabidopsis *FRO* genes are tightly regulated by Cu status is novel for plants. Previous data has shown that *FRO3* responds to Cu status, and FRO2, which is localized to the plasma membrane, can reduce Cu under Fe deficiency (Robinson et al, 1999; Muhkerjee et al, 2006). However, *frd1* (*fro2*) mutants do not show Cu deficiency (Robinson et al, 1999). Even though *FRO3* responds to Cu deficiency, recent hypotheses suggest that FRO3 may function as a mitochondrial reductase (Jain and Connolly, 2013; Jain et al, 2014). Thus, it appears that FRO4 and FRO5 are the only FRO family members that function in reduction of Cu at the root surface for subsequent uptake by plants. It is perhaps unsurprising that FROs can act as dual reductases for both Fe and Cu, as the yeast reductases, FRE1 and FRE2, function to reduce both Fe and Cu prior to import (Martins et al, 1998).

Future directions:

The data presented in this thesis and in our recent collaborative publication shows strong support for the hypothesis that FRO4 and FRO5 are Cu reductases that are located at the root surface during Cu deficiency. Work could still be done to determine if FRO5 could function as a Fe/Cu reductase in leaf cells, or if these genes play a role in Cu remobilization during senescence and seed formation, as some data has suggested that

they may be expressed in reproductive tissue (Wu et al, 2005). Subcellular localization needs to be definitively determined, as well as spatial and temporal expression patterns. *FRO2* mRNA levels are highest 72h after transfer to Fe deficient conditions (Robinson et al, 1999; Connolly et al, 2003). It would be interesting to see how rapidly transcripts of *FRO4* and *FRO5* accumulate after plants are shifted to Cu deficient media. Studying protein accumulation and turnover is difficult; due to the fact antibodies specific to FRO4 and FRO5 are not available.

While agricultural Cu deficiency is not as widespread Fe deficiency, our understanding of global Cu homeostasis in plants is important due to how intertwined Cu and Fe homeostasis are in plants and animals. The work presented here increases our understanding of the Cu homeostasis network, and shows that reduction of Cu by FRO4/5 is an essential part of the high affinity Cu uptake pathway.

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