MOLECULAR GENETIC ASPECTS OF IRON AND COPPER CROSS-TALK IN Arabidopsis thaliana

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Winter 12-2-2015

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MOLECULAR GENETIC ASPECTS OF IRON AND COPPER CROSS-TALK IN *Arabidopsis thaliana*

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University of Nebraska, 2015

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Iron (Fe) and copper (Cu) are essential micronutrients for plants and humans who consume plants. Their functions are tightly linked. For example, both of Fe and Cu work as cofactors in superoxide dismutase proteins to prevent reactive oxygen species damage. When Fe is removed from nutrient solution, plants uptake more Cu, but how plants sense and respond to Fe status is not fully understood. This thesis includes two projects that focus on Fe and Cu interaction, homeostasis and cross-talk in *Arabidopsis thaliana*.

Two *Arabidopsis* ecotypes, Kas-1 and Tsu-1, have difference in timing of Cu accumulation in rosettes under Fe deficiency. In the first study, we utilized a Kas-1 and Tsu-1 recombinant inbred line population form QTL mapping. Based on QTL composite interval mapping, one significant QTL was identified on the chromosome one [C1_23318972, C1_25175851] which explained 10% of the variation. We identified candidate genes based on their functions and amino acids differences between Kas-1 and Tsu-1. A time course experiment suggested that two candidate genes, *HMA5* and *ATX1*, can’t account for differences between Kas-1 and Tsu-1 for timing of Cu accumulation.
Genome-wide transcriptional profiling with Illumina HiSeq technology was used to reveal Fe/Cu cross-talk gene lists. The use of SQUAMOSA Promoter Binding Protein-Like7 (SPL7) mutant plants in Fe and Cu deficiencies addressed the research hypothesis that the uptake of Cu under Fe deficiency is independent of the normal Cu uptake system, and we identified specific SPL7 regulated genes under Cu deficiency. Wild-type Col-0 and mutant spl7 showed different patterns of classic bHLH family genes bHLH38, bHLH39, bHLH100, and bHLH101. Several ETHYLENE RESPONSIVE ELEMENT BINDING FACTORS (ERFs) were downregulated in Col-0, not in spl7. The wild type Col-0 and spl7 mutant transcriptional profiling would give significant insight into Fe and Cu homeostasis.
DEDICATION

This thesis is dedicated to my parents Xiang Yang & Xu Li, my dear grandpa

Xu Chunkun, and grandma Tian Yingjie
ACKNOWLEDGEMENTS

Firstly I would like to thank my advisor Dr. Brian Waters for his unselfish teaching and training. His critical thinking and research spirit will guide me wherever I am in future. And I appreciate his well considering for his students’ feeling.

It is my honor to have Dr. Paul Staswick and Dr. Stephen Baenziger for serving on my committee. I thank them for their illuminating suggestions and valuable criticisms.

Also I thank to our ‘iron man’ group members. Thanks Mary Guttieri, Raghu Prakash and En-jung Hsieh teaching me from beginning with patience and guiding me being better. Thanks to our lovely colleagues Caixia Liu, Tori Hinrichs, Yuzhi Chen Erin Kinley for running our lab more efficient.

Finally I thank to my parents Xiang Yang and Xu Li for loving and supporting me unconditionally.
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Chapter 1 Literature review

1. The importance of Plant Iron and Copper nutrition

Iron (Fe) and copper (Cu) are essential micronutrients in plants. Deficiency or excess of Fe and/or Cu can negatively affect growth and metabolism. Imbalanced metabolism of these nutrients results in nutrient deficiency or toxicity. Because plants are a primary source of iron for humans and animals, plant nutrition is also closely related to human health. The United Nations Food and Agriculture Organization has estimated that iron deficiency negatively affects about 2 billion people in the world and can be serious as anemia (Fourth report on the world nutrition situation, 2000). The majority of the population at risk are women and children, especially younger children and pregnant women. Iron deficiency can bring younger children cognitive dysfunction or even blindness (Grantham-McGregor and Ani, 2001). Moreover, iron deficiency may lead to oxidative DNA damage, brain maldevelopment, and immune dysfunction (Ames, 2001).

Scientists still have a big mission to develop and introduce varieties of seed and grain crops that contain high bioavailable iron (Guerinot, 1994).

The study of iron and copper uptake mechanisms in plants will help to provide information to construct iron efficient plant varieties, to enhance corresponding genes’ regulation, thus to increase the micronutrients in edible portion of fruits and vegetables.

1.1 Iron availability and its physiological functions

Iron is abundant at the Earth’s crust, but oxygenated environments and calcareous soil makes iron poorly soluble and low in bioavailability; further, a high level
of bicarbonate in the soil will decrease uptake of iron by plants. The availability of iron for plant uptake depends on soil pH because Fe activity is highly depressed on the alkaline soil (Lindsay and Schwab, 1982; Mengel, 1994). Fe uptake from the root apoplast into the cytosol of root cells is the critical process (Mengel, 1994). And Fe transport across the plasma membrane is initiated by Fe (III) reduction to reduce Fe (III) to Fe (II) through ferric-chelate reductase (Robinson et al., 1999a).

Understanding the mechanisms than control plant iron homeostasis is crucial for improving the growth of crops in the alkaline soil (Walker and Connolly, 2008). Iron has many physiological functions. Firstly, iron can be a cofactor for a wide range of redox enzymes, including cytochrome (cyt) oxidase, peroxidase, catalase, iron-sulfur proteins, and ferredoxin (Guerinot, 1994). Iron Fe (III) and Fe (II) acts as electron acceptors and donors that function in many important redox systems (Palmer and Guerinot, 2009). Iron has an integral role in respiration and photosynthetic electron transport system and acts as a cofactor in electron transport chain carriers such as Cyt f, Cyt b_{559}, and Cyt b_{563} (Cramer, 2004). Iron proteins in leghemoglobin and nitrogenase enzymes in leguminous crops are crucial for nitrogen fixation (Phillips, 1980). Secondly, iron is an activator in the process of chlorophyll synthesis. Lack of iron in plants results in inhibition of chloroplast development (Nishio et al., 1985). Iron is essential for specific thylakoid and chlorophyll synthesis (Nishio et al., 1985).

**1.2 Copper physiological functions**

Copper is also an essential nutrient in plants, and copper-containing proteins have important functions in photosynthesis (e.g. plastocyanin) (Weigel et al., 2003),
respiration (cytochrome c oxidase) (Carr and Winge, 2003; García et al., 2014), reactive oxygen metabolism (Cu/ZnSOD) (Bowler et al., 1992; Kliebenstein et al., 1998), perception of ethylene (ethylene receptors) (Rodríguez et al., 1999; Chen et al., 2002), cell wall modeling (laccase) (Gavnholt and Larsen, 2002; Nakamura and Go, 2005), defense systems include wound healing, response to pathogens (amine oxidase) (Rea et al., 2002; An et al., 2008; Angelini et al., 2008), and conversion of monophenol to diphenols (polyphenol oxidase) (Arnon, 1949; Mayer, 2006).

The function of Cu in biochemical reactions results in cycling between oxidized Cu(II) and reduced Cu(I) in electron carriers and enzymes (Vella, 1995). Copper can catalyze redox reactions that involve oxygen (O’Dell, 1976; Linder and Hazegh-Azam, 1996). Excess Cu is toxic because it stimulates production of free radicals, so Cu homeostasis is tightly controlled (Burkhead et al., 2009a). Cu deficiency can decrease growth rate and the symptoms appear at young leaves first. And Cu deficiency can influence cell wall formation and pollen production. Copper functions in cytochrome, polyphenol oxidase and in ascorbic acid oxidase as a catalyst (Stotz, 1937) where it relates to respiration. Most copper in plants is contained in plastocyanin, a protein with a function in electron transfer from the cytochrome b6f complex to photosystem I (PSI) in the thylakoid lumen of chloroplasts (Yamasaki et al., 2007a; Palmer and Guerinot, 2009). Copper is a component of another major copper protein, CuSOD, which participates in scavenging reactive oxygen species (ROS). Among the CuSOD proteins, CSD1 localizes to the cytoplasm, CSD2 localizes to stroma of chloroplasts, and CSD3 localizes to peroxisomes (Burkhead et al., 2009a). Cu deficient plants have reduced
electron transport capacity in photosynthesis. Plant optimal growth is closely related to balanced delivery of copper cofactors in cytosol, endoplasmic reticulum, mitochondrial inner membrane, chloroplast stroma, thylakoid lumen and the apoplast (Pilon et al., 2006). If any of the processes are unbalanced, metabolic damage will occur in plants.

2. Arabidopsis iron homeostasis

When plants have unbalanced metabolism, changes in metal chelation, uptake, transport and distribution are necessary to maintain stabilities of physiological regulation, or homeostasis. Arabidopsis maintains iron homeostasis by regulation of multiple gene family members. FERRIC REDUCTASE-OXIDASE(FRO) family, and ZRT, IRT-LIKE PROTEINS (ZIP) family are responsible for Fe acquisition and transport in roots (Eide et al., 1996; Robinson et al., 1999b; Vert et al., 2002a; Mukherjee et al., 2006a).

NATURAL RESISTANCE ASSOCIATED MACROPHAGE PROTEINS (NRAMP) family, YELLOW STRIPE LIKE (YSL) family function in the intracellular and intercellular epidermal cells in roots. Also, BASIC HELIX LOOP HELIX (bHLH) family, FE-DEFICIENCY INDUCED TRANScription FACTOR 1 (FIT) can regulate genes expression under iron deficiency. And, iron storage homeostasis protein ferritin is close related to iron toxicity regulation.

2.1 Strategy I plant iron uptake mechanism

Understanding the mechanisms of plant iron uptake is important for biofortification efforts. Angiosperm plant species have two different strategies to uptake iron from soil. Dicot and non-graminaceous species use strategy I Fe uptake. This is a reduction-based strategy where they reduce Fe (III) before transport into roots.
Graminaceous plants use strategy II Fe uptake, where they release chelators, called phytosiderophores, (Marschner et al., 1986) into the rhizosphere to bind Fe (III) for iron uptake (Palmer and Guerinot, 2009). This allows strategy II plants to solubilize inorganic Fe (III) compounds in the rhizosphere even in a high pH environment (Römheld and Marschner, 1986).

*Arabidopsis* uses strategy I for Fe uptake (Fig 1.1). The rhizosphere acidification results from plant roots pumping protons, which lowers the rhizosphere pH, and solubilizes iron (Guerinot & Yi, 1994) (Walker and Connolly, 2008). Acidification of the rhizosphere requires proton-ATPase which belongs to *ARABIDOPSIS* H⁺-ATPASE (AHA) gene family (Curie and Briat, 2003). Root epidermal cells can regulate ferric-chelate reductases the ferric chelate-reductase FRO2, FRO3 to convert Fe (III) to Fe (II) (Robinson et al., 1999a; Wu et al., 2005; Mukherjee et al., 2006b). FRO2 transfers electrons across the plasma membrane from the cytoplasmic NADH, and Fe (III) loses one electron (Robinson et al., 1999b). Under iron deficiency, plants upregulate *FRO2* gene expression and increase *FRO2* mRNA accumulation (Connolly et al., 2003).

Also, FRO2 is able to reduce Cu II to Cu I, however, *FRO2* gene expression is not increased by copper deficiency (Palmer and Guerinot, 2009). Therefore FRO2 functions to reduce Fe (III), and reduction of Cu (II) is a non-specific effect. However, simultaneous iron and copper deficiency induce ferric reductase enzyme activity synergistically (Cohen et al., 1997; Romera et al., 2003). IRON-REGULATED TRANSPORTER 1 (IRT1), a Fe (II) high-affinity uptake transporter, transports Fe(II) into root cells (Henriques et al., 2002; Varotto et al., 2002; Vert et al., 2002b). *irt1* knockout mutants can be rescued by iron
fertilization, which indicates that an additional low-affinity iron uptake mechanism may exist (Curie and Briat, 2003). While FRO2 provides ferrous iron and substrate for IRT1 coordinately, FRO2 and IRT1 orthologs have been identified in cucumber, tomato, and pea (Eckhardt et al., 2001; Waters et al., 2002, 2007; Connolly et al., 2003; Cohen et al., 2004; Li et al., 2004).

2.2 FIT and Iron Homeostasis

The transcription factor FER was the first to be identified in higher plants that is involved in Fe homeostasis (Ling et al., 2002). The fer mutant has an iron uptake defective phenotype in tomato (Ling et al., 1996). The FER orthologue gene FIT was identified in Arabidopsis thaliana (Colangelo and Guerinot, 2004; Jakoby et al., 2004; Yuan et al., 2005). FIT is a key transcription factor for iron homeostasis that regulates FRO2 and IRT1 gene expression (Bauer, Ling, & Guerinot, 2007; Colangelo & Guerinot, 2004; Yuan et al., 2005). FIT functions as a heterodimer with certain bHLH family proteins (Yuan et al., 2008). bHLH members AtbHLH38, AtbHLH39 were confirmed to interact with FIT, activating ferric chelate reductase (Yuan et al., 2008); AtbHLH100, AtbHLH101 could interact with FIT in yeast cells (Wang et al., 2013). Overexpression of FIT with either bHLH38 or bHLH39 can increase IRT1 protein accumulation and ferric chelate reductase activity under iron deficiency (Yuan et al., 2008). This indicated that IRT1 and FRO2 are directly regulated by FIT/bHLH38 complex and FIT/bHLH39 complex. Under Fe deficiency overexpressing 35S-FRO2 transgenic plants showed higher ferric chelate reductase activities compared to WT and can grow better on iron deficient soil compared to wild type Col-0, which indicated Fe reduction is the rate-limiting step for Fe
uptake (Connolly et al., 2003). bHLH100 and bHLH101 also function in iron deficiency response and uptake (Sivitz et al., 2012; Wang et al., 2013). And, overexpression of FIT with bHLH101 showed constitutive expression of Fe uptake genes FRO2 and IRT1 in roots (Wang et al., 2013).

2.3 Hormones related to iron signaling

Hormones and other small molecules can play critical roles on iron deficiency signaling (Hindt and Guerinot, 2012). It was hypothesized that auxin, ethylene, and nitric oxide (NO) are positive regulators at an increased level under iron deficiency; while cytokinin and jasmonate are negative regulators that have a decreased level under iron deficiency (Hindt and Guerinot, 2012). Under Fe deficiency, ethylene acts as the activator with the increased production can influence the increasing of ferric reductase, iron transporter and H^+\text{-}ATPase gene expression and ethylene precursors (ACC and ethephon) promote the responses (Romera and Alcántara, 2004; Lucena et al., 2006; García et al., 2015). Recently, their physiological link was elucidated. Transcription factors ETHYLENE INSENSITIVE3 and ETHYLENE INSENSITIVE3-LIKE1 (EIN3/EIL1) are activated through ethylene signaling pathway (Chao et al., 1997). EIN3/EIL1 can stabilize FIT, and it was a positive signal for FIT accumulation (Lingam et al., 2011; Hindt and Guerinot, 2012). Nitric oxide participates in abiotic and biotic stresses (Durner and Klessig, 1999; Wendehenne et al., 2004). Like auxin and ethylene, NO is also a positive signal. NO has been identified as a stabilizing stimulus that participated in the FIT protein accumulation (Meiser et al., 2011). The negative factor cytokinin can decrease root elongation, and limit root nutrient uptake. It is the inhibited root growth that
represses *FIT* expression whatever iron supply (Séguéla *et al.*, 2008; Hindt and Guerinot, 2012). Jasmonate, a stress hormone, is involved in response to various stimuli (Shah, 2009; Wasternack and Kombrink, 2010). Also, jasmonate inhibited *FRO2, IRT1* mRNA expression level whenever it is in wild type or *fit* mutant, which indicated that this process is not *FIT* dependent (Maurer *et al.*, 2011; Hindt and Guerinot, 2012).

3. Copper uptake, transport and homeostasis in *Arabidopsis*

3.1 Copper transport family

Copper transporter *COPT2* transports Cu I into root cells under Cu deficiency (Sancenón *et al.*, 2003), after *FRO4, FROS* reduced Cu II to Cu I (Bernal *et al.*, 2012b) (Fig 1.1). *FRO3* can be induced under Cu deficiency especially in lateral roots and vasculature (Mukherjee *et al.*, 2006b). *COPT1* and *COPT2* were high affinity copper transporters, which were down-regulated under copper excess in *Arabidopsis* cells (Sancenón *et al.*, 2003). *COPT3* and *COPT5* were secondary copper transporters and showed intermediate copper transport rate (Yruela, 2005a). *COPT3* may function copper mobilization from intracellular stores (Burkhead *et al.*, 2009a). *COPT5* functioned in copper scarcity, and may act as internal stores in photosynthesis (Garcia-Molina *et al.*, 2011). Recently, it was found that *COPT6* was a high affinity copper transporter similar to *COPT1* and *COPT2*; and functioned as copper redistribution in shoots (Garcia-Molina *et al.*, 2013). Among *COPT* family members, *COPT2* acts as copper and iron homeostasis functions. *COPT2* mRNA is induced under Fe deficiency and can help increases copper superoxide dismutase enzymes through transporting copper I.
3.2 Copper transporting P-type ATPase family

Copper transporting P-type ATPases function in transport of Cu ion across membranes (Burkhead *et al.*, 2009a). Three of these transporters, HMA6, HMA7, and HMA8 were also named PAA1, RAN1, and PAA2, respectively. HMA7 transports Cu to the secretory pathway, also, is involved in the formation of functional ethylene receptors which are required for the ethylene-signaling pathway (Woeste and Kieber, 2000). HMA6 is responsible for copper transport into the chloroplast and its mutant has impaired photosynthetic electron transport that can cause a high chlorophyll fluorescence phenotype (Shikanai *et al.*, 2003). HMA6 functions as a cofactor in delivery Cu to plastocyanin and to Cu/ZnSOD (Shikanai *et al.*, 2003). HMA8 mediated Cu delivery into thylakoids and supplied copper to plastocyanin (Tapken *et al.*, 2012). HMA5 interacted with metallochaperones ATX1, and may function in copper compartmentalization and detoxification, mainly in roots (Andrés-Colás *et al.*, 2006). HMA1, HMA2, HMA3 and HMA4 can transport divalent cations in *Arabidopsis*. Overexpression of *HMA1* in *HMA6* (PAA1) mutant background *Arabidopsis* showed copper accumulation in rosettes (Boutigny *et al.*, 2014).

3.3 Copper chaperones involved in copper homeostasis

There are three copper chaperones, ATX (CCH and ATX1), COX17, and a copper chaperone for SOD (CCS) involved in copper homeostasis in plants at different levels. CCH functions were first found in tomato. It functions in oxidative stress, senescence, and is related to copper homeostasis and plant defense system which is infect with pathogen *Botrytis cinerea* (Company and González-Bosch, 2003). Cu delivery to Cu-
transporting ATPases for thylakoid involved ATX1-like metallochaperones (Banci et al., 2006). ATX1 shuttles Cu to a P-type ATPase for translocating Cu into the secretory pathway (Pufahl et al., 1997). Arabidopsis AtCOX17 gene expression was highly expressed under excess copper supply which suggests that it has tolerance function to heavy metals (Zhou and Goldsbrough, 1994). Also, COX17 chaperone could supply copper to mitochondria and function for cytosolic oxidase enzymes CuSOD (Yruela, 2005b, 2009). CCS delivers Cu to Cu/ZnSOD in chloroplast and cytosolic and its transcriptional level is regulated by copper (Cohu et al., 2009).

4. Iron and copper homeostasis in strategy I plants

Because Fe and Cu are essential metals that play key roles in respiratory and photosynthetic electron-transport chains in mitochondria and chloroplasts, respectively, their functions are closely related and plant responses to these metals interact in various ways. Fe(III) chelate reductase performs a general function in uptake of cations Fe(III) and Cu(II) in the dicot pea (Pisum sativum L.) roots (Cohen et al., 1997). It was suggested that there was synergistic effects that simultaneous Fe and Cu deficiency enhanced ferric reducing capacity (Romera et al., 2003). Red clover (Trifolium pretense L.) root reductase experiments found Cu deficiency induced a different reductase system than Fe deficiency (Shao et al., 2005). Waters (2012) elucidated Fe deficiency enhanced Cu accumulation in rosettes in Arabidopsis, as the increased CuSOD can replace FeSOD in case of future oxidative stress (Waters et al., 2012). The Cu and Fe cross talk and metal sensing mechanism details are poorly documented in plants so far (Ravet and Pilon, 2013).
4.1 Iron and copper trade off hypothesis

The iron (Fe) and copper (Cu) trade off hypothesis suggests that if either Fe or Cu is limiting, the other metal will be over accumulated to partially compensate for its deficiency. In Arabidopsis, rosettes would increase CuSODs accumulation under low Fe supply to replace FeSODs protein in case of oxidative stress caused by reactive oxygen species (ROS) (Abdel-Ghany et al., 2005; Waters et al., 2012). The hypothesis continues that Fe deficient plants increase Cu accumulation to synthesize CuSODs proteins. In Fe deficient cucumber (Cucumis sativus), plants grown in -Cu solution had the highest ferric reductase activity compared to treatments over a range of Cu supply (Waters and Armbrust, 2013). This suggested that Cu deficiency can stimulate ferric reductase activity. In melon, the fefe mutant lacks proper regulation of the normal Fe uptake pathway, and can’t upregulate FIT, FRO1, and IRT under Fe deficiency (Waters et al., 2014). But, when Cu is limiting, the ferric reductase activity was upregulated, and so was FRO2, COPT2, and ZIP2 (Wintz et al., 2003; Waters et al., 2014).

4.2 microRNAs regulate iron and copper homeostasis

MicroRNAs (miRNAs) are a class of regulatory RNAs of ~21-nucleotide that function primarily as negative regulators in plants and animals at the posttranscriptional level (Abdel-Ghany and Pilon, 2008). They play critical roles in nutrient uptake/homeostasis, and in phloem-mediated long distance transport in varying plant physiological processes, such as Fe and Cu stress responses (Ambros, 2001; Zeng et al., 2014). MicroRNAs have important roles in regulation of Fe and Cu cross talk, and the tradeoff hypothesis by regulating some of the genes involved in these processes.
miR397a, miR398a and miR398b/c that regulate Cu containing gene expression were down regulated by Fe deficiency (Waters et al., 2012). MiRNA398 can mediate down-regulation of CuSOD, ZnSOD genes in plastocyanin when Cu is deficient (Yamasaki et al., 2007a). Further, miRNA398 targets include the copper chaperone for SOD (CCS) that encodes metallochaperone, and cytochrome c oxidase (COX-5b) for encoding plant homolog of the Zn binding subunit of mitochondrial copper enzyme is regulated by miRNA398 (Bonnet et al., 2004; Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004; Yamasaki et al., 2007a; Cohu et al., 2009; Beauclair et al., 2010).

The Cu enzyme regulation is necessary for using Cu economically in green photosynthetic tissues. At limited Cu supply or no Cu supply, miRNA398 is upregulated and degrades CuSODs, while FeSODs were accumulated in all the tissues; and, at high Cu supply FeSOD protein was diminished (Abdel-Ghany and Pilon, 2008), (Stein and Waters, 2012). Beside FeSODs replace of CuSODs under Cu deficiency, additional microRNAs miR397, miR408, and miR857 were predicted to target in Cu protein plantacyanin and laccase (LAC) copper protein family (Yamasaki et al., 2007a, 2009; Abdel-Ghany and Pilon, 2008). The siRNA/miRNA methyltransferase Hua enhancer (HEN1) mutant hen1-1 plants can’t alter Cu homeostasis and FeSOD protein didn’t accumulate under low Cu supply (Abdel-Ghany and Pilon, 2008). Sucrose induced miR398 expression (Dugas (2012), and miR398 can act as a translational repressor (Dugas and Bartel, 2008). This indicated the regulation of Cu-Fe cross talk was complicated. Fe and Cu cross talk has different layers to regulate included posttranscriptional regulations and miRNA and miRNA interactions.
4.3 SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 7 (SPL7) and copper homeostasis

In the unicellular green algae *Chlamydomonas reinhardtii*, the copper response regulator (CRR1) targets genes of the copper and hypoxia sensing pathways (Merchant *et al.*, 1991, 2006). CRR1 sequences include two candidate metal-binding domains, SBP domain and Cys-rich sequence (Kropat *et al.*, 2005). And it can sense copper and it has multiple layers to regulate CRR1 functions, including metal sensing, transcription factor trafficking and degradation (Rutherford and Bird, 2004; Kropat *et al.*, 2005). The genes targeted by CRR1 also contain GTAC motifs in their promote regions. In plants, the GTAC motifs were frequently found in the promoters of Cu-microRNAs and FeSODs. In *Arabidopsis* the transcription factor SPL7 protein has good sequence similarity to the CRR1 protein (Cardon *et al.*, 1999; Burkhead *et al.*, 2009b).

Under Cu deficient condition, plants minimize the Cu protein utilization through regulating Cu-microRNAs. Cu associated microRNAs that mediate down regulation of *CuSODs*, plastocyanin, and laccase genes, have GTAC motifs in their promoters that transcriptionally interact with SPL7 under Cu deficiency (Fig 1.2). Yamasaki et al. (2009) presented evidence that SPL7 was a central Cu regulator that activated miR398, copper transporters, and Cu chaperone (Yamasaki *et al.*, 2009). On the other hand, the SPL7 SBP domain binds GTAC motifs in other target gene promoters to upregulate Cu uptake and delivery transport (Fig 1.2). For example, *COPT1, COPT2, ZIP2* and *FRO3* were upregulated under Cu deficiency in wild type, but remain low expression in *spl7* mutant. Moreover, *copper chaperone (CCH)* mRNA remains low in *spl7* mutant, and *antioxidant1 (ATX1)* which was constitutively expressed in wild type, was not detected in *spl7*. 
Besides studying single GTAC box interaction with SPL7, a putative GTAC box and Cu regulation didn’t have a correlation (Andrés-Colás et al., 2013). And, the putative Cu deficiency responsive cis regulatory element that contains ≥ 3 GTAC boxes in ≤ 65bp in the 500 bp upstream of the ATG are considered more important regulates Cu deficiency (Andrés-Colás et al., 2013). Genes that include putative Cu deficiency responsive cis regulatory elements in their promoter regions are FeSOD1 (FSD1), miR398b, ZIP2, COPT2, YSL2, CCH, Myb-related transcription factor (LHY), chloroplast-localised calcium sensor (CAS), and component of the SMC5/6 complex (SMC6B). Although response to Cu deficiency, transcriptional regulation of Cu microRNAs and SPL7 functions knowledge have been progressively increased, the picture is far from complete (Peñarrubia et al., 2010a).
References


Ames BN. 2001. DNA damage from micronutrient deficiencies is likely to be a major cause of cancer. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 475, 7–20.


Books:


Fig 1.1 Important Fe and Cu transport genes in *Arabidopsis*. Fe (III)-chelates were reduced before Fe(II) is transported across the plasma membrane (Schmidt, 2003a). ZIP2 transports Cu (II) (Grotz et al., 1998; Wintz et al., 2003) and COPT1 transports Cu (I) (Kampfenkel et al., 1995; Sancenón et al., 2004). FRO4/FRO5 convert Cu (II) to Cu (I). Arrows indicate the directions of genes’ transport (Bernal et al., 2012a). AHA2, H+–ATP2; FRO2, ferric reductase oxidase 2; IRT1, iron-regulated transporter; ZIP2, ZRT-IRT like protein 2; COPT1, copper transporter 1; FRO4/FRO5, ferric reductase oxidase 4,5. Drawing the picture took the references of Schmidt (2003) Fig. 1 and Puig (2007) Fig. 1 (Schmidt, 2003b; Puig et al., 2007).
**Fig 1.2** Model for Cu deficiency in *Arabidopsis*. *SQUAMOSA* promoter binding like protein 7 (SPL7) mediates the transcriptional reaction of the genes involved in Cu transports, interactions and homeostasis. SPL7 binds *SQUAMOSA* to the GTAC motifs on the promoters of the target genes (Yamasaki et al., 2009). Under Cu deficiency SPL7 targets high affinity Cu uptake transports COPT and FeSODs which can substitute CuSODs in chloroplasts (Peñarrubia et al., 2010b). Cu deficiency down-regulated CuSODs is through micro398 to degrade the Cu-miRNA message RNAs, thus degrades CuSODs (Yamasaki et al., 2007b). Drawing the picture took the references of Burkhead (2009) Fig. 5 and Penarrubia (2009) Fig. 2 (Burkhead et al., 2009b; Peñarrubia et al., 2010b).
Chapter 2. QTL mapping of Cu uptake genes that sensing Fe deficiency

Introduction

Essential transition metals Iron (Fe) and copper (Cu) are important nutrients in many physiological processes, such as photosynthesis, respiration and oxidative stress protection. In order to maintain normal biological functions, their homeostasis functions are highly linked. Fe and Cu homeostasis functions require metal transporters, transcription factors, microRNAs and metal homeostasis proteins to regulate plants physiological functions. Fe uptake is tightly regulated by the plant, and gene expression to adjust Fe uptake capacity is altered dynamically depending on internal Fe status (Walker and Connolly, 2008). Cu homeostasis is tightly linked to Fe deficiency responses. Ferric reductase enzyme FRO3 and copper transporter COPT2 were up-regulated by both Fe and Cu deficiency in roots (Yamasaki et al., 2009b). ZRT/IRT-Like protein 2 (ZIP2) was up-regulated under Cu deficiency (Palmer and Guerinot, 2009) and down-regulated under Fe deficiency (Yang et al., 2010; Stein and Waters, 2012). Eight microRNAs were responsive to Fe deficiency and three of them (miR397a, miR398a, and miR398b/c) can regulate transcripts of Cu-containing proteins (Kong and Yang, 2010). Fe deficiency resulted in Cu accumulation, down regulated microRNA (miR397a, miR398a, miR398b/c), and up regulated copper superoxide dismutase (CuSOD) genes respond to prevent damage from superoxide (Stein & Waters, 2012). In Arabidopsis, rosettes increase Cu accumulation under Fe deficiency, and Cu uptake is an important component of the Fe stress response. Fe deficient plants down-regulate Fe containing
FeSOD, and the function of chloroplast-localized CuSOD can be substituted by FeSOD. Thus, the accumulation of Cu by Fe deficient plants is an important adaptation mechanism that helps prevent oxidative stress, and rosette Cu accumulation can be considered a chemical marker for Fe status.

Iron deficient plants have symptoms including chlorotic leaves, less biomass, acidification of the nutrient solution, and smaller roots. Under Fe deficient conditions, *Arabidopsis* increases ferric reductase activity in roots. To reduce ferric Fe (III) to ferrous Fe (II) as part of the reduction strategy of Fe uptake, also ferric reductase enzymes FRO4/FRO5 can reduce Cu (II) (Mukherjee *et al.*, 2006b; Bernal *et al.*, 2012; Pestana *et al.*, 2013). The reduced Fe (II) is transported into the roots by the plasma-membrane divalent cation transporter IRT1 (Eide *et al.*, 1996; Eckhardt *et al.*, 2001). And Fe (II) is solubilized through the H⁺-ATPase AHA2 that is involved in rhizosphere acidification (Santi and Schmidt, 2009). The bHLH transcription factor FIT and its partner bHLH proteins are needed for proper regulation of these genes (Colangelo and Guerinot, 2004; Wang *et al.*, 2007; Yuan *et al.*, 2008).

Previously, Cu accumulation in rosettes under Fe deficiency in *Arabidopsis* was attributed to non-specificity of the Fe uptake system (Mukherjee *et al.*, 2006b; Puig *et al.*, 2007a). Iron and Cu uptake systems share the common gene family FRO (Robinson *et al.*, 1999; Mukherjee *et al.*, 2006a; Puig *et al.*, 2007b). However, recently through time course experiments, it has been elucidated that Cu uptake and accumulation in rosettes had already occurred prior to a measurable up-regulation of ferric chelate reductase activity, depression in bulk Fe concentration, or maximal induction of *FIT*,
Moreover, the altered expression of Cu transporting proteins or Cu homeostasis genes in roots (CCH, OPT3, COPT2, ZIP2), and rosettes (CCS1, OPT3, COPT2) could be part of rosette Fe deficiency response. Thus, it suggests Cu uptake under Fe deficiency is a Fe regulated and specific process that uses machinery separate from the Fe uptake system. Here, we hypothesize that Cu uptake and accumulation under Fe deficiency is the result of a specific Fe regulated Cu uptake system.

*Arabidopsis* Cu concentration is the final result of uptake and translocation of Cu from the rhizosphere, as influenced by the environment and the plant’s own genome (Ryan *et al.*, 2013). Cu uptake and accumulation is tightly regulated by plants (Yruela, 2005a), and the protein polymorphisms very by genotypes may influence Cu uptake gene expression. The genetic differences in Cu uptake gene promoters and proteins may have different effects on the efficiency of Cu acquisition (Yruela, 2005b; Waldron *et al.*, 2009), thus resulting in differences in expression of genes involved in Cu homeostasis (Clemens, 2001; van de Mortel *et al.*, 2006). For example, under Cu deficiency the Cu homeostasis activator SPL7 interacts with genes promoter region through its GTAC motifs can enhance the efficiency of Cu acquisition (Yamasaki *et al.*, 2009a). *Arabidopsis thaliana* is molecularly and genetically well characterized to study genetic factors underlying the natural variations (Shindo *et al.*, 2007; Singh *et al.*, 2013). Most phenotypes and trait variation in nature arise from the combination of several altered genes (Benfey and Mitchell-Olds, 2008). The multigenic effects (one phenotype is caused by multiple genes) can vary depending on the different allelic combinations that
can lead to a continuous variation of trait values (Barton and Keightley, 2002). The QTL mapping approach that we will use in this experiment could pinpoint unknown genes involved in Fe deficiency responses by using natural variation.

Natural variation between *Arabidopsis* ecotypes occurs for rhizosphere acidification (Santi and Schmidt, 2009), root ferric reductase activity (Saleeba and Guerinot, 1995; Stein and Waters, 2012) and Fe regulated gene expression (Yang *et al.*, 2010; Stein and Waters, 2012). *Arabidopsis thaliana* Tsu-1 is from a warm and humid region of Tsushima, Japan and Kas-1 is from cold and dry high elevations of Kashmir, India are two physiologically extreme ecotypes (Juenger *et al.*, 2010). Previous studies have used those two ecotypes to detect candidate genes responsible for physiological differences (McKay *et al.*, 2008) in carbon isotope discrimination (McKay *et al.*, 2003), flowering time (Werner *et al.*, 2005), drought adaptation (McKay *et al.*, 2008), gene expression under drought (Juenger *et al.*, 2010), mineral accumulation (Buescher *et al.*, 2010) and Fe uptake genes expression (Stein and Waters, 2012). Kas-1 and Tsu-1 have showed substantial differences in timing and magnitude of physiological and molecular Fe deficiency responses (Stein and Waters, 2012). After removal of Fe from nutrient solution, Kas-1 exhibited upregulated Fe deficiency responses significantly earlier than Tsu-1, including rapid *FRO2* and *IRT1* gene expression (Waters *et al.*, 2012; Stein and Waters, 2012). In the Kas-1 and Tsu-1 time course experiment, after removing Fe, Kas-1 accumulated Cu rapidly than Tsu-1 in rosettes and the difference of Cu accumulation increased until the 24 hour time point (Fig 2.1). At the 48 time point Tsu-1 catch up with Kas-1 on Cu accumulation in rosettes (Fig 2.1). These differences in timing of response
to Fe deficiency under certain time point can be exploited to understand the factors that regulate plant adaptation to Fe deficiency stress.

In order to get acceptable accuracy and power of QTL detection, a large RIL population (>300 individuals) is necessary for identification of recombination between tightly linked markers (Darvasi et al., 1993; Charmet, 2000; Loudet et al., 2002). Kas-1 and Tsu-1 were used to make a large (302 line) F9 recombinant inbred line (RIL) population (McKay et al., 2008). Copper accumulation in rosettes of RILs at 24 hours removal of Fe was phenotyped. The QTL mapping Kas-1 and Tsu-1 RIL population would reveal one or more unknown Fe regulated Cu uptake genes.

Materials and Methods

Plant materials and growth

Seeds of the *A. thaliana* ecotypes used in this study, Kas-1 (CS28377), Tsu-1 (CS28782), Col-0 (CS28168), mutant *hma5* (SALK_040252C) and mutant *atx1* (SALK_026221C) were obtained from the *Arabidopsis* Biological Resource Center (The Ohio State University). Kas-1 and Tsu-1 recombinant inbred lines (RIL) ordered from *Arabidopsis* Biological Resource center and marker phenotypes were obtained from Dr. John McKay at Colorado State University. For ecotypes Kas-1, Tsu-1 and RILs, seeds were imbibed in 0.1% agar at 4°C for 3d. Seeds were planted onto rockwool loosely packed into 1.5ml centrifuge tubes with the bottoms removed. The tubes were inserted into trays with 48 holes. And the trays with rockwool tubes were set into trays with 3 L nutrient solution, composed of: 0.8 mM KNO$_3$, 0.4mM Ca(NO$_3$)$_2$, 0.3 mM NH$_4$H$_2$PO$_4$, 0.2
33 mM MgSO₄, 25 μM Fe(III)-EDDHA, 25 μM CaCl₂, 25 μM H₃BO₃, 2 μM MnCl₂, 2 μM ZnSO₄, 0.1 μM CuSO₄, 0.5 μM Na₂MoO₄, and 1 mM MES buffer (pH 5.5). Lighting was provided at a photoperiod of 16h of 150umol m⁻² s⁻¹ 4100K fluorescent light (on at 06:00h and off at 22:00h). Each tray had three replications. The parents Kas-1 and Tsu-1 were grown in each tray. Each RIL was grown in three separate trays and rosettes Cu accumulation was measured after Fe deficiency treatment for 24 hours. Data was normalized across experimental blocks using the parental Kas-1 and Tsu-1 common within each block. On the 11th day, the roots grew out from the tubes and the upper trays with seedlings were transferred to deep trays with 11 L of the nutrient solution described above. Seedlings were grown an additional 14 d with constant aeration before Fe deficiency treatment. Then the upper trays with plants were moved to new nutrient solution without Fe (other micronutrient were the same as described above). All treatments were initiated at 14:00pm, which was 8h before the end of the photoperiod. Rosettes were collected after 24 h treatment. In the candidate genes test experiment, four replications of each variety of Col-0, hma5 and atx1’s rosettes were collected at the 0, 8, 16, 24 and 48 time points after switch to treatment. The pretreatment, treatment and Cu measurement were the same as the RIL QTL mapping.

**Mineral measurements:**

Rosettes were dried at 60 °C for at least 72h and weighed to determine the dry weight. Samples were digested with 3ml of concentrated HNO₃ (VWR, West Chester, PA, USA, Trace metal grade) at room temperature overnight then at 100 degree C for 1.5h, followed by addition of 2ml of 30% H₂O₂ (Fisher Scientific, Fair Lawn, NJ, USA) and
digestion for 1 h at 125 °C, and finally heating the samples to dryness at 150 °C. Dried samples were then resuspended in 3ml of 1% HNO$_3$, and Cu was quantified by bathocuproinedisulfonic acid (BCS) protocol. The BCS working solution (to take 10ml as an example) needs 0.015g bathocuproine disulfonic acid and 0.16g ascorbic acid dissolved in 3.5ml water and 6.5ml acetate buffer. The absorbance of resuspend samples and a Cu standard curve were measured at 483 nm. Then 100ul volume BCS working solution was added to each 200ul sample and absorbance at 483 nm was measured again. The first plate’s A483 was subtracted from the final plate’s A483) and μg Cu in samples was calculated using the equation from the standard curve, and divided by dry weight (g) to calculate the concentration in the tissue samples (μg Cu/g DW = ppm).

**Statistical design and analysis:**

The experiment was a Randomized Complete Block Design (RCBD) for experiment design. Totally the 302 RIL lines were grown seven times. Every line had three replicates in three trays separately. Parents Kas-1 and Tsu-1 were grown in each tray. To normalize sample Cu concentration across experimental blocks, the statistical model was $y_{ij} = \mu + \tau_i + bj + \tau bj + e_{ijk}$, where $\mu$ denotes the intercept, $\tau_i$ denotes the mean effect of the $i^{th}$ treatment, $bj$ denotes the block effect of the $j^{th}$ treatment, $\tau bj$ denotes the treatment and block interaction effect, and $e_{ij}$ represents the residual, associated with the $j^{th}$ observation on the $i^{th}$ treatment. Each plant was considered as one experimental unit. The statistical analysis was used to adjust the treatment effects of the seven
growth times through SAS PROC MIXED. The adjusted RIL Cu accumulation were used for QTL mapping.

**QTL mapping and candidate genes collection:**

The Kas-1 and Tsu-1’s RIL population has been genotyped at a moderate density with SSR and SNP markers (McKay *et al.*, 2008). QTL for Cu accumulation was mapped by composite interval mapping using WinQTL CARTOGRAPHER (Wang *et al.*, 2007). This map was used for QTL analysis of Cu accumulation during the 24 hours Fe deficiency. Totally there were 1070 markers, and those markers and positions were obtained from John McKay at Colorado State University. The genetic map spanned approximately 450.651cM of the *Arabidopsis* genome with an average distance between markers of 0.3 cM. A likelihood ratio (LR) significance threshold of P=0.05 was determined for Cu accumulation trait by performing 1000 permutations before mapping. Annotated genes known to be involved with mineral uptake or mineral homeostasis (or family members of such genes that fell within these confidence intervals were considered to be candidate genes (Waters and Grusak, 2008). Moreover, the specific candidate genes were isolated based on their amino acid differences between Kas-1 and Tsu-1. The *Arabidopsis* amino acid sequences are available on 1001 Genomes Project at http://signal.salk.edu/atg1001/3.0/gebrower.php.

**Results**

**Cu accumulation & Dry weight**

It was assumed that as plants grew bigger, it would have more Cu content in rosettes. While if the rosettes weight can influence Cu accumulation or concentration in
rosettes need to know before QTL mapping. By measuring the RIL individual rosettes’ dry weights and their Cu concentration can indicate if the growth rate influence the Cu concentration phenotype. There was no correlation ($R^2=0.005$) between the rosette dry weight and their Cu concentration (Fig 2.1), which indicated the Cu accumulation phenotype was driven by genetics and not growth rate. Rosettes from a geographically diverse panel of recombinant inbred 302 Arabidopsis (Kas-1 × Tsu-1’s KT101-KT259 and Tsu-1 × Kas-1’s TK001-TK203) was screened in an effort to identify altered Cu concentration in rosettes (Fig 2.2). Based on the survey, the rosettes Cu accumulation in Arabidopsis is under genetic control and there was nearly 10-fold difference between the highest and lowest Cu accumulation across RIL of Kas-1 and Tsu-1. By using ecological genotypes Kas-1 and Tsu-1 in each experimental blocks, the normalized Cu concentration varied across the 302 accessions from 5.1 to 50.7 μg/g dry weight (Fig 2.3). 25 RIL plants contained the extreme high side of the Cu distribution (rosettes Cu > 30 μg/g dry weight) and 30 RIL plants contained the extreme low side of the Cu distribution (rosettes Cu < 10 μg/g dry weight). The continuous Cu accumulation from the 5.1μg/g to 50.7μg/g suggest the phenotype was determined by quantitative genetics (Fig 2.3).

**QTL mapping and candidate genes selection**

Since Cu regulation and accumulation under Fe deficiency was a specific Fe regulated Cu uptake system, we phenotype Cu accumulation in RILs under Fe deficiency. Genetic factors determined by the linkage between markers and phenotypes influenced by Cu availability should be indicated by QTL mapping. The distribution of rosettes Cu
accumulation across RILs did not fit a normal distribution (P=0.0145), which suggest a small number of genetic factors regulate Cu accumulation under Fe deficiency across RILs (Fig 2.4). We discovered one significant QTL on chromosome 1 was associated with Fe regulated Cu accumulation in rosettes with a LOD score of 2.82, explained 10% of the variation (Fig 2.5 (a)). The QTL was detected near the SNP marker F5I14 at the position of 79.5cM. With the three replications of the experiment, the interval was from C1_23318972 to C1_25175851 with α=0.01 (Fig 2.5(b)). Four candidate genes were selected within this region (8766 genes in between C1 73.309cm and 81.805cm) based on their biological and molecular functions. Also, their amino acids sequences have differences between Kas-1 and Tsu-1 (Fig 2.6). The four genes were At1g60960 (IRT3, iron regulated transporter3), At1g63440 (HMA5, heavy metal ATPase5), At1g65730 (YSL7, yellow stripe like7), At1g66240 (ATX1, homolog of anti-oxidant1).

**Candidate gene test**

Previously, time course experiment of Kas-1 and Col-0 rosettes Cu concentration reached more than double 24 hours after removal of Fe, whereas Tsu-1 didn’t accumulate Cu until 48 hours (Waters et al., 2012). Here we used hma5 and atx1 mutants which obtained from Col-0 background with T-DNA insertion. The two mutants and Col-0 would be test Cu accumulation in rosette under certain time points after removed Fe from nutrient solution. If the two mutants could accumulate Cu differ from Col-0 at 24 hour time point, then it would gave more evidence that candidate genes account for the Fe regulated Cu uptake. However, in our result, the curves of the three genotypes accumulated Cu didn’t have apparent differences in between each other (Fig
2.7. As a result, the candidate genes HMA5 and ATX1 didn’t result in rosette Cu accumulation in Kas-1 that differ from Tsu-1. Col-0, hma5 and atx1 mutants showed same Cu accumulation in rosettes after moving to Fe deficient nutrient solution at 24 hour time point. At the earlier 8 h time point, Col-0 rosettes accumulated near doubled Cu 10.25μg/g than 0 h time point 6.56μg/g with t test 0.00992 (<0.05). While the mutants’ hma5 and atx1 didn’t have Cu accumulation difference between 8 h time point and 0 h time point. Among the three genotypes, Col-0 rosettes Cu accumulation had the maximum at the 48 h time point 19.7±1.6 uM and the minimum 0 h time point 6.5±1.9. The Col-0 rosettes accumulated most Cu than the two mutants, this indicated Fe deficiency might interact with HMAS and ATX1. At the 24 hour time point, there was no difference among hma5, atx1 and Col-0 on Cu accumulation in rosettes.

**Discussion**

Using QTL mapping on 302 A. thaliana RI population from parent accessions Kas-1 and Tsu-1 and approximately 1070 SSR and SNP markers, we identified one relatively strong peak associated with Fe regulated Cu accumulation in rosettes. The Kas-1 and Tsu-1 RIL population has Cu accumulation in rosettes that range from 8 μg/g to 48 μg/g. QTL mapping allowed us to detect the linkages between Cu accumulation and markers. The most highly associated marker in the peak accounted for 10% of the total variance in the rosette Cu accumulation.

To identify the certain candidate genes from metal uptake genes in the list, we compared the amino acid sequence of the proteins encoded by genes (IRT3, HMA5, YSL7, ATX1) with Kas-1 and Tsu-1 and conducted the time course experiment by testing
certain time points’ Cu accumulation in rosettes after removing Fe from nutrient solution. ZIP family IRT3 functioned in plasma membrane as a Fe and Zn transporter in Arabidopsis (Lin et al., 2009), and it couldn’t respond to Cu excess (Talke et al., 2006). YSL family was hypothesized to transport metals in plants (DiDonato et al., 2004), and YSL7 was supposed to enhance stamen and pollen development (Takahashi et al., 2003; Curie et al., 2009) HMA5 is expressed in roots and strongly and specifically induced by Cu (Andrés-Colás et al., 2006a; Kobayashi et al., 2008), also it influenced Cu accumulation in rosettes. ATX1 Cu chaperones, a specific domain of the CCH Cu chaperone, intact with HMA5 and specifically delivers Cu to heavy metal P-type ATPase (Andrés-Colás et al., 2006a; Puig et al., 2007c). Kas-1 and Tsu-1, extremely different ecotypes, have showed differences in timing of upregulation of ferric reductase activity, FIT and FRO2 gene expression and Cu accumulation under Fe deficiency (Stein and Waters, 2012). This suggests that Kas-1 has genetically determined differences in Fe status perception or signaling compared to Tsu-1. In our result, there was no strong QTL peak (phenotype explained above 10% of the total variance) that demonstrated linkage between Cu accumulation and markers. The missing data of SSR markers might be reason account for the low LOD score. Also the lack of complementary pattern of positive and negative allelic “Fe and Cu crosstalk” effects in between Kas-1 and Tsu-1 may cause the low LOD score. Usually there were multiple allel contribute a QTL. If some allel located at Kas-1 and some allel located at Tsu-1, and those allel didn’t link to each other, it would be hard to see the phenotypic differences between Kas-1 and Tsu-1. Notably behind the main QTL on chromosome one 79.5cM, near 5cM there was a
weak QTL at chromosome one 83.7~85.6cM (Fig 2.4). The weak QTL may reflect the quantitative nature of Cu uptake and accumulation where some genes may have small additive effects or interaction on the process of Cu uptake, Cu and Fe interaction, and Cu accumulation in rosettes. Another reason gave rise to QTLs were not strong was experiments could be there were many genes involved in plant mineral nutrition in gene families with overlapped functions and expression patterns among individual members (Waters and Grusak, 2008).

A gene within QTL confidence intervals is probably not the only determinant of the trait, and there are often multiple genes that affect the trait of interest through individual additive effects or interactions (Flint and Mott, 2001; Tonsor et al., 2005; Mitchell-Olds and Schmitt, 2006). Fe deficiency stimulated Cu accumulation in rosettes, and this process involved multiple gene families to regulate and uptake minerals from rhizosphere into rosettes. For example, the Fe (III) chelate reductase family FRO genes FRO2 (Robinson et al., 1999), Fe (II) transporter family ZIP genes IRT1 and IRT2 (Eide et al., 1996; Vert et al., 2001, 2002), NRAMP genes NRAMP1, NRAMP3, NRAMP4 (Curie et al., 2000; Lanquar et al., 2005) and MATE gene FRD3 (Rogers et al., 2000; Green and Rogers, 2004), Fe-nicotianamine (NA) transporter family OPT genes YSL1, YSL2 (DiDonato et al., 2004; Waters et al., 2006), and Fe regulated transcription factor FIT (Colangelo and Guerinot, 2004; Jakoby et al., 2004) are all Fe homeostasis factors and can respond to Fe deficiency. And Cu homeostasis factors include ZIP family genes ZIP2, ZIP4 (Grotz et al., 1998; Wintz et al., 2003), COPT family genes COPT1, COPT2 (Kampfenkel et al., 1995; Sancenón et al., 2003, 2004), HMA family genes HMA1, HMA5.
(Andrés-Colás et al., 2006a; Boutigny et al., 2014), and ATX family genes CCH, ATX1 (Himelblau et al., 1998; Puig et al., 2007c). Among the Fe and Cu homeostasis genes, single mutant genotypes fit, irt1 results in detectable phenotype. And, most of single-gene knockouts have no detectable mutant phenotype, for example hma5 and atx1. Since HMA5 interacts with Arabidopsis ATX1-like Cu chaperone that is important in Cu transport and detoxification (Andrés-Colás et al., 2006b), the HMA5 and ATX1 genes knock-out double mutant hma5atx1 that from different family members may result in severe mutant phenotype (Hussain et al., 2004; Waters et al., 2006) and might be helpful to detect Fe specific Cu uptake roles.

In the time course experiment, we couldn’t verify that HMA5 or ATX1 resulted in Kas-1 accumulating Cu more rapidly than Tsu-1 at 24 h, thus functioned as Fe deficiency specifically regulated Cu accumulation in rosettes. Because hma5 and atx1 didn’t have different rates of Cu accumulation in rosettes than Col-0 at 24 hour time point. The ATX1-like Cu chaperones have the predominant function as Cu delivery to P-type ATPase (Puig et al., 2007c), and HMA5 involves in Cu translocation from roots to shoots (Kobayashi et al., 2008). While the mechanism of HMA5 and ATX1 responded to Fe deficiency at the earlier hours are unknown. Cu uptake is an important component of the Fe stress response, while single mutants may not contribute to the specific Cu accumulation in rosettes. When transferred from +Fe to –Fe, the Fe uptake mutants irt1, fro2, irt3 and Cu uptake mutants copt2 and fro3 accumulated additional Cu (our unpublished data). Overall, under Fe deficiency Arabidopsis copper accumulation in
rosettes is not a result of lack of specificity of Fe uptake, but is an important adaptation to Fe deficiency.
References


comparative genomic hybridization and gene expression studies of Kas-1 and Tsu-1 accessions of *Arabidopsis thaliana*. Plant, cell & environment **33**, 1268–84.


Waters BM, Armbrust LC. 2013. Optical copper supply is required for normal plant iron deficiency responses. Plant Signaling and Behavior, 8:12, e26611.


Fig 2.1 Cu accumulation increased rapidly in Kas-1 that compared with Tsu-1 on the 24 hour time point. The genetic difference on the 24 hour time point in between Kas-1 and Tsu-1 would be phenotyped by using Kas-1 and Tsu-1 recombinant inbred (RI) population.
Fig 2.2 The Cu concentration (μg/g) in rosettes plotted against dry weight (DW) in Kas-1, Tsu-1 and 302 recombinant inbred (RI) population. Each dot represented one sample. Cu accumulation did not correlate with rosette DW (R²=0.005). Cu accumulation variables and dry weight variables have no relationship. This indicated the phenotype was not driven by growth rate.
Fig 2.3 Rosettes Cu accumulation in Kas-1, Tsu-1 and a total of 302 Kas-1/Tsu-1 RI population were grown in the absence of Fe for 24 hours. 48 varieties were grown in one tray, and each tray contained parents Kas-1 and Tsu-1 as references or indicators. Each RI line had three real replications in each time growing. It took seven times to finish all the RI populations. Block effects and treatment effects would be adjusted based on references Kas-1 and Tsu-1 in each time growing in each tray.
**Fig 2.4** The frequency distribution of rosettes Cu concentration in Kas-1 and Tsu-1’ 302 RI population grown in the absence of Fe nutrient treatment for 24 hours. Horizontal bars represent the ranges of two accessions Kas-1 and Tsu-1 grown in all trays. Over half of varieties have Cu accumulation range from 15~25μg/g. In average Kas-1 had more Cu accumulation in rosettes than Tsu-1’s, while there was no statistical difference between Kas-1 and Tsu-1 Cu accumulation.
Fig 2.5 Chromosome one genetic architecture and predicted physical position of QTL for phenotyping rosettes Cu accumulation in Kas-1/Tsu-1 RI population. (a) Chromosome one’s genetic architecture of the rosette Cu accumulation in the Kas-1/Tsu-1 RI population. The dotted line marked by red triangle indicates LOD score curve’s peak obtained by the CIM method. The percentage of phenotypic variance was 10, and the additive effect of Cu accumulation was indicated from QTL detection through CIM method. Significant threshold for LOD with 95% confidence interval (α=0.05) corresponding to markers’ position was from C1_23318972 to C1_25175851, which refers to genes from AT1G58350.1 to AT1G67260.2. (b) The predicted physical position of QTL on the chromosome one with showing appropriate marker distance. The selected marker position (showed with red triangle) around QTL was indicated on the genetic linkage map of the Kas-1/Tsu-1 RI population and Col-0 physical genomic position. The blue shaded region indicates the position of QTL.
Fig 2.6 Four candidate genes' haplotype structures compared with Kas-1 and Tsu-1. Four genes were selected from the range (AT1G58350.1, AT1G67260.2) by comparing the haplotype structure with parents Kas-1 and Tsu-1 on the Genome Brower on Arabidopsis 1001 genomes. (a) Kas-1 and Tsu-1 have differences of three haplotypes on IRT3 exon. (b) Kas-1 and Tsu-1 have differences of four haplotypes on HMA5 exon. (c) Kas-1 and Tsu-1 have differences of multiple haplotypes on YSL7 exon. (d) Kas-1 and Tsu-1 have differences of one haplotype on ATX1 exon. Under Fe deficiency, the IRT3 can be stimulated and increased Fe transport on the plasma membrane. The copper chaperone ATX1 cooperated with HMA5 enhanced Cu ion binding and involved in Cu detoxification. The metal-nicotianamine transporter YSL7 may also respond to Fe deficiency and enhance the amino acid transport on the plasma membrane. CuAO1 contributed to oxidation-reduction process and nitric oxide (NO) synthesis. And it may also involve in Fe deficiency since NO is a positive signal under Fe deficiency.
Fig 2.7 Time course experiment of ecotype Col-0, mutant hma5 and atx1 Cu accumulation. Four rosettes of each variety were collected at 0, 8, 16, 24, 48 time point after transferred from +Fe to −Fe solution. Error bars stand for the standard deviation.
Chapter 3. RNA sequencing build up Fe/Cu cross talk gene network and iron SOD, copper SOD genes expression

Introduction

Iron (Fe) and copper (Cu) are essential micronutrients for their redox properties. Both metals play critical roles in electron transport; for photosynthesis in chloroplasts, and for respiration in mitochondria. Additionally, Fe is required for sulfate assimilation, nitrogen fixation, and DNA synthesis and repair (Aravind and Koonin, 2001). Cu is involved in cell wall metabolism, ethylene perception, oxidative stress protection and molybdenum cofactor biosynthesis (Kuper et al., 2004).

Fe deficiency is a widespread nutrient imbalance problem since its bioavailability is limited in alkaline and calcareous soils, which comprise one third of the Earth (Chen and Barak, 1982). Plants have developed strategies to acquire Fe ion. In the reduction-based strategy, Fe(III) is reduced before the Fe(II) is transported across the plasma membrane (Schmidt, 2003). Under metal-limiting conditions, reduction-based strategy plant roots use ATPase activity to extrude protons in the rhizosphere to decrease the pH of the soil (Guerinot and Yi, 1994). Also, they increase expression of the metal transporter gene IRT1, and the ferric-chelate reductase gene FRO2 (Robinson et al., 1999; Vert et al., 2002; Sancenón et al., 2004). In Arabidopsis, Fe uptake genes are upregulated by the FIT transcription factor and certain other BASIC HELIX-LOOP-HELIX (bHLH) transcription factors in response to Fe deficiency. FIT interacts with bHLH family lb subgroup proteins and directly regulates target genes FRO2 and IRT1 expression under Fe deficiency (Wang et al., 2007, 2013a). The bHLH gene family lb subgroup has
four members in Arabidopsis: bHLH38, bHLH39, bHLH100, and bHLH101. These four genes seem to have redundant functions for Fe deficiency responses and uptake (Wang et al., 2007, 2013a). Under Fe deficiency, double mutants and triple mutants of bHLH38, bHLH39, bHLH100, bHLH101 genes knock-out have reduced FRO2 and IRT1 gene expression in roots and reduced Fe content in shoots (Wang et al., 2013a).

Overexpressing FIT and bHLH101 (OxFIT/101) together can result in constitutive FRO2 and IRT1 genes expression and increased Fe accumulation in shoots (Wang et al., 2013a).

In strategy I plants, the ethylene production is increased in Fe deficiency stress conditions (Waters and Blevins, 2000; Li et al., 2004). Treatments with ethylene precursors increased ferric reductase activity, and treatments with synthesize ethylene inhibitors decreased ferric reductase activity (Romera and Alcantara, 1994; Robinson et al., 1999; Waters et al., 2007). The ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR (ERF) family in Arabidopsis comprises 122 members in 12 groups, and the biological functions of the majority of these genes are unknown (Moffat et al., 2012). ERFs participate ethylene-activated signaling pathway and intracellular signal transduction (Lorenzo et al., 2003; Zhang et al., 2009), still their signals for Fe stress remain elusive.

Fe and Cu homeostasis are tightly linked. The Cu storage protein Cu chaperone (CCH) is present at phloem-enucleated sieve elements and chloroplast where it functions in cellular Cu ion homeostasis (Himelblau et al., 1998; Mira et al., 2001a,b). An example of Fe and Cu homeostasis is the alternative use of a Fe-containing enzyme or a Cu-containing enzyme to catalyze the same biological reaction in the chloroplast. In
Arabidopsis rosettes, the expression of the FeSOD gene FSD1 and CuSOD genes CSD1 and CSD2 is regulated by Cu supply (Cohu and Pilon, 2007; Buckhout et al., 2009). Low Cu supply induces FSD1 gene expression and CSD1 and CSD2 expression are low, while high Cu increases CSD1 and CSD2 gene expression and FSD1 expression is low (Cohu and Pilon, 2007; Buckhout et al., 2009). In chloroplasts, Fe superoxide dismutase genes (FeSOD) are replaced by Cu superoxide dismutase genes (CuSOD) when Fe is deficient (Abdel-Ghany and Pilon, 2008; Waters et al., 2012). Whereas, CuSOD can be substituted by FeSOD during Cu deficient condition (Puig et al., 2007a). However, the mechanisms of cross talk between ROS-sensing and metal sensing mechanisms are poorly documented in plants so far (Ravet and Pilon, 2013a).

The iron (Fe) and copper (Cu) trade off hypothesis states that if either Fe or Cu is limiting, the other metal will be accumulated to compensate (Waters et al., 2012). Besides the chloroplast alternative use of FeSOD and CuSOD in Arabidopsis, other species also have showed Fe and Cu cross talk. Under Fe deficiency, cucumber (Cucumis sativus) plants grown in Fe deplete solution have higher ferric reductase activity at higher concentrations over a range of Cu supply (Waters and Armbrust, 2013). This suggests that Cu deficiency can stimulate ferric reductase activity. In melon, the fefe mutant lacks normal Fe uptake, and can’t upregulate FIT, FRO1, and IRT under Fe deficiency (Waters et al., 2014). But, when Cu was withheld, the ferric reductase activity was upregulated and so was FRO2 and COPT2 (Waters et al., 2014).

Moreover, Fe and Cu homeostasis can optimize the utilization of scarce metals. In Arabidopsis chloroplast, the alternative use of CuSOD versus FeSOD will benefit the
economic use of Cu. Putative cupric chelate reductases FRO4 and FRO5 are required for high affinity Cu uptake under Cu deficiency in Arabidopsis roots, in a SPL7 dependent manner (Bernal et al., 2012b). While ZINC-REGULATED TRANSPORTER IRON-REGULATED TRANSPORTER PROTEIN2 (ZIP2) was involved in Cu transport (Wintz et al., 2003), it was down regulated under Fe deficiency (Yang et al., 2010; Stein and Waters, 2012). COPPER TRANSPORTER2 (COPT2) expression has synergic up-regulated expression in response to Fe and Cu deficiency in roots (Perea-García et al., 2013), while it is down regulated by Fe deficiency in rosettes (Sancenón et al., 2003a). This up-regulated in roots and down-regulated in rosettes under Fe deficiency may reflect Cu accumulation in rosettes (Yamasaki et al., 2009; del Pozo et al., 2010; Waters et al., 2012).

Under Cu deficiency, Cu microRNAs (miRNAs) downregulate the abundance of target Cu dependent proteins through cleavage of target transcripts (Yamasaki et al., 2007, 2009; Abdel-Ghany and Pilon, 2008). MiR397a, miR398a and miR398b/c regulate Cu containing proteins (Yamasaki et al., 2007), and these three microRNAs were down regulated by Fe deficiency (Waters et al., 2012). MiRNA398 can mediate down-regulation of CuSOD and plastocyanin when Cu is deficient, so that CuSODs were hardly detected and FeSODs accumulated in the chloroplast (Yamasaki et al., 2007; Abdel-Ghany and Pilon, 2008). Additional microRNAs that respond to Cu; miR397, miR408, and miR857, target plantacyanin and the laccase (LAC) copper protein family (Yamasaki et al., 2007, 2009; Abdel-Ghany and Pilon, 2008). CSD2 is regulated at the post-transcriptional level rather than the transcriptional level, through miRNA398 (Yamasaki et al., 2007). Also miR398 can regulate CSD1 and Cu chaperone for superoxide
dismutase (CCS1) under Cu deficiency, where its mRNA cleavage is induced by miR398 (Sunkar et al., 2006; Beauclair et al., 2010; Mendoza-Soto et al., 2012).

In Arabidopsis, the uptake and use of Cu is dependent on the transcriptional regulator SQUAMOSA PROMOTER BINDING PROTEIN-LIKE7 (SPL7). Under Cu stress, SPL7 directly binds GTAC motifs in the promoters of miR398 and downregulated copper transporters and Cu chaperone (Yamasaki et al., 2009). Genes for Cu uptake and transport, for example COPPER TRANSPORTER COPT1, COPT2, FRO3, YELLOW STRIPE-LIKE2 (YSL2) and ZIP2 were upregulated under Cu deficiency in wild type, but not in spl7 mutant (Wintz et al., 2003; Mukherjee et al., 2006a; Yamasaki et al., 2009). Moreover, the metallochaperone COPPER CHAPERONE (CCH) mRNA remains low in the spl7 mutant, and Heavy Metal Transport Protein antioxidant1 (ATX1) expression was not detected in spl7, while it was constitutively expressed in wild type (Burkhead et al., 2009; Shin et al., 2012). Although knowledge of response to Cu deficiency and transcriptional regulation of Cu microRNAs and SPL7 functions have been progressively increased, the picture is far from complete (Peñarrubia et al., 2010).

It is clear that either Fe or Cu deficiency can influence uptake of the other metal, but the signals and genes involved are not known. The normal Cu uptake system (0.5 μM Cu) does not function in the spl7 mutant (Fig 3.1). The spl7 mutant has less Cu content, loss of ferroxidase (catalyzes oxidization of Fe(III) to Fe(II)) activity and has a defect in root-to-shoot Fe movement under Cu deficiency (Bernal et al., 2012b). Under normal Cu and Fe condition (0.5 μM Cu and 25 μM Fe), spl7 mutant has only half rosette mass than Col-0, one third Cu content than Col-0 and less Fe content in rosette
compared with Col-0 (Fig 3.1). Given Fe deficient (0.5 μM Fe), the spl7 mutant can increase Cu uptake and increase Cu accumulation (in a sense of content, not the concentration) in rosettes (Fig 3.2). This result suggests that Cu uptake under Fe deficiency is independent of the normal Cu uptake system. Thus, the some metal transporter gene that has different expression in spl7 and Col-0 under Fe deficiency might be responsible for Cu uptake. We would like to detect the genes that are regulated by SPL7 or independent of normal Cu uptake in spl7, especially the metal transporters, and transcriptional factors related to Fe and Cu regulation. Other treatments (- Cu, -Fe-Cu) will help to define the SPL7-dependent and independent genes. The combination of the results in Col-0 and spl7 will provide a catalog of genes regulated by Fe deficiency, without confounding effect of secondary Cu deficiency effects in spl7 mutants, because spl7 was grown in high Cu conditions to rescue its growth prior to treatments. Moreover, using spl7 mutant could uncover new synergistic interactions between Fe deficiency, Cu deficiency, and simultaneous Fe and Cu deficiency in metal uptake, interaction, homeostasis processes.

Our results present a genome-wide analysis of the transcriptional profiling to reveal Fe/Cu cross-talk gene lists, based on the Illumina HiSeq technology. The use of spl7 Fe and Cu deficiency conditions addressed the research hypotheses: The uptake of Cu under Fe deficiency is independent of the normal Cu uptake system; and Cu deficiency can cause the secondary physiological Fe regulation that is dependent on SPL7, as spl7 had higher Fe accumulation than Col-0 if didn’t supply adequate Cu (Fig 3.2). We identified 3072 genes that had at least two-fold changes of either upregulated
or downregulated upon Fe deficiency, Cu deficiency, Fe and Cu deficiencies. Col-0 and spl7 showed different patterns of classic bHLH family genes bHLH38, bHLH39, bHLH100, bHLH101. In our results, the bHLHs didn’t respond to Cu deficiency in roots in spl7 while they were downregulated in Col-0. And spl7 mutant upregulated bHLHs under simultaneous Fe and Cu deficiencies, whereas Col-0 downregulated those genes.

Further, we measured rosettes Cu and Fe concentration in triple mutant bhlh39bhlh100bhlh101 and Col-0. Several ERFs were downregulated in Col-0, not in spl7. The Col-0/spl7 transcriptional profiling would give significant insight into Fe and Cu homeostasis.

**Materials and methods**

**Plant growth and materials**

Seeds of *Arabidopsis thaliana* ecotype Col-0 and mutant spl7 (SALK_109908c) were obtained from the *Arabidopsis* Biological Resource Center (The Ohio State University). The triple knock out mutant bHLH39, bHLH100, bHLH101 (k39k100k101) was obtained from Dr. Hong-Qing Ling, Beijing, China. Seeds were imbibed in 0.1% agar at 4°C for 3d. Seeds were planted onto rockwool loosely packed into 1.5ml centrifuge tubes with the bottoms removed. The tubes were inserted in trays in containers of nutrient solution, composed of: 0.8 mM KNO₃, 0.4mM Ca(NO₃)₂, 0.3 mM NH₄H₂PO₄, 0.2 mM MgSO₄, 25 μM Fe(III)-EDDHA, 25 μM CaCl₂, 25 μM H₃BO₃, 2 μM MnCl₂, 2 μM ZnSO₄, 0.1 μM CuSO₄, 0.5 μM Na₂MoO₄, and 1 mM MES buffer (pH 5.5). Lighting was provided at a photoperiod of 16h of 150umol m⁻² s⁻¹ 4100K fluorescent light (on at 06:00h and off at 22:00h). After 10d, seedlings and the tubes were transferred to wells
in lids of containers containing 0.75 liters of the same nutrient solution with constant aeration for an additional 14 before plants were transferred to treatments. For spl7 mutant and wild type Col-0 RNA sequencing experiment, and bHLH triple mutant \textit{k39k100k101} along with Col-0 ICP-MS experiment, normal pretreatment of Col-0 and spl7 were 25uM Fe and 0.1uM Cu, and “rescue” pretreatment for spl7 was 25 uM Fe and 2.5 uM Cu. The normal spl7 plants are only half the size of Col-0 unless they are “rescued”. The pretreatment lasted 14 days. In the spl7 RNA-seq experiment, the plants were switched to four treatments: 25 uM Fe, 0.5 uM Cu (control); 25 uM Fe, 0 Cu; 0 Fe, 0.5 uM Cu; 0 Fe, 0 Cu for the last 3 days. The remaining micronutrients were at the same concentrations described above. Shoots and roots were collected into liquid nitrogen from different containers for biological replications. The triple mutant \textit{k39k100k101} and Col-0 had six treatments for 14 days: 0 Cu, 0.5 uM Fe; 0 Cu 25 uM Fe; 0.5 uM Cu, 0.5 uM Fe; 0.5 uM Cu, 25 uM Fe; 2 uM Cu, 0.5 uM Fe; and 2 uM Cu, 25 uM Fe. Rosettes were collected from four containers for each nutrient treatment, and measured Cu and Fe concentration through ICP-MS in future.

For RT-PCR experiment, Col-0 seeds were grown for the first 10 days in the nutrient solution described above, except used 0.5 uM Cu instead of 0.1 uM Cu. Then seedlings were transferred into 0.75 liter containers with 10 treatments for 11 days. The 10 treatments included two Fe concentrations (0.5 uM and 25 uM) and five Cu concentrations (0, 0.1 uM, 0.5 uM, 1 uM and 2 uM). Then rosettes were collected into liquid nitrogen for isolation of RNA for RT-PCR.

\textit{Experimental design and Statistical analysis}
All three experiments were factorial treatment design. Col-0 and spl7 rescue had four nutrient treatments and spl7 normal was grown only in the control treatment. Each plant was considered as one experimental unit. Three samples of each genotype were collected from three separate containers for each treatment. Similarly the triple mutant k39k100k101, Col-0 ICP-MS experiment was conducted with the same treatments, but each container had four experimental units for each genotype, and there were four containers of each treatment. For the Col-0 RT-PCR experiment, 10 treatments were applied with three replications. The statistical analysis used SAS PROC GLIMMIX procedure to test least square mean for each genotype under three treatments.

**Mineral analysis**

Rosettes were dried at 60 °C for at least 72h before determining dry weight. Samples were digested with 3ml of concentrated HNO$_3$ (VWR, West Chester, PA, USA, Trace metal grade) at room temperature overnight then at 100 degree Celcius for 1.5h, followed by addition of 2ml of 30% H$_2$O$_2$ (Fisher Scientific, Fair Lawn, NJ, USA) and digestion for 1 h at 125 °C, and finally heating the samples to dryness at 150 °C. Dried samples were then resuspended in 3ml of 1% HNO$_3$. Fe and Cu concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS).

**Real-time reverse transcriptase-PCR**

Total RNA was extracted from shoots and roots (RNA-seq experiment), or shoots (Col-0 RT-PCR experiment) using the RNeasy Plant Kit (Qiagen, Chatsworth, CA, USA). RNA quality and concentration were determined by UV spectrophotometry. DNase (RNase-free DNase I, New England Biolabs, Ipswich, MA, USA) treated RNA (1ug) was
used for cDNA synthesis, using the High Capacity cDNA Reverse Transcription kit (ABI, Foster City, CA, USA) with random hexamers at 2.5 uM final concentration. cDNA corresponding to 10 ng of total RNA was used in a 15 ul real-time PCR reaction performed in a MyiQ (Bio-Rad, Hercules, CA, USA) thermal cycler using SYBR GreenER qPCR SuperMix (Invitrogen Technology, Carlsbad, CA, USA) and 10 uM gene specific primers (see below). The following standard thermal profile was used for all PCRs: 50 ° C for 2 min, 95 ° C for 8 min, followed by 40 cycles of 95 ° C for 15 s and 60 ° C for 30 s. At the end of each reaction, a dissociation curve was performed to ensure primer specificity. The Ct values for all genes were calculated using BioRad IQ5 System Software version 2.0 (BioRAD, Hercules, CA, USA) and normalized to the Ct value of ubiquitin (UBQ10) using the equation $Y = 2^{-\Delta Ct}$, where $\Delta Ct = \frac{1}{4} \text{CtUBQ10} - \text{Ct target gene}$. 

**RNA sequencing and bioinformatics**

Total RNA was isolated from shoots and roots using the RNeasy Plant Kit (Qiagen, Chatsworth, CA, USA). RNA-seq was performed using an Illumina HiSeq 2500 instrument for 100bp paired end read at the University of Nebraska Medical Center. Reads were mapped to the transcriptome and normalized by Dr. Chi Zhang, UNL Department of Biological Sciences. The lists of genes’ short names and descriptions were checked on the PANTHER – Gene List Analysis.

**Results**

**Transcriptomic characterization of Fe and Cu cross talk**

**Under Fe deficiency**
The Fe deficiency treatment on Col-0 and spl7 mutant will facilitate identification of the Fe deficiency regulated Cu uptake system or how Fe deficiency plants uptake Cu. The spl7 mutant can’t uptake Cu with the normal Cu uptake system, while when made Fe deficient it can take up more Cu (Fig 3.2(c)). Therefore the Fe-deficiency stimulated Cu uptake system is independent of spl7 and is not the normal Cu uptake pathway. Under Fe deficiency, we should be able to find upregulated and downregulated genes in both spl7 and Col under Fe deficiency that are responsible for Cu uptake (Fig 3.3). The upregulated common genes in roots (176) and shoots (55) were bHLH38, bHLH39, bHLH100, bHLH101 and IRT1. The downregulated genes in roots (26) has ZIP3, and in shoots (24) have FER1, FER3, FER4, YSL1, VTL1, and VTL2. The blue copper protein (BCB) gene was found in the upregulated genes in roots (176), while it’s not clear how the Fe deficiency regulate Cu protein accumulation BCB in roots.

**Under Cu deficiency**

Under Cu deficiency, genes that were regulated in WT but were not regulated in spl7 mutants may be SPL7 regulatory targets (Fig 3.4). In roots, genes that were upregulated only in WT (29 genes) included FSD1, FRO4, FRO5 and COPT2, known SPL7 dependent targets (Table 1). While bHLH38, bHLH39, bHLH100, bHLH101 were downregulated under Cu deficiency in Col-0 roots, which was not seen in literature before. And if those four genes were related to SPL7 function is unknown. Under Cu deficiency in shoot, three copper proteins’ coding genes; BCB, CSD1 and CCS, were downregulated in WT, while not in the spl7 mutant.

**Under Fe and Cu simultaneous deficiency**
In root, under Fe and Cu simultaneous deficiency we were interested in Fe and Cu co-regulated SPL7 targets (upregulated 85, downregulated 183) and opposite regulation (27) between two genotypes (Fig 3.5 (a)). In shoot, under Fe and Cu simultaneous deficiency we were interested in Fe and Cu co-regulated SPL7 targets (44, 443) and opposite regulation (49) between two genotypes (Fig 3.5 (b)). 10 ethylene response factors were downregulated in WT. This may suggest SPL7 downregulated ERF though a transcriptional repressor or miRNA to limit Fe and Cu consumption in shoot.

**Summary of RNA-seq genes related to Fe and Cu cross talk**

To elucidate the important genes (those that have different fold changes in between spl7 and Col-0) allow us to track the previous study and add something new. We selected key metal transporter genes, Cu storage genes, Fe homeostasis genes, and important unknown genes related to Cu and Fe crosstalk to analyze. All the genes are presented as fold change in the Table 3.2, Table 3.3, and Table 3.4.

**The key metal transporter genes**

As the spl7 mutant has a lack of normal Cu uptake, Cu uptake under Fe deficiency is independent of key Cu homeostasis gene SPL7. Second, the spl7 mutant can be rescued by extra Cu (2.5uM) and normal Fe (25uM) supply so that spl7 mutants have the same Cu and Fe accumulation in rosettes as wild type Col-0. If the same metal uptake genes have different responses in the two genotypes system would be compared.

The SPL7 transcription factor interacted with the “GTAC” motif in promoter regions or target genes to regulate gene expression in Cu homeostasis (Yamasaki et al.,
The recurrence of “GTAC” motif boxes (≥3) in short regions of ≤ 65 bp may seen as the potential cis regulatory elements (Andrés-Colás et al., 2013). Genes containing “GTAC” motif were FRO4 (five), FRO5 (eleven), COPT2 (four), and ZIP2 (five) (Bernal et al., 2012b; Andrés-Colás et al., 2013). These four genes showed upregulated fold change under Cu deficiency in Col-0 roots, while they were not upregulated in the spl7 mutant (Table 3.2). This result is consistent with the previous study and give strong evidence that “GTAC” motif closely relate to Cu homeostasis theory. ZINC TRANSPORTER 8 PRECURSOR (ZIP8) had an opposite expression in Col-0 roots and spl7 roots. ZIP8 showed upregulation under Fe deficiency (Table 3.2) in Col-0. Interestingly, ZIP8 was down-regulated under Cu deficiency and simultaneous Fe and Cu deficiency, which was in contrast with spl7. The Fe homeostasis four bHLH family genes bHLH38, bHLH39, bHLH100, bHLH101 showed similar gene expression patterns to each other. They all have opposite expression pattern between Col-0 and spl7 in roots under -Fe-Cu conditions (Table 3.5). Also, they were downregulated under Cu deficiency in Col-0, and didn’t respond to Cu deficiency in spl7.

Cu/Fe storage and homeostasis genes

In Col-0, CCH was upregulated in roots under Cu deficiency and downregulated in shoots under Fe deficiency (Table 3.3). While in spl7, CCH was not significantly differentially regulated in either condition. Cu chaperone for SOD1 (CCS) was downregulated under Cu deficiency in Col-0 shoots, but not in spl7. This suggest that lack of Cu uptake in spl7 might influence CCH and CCS regulation. FSD1 was down-regulated under Fe deficiency and up-regulated under Cu deficiency in Col-0 (Table 3.3),
while in spl7 FSD1 was not downregulated. This result might reflect the higher Fe accumulation in spl7 shoots than the wild types’ (Fig 3.2). CSD1 and CSD2 were downregulated under Cu deficiency in Col-0 shoots, but not in spl7 shoots (Table 3.3). This indicated the spl7 mutant has faulty Cu homeostasis and can’t flexibly switch FeSOD and CuSOD like Col-0. Ferritin was regulated at transcriptional level and post-transcriptional level by Fe (Briat and Lobreaux, 1997; Thomine et al., 2011; Ravet and Pilon, 2013b). Both Col-0 and spl7 down-regulated FER1, FER3 and FER4 under Fe deficiency, while spl7 showed much stronger downregulation than the Col-0 (Table 3.3). Moreover spl7 can down-regulate FER genes under -Fe,-Cu condition. This may reflect the extra Fe accumulation in spl7 rosettes in nature when given the same Fe concentration (Fig 3.2).

**The ERF and large fold change in unknown genes in term of Fe and Cu signaling**

In the Venn diagram analysis, the *Ethylene Responsive Element Binding Factor* (ERF) genes have got our attention. Because of ethylene would has increased production under Fe deficiency and ERF transcription factors can act as activate or repress roles to ethylene-response genes (Lynch and Brown, 1997; Wang et al., 2013c; Thirugnanasambantham et al., 2015). *ERF* family encode transcriptional regulators with variety functions have involved in physiological and developmental processes in plants (Nakano et al., 2006). While in our RNA-seq results, we see *ERF5, ERF6, ERF11, ERF14, ERF104, ERF109* were down-regulated in Col-0 shoots under Fe deficiency and Fe, Cu deficiencies, not in the spl7 shoots (Table 3.4). This opposite regulation led us to think of the ERF may relate to Fe and Cu crosstalk.
The three unknown genes showed in Table 3.4 were annotated as unknown based on unknown functions, and unknown biological process. AT1G47395 located in endomembrane, AT2G30766 located unknown, and AT2G14247 located in chloroplast. The three unknown genes were highly induced in spl7 shoots under Fe, Cu deficiencies and were downregulated in shoots under Cu deficiency in Col-0. Also we discovered five transposons (TE) that have consist expression in Col-0 -Fe, -Fe-Cu treatments and in spl7 mutant -Fe treatment in root and shoot (Table 3.4). We may assume spl7 knock out can alter DNA in the genome in other chromosomes through transposons.

**RT-qPCR of FeSODs, CuSODs and COPT2**

We used real-time PCR to measure expression of the *Arabidopsis* rosette *FSD1*, *FSD2*, *CSD1*, *CSD2* to determine FeSOD and CuSOD regulation by both Fe and Cu; and *COPT2* genes to determine if Fe and Cu deficiencies acted synergistically. Overall, *FSD1* has higher expression at normal Fe (25uM) supply than in the Fe deficient condition (0.5uM) at all quantities of Cu supply (Fig 3.6). And at the normal Cu 0.5 μM and high Cu supply of 1 μM and 2 μM, *FSD1* under normal Fe condition was upregulated much more than in the Fe deficient condition. This indicated that *FSD1* was regulated by Fe status strongly. Moreover, *Arabidopsis* rosettes use Fe primarily as SOD composition and they use Cu frugally (Puig *et al.*, 2007b; Ravet and Pilon, 2013b). While, in the very low Cu conditions, *FSD1* was upregulated higher than in the replete Cu conditions. This indicated *FSD1* was downregulated by Fe deficiency and upregulated by low Cu supply.

At the very low Cu to normal Cu supply (0, 0.1 μM and 0.5 μM), there was no big difference in *FSD2* expression between different Fe supply (Fig 3.6). Notably, at 1 μM Cu
supply, \textit{FSD2} was upregulated under the Fe deficient condition. This may be one of responses when \textit{Arabidopsis} rosettes have Cu toxicity. Overall, \textit{FSD2} was regulated by Fe at 1 \(\mu\)M Cu concentration and wasn’t regulated by Cu. The \textit{CSD1} expression depended upon the Cu supply in the two Fe conditions. \textit{CSD1} was upregulated by 0.5 \(\mu\)M Fe under 0, 0.1 \(\mu\)M Cu conditions. Similar to \textit{CSD1}, \textit{CSD2} was upregulated by low Fe supply at 0, 0.1 \(\mu\)M and 0.5 \(\mu\)M Cu. And under high Cu supply, especially 2.0 \(\mu\)M Cu, \textit{CSD2} was higher under normal Fe than in the Fe deficient condition. This indicated \textit{Arabidopsis} rosettes might have difficulties to utilize CuSODs under Cu toxicity conditions. Except the 0 Cu condition, there was no big difference between \textit{COPT2} expressions under different Fe supply (Fig 3.6). Under 0 Cu condition, \textit{COPT2} was upregulated under Fe deficiency. Thus, \textit{COPT2} gene expression was upregulated by Fe and Cu deficiency simultaneously induced through the synergistic regulation.

\textit{Triple mutant k39k100k101 and Col-0 Fe and Cu concentration test}

In the \textit{spl7} RNA-seq results, \textit{bHLH38}, \textit{bHLH39}, \textit{bHLH100} and \textit{bHLH101} showed downregulation under Cu deficiency in Col-0, while not in the \textit{spl7} mutant, suggesting that in roots the four \textit{bHLHs} can respond to Cu supply. Whether this response reflects shoots Cu accumulation was unknown. There were six treatments in terms of multiple Cu and Fe conditions (Fig 2.7). There were treatments’ differences for Cu accumulation. While there was no difference of Fe accumulation between any two treatments. Both genotypes accumulated more Cu in rosettes under low Fe, high Cu (0.5 \(\mu\)M Fe, 2 \(\mu\)M Cu) compared to none Cu toxicity conditions. Under the low Cu condition, for Col-0 Fe
deficiency plants can accumulate more Fe than the Fe replete condition while their Cu accumulation were similar (Fig 3.7b Fig 3.7c) left two bars.

**Discussion**

The main objective of spl7 and Col-0 RNA-seq analysis was to understand spl7 mutant specific metal regulation and how it influences Fe and Cu cross talk. Here we compared under Cu deficiency the specific SPL7 upregulated genes with two previous publications (Table 3.1); also, showed spl7 specific up-regulated genes under Fe deficiency which were not observed in the Cu deficiency condition (Fig 3.3). Also, the genes that were not upregulated in spl7 may be part of an undefined Cu uptake pathway. Thus SPL7 as the important Cu homeostasis transcription factor functions not only has effects on Cu homeostasis genes, but also influence Fe uptake and Fe Cu cross talk (Table 3.2, Table 3.3, and Table 3.4). Moreover, we showed that FeSOD is regulated by both Fe and Cu. And, additional Cu is needed in Fe deficiency plants, also CuSODs are regulated by Fe directly which is not just by Cu (Fig 3.6, Fig 3.8).

*Knock out SPL7 stimulates genes’ upregulation in roots and shoots*

Under Fe deficiency the ethylene upregulation was related to Fe acquisition genes, known as iron deficiency-induced ethylene-dependent (IDED) genes, for example, *FIT, FRO2* and *IRT1* (Lucena et al., 2007). While IDED genes that were induced by Fe deficiency also were negatively affected by ethylene inhibitors, like Co and STS (Garcia et al., 2010). IDED genes included *FIT, FRO2, IRT1, bHLH38, bHLH39*, et al. nineteen genes (Garcia et al., 2010) and in our result fifteen IDED genes have upregulation under Fe deficiency (Table 6). Previously, In Col-0 ERF family *ERF5, ERF6,
ERF11, ERF14, ERF104, ERF109 were down-regulated in shoots under Fe deficiency and Cu Fe deficiency, while they were not downregulated in the spl7 shoots. This suggest those ERF genes may have an interaction with SPL7 as the DNA-binding repressors. When SPL7 is present and without corresponding inductive substances, ERF can bind to target promoters and block the RNA polymerase to the promoters. Then the downstream genes can’t be regulated. Given the SPL7 knock out, spl7 mutant up-regulated transposons and the transposons may interact with ERF promoter regions under the certain conditions and prevent ERF bind to the operators. Thus a much more genes can be up-regulated in spl7 mutant.

Fe and Cu homeostasis genes regulation in spl7 mutant under Fe, Cu, Fe Cu deficiency

In Arabidopsis wild type the transcription factor bHLH38, bHLH39, bHLH100, bHLH101 interact with FIT to regulate downstream genes such as FRO2 and IRT1 (Yuan et al., 2008; Sivitz et al., 2012; Wang et al., 2013b). It seemed that SPL7 transcription factor did influence the transcript level of subgroup Ib bHLHs because in the spl7 mutant under Fe deficiency those bHLHs were up-regulated more strongly than in Col-0, both in roots and shoots. Interestingly, bHLH100 showed a big upregulation under Cu deficiency in spl7 rescue while not in the Col-0. It seemed that the upregulation level of bHLH100, bHLH101 can reflect Fe accumulation status in rosettes as spl7 can accumulate much more Fe in rosettes than Col-0 (Fig 3.2). To conduct a follow up experiment to determine Fe and Cu regulation on the bHLH genes, bHLH39, bHLH100 and bHLH101 knock out triple mutant k39k100k101 and wild type Col-0 were grown under multiple Fe and Cu conditions (Fig 3.7). This indicated that not only can Cu deficiency influence
bHLHs genes regulation in roots, but can make a difference in Cu accumulation in rosettes.

Compared with Col-0, the spl7 mutant had altered regulation of metal transporter genes. Under Cu deficiency, the FRO4, FRO5, COPT2 and ZIP2 that were up-regulated in the Col-0 roots were not regulated in the spl7. Cu deficiency marker FRO4 and FRO5 that have showed dependent of Fe homeostasis (regulated by FIT) in a SPL7 dependent manner in roots (Bernal et al., 2012b). While in shoots FRO4 is not SPL7 dependent, FRO4 and FRO5 reduce Cu (II) to Cu (I) before its cellular uptake in the shoots (Wu et al., 2005; Mukherjee et al., 2006b; Yamasaki et al., 2009). Besides the major reductase role of FRO4 and FRO5, the Cu (II) transporter the ZIP2 also assist in the Cu uptake process (Wintz et al., 2003; Puig et al., 2007c; Burkhead et al., 2009; Yamasaki et al., 2009). It might be also dependent on SPL7 as its promoter region contains GTAC motifs (Andrés-Colás et al., 2013). The high affinity Cu transporter COPT2 is up-regulated in roots by both Fe and Cu deficiencies (Sancenón et al., 2003b; Colangelo and Guerinot, 2004; Waters et al., 2012). In spl7, we also observed COPT2 was up-regulated by both Fe and Cu deficiencies in roots. Thus, the SPL7 regulatory GTAC element in its promoter region cannot be the only standard to determine if a gene is regulated by Cu.

**Fe deficiency, Cu deficiency and Fe, Cu deficiencies effect on metal homeostasis proteins**

The spl7 mutant and Col-0 RNA-seq experiment provides new insights into Fe and Cu cross talk. First the trade-off Fe and Cu hypothesis (Fig 3.8) has demonstrated that Cu deficiency led to FeSOD genes upregulation by SPL7 and CuSOD genes
downregulation through degradation mediated by miR398, and this can be understood of FeSODs replaced of CuSODs to maintain SOD functions (Waters et al., 2002; Yamasaki et al., 2007; Abdel-Ghany and Pilon, 2008; Bernal et al., 2012b). Besides, the FSD1 was regulated by both Fe and Cu, the CuSODs are regulated by Fe directly, not just by Cu. Under Cu deplete conditions, the CuSODs were up-regulated under Fe deficiency more than the Fe replete condition. Under Cu replete conditions, Fe replete condition can up-regulated CuSODs. These evidences suggest chloroplasts use Fe and Cu in a balanced way to avoid metal toxicity. AT4G31940 (Cytochrome P450) involved in oxidation reduction was down-regulated under Fe and Cu deficiencies in Col-0, while was up-regulated in spl7. This indicated Col-0 and spl7 rescue mutant might have different oxidative stresses even under the equal size and the same nutrient condition.

In Arabidopsis, Fe storage in vacuole is an important emerging component of Fe homeostasis, and Nramp4 is one of the important Fe transporters in vacuole (Lanquar et al., 2005; Kim et al., 2006). Nramp4 transporter takes an important role in Fe storage and mobilization, also it has showed upregulation under Fe deficiency in both shoots and roots in spl7 mutant. In the recent studies, ferritin regulation has showed a tight interaction with the efficient Fe usage and with the Fe mediated oxidative stress protection (Arnaud et al., 2006; Ravet et al., 2009, 2011). Ferritin was regulated by Fe and H$_2$O$_2$ through both transcriptional and post transcriptional levels (Ravet and Pilon, 2013c). Under Fe deficiency in shoots, spl7 mutant was down-regulated FER1, FER3 and FER4 genes much stronger than it was in the Col-0. As ferritin functions in the ROS-
detoxifying system, the strongly down-regulated \textit{FER} genes under Fe deficiency might reflect the less severe the \textit{spl7} rescue mutant was when compared with Col-0.
References


Shin L-J, Lo J-C, Yeh K-C. 2012. Copper Chaperone Antioxidant Protein1 Is Essential for Copper Homeostasis. PLANT PHYSIOLOGY 159, 1099–1110.


Wild type Col-0 and mutant spl7 grew on multiple Fe and Cu nutrient solutions. The normal condition 25 uM Fe and 0.5 uM Cu condition (showed in yellow box), spl7 had only half size of Col-0, and had 34% lower Cu concentration and 49% lower DW than Col-0 (see Fig 3.2). While spl7 can be rescued by either higher Cu, normal Fe condition or lower Fe, normal Cu condition supply. In the red box, Col-0 and spl7 have same rosettes mass, and Cu, Fe accumulation in rosettes (see Fig 3.2), which indicated 2.5 uM Cu was adequate to restore growth. When decreasing Fe concentration to 5 uM, spl7 fully restored growth (showed in blue box).
Fig 3.2 Col-0 and spl7 rosettes dry weight (DW), Fe and Cu concentration under multiple Fe and Cu concentrations’ treatments. (a). With the lower Cu 0.1 uM, Col-0 plants have grown much bigger than the spl7 mutants. While spl7 plants can increased dry weight as decreasing Fe supply. Under the normal Fe 25 uM and Cu 0.5 uM supply, spl7 has only half dry weight than the Col-0. Decreased Fe supply (25 uM to 5 uM) can rescue spl7 growth. Given extra Cu supply spl7 plants’ DW was as big as Col-0’s. (b). With the 0.1 uM Cu, Col-0’s Fe accumulation was depended on Fe supply, while not for spl7. Under 0.5/25 uM Cu/Fe supply, spl7 can accumulate more Fe than Col-0. In the Cu rescue condition, they accumulated same Fe. (c). Since spl7 was a Cu uptake/homeostasis defect genotype, given the normal Cu 0.5 uM Col-0 accumulated more Cu
in rosettes than the spl7 did. Values were the means plus SD of at least five biological replicates. The symbol "*" and "**" above the bars represent significant differences (P < 0.05).
Fig 3.3 Venn diagrams for Fe regulated genes in wild type Col-0 and mutant spl7. Genes of interest are shown in the appropriate set or overlap of sets. (a) In roots two genotypes upregulated/downregulated genes under Fe deficiency. (b) In shoots two genotypes upregulated/downregulated genes under Fe deficiency. (WT_dn_-Fe is short for wild type downregulated genes under Fe deficiency; spl7r_dn_-Fe is short for spl7 rescue downregulated genes under Fe deficiency, the rest shorts are in the similar analogy.)
Fig 3.4 Venn diagrams for copper (Cu)-regulated genes in Arabidopsis wild type Col-0 and mutant spl7. Genes of interest are shown in the appropriate set or overlap of sets. (a) In roots two genotypes upregulated/downregulated genes under Cu deficiency. (b) In shoots two genotypes upregulated/downregulated genes under Cu deficiency. (WT_dn_-Cu is short for wild type downregulated genes under Cu deficiency; spl7r_dn_-Cu is short for spl7 rescue downregulated genes under Cu deficiency, the rest shorts are in the similar analogy.)
Fig 3.5 Venn diagrams for iron (Fe) and copper (Cu)-regulated genes in Arabidopsis wild type Col-0 and mutant spl7. Genes of interest are shown in the appropriate set or overlap of sets. (a) In roots two genotypes upregulated/downregulated genes under Fe and Cu deficiency. (b) In shoots two genotypes upregulated/downregulated genes under Fe and Cu deficiency. (WT_dn_-Fe-Cu is short for wild type downregulated genes under Fe, Cu deficiency; spl7r_dn_-Fe-Cu is short for spl7 rescue downregulated genes under Fe, Cu deficiency, the rest shorts are in the similar analogy.)
Fig 3.6 The phenotypes of Col-0 under ten treatments (a) and FSD1 (b), FSD2 (c), CSD1 (d), CSD2 (e), and COPT2 (f) gene expression using quantitative real time PCR of Col-0 under multiple Fe and Cu treatments. Rosettes RNA was isolated from rosette of plants grown under the treatments for 11 days in hydroponic system. For normalization across samples expression of UBQ10 was used and the relative expression of FSD1, FSD2, CSD1, CSD2 and COPT2 calculated
using the $2^{-\Delta CT}$ method. Presented data were the means of three biological replicates, and each was analyzed 3 times by qRT-PCR. Error bars represent ±SE.
Fig 3.7 (a). Triple mutant k39k100k101 and Col-0 phenotypes under six treatments. Cu (b) and Fe (c) concentration of rosettes of triple mutant and Col-0 plants grown in multiple Fe and Cu treatments. Data was summarized from an average of at least 6 biological replicate plants’ rosettes for each line at each treatment. No significant differences were observed in the rosettes Cu and Fe accumulation between two genotypes. Plants were grown under the treatments for 14 days in the hydroponic system. Number of stat(s) above each treatment indicate statistically significant groups using T Grouping ANOVA with a 95% confidence interval.
Fig 3.8 Substitution of chloroplastic SOD enzymes depending on Cu bioavailability in Col-0. Cu/ZnSOD is the predominant SOD within the chloroplast during Cu-replete conditions, while be substituted by FeSOD during Cu limitation.
Table 3.1 Partial SPL7 target genes – under Cu deficiency the upregulated genes annotation in Log2 FC (fold change) in WT in roots

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| Shoot | | | | |
|-------| | | | |
| AT2G39030 | L-ornithine N5-acetyltransferase NATA1 | NATA1 | 1.8 | |
| AT5G03545 | INDUCED BY STARVATION 2                | ATIPS2 | 1.1 | |
| AT2G23910 | NAD(P)-binding Rossmann-fold superfamily protein |         | 1.2 | |
| AT1G80660 | ATPase 9, plasma membrane-type           | AHA9    | 1.2 | |
| AT5G37970 | Probable S-adenosylmethionine-dependent methyltransferase |         | 1.3 | |
| AT3G28780 | Uncharacterized protein                  |         |         | |

In our data only showed the wild type Col-0 upregulated genes under Cu deficiency which were not included spl7 mutants'. These genes were compared with previous paper’s RNA-sequencing results, Bernal 2012 (supplemental data set 1’s root and shoot sheets) and Zhang 2014 (supplemental data set 1’s sheet 5 WT-dc/WT-sc under Cu deficiency) (Bernal et al., 2012a, Zhang et al., 2014).
Table 3.2 Summary of important metal homeostasis genes under Fe deficiency, Cu deficiency and Fe, Cu deficiencies in Col-0 and spl7

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Arabidopsis WT Col-0 and spl7 mutant were grown in the hydroponic solution containing normal concentration of 25 uM Fe, 0.5uM CuSO4; iron deficiency concentration of 0 Fe, 0.5uM CuSO4; copper deficiency concentration of 25 uM Fe, 0 CuSO4; and 0 Fe, 0 CuSO4 for three days before collected. Genes in the fold changes are shown that root and shoot transcript abundances have (1) log2FC (average of WT -Fe) ≥ 2 log2FC (average of WT +Fe) for positive log2 FC (WT – Fe versus WT +Fe); log2FC (average of spl7 -Fe) ≤ 2 log2FC (average of spl7 +Fe) for negative log2 FC (spl7 – Fe versus spl7 +Fe); (2) log2FC (average of WT -Cu) ≥ 2 log2FC (average of WT +Cu) for positive log2 FC (WT – Cu versus WT +Cu); log2FC (average of spl7 -Cu) ≤ 2 log2FC (average of spl7 +Cu) for negative log2 FC (spl7 – Cu versus spl7 +Cu); (3) log2FC (average of WT -Fe-Cu) ≥ 2 log2FC (average of WT +Fe+Cu) for positive log2 FC (WT –Fe+ Cu versus WT +Fe+Cu); log2FC (average of spl7 -Fe-Cu) ≤ 2 log2FC (average of spl7 +Fe+Cu) for negative log2 FC (spl7 –Fe+ Cu versus spl7 +Fe+Cu).
Table 3.3 Summary of genes for Cu/Fe storage and homeostasis under Fe deficiency, Cu deficiency and Fe, Cu deficiencies in Col-0 and spl7

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*Arabidopsis* WT Col-0 and spl7 mutant were grown in the hydroponic solution containing normal concentration of 25 uM Fe, 0.5uM CuSO₄; iron deficiency concentration of 0 Fe, 0.5uM CuSO₄; copper deficiency concentration of 25 uM Fe, 0 CuSO₄; and 0 Fe, 0 CuSO₄ for three days before collected. Genes in the fold changes are shown that root and shoot transcript abundances have (1) log2FC (average of WT -Fe)≥ 2 log2FC (average of WT +Fe) for positive log2 FC (WT – Fe versus WT +Fe); log2FC (average of spl7 -Fe)≤ 2 log2FC (average of spl7 +Fe) for negative log2 FC (spl7 – Fe versus spl7 +Fe); (2) log2FC (average of WT -Cu)≥ 2 log2FC (average of WT +Cu) for positive log2 FC (WT – Cu versus WT +Cu); log2FC (average of spl7 -Cu)≤ 2 log2FC (average of spl7 +Cu) for negative log2 FC (spl7 – Cu versus spl7 +Cu); (3) log2FC (average of WT -Fe-Cu)≥ 2 log2FC (average of WT +Fe+Cu) for positive log2 FC (WT –Fe- Cu versus WT +Fe+Cu); log2FC (average of spl7 -Fe-Cu)≤ 2 log2FC (average of spl7 +Fe+Cu) for negative log2 FC (spl7 –Fe- Cu versus spl7 +Fe+Cu).
Table 3.4 Summary of important unknown genes in term of Fe and Cu homeostasis under Fe deficiency, Cu deficiency and Fe, Cu deficiencies in Col-0 and spl7

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<th>Roots</th>
<th>Roots</th>
<th>Roots</th>
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</table>

Arabidopsis WT Col-0 and spl7 mutant were grown in the hydroponic solution containing normal concentration of 25 uM Fe, 0.5uM CuSO₄; iron deficiency concentration of 0 Fe, 0.5uM CuSO₄; copper deficiency concentration of 25 uM Fe, 0 CuSO₄; and 0 Fe, 0 CuSO₄ for three days before collected. Genes in the fold changes are shown that root and shoot transcript abundances have (1) log2FC (average of WT -Fe) ≥ 2 log2FC (average of WT +Fe) for positive log2 FC (WT – Fe versus WT +Fe); log2FC (average of spl7 -Fe) ≤ 2 log2FC (average of spl7 +Fe) for negative log2 FC (spl7 – Fe versus spl7 +Fe); (2) log2FC (average of WT -Cu) ≥ 2 log2FC (average of WT +Cu) for positive log2 FC (WT – Cu versus WT +Cu); log2FC (average of spl7 -Cu) ≤ 2 log2FC (average of spl7 +Cu) for negative log2 FC (spl7 – Cu versus spl7 +Cu); (3) log2FC (average of WT -Fe-Cu) ≥ 2 log2FC (average of WT +Fe+Cu) for positive log2 FC (WT –Fe- Cu versus WT +Fe+Cu); log2FC (average of spl7 -Fe-Cu) ≤ 2 log2FC (average of spl7 +Fe+Cu) for negative log2 FC (spl7 –Fe- Cu versus spl7 +Fe+Cu).
Table 3.5: Read counts for the \textit{bHLH38}, \textit{bHLH39}, \textit{bHLH100}, \textit{bHLH101} in Col-0 and \textit{spl7}

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<thead>
<tr>
<th>Genes</th>
<th>Short name</th>
<th>roots WT</th>
<th>roots \textit{spl7 rescue}</th>
<th>rosettes WT</th>
<th>rosettes \textit{spl7 rescue}</th>
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<td></td>
<td></td>
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<td>Fe-</td>
<td>Fe+</td>
<td>Fe-</td>
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<td></td>
<td></td>
<td>Cu+</td>
<td>Cu-</td>
<td>Cu+</td>
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\textit{Arabidopsis} WT Col-0 and \textit{spl7} mutant were grown in the hydroponic solution containing normal concentration of 25 uM Fe, 0.5uM CuSO$_4$; iron deficiency concentration of 0 Fe, 0.5uM CuSO$_4$; copper deficiency concentration of 25 uM Fe, 0 CuSO$_4$; and 0 Fe, 0 CuSO$_4$ for three days before collected. Genes in the fold changes are shown that root and shoot transcript abundances have (1) log2FC (average of \textit{WT} -Fe)≥ 2 log2FC (average of \textit{WT} +Fe) for positive log2 FC (\textit{WT} – Fe versus \textit{WT} +Fe); log2FC (average of \textit{spl7} -Fe)≤ 2 log2FC (average of \textit{spl7} +Fe) for negative log2 FC (\textit{spl7} – Fe versus \textit{spl7} +Fe); (2) log2FC (average of \textit{WT} -Cu)≥ 2 log2FC (average of \textit{WT} +Cu) for positive log2 FC (\textit{WT} – Cu versus \textit{WT} +Cu); log2FC (average of \textit{spl7} -Cu)≤ 2 log2FC (average of \textit{spl7} +Cu) for negative log2 FC (\textit{spl7} – Cu versus \textit{spl7} +Cu); (3) log2FC (average of \textit{WT} -Fe-Cu)≥ 2 log2FC (average of \textit{WT} +Fe-Cu) for positive log2 FC (\textit{WT} –Fe- Cu versus \textit{WT} +Fe+Cu); log2FC (average of \textit{spl7} -Fe-Cu)≤ 2 log2FC (average of \textit{spl7} +Fe-Cu) for negative log2 FC (\textit{spl7} –Fe- Cu versus \textit{spl7} +Fe+Cu).
Table 3.6 WT Col-0 and spl7 iron deficiency-induced ethylene-dependent (IDED) genes in fold changes

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>WT-Fe</th>
<th>spl7-Fe</th>
<th>Annotation</th>
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<td>AT4G19690</td>
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<td>2.0</td>
<td>IRT1, Fe(II) transporter</td>
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<td>CCCL1, CCC1-like protein, putative metal transporter</td>
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<tr>
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<td>-2.3</td>
<td>CCCL2, CCC1-like protein, putative metal transporter</td>
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<tr>
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<td>CCCL3, CCC1-like protein, putative metal transporter</td>
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<td>FRD3, MATE efflux family protein</td>
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<td>FRO2, Ferric chelate reductase oxidase 2</td>
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<td><strong>Amino acid transport</strong></td>
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<td>AAT, amino acid transporter family protein</td>
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<td><strong>Transcription factor</strong></td>
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<td>bHLH38, basic helix-loop-helix 38</td>
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<td>bHLH39, basic helix-loop-helix 39</td>
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IDED genes listed on the table has been confirmed by conducting ethylene inhibitor Co and STS that can significantly decreased Fe acquisition genes fold changes (Garcia, et al., 2010). Data in the table present three biological replicates. And genes were ordered through physiological responses to iron deficiency.
## Appendix 3.1 Gene description for Fig 3.3

<table>
<thead>
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<th>Tissue</th>
<th>Genotype/Regulation</th>
<th>Gene locus</th>
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<td><em>bHLH39</em>, cellular response to iron ion starvation, iron homeostasis, regulation of transcription</td>
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## Appendix 3.2 Gene description for Fig. 3.4

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<th>Description</th>
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<td>cellular iron ion starvation, iron homeostasis, regulation of transcription</td>
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oxidation-reduction process, removal of superoxide radicals, response to copper/iron
AT1G12520  COPPER CHAPERONE FOR SOD1 (CCS), cellular copper ion homeostasis, intracellular copper transport, removal of superoxide radicals
### Appendix 3.3 Gene description for Fig 3.4

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**Shoot**       **WT up**

| AT5G49740 | FERRIC REDUCTION OXIDASE 7 (FRO7), ion transport, photosynthetic electron transport chain |
| AT2G28190 | COPPER SUPEROXIDE DISMUTASE 2 (CSD2), cellular response to UV-B/high light intensity/oxidative stress/salt stress/sucrose stimulus, removal of superoxide radicals, response to copper/iron |