DISSERTATION

REGULATION OF COPPER HOMEOSTASIS IN PLANTS: A FOCUS ON CHLOROPLASTIC SUPEROXIDE DISMUTASES AND COPPER DELIVERY MECHANISMS

Submitted by

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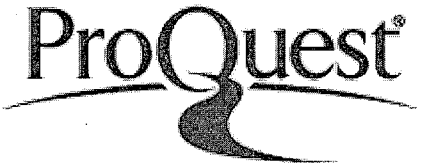

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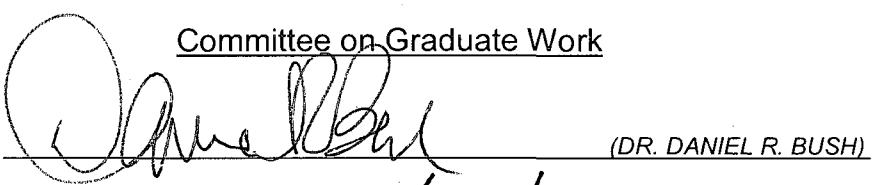

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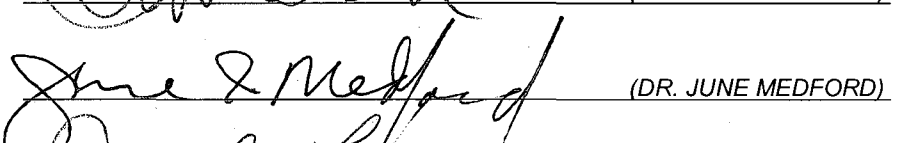

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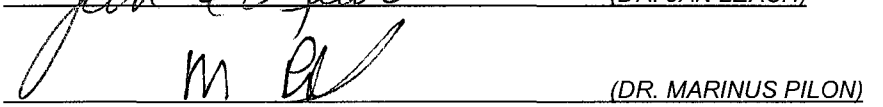

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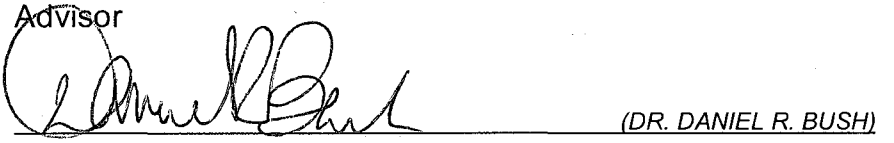

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ABSTRACT OF DISSERTATION

REGULATION OF COPPER HOMEOSTASIS IN PLANTS: A FOCUS ON CHLOROPLASTIC SUPEROXIDE DISMUTASES AND COPPER DELIVERY MECHANISMS

Copper (Cu) is an essential micronutrient for higher plant growth and is found in proteins that are important in photosynthesis and respiration. As a cofactor, this trace element is associated with many proteins including plastocyanin, Cu/Zn superoxide dismutase (Cu/ZnSOD), and mitochondrial cytochrome-*c* oxidase. Due to its redox-active role, Cu is essential for plant life, yet Cu is also dangerous as a free cellular ion and even toxic if in excess. Therefore, delivery and sequestration of Cu must be tightly regulated. The research of this dissertation indicates that sensory mechanisms and signaling pathways exist to coordinate Cu transport and target protein expression based on Cu status. For *Arabidopsis* and crop species, chloroplastic Cu/ZnSOD is down-regulated during limited Cu availability while at the same time FeSOD is up-regulated. During Cu-limited growth, when Cu/ZnSOD is down-regulated, plastocyanin levels do not change. We suggest that this reduction in Cu/ZnSOD allows for preferential Cu delivery to plastocyanin, which is essential for photosynthesis, while also maintaining chloroplast SOD activity. Cu delivery to Cu/ZnSOD is accomplished by the Cu Chaperone for SOD (CCS). When a CCS loss of function mutant was grown on Cu supplemented soil Cu/ZnSOD and FeSOD activity was not detected.

chloroplast did not exhibit an observable phenotype or photosynthetic deficiencies, even after high light stress treatments. Recent studies have shown that Cu/ZnSODs in the cytosol and chloroplast, along with other Cu proteins, are regulated by Cu via microRNA directed cleavage of Cu protein mRNA. It has also been determined that during Cu-limited growth the SPL7 transcription factor plays a central role in activating Cu-microRNAs and possibly Cu transporters. The research of this dissertation indicates that CCS is also regulated by Cu, mediated by microRNA398, which was not previously predicted by bioinformatic algorithms. Furthermore, data is presented to suggest that SPL7 likely regulates the promoter of FeSOD by activating transcription during limited Cu availability.

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TABLE OF CONTENTS

CHAPTER 1

Scope of dissertation

Plants must acquire all of their nutrients from the surrounding soil without the ability to move to a more desirable location; therefore, a plant must alter its physiology as a means of acclimation to gain control over fluctuating environmental conditions for survivability. Copper (Cu) is essential for photosynthesis in trace quantities while also being toxic when in excess (1). Therefore, it is important for a plant to maintain adequate Cu levels so that primary production and proper development continues while also avoiding potential toxic effects. Agricultural soils in many areas could contain high levels of Cu from fungicides, animal feed slurries, and various human wastes; other soils may have low Cu availability due to extensive agricultural use or leaching (1-3). A better understanding of micronutrient homeostasis in higher plants could help develop sensitive tools for determining crop Cu status, increase primary productivity and plant health, and possibly play a role in environmental metal cleanup or nutrient enrichment of foods.

The aim of my dissertation is to examine how plants respond to different Cu availabilities by regulating its delivery in a hierarchal fashion to ensure allocation to vital proteins, while also maintaining normal metabolic functions. Focus is on chloroplast Cu transport, allocation, and signaling. Specifically, I examined how

Cu delivery is prioritized to the essential chloroplast protein plastocyanin through the regulation of Cu delivery mechanisms and other Cu proteins. The present state of the field of Cu homeostasis is discussed in chapter 2 which provides background information on the functions of Cu proteins and gives insight into cellular transport and homeostasis mechanisms in plants.

This dissertation has four experimental chapters that are outlined below. For each of these, design, research, and writing were conducted by Christopher M. Cohu, unless otherwise specified below, with guidance and mentoring by Marinus Pilon.

The four main research objectives are:

1. Determine the prioritization of chloroplastic Cu delivery to target proteins during Cu limited growth for *Arabidopsis* and crop species (Chapter 3, Cohu and Pilon, 2007).
2. Chloroplastic Cu/Zn-superoxide dismutase (Cu/ZnSOD) and iron-superoxide dismutase (FeSOD) exhibit reciprocal regulation based on Cu availability. The objective is to identify if a dramatic decrease in chloroplastic Cu/ZnSOD and FeSOD activity affects photosynthetic efficiency, plant growth, development, and reproduction (Chapter 4). Backcrossing and initial observation of CCS knock-out lines was conducted by Kathryn A. Gogolin-Reynolds and Dyllon Martini. Salah E. Abdel-Ghany and Alexander M. Onofrio helped with plant growth and assisting on stress experiments. Jared R. Bodecker conducted initial experiments on CCS mRNA, and Jeffrey A. Kimbrel assisted with CCS

antibody production. Krishna K. Niyogi hosted me in his lab at UC Berkeley for the HPLC analysis.

3. Characterize Cu chaperone for SOD (CCS) expression patterns in relation to Cu availability and determine how CCS is regulated by Cu. Also, determine CCS spatial and temporal promoter activity (Chapter 5). CCS over-expresser line was constructed by Salah E. Abdel-Ghany. Alexander M. Onofrio and Ryan Tam assisted with histochemical and fluorogenic analysis on *CCSpro::GUS* lines. Loss of function and over-expresser miR398 lines were a generous gift from Bonnie Bartel, Rice University.
4. Characterize FeSOD expression patterns in relation to Cu availability and determine how FeSOD is regulated by Cu. Also, determine FeSOD spatial and temporal promoter activity (Chapter 6). Alexander M. Onofrio assisted with fluorogenic, histochemical, and immunoblot analysis. SPL7 mutant lines were a generous gift from Toshiharu Shikanai, Kyoto University, Kyoto, Japan.

Finally, the collective results are summarized and their implications discussed (chapter 7).

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CHAPTER 2

Cell biology of copper

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CHAPTER 2

Cell biology of copper

Summary

The transition metal copper (Cu) is an essential micronutrient for normal plant growth and development. Copper is a cofactor of proteins involved in photosynthesis, respiration, ethylene perception, removing superoxide radicals, and cell wall modification. The biochemical reactions catalyzed by most Cu enzymes in plants are known. However, in many cases we are not yet sure about the biological function of these Cu proteins. Copper delivery to Cu proteins has evolved with a set of evolutionarily conserved transporters and metallo-chaperones. Analysis of Cu transporter and metallo-chaperone loss of function mutants has increased our understanding of the localization and biological function of many Cu delivery mechanisms and target Cu proteins. Studies examining the regulation of Cu transporters, metallo-chaperones, and Cu proteins have revealed an elegant system to regulate Cu homeostasis. Copper in excess is toxic while Cu deficiency can lead to decreased photosynthetic activity and reproductive success. To avoid Cu deficiency or toxicity symptoms in a sub-optimal environment, plants are capable of directing Cu delivery based on needs via regulation of Cu proteins and delivery systems. For many Cu proteins, a

network of Cu microRNAs, under the control of a SPL7 transcription factor, orchestrates the prioritization of Cu delivery based on Cu availability.

Introduction

Copper (Cu) is an essential micronutrient for life, and it is important for many cellular processes in numerous organelles and compartments. In cells, Cu is found in two common states, Cu(I) (reduced) or Cu(II) (oxidized). Cu ions often act as cofactors in Cu-proteins that are associated with electron transfer reactions and redox reactions involving oxygen (1). However, the redox-active properties of Cu could also cause unwanted and uncontrolled reactions if left alone as a free ion in the cell. Free Cu ions could lead to the formation of toxic hydroxyl radicals which then are capable of damaging macromolecules (2). Therefore, an extensive network of Cu transporters and metallo-chaperones has evolved to bind and shuttle Cu in a manner that ensures proper Cu delivery to Cu proteins in various cellular organelles and compartments. A complete list of transporters, Cu delivery, and Cu target proteins is illustrated in table 1.

Functions of Cu proteins in plants

We know the biochemical reactions catalyzed by most Cu enzymes in plants. Remarkably, in many cases we are not yet sure about the biological function of these Cu proteins. Before describing how Cu is delivered to Cu proteins in plants we like to discuss what we know about the functions of the Cu proteins.

Plastocyanin - The plastocyanin protein was first discovered in Algae (3). Plant (Poplar) plastocyanin was one of the first Cu proteins with a crystal structure known which showed that the protein binds its Cu via a cysteine, a methionine and two histidine ligands (4). Plastocyanin is the most abundant protein in the thylakoid lumen where it functions as a mobile carrier of electrons from the cytochrome-b6/f complex to PSI (5,6). Therefore, it has a critical role in both linear and cyclic electron flow (7). A higher plant (*Silene pratensis*) plastocyanin cDNA sequence was first published in 1985 (8). Arabidopis has two plastocyanin genes (9,10) that encode proteins with highly similar secondary structure and seemingly redundant function (11). Unlike some cyanobacteria and green algae, plastocyanin is the only protein that can accept electrons from the cytochrome-b6/f complex in higher plants (12), and *Arabidopsis* mutants with insertions in both plastocyanin genes are seedling-lethal on soil (13). Arguably, plastocyanin is the most important Cu protein in photosynthetically growing plants.

Cytochrome c oxidase - In mitochondria, Cu is required for the function of cytochrome *c* oxidase (COX), the proton-pumping terminal oxidase in the inner membrane (14). This multi-subunit protein contains three Cu ions as cofactors in two Cu sites in addition to heme. At least ten subunits make up the cytochrome *c* oxidase of higher plants. In yeast, three conserved mitochondrial encoded subunits (COX1, 2, and 3) form the core of the enzyme and contain the Cu binding sites (14). Coordinated expression of nuclear and mitochondrial encoded COX genes is required for activity of cytochrome *c* oxidase. Nuclear encoded

COX subunit genes may help control tissue specific activity of this enzyme (15). It is well established that a lower cytochrome *c* oxidase activity is one effect of severe Cu deficiency in plants (16). In addition to COX, plants have the Fe-containing alternative oxidase (AOX). This protein accepts electrons from ubiquinone and reduces O_2 to water without proton pumping. AOX activity may prevent over-reduction of the quinone pool in the case that downstream electron transport is less efficient due to a stressful environment (17).

Cu/Zn superoxide dismutase - Together with Zn, Cu is also a cofactor of Cu/Zn superoxide dismutase (Cu/ZnSOD) proteins that function in reactive oxygen species metabolism (18). SOD enzymes catalyze the conversion of two superoxide ions and two protons to peroxide and molecular oxygen. Three genes encode for Cu/ZnSOD in the *Arabidopsis* genome: CSD1 is active in the cytosol, CSD2 is active in the stroma, and CSD3 is active in peroxisome (19). Homologs of these Cu/ZnSOD genes have been found in the genomes of other plant species but they are not found in *Chlamydomonas*.

Ethylene receptors - The ethylene receptors are Cu-binding proteins (20) that are active in an early endomembrane system compartment, most likely the endoplasmic reticulum (21). For proper ethylene perception and responses, Cu must be delivered to the lumen of the endomembrane system (22).

Phytocyanins - The phytocyanins constitute a plant specific blue Cu protein family. Phytocyanins have structural similarity to plastocyanin and bind a single Cu per polypeptide. Phytocyanins include plantacyanin, stellacyanin, and uclacyanin (23). These proteins differ from plastocyanin in that their precursors

harbor signal peptides that direct them into the endoplasmic reticulum and secretory pathway. The biological role of these phytocyanins is not fully clear. It was suggested that some may mediate lignin polymerization (23). In Lilly, plantacyanin functions as a signaling molecule in the transmitting tract of the pistil (24) and evidence in *Arabidopsis* also suggests a role in reproduction (25).

Laccase and ascorbate oxidase - Multi-copper oxidases (MCO) form a super-family of proteins that bind four Cu ions (26) and include the ferroxidases (in yeast and *Chlamydomonas*), ceruloplasmin (in mammals), ascorbate oxidase, and laccases (plants and fungi). In plants, apoplastic ascorbate oxidases were shown to have roles in cell expansion, plant biomass production, and salt tolerance (27,28). Laccase catalyzes the oxidation of a suitable substrate molecule (phenols and aromatic or aliphatic amines) to the corresponding reactive radicals with the production of water and oligomers (29). In plants, laccases are apoplastic and encoded by a multi-gene family with 17 genes in *Arabidopsis* (30,31). Different laccases are expressed in different organs and at different developmental stages of growth in *Arabidopsis* (30). Insertion mutants for most laccases do not show a phenotype except for Lac2 (altered root elongation during de-hydration), Lac8 (early flowering), and Lac 15 (altered seed color) (31). The mutation in Transparent Testa-10 (TT10), an *Arabidopsis* mutant with a lack in seed color, was mapped to Lac15 which is expressed in the developing seed and functions in the formation of proanthocyanidin or tannin (32). Another possible role of laccase is in Fe acquisition (33). All other proposed biological functions of laccases in plants can be summarized as "cell wall

modeling"; this includes roles in lignin synthesis, maintenance of cell wall structure and integrity, response to stress, and wound healing (34-38).

Polyphenol oxidase - Polyphenol oxidase (PPO) or tyrosinase was the first discovered Cu enzyme in plant plastids (39). It is found in the thylakoid lumen and contains a dinuclear Cu center. PPO catalyzes the conversion of monophenols to ortho-diphenols and ortho-dihydroxyphenols to ortho-quinones, resulting in black or brown pigment deposits (40). In tomato, the PPOs are encoded by a gene family with seven members (41) that are differentially expressed (42). Wounding, stress, pathogen, and herbivore attack have been shown to induce PPO activity in different plant species, suggesting a role for PPO in plant resistance to stress and pathogens (40). PPO is not ubiquitous and there is no homolog for PPO in *Arabidopsis* (6).

Amine oxidase - Amine oxidases contain a single Cu as well as a special topa quinone cofactor that is formed by post-translational modification of a conserved tyrosine residue (43). These secreted enzymes catalyze the oxidative deamination of primary amines to aldehydes in a reaction that requires free radicals and also produces hydrogen-peroxide (44). Poly-amines such as spermine are likely substrates for these enzymes. The Cu atom is bound by three histidine residues and is required not only for the post-translational formation of the topa quinone cofactor but also for the regular catalytic cycle (43). Amine oxidases are reported to be the most abundant Cu proteins in the apoplastic space of peas. Proposed functions include roles in cell wall differentiation, which in turn could be significant for stomatal closure (45), wound healing (46,47), and

responses to pathogen attack (46,48). The observed amine oxidase expression pattern and timing in tobacco would be consistent with a role in either peroxide dependent protein cross-linking or lignification (49).

Other roles of Cu in plants - While determining the structure of CNX1, an enzyme that functions in molybdenum cofactor synthesis, it was found that a Cu ion temporarily occupies the site for molybdenum insertion in the bound molybdopterin substrate (50). This observation now links Cu metabolism to nitrogen assimilation and phytohormone biosynthesis, which are functions of molybdenum cofactor requiring enzymes. Cu may also play a role in thylakoid grana stacking (51). Unlike what has been found for yeast and *Chlamydomonas*, there is no direct requirement for Cu in Fe acquisition.

Cu movement in and out of root cells

Cu uptake - In root cells, Cu enters the cytosol by a cell membrane COPT-family transporter (52). The family of COPT transporters belongs to a highly conserved Ctr-like Cu transporter family also found in yeast and humans (53). COPT transporters have three transmembrane domains, a likely N-terminal metal binding domain, and an essential MXXXM transmembrane domain (54). *Arabidopsis* encodes five COPT transporters (COPT1 - 5). Of these, four are expressed and these proteins likely import Cu into the cytosol, though their sub-cellular location is not determined. Information on COPT1 promoter fusion and antisense lines suggest that COPT1 is involved in Cu uptake from the surrounding growth medium at root tips (55). COPT1 antisense lines exhibited

elongated root growth when compared to wild-type plants, and the phenotype could be partially restored upon Cu feeding in the medium (55). COPT2 is also likely involved in cellular uptake considering its expression in root and leaf tissues, along with up-regulation of transcripts during limited Cu growth, similarly seen for COPT1 (56). COPT3 and COPT5 are highly expressed in aerial tissues (56), and may serve to transport Cu from intracellular stores.

Ctr-like proteins transport Cu in its reduced form (57), but most extracellular Cu in soil is oxidized as Cu(II). *Arabidopsis* and dicot species utilize root surface ferric reductases, such as FRO2, for uptake of Fe in its reduced form (58). It is also possible that ferric reductases could reduce Cu for import (59). When plants are fed an excess of Cu, Fe concentrations decrease; the opposite is also true during limited Cu growth (59,60). Interestingly, FRO3, localized in roots and vasculature, exhibits increased expression during Cu deficient growth (61). However, FRO activity has not been reported to reduce Cu(II). In addition to COPT transporters, ZIP2 and ZIP4 (ZIP family transporters) have been reported to complement the yeast *ctr1* mutant, which is deficient in Cu uptake (62). ZIP2 transcript expression is highest in root tissue while ZIP4 expression is high in both root and leaf tissue, and they respond to Cu status (62).

Cu export and intercellular reallocation - While HMA5 (Heavy Metal Associated) Cu-transporter likely supplies Cu to apoplastic Cu oxidases and laccases, it also plays an important role in removing excess Cu from the cytosol of root tissues (63,64). Root tissues in *hma5* loss of function mutants accumulate elevated levels of Cu when compared to wild-type plants, and *hma5* mutants

were more sensitive to Cu feeding (63). Delivery of Cu within the cytosol of plants to RAN1 and HMA5 may be accomplished by two homologs of the yeast Atx1 Cu chaperone, ATX1 (63,65) and CCH (66). Both ATX1 and CCH from *Arabidopsis* are able to complement the yeast *atx1* mutant, and they interact with the N-terminal domain of *Arabidopsis* HMA5 and RAN1 (ATX1 only) in a yeast two hybrid system (63,65). The yeast Atx1 and ATX1 in *Arabidopsis* are similar, however, CCH contains an added plant specific C-terminal extension (65,67). Interestingly, this C-terminal addition negatively affects interactions with HMA5, but a positive interaction was observed when the C-terminal region of CCH was removed (63,65). CCH has been found in phloem-endonucleated cells, and it is possible that the additional C-terminal region allows for symplastic intercellular Cu trafficking through plasmodesmata (63,68). Up-regulation of ATX1 and CCH has been reported for plants undergoing Cu deficiency, senescence, mechanical and oxidative stress, along with jasmonic acid treatments in *Arabidopsis* (65, 66,68), and in poplar (69).

Root to shoot Cu translocation - Because HMA5 is involved with Cu movement from the symplast to apoplast, and is highly expressed in roots, it is possible that HMA5 is also involved in transporting Cu into the xylem. If so, it is not the only mechanism to load Cu into the xylem considering that *hma5* loss of function mutants were able to maintain much of the Cu translocation to shoot tissues (63). No other mechanism for Cu loading into the xylem has been suggested or identified. Once in the xylem, long distance Cu translocation to aerial tissues may involve the chelator nicotianamine. As a methionine-derived

compound, nicotianamine chelation of Fe in xylem sap for translocation has been suggested (70). Nicotianamine has also been shown to have a high affinity for Cu binding in tomato xylem sap, and less than 0.5% of total xylem Cu was found as free Cu(II) ions (71). This suggests that Cu in xylem sap is mostly chelated. The tomato mutant *chloronerva*, which lacks nicotianamine, also supports the idea that nicotianamine is involved with long distance transport of heavy metals. *chloronerva* mutant plants exhibit increased Cu concentrations in root tissues and decreased xylem and shoot levels compared to wild-type (72). Upon application of nicotianamine to these mutants it was observed that root Cu concentrations decreased while xylem and shoot levels increased, especially in young leaves (72). In addition, tobacco plants over-expressing a nicotianamine aminotransferase (NAAT) gene, which creates a nicotianamine shortage in tobacco, led to Cu deficiencies in leaves and problems associated with reproduction (73).

Excess Cu - In some cases plant cells may have to deal with excessive Cu. Plants such as *Arabidopsis* do not accumulate high levels of Cu in tissues and are often sensitive to elevated Cu. During sub-toxic Cu feeding, plants may be able to chelate Cu using a cysteine-rich metallothionein (MT). *Arabidopsis* contains several MT genes, some of which are up-regulated during Cu excess (74,75). Another possible Cu chelator in the cell is phytochelatin, which is derived from glutathione (76). When plants lack both MT1a/MT2b and phytochelatin they exhibit a more severe phenotype on elevated Cu than MT or phytochelatin mutants alone (77). Simply moving Cu out of the cell may also help maintain

normal cellular Cu levels. Considering that *hma5* mutants are sensitive to Cu feeding, HMA5 is likely involved in detoxifying cells of excess Cu by moving the ions into extracellular spaces (63,64). HMA5, COPT1, and COPT2 transporters are regulated by Cu differently. HMA5 increases during Cu excess (63) while the Cu importers COPT1 and COPT2 decrease (55,56); consistent with the role of HMA5 acting to prevent excess ions in the cell while also avoiding Cu toxic conditions for neighboring cells.

Intracellular Cu delivery to Cu protein targets

Chloroplast - Cu import into the chloroplast stroma and thylakoid lumen is the most understood of any organelle in plants. The inner envelope membrane contains a metal-transporting P-type ATPase for *Arabidopsis*, PAA1 (HMA6), and is responsible for Cu import into the stroma (78,79); while PAA2 (HMA8) imports Cu from the stroma into the thylakoid lumen (80,81). Both are P_{1B} type pumps and members of the Heavy Metal Associated (HMA) transporter family (82,83). There are eight members in the HMA family. HMA1 to 4 are classified as possible Zn, Cd, Co, and Pb transporters; while HMA5 to 8 are classified as Cu and Ag transporters (83,84). Both PAA1 and PAA2 Cu transporters have sub-cellular targeting information in the N-terminal region of the peptide, but the mechanism of protein import is not yet identified (80). PAA1 and PAA2 Cu transporters have eight predicted transmembrane domains with a heavy metal binding motif in the N-terminal region. In addition, they contain ATP binding, phosphatase, phosphorylation, and transmembrane CPC (amino acid) ion

transduction domains (80,85). Upon Cu metal binding and phosphorylation of P_{1B} type transporters, the Cu ion is transported across the membrane through changes in protein conformation (86,87). Transport of heavy metals in most of these P_{1B} type transporters is thought to initiate in the sub-cellular compartment containing the heavy metal binding N-terminal region (86,87). This would place the N-terminal domains for PAA1 and PAA2 in the chloroplast envelope intermembrane space and stroma respectively. However, the orientation and mechanism for accepting and donating Cu is not yet known for PAA1 and PAA2.

Plastocyanin import into chloroplasts and thylakoids is conducted using the Tic/Toc and SecA/SecY-mediated pathways (88), which translocate proteins in an unfolded state. Upon import, plastocyanin acquires its Cu cofactor for final assembly and stability. Mutants with impaired Cu transport (*paa1* and *paa2* loss of function mutants) exhibited reduced plastocyanin accumulation even though transcript levels remained high (80); supporting that plastocyanin requires Cu for final assembly and for stability, as similarly suggested for *Chlamydomonas* (89). Interestingly, even though both plastocyanin forms are seemingly similar in function (11), new evidence suggests that plastocyanin 2 (PC2) accumulates during increasing Cu feeding even though photosynthetic benefits were not observed. Plastocyanin could, therefore, have a secondary role as a Cu buffer (90). The mechanism for Cu delivery between PAA2 and plastocyanin has not been determined, and a Cu chaperone in the thylakoid lumen has not been identified. It is possible that plastocyanin receives its Cu directly from PAA2 or from a Cu pool. In addition to PAA1, another possible Cu transporter in the

chloroplast envelope membrane is HMA1, which may supply some Cu to CSD2 in the stroma (91). Though HMA1, PAA1, and PAA2 are in the HMA family, they do contain some differences. Unlike PAA1 and PAA2, HMA1 does not have a conserved MxCxxC N-terminal heavy metal binding domains; instead it contains a poly-histidine domain. In addition, HMA1 contains a SPC ion transduction domain instead of CPC (amino acid) found in PAA1 and PAA2 (82). Chloroplast Cu concentrations and CSD2 activity levels decrease in *hma1* mutants, and a photo-oxidative stress phenotype was reported when plants were grown in elevated light (91). However, *hma1* plants did not exhibit defects in total plastocyanin levels. Instead, it was suggested that a decrease in CSD2 activity led to the phenotype observed (91). A defect in plastocyanin levels was observed in a *paa1* mutant that also exhibited an electron transport phenotype (79,80). In the case of *paa1*, the phenotype was partially restored by Cu feeding (79,80), but the *hma1* mutant phenotype not was restored by Cu feeding (91). It is possible that HMA1, with lower Cu transport activity, mediates the delivery of Cu to plastocyanin in *paa1* mutants during Cu feeding, but the link, if any, between HMA1 and Cu transport to plastocyanin is still unclear.

Like plastocyanin, Cu/ZnSODs also require Cu for final assembly, activity, and stability. When Cu delivery to the chloroplast stroma is disrupted by a *paa1* loss of function mutant, CSD2 proteins do not accumulate to wild-type levels while CSD2 transcript levels increase (80). The Cu Chaperone for SOD (CCS) delivers Cu to Cu/ZnSODs (92), and is also active in the cytosol and plastids of plants (93). The stromal and cytosolic versions of CCS are encoded by one gene

in *Arabidopsis* with two in-frame ATG sites that span a chloroplast transit peptide (93). In a T-DNA knock-out mutant of *CCS* (CCS-KO), Cu delivery to Cu/ZnSODs was dramatically reduced and Cu/ZnSOD proteins did not accumulate, further suggesting that Cu/ZnSODs require Cu delivery for protein stability (93). It is not known if CCS in stroma acquires Cu directly from PAA1 for delivery to Cu/ZnSOD.

While CSD1 and CSD2 receive their Cu from CCS in the compartment in which they are active, it is likely that CSD3 in the peroxisome does not. CSD3 has a peroxisomal targeting sequence but it likely receives its Cu cofactor in the cytosol prior to import because the peroxisome can import proteins in a folded state. Complementation of a CCS loss of function mutant (CCS-KO) using a CCS version without the chloroplast targeting sequence rescues both CSD1 and CSD3 activities, but not CSD2 in the stroma (93).

Mitochondria – The Cu delivery mechanisms involved in the mitochondria are known mainly for yeast and mammalian cells; however, some homologous proteins have been found in plants. Delivery of Cu to cytochrome c oxidase in yeast is accomplished by Cox11, Cox17, Cox19, and Sco1 (14). The mitochondrial matrix in yeast stores Cu as a pool of soluble low molecular weight ligand complexes (94). It was suggested that the Cu pool in the matrix may supply Cu to the intermembrane space to Cox17. Cox17 is a metallo-chaperone that delivers Cu to Cox11 and Soc1 in the intermembrane space, which in turn deliver Cu to different cytochrome *c* oxidase subunits (95). *Arabidopsis* functional homologs for AtCOX17 (96) and AtCOX19 (97) have been identified. However,

homologs of Cox11 and Sco1 chaperones in plants, along with Cu transport across the mitochondria membranes, have not been characterized.

Endomembrane and Secretory Pathway - The RAN1 (HMA7) (responsive-to-antagonist 1) Cu transporter is a functional homolog of yeast and human P-type ATPase Cu transporters active in the endomembrane system (22). Homologs of RAN1 in yeast and mammals act in Cu transport from the cytosol into the secretory pathway (98). A mild *ran1* loss of function mutant in *Arabidopsis* lowered the plant's ability to respond to an antagonistic ethylene signal. This suggests that RAN1 is involved in Cu delivery to ethylene receptors (22). A dramatic loss of RAN1 function led to phenotypes associated with reduced cell wall elongation (99), perhaps due to defects in extracellular Cu oxidases and laccases.

Another member of the HMA family of Cu transporters, homologous to RAN1, is HMA5 (100). HMA5 is mainly localized in root and flower tissues. In *hma5* loss of function mutants a phenotype associated with cell wall elongation was observed. However, *hma5* had no defects in ethylene reception, as observed for *ran1* (22,63,99). Together, RAN1 and HMA5 could be involved in supplying Cu to many or all extracellular Cu proteins, but their specificity likely involves organ and intracellular locations relative to where extracellular oxidases and laccases receive their Cu.

Senescence, reallocation, and delivery to reproductive tissues

Copper is not readily reallocated from older leaves to younger tissues. During Cu deficiency, young leaves, shoot meristems, and reproductive tissues are affected before older leaves show signs (16). Therefore, a significant proportion of the Cu allocated to reproductive tissues likely comes directly from the roots (101). However, there are several indications that some Cu from older leaves is reallocated to newer leaves and reproductive tissues by chelators via symplastic movement. During senescence, the transcripts of the CCH chaperone increase (68). As a chelator with a putative C-terminal peptide to facilitate symplastic movement, CCH may chelate Cu in the cytosol during senescence for movement to vascular bundle tissues or movement within the phloem (63,68). Up-regulation of metallothionein (MT1) has also been reported during senescence (102), and it is possible that MT1a and MT2b are involved in phloem reallocation of Cu (75).

Another mechanism for reallocating Cu likely involves nicotianamine and the Yellow Stripe-Like (YSL) transporters. In addition to the xylem, nicotianamine is also found in phloem sap. Nicotianamine is a precursor of phytosiderophores which together with YSL transporters is involved in the strategy-II Fe uptake in monocot roots (70). Dicots, like *Arabidopsis*, use a strategy-I Fe uptake system that utilizes the root surface FRO2 ferric reductase and the IRT1 ZIP-family transporter, yet *Arabidopsis* encodes eight YSL transporters (70). These YSL transporters likely function to import nicotianamine metal complexes (103,104), which could then act as a metal ion redistribution system between tissues via the phloem (70,101). In a *ysl1 ysl3* double loss of function mutant, Cu concentrations

in seeds were reduced by 82% when compared to the parental line (105). In addition, this mutant line did not efficiently reallocate Cu and Fe from rosette and cauline leaves (101).

For proper seed set adequate Cu is required (16,106). Cu delivery to cells involved in reproduction likely receives most of their Cu directly sent from the roots, but it appears that Cu delivery is via the xylem and the phloem (see above). Cu, along with other metals, bound to nicotianamine could be imported by YSL transporters, and moved through the symplast by CCH. Cells that do not have plasmodesmata for intercellular Cu trafficking by CCH, like pollen, would require COPT1, which is highly expressed in pollen (55). COPT1 mutant plants with reduced expression exhibit defects in pollen development (55). Extracellularly, plantancyanin has been implicated in pollen tube guidance (24,25), and HMA5 is highly expressed in flowering tissues, most likely pollen (63). HMA5 may deliver Cu to plantacyanin in pollen, but this connection between the two has not been verified experimentally.

Regulation of Copper Homeostasis

Plants that are Cu deficient exhibit photosynthetic deficiencies, shoot apical meristem death, curling of leaves, and poor seed set (16, 107). To avoid Cu deficiency or toxicity symptoms in a sub-optimal environment, plants are capable of directing Cu delivery based on needs via regulation of Cu delivery systems. Copper toxicity leads to increased expression of some transporters and Cu chelators (see above). However, during Cu deficiency, post-transcriptional

regulation of many Cu proteins is mediated by microRNA directed cleavage of Cu protein mRNAs. Transcriptional activation of microRNAs, and possibly transporters, during Cu limited growth is mediated by a SPL7 (*SQUAMOSA* promoter-binding protein like 7) transcription factor. Together, this mechanism to down-regulate Cu proteins and delivery systems may allow for prioritized delivery to the most essential Cu proteins during limited Cu availability.

Transcription factors – Transcriptional responses to Cu require transcription factors that can sense Cu. *Chlamydomonas* are capable of switching between two functionally similar photosynthetic proteins, cytochrome *c6* (heme protein) and plastocyanin (Cu-protein), when Cu levels are limited or sufficient, respectively. This switch is mediated by the transcription factor Cu Response Regulator (CRR1) that activates transcription of the cytochrome *c6* when Cu is limited (108). Interestingly, *Crr1* mRNA and expression is not regulated by Cu. A conformational change in the CRR1 protein in response to Cu availability was suggested to act as an activation mechanism (108). While higher plants cannot substitute plastocyanin with cytochrome *c6*, Cu/ZnSOD and FeSOD regulation exhibit a similar reciprocal expression pattern (80,109,110).

Higher plants contain a homolog to CRR1 know as SPL7 that has recently been shown to be a key regulator of Cu homeostasis by binding to GTAC promoter core motifs (111). There are 12 members in the *Arabidopsis* SPL family that contain a conserved SBP (*SQUAMOSA* promoter-binding protein) DNA binding domain and a nuclear localization signal (112). SPL transcription factors have been reported to be involved in development and nutrient homeostasis. In

Arabidopsis, SPL7 likely mediates regulation of some Cu, Zn, and Fe transporters. Wild-type plants increase *COPT1*, *COPT2*, *ZIP2*, *FRO3*, and *YSL2* mRNA when Cu is limited, but in a *spl7* mutant the mRNA of these transporters did not increase (111). The *YSL2* promoter contains 5 GTAC core motifs indicating that the transporter may be directly regulated by Cu via SPL7. The Cu-chaperone CCS, which has been shown to decrease during Cu deficiency (62), did not decrease in the *spl7* mutant (111). When the *spl7* mutant was grown on low Cu it exhibited a severe growth phenotype, supporting that SPL7 is an important regulator during Cu-limitation. On the other hand, HMA5 and FRO6, which are regulated by Cu, and ATX1 (constitutively expressed), were not identified as being regulated by SPL7 (111). It is possible that yet another Cu sensitive regulatory mechanism for HMA5 and FRO6 exists. While SPL7 may regulate some Cu transporters and chaperones directly, SPL7 has also been shown to activate specific microRNA transcription during Cu limited growth leading to the cleavage of many Cu protein mRNAs (111). In the *spl7* mutant miR397, miR398, miR408, and miR857 were not detected even when Cu was limited.

The Cu microRNAs – microRNAs belong to a highly conserved group of small 20-21-nt RNAs that disrupt mRNA translation by guiding the cleavage of target mRNAs (113,114). miR398 was the first microRNA shown to target mRNAs that encode Cu/Zn superoxide dismutases in the cytosol (CSD1) and the chloroplast (CSD2) of *Arabidopsis* (114). Oxidative stress was shown to reduce mature miR398 levels that led to increased CSD1 and CSD2 mRNA and enzyme activity.

During non-stress growth conditions, Cu availability was also shown to regulate Cu/ZnSOD expression and activity (80,109). Linking Cu availability with the regulation of CSD1 and CSD2 by miR398 was established when Cu-supplemented *Arabidopsis* plants demonstrated an absence of miR398 while CSD1 and CSD2 mRNA abundance increased (110). The transcripts of plantacyanin and several members of the laccase family were identified as targets of miR397, miR408, and miR857 directed cleavage during Cu-limited growth (116). Together these studies suggest that Cu microRNA mediated down-regulation of many Cu-proteins is a mechanism to allow for Cu delivery to the most essential of the Cu-proteins, such as plastocyanin. Another interesting observation of microRNA in Cu homeostasis was the observation that sucrose in tissue culture medium elevated miR398 levels regardless of Cu levels (117). This suggests that there is additional regulation on Cu microRNAs from other signaling sources.

SPL7 is mainly found in the roots yet microRNAs are found throughout the plant and sometimes only in above-ground tissues (111,116). High expression of SPL7 in the roots indicates a role in detecting Cu availability at the site of Cu entry, then orchestrating whole plant Cu delivery. Recently, miR398, among other microRNAs, was found in the phloem of *Brassica napus* (118), rapeseed and pumpkin (119), which suggests that Cu homeostasis signals could originate from source tissues. This method of signal delivery could be very important for young developing leaves during Cu-limitation so that proper Cu delivery to essential Cu-proteins is maintained during initial development.

Overview

Transition metal homeostasis is perhaps more completely understood for Cu than any other metal in plants. Identification of Cu transporters and metallo-chaperones that are important in Cu delivery to ethylene receptors, for photosynthesis, and Cu movement into and out of the cell has provided a more complete understanding of Cu homeostasis mechanisms. Information on Cu delivery mechanisms has also allowed for studies that examine how Cu homeostasis is regulated during development and changing Cu status, both at a cellular and whole plant level. While Cu delivery mechanisms and regulation of Cu homeostasis is becoming clearer, the biological function of Cu proteins remains unclear in many cases. As the regulation pattern for Cu proteins and delivery mechanisms is resolved, it may help in identifying the biological role of many Cu proteins, both essential and seemingly non-essential.

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Protein	Most Likely Location	Proposed Function
Transporters		
COPT1	Plasma membrane	Cu uptake
COPT2	Plasma membrane	Cu uptake
COPT3, 4, and 5	?	Intracellular re-allocation
HMA1	Plastid envelope membrane	Ion transport
HMA5	Plasma membrane	Cu export
HMA6 (PAA1)	Plastid envelope membrane	Cu import to stroma
HMA7/RAN1	Secretory pathway	Cu import to secretory pathway
HMA8 (PAA2)	Thylakoid membrane	Cu import to thylakoids
ZIP2, ZIP4	Plasma membrane	Zn uptake
FRO3	Plasma membrane	Fe(III) reductase
YSL1, 2, and 3	Plasma membrane	Chelated ion import/export
Cu Delivery and Binding		
CCH and ATX1	Cytosol, phloem	Symplastic Cu delivery
CCS	Cytosol and stroma	Cu delivery for Cu/ZnSOD
Metallothionein	Cytosol	Ion chelating
Phytochelatin	Cytosol	Ion chelating
Nicotianamine	Xylem, phloem, cytosol	Long distance ion transport
Major Cu Proteins		
Plastocyanin	Thylakoid lumen	Photosynthetic electron transport
Cytochrome *c* oxidase	Mitochondrial innermembrane	Electron transport, proton pumping, terminal oxidase
Cu/ZnSOD	Cytosol, stroma, and peroxisome	Dismutation of superoxide radicals
Ethylene receptors	Edoplasmic reticulum	Ethylene signal perception
Phytocyanins	Apoplast	Reproduction, lignin polymerization
Laccase	Apoplast (secretory pathway)	Cell wall modeling, phenolic metabolism
Polyphenol oxidase	Thylakoid lumen	Conversion of monophenols to diphenols; conversion of dihydroxy phenols to ortho-quinones
Amine oxidase	Apoplast	Cell wall differentiation, wound healing, response to pathogens

Table 1. Identification, most likely location, and proposed function of transporters, Cu delivery and major Cu target proteins.

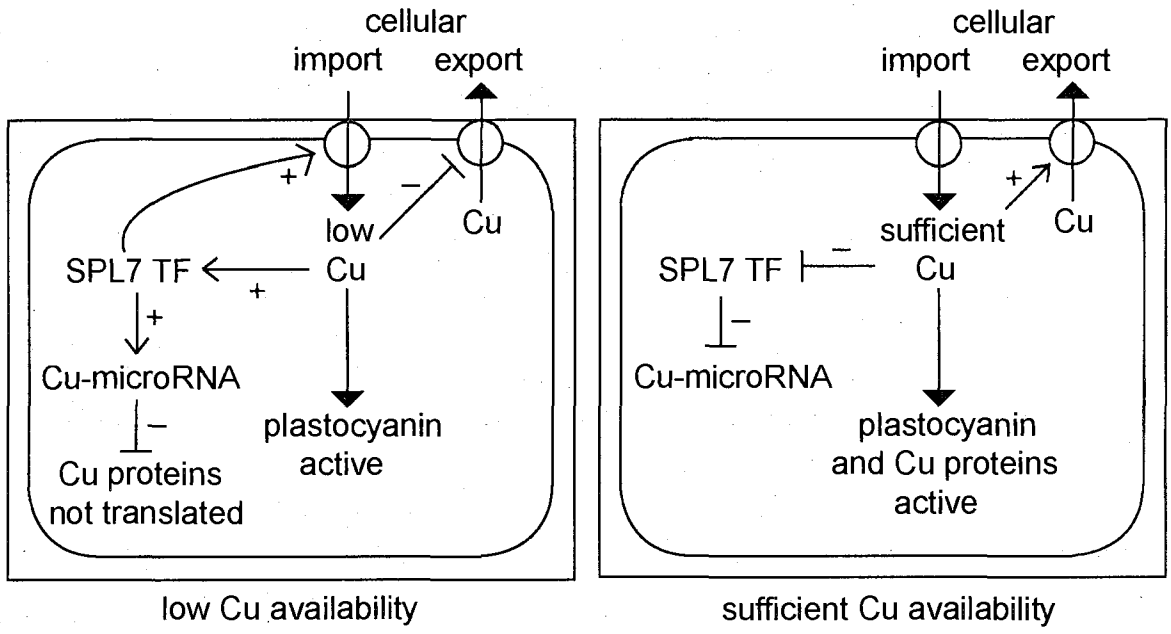

Figure 1. Model of Cu homeostasis regulation during low and sufficient Cu availability. *SQUAMOSA* promoter-binding protein like-7 transcription factor (SPL7 TF) and open circles for plasma membrane transporters are indicated. Cu uptake, export, and delivery are represented with closed arrow heads. Up-regulation of Cu delivery proteins is represented by open arrows and (+) to indicate activation, while down-regulation of proteins is represented by a perpendicular line and a (-).

CHAPTER 3

Regulation of superoxide dismutase expression by copper availability

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CHAPTER 3

Regulation of superoxide dismutase expression by copper availability

Abstract

The most abundant copper proteins in green tissues are plastocyanin in thylakoids and copper/zinc superoxide dismutase (Cu/ZnSOD) of which the major isoforms are found in the cytosol and in the chloroplast stroma. An iron superoxide dismutase (FeSOD) can also be found in the stroma. The expression of superoxide dismutases (SODs) has been studied mainly in the context of abiotic stress. However, the availability of metal co-factors may also determine SOD expression patterns. Indeed in *Arabidopsis thaliana* Cu/ZnSOD enzymes were only expressed when copper was sufficient. This observation was made for plants grown on sucrose-containing tissue culture media and regulation of SOD expression by copper has not been tested for other species. To investigate the effect of copper on SOD expression we used a hydroponic setup in which plants grew without any evident stress symptoms. We observed that, *A. thaliana*, *Brassica juncea*, *Lycopersicum lycopersicum*, *Zea mays*, and *Oryza sativa*, all down-regulated Cu/ZnSOD in response to copper limitation. Under this condition FeSOD expression was up-regulated to replace Cu/ZnSOD in the stroma in all plants except *Z. mays*, in which FeSOD was not detectable. Copper limitation did not affect plastocyanin accumulation in any of the plants except *Z. mays*.

Comparisons of leaf copper contents and superoxide dismutase expression suggest that Cu/ZnSOD and FeSOD expression levels are good indicators of impending copper deficiency. Plants that down-regulate Cu/ZnSOD and up-regulate FeSOD under copper limitation can maintain superoxide scavenging and save copper for use in plastocyanin, which is essential for photosynthesis.

Abbreviations- Cu/ZnSOD, Cu-Zn superoxide dismutase; FeSOD, Fe superoxide dismutase; MnSOD, Mn superoxide dismutase; PC, plastocyanin

1. Introduction

The trace element copper (Cu) is required as a cofactor for several processes including photosynthesis, respiration, ethylene perception, oxidative stress reduction, cell expansion, and cell wall lignification (1). In plant chloroplasts two major Cu containing proteins are found, plastocyanin (PC) and Cu/Zn superoxide dismutase (Cu/ZnSOD). Plastocyanin is one of the most abundant proteins in the thylakoid lumen (2,3) and is essential for electron transfer between the cytochrome b_6f complex and photosystem I (4). Polyphenol oxidase is another Cu protein found in the thylakoids of some plants, such as spinach (2), but not in other species such as *Arabidopsis thaliana* (3). In the chloroplast stroma, Cu/ZnSOD requires Cu, along with Zn, as co-factors to catalyze the dismutation of superoxide radicals ($O_2^{\cdot-}$) into H_2O_2 (5). In *A. thaliana*, seven SOD genes have been identified, with the major activities coming from CSD1, CSD2, FSD1, and MSD1 (6). CSD1 and CSD2 both encode a Cu/ZnSOD with CSD1 activity in the

cytosol and CSD2 activity in the stroma. FSD1 encodes a chloroplast localized SOD that utilizes Fe as the co-factor (FeSOD), while MSD1 encodes a mitochondrial SOD (MnSOD) that utilizes Mn as the co-factor (6). Other important Cu proteins in plant cells include cytochrome-*c* oxidase in the mitochondria, the ethylene receptors in the endomembrane system, and various apoplastic oxidases (for a review see 7).

Because of its redox potential, Cu can exist in both the Cu^{2+} and Cu^{1+} form in living organisms. The reversible oxidation-reduction of Cu makes it very useful as a co-factor in electron transfer reactions. However, the redox activity of Cu could also result in oxidative stress if Cu was present as a free ion. In yeast, the estimated free Cu ion concentration in the cell was at least 12 orders of magnitude below the total Cu concentration (8). To allow efficient delivery of essential metal ions and at the same time avoid toxic excess, all organisms have evolved transport and sequestration systems (9). Copper can be limiting to plant productivity in crop species when below 5 μg g^{-1} dry weight (DW) whereas toxicity is reported above 30 μg g^{-1} DW (1). The transport and delivery of Cu into and within plant cells has been reviewed recently (7). Plant cells acquire Cu through CopT family transporters (10), while Cu is removed from the cytosol by the HMA5 transporter (11). Within the cell, transport in chloroplasts is the best understood. Two Cu-transporting P-type ATPases, PAA1 and PAA2, are required for efficient delivery of Cu to PC in chloroplasts. A detailed biochemical and phenotypic analysis of mutations for PAA1 and PAA2 indicated that the transporters function in the chloroplast envelope and thylakoid membrane,

respectively. A third component of the Cu-delivery system in plant plastids is the Cu-chaperone CCS, a functional homologue of a yeast metallochaperone for Cu (12). Interestingly, CCS in plants has dual localization, in both cytosol and chloroplasts, and is required for activity of both CSD1 and CSD2 (13). Cu homeostasis not only requires mechanisms by which Cu is delivered to sub-cellular targets, but also regulated expression of targets for Cu delivery. Availability of Cu was found to be a major determinant of Cu/ZnSOD and FeSOD expression in *A. thaliana* (12,14). When Cu supply was sufficient, CSD1 and CSD2 were expressed and became active, yet their expression and activity diminished when Cu supply was limited. It was proposed that when Cu supply is limited, Cu is shuttled to PC rather than to CSD1 and CSD2, because PC is essential (14). However, during low Cu conditions, SOD activity may still be important for the scavenging of superoxide radicals produced at photosystem I, so an alternative SOD isoform, FSD1, is used. In the photosynthetic alga *Chlamydomonas reinhardtii*, PC is not essential because a heme-containing cytochrome c_6 can functionally replace it under Cu limitation (15). Thus, the hierarchy of Cu use and the mechanism to reduce the effects of Cu limitation is different in *Arabidopsis* and *Chlamydomonas*.

The reciprocal expression of Cu/ZnSOD and FeSOD in response to Cu availability during non-stress conditions was only demonstrated for *A. thaliana* plants grown on tissue culture medium. Thus far, regulation has not been verified in other growth conditions, and it has not been examined in other plant species. Information about micronutrient use at the cellular level could benefit agricultural

crop production through a better understanding of the genetic programs by which plants optimize photosynthetic activity in their green tissues during sub-optimal Cu growth conditions. In this study we present evidence that supports the model in which Cu is allocated preferentially to PC over cytosolic and stromal Cu/ZnSOD during Cu limited growth for a variety of crop species.

2. Materials and Methods

Plant species and growth conditions. The following species were used: *A. thaliana (col-0), Brassica juncea (accession no. 173874), Zea mays (var sugosa), Oryza sativa (sub. indica* IR64), and *Lycopersicon lycopersicum* (Pole Brandywine). Plants were germinated on Whatman® filter paper soaked in double distilled H_2O (ddH_2O). Seedlings (4 to 7 days old) were placed on 1/5X Hoagland's hydroponic solution (16). For growth under Cu limitation, Cu was omitted from the medium, while 0.05 µM Cu was added for Cu sufficient conditions. Hydroponic growth medium was contained in black plastic tubs 25 cm by 40 cm (6.8 L volume) and used in conjunction with a non-transparent acrylic cover having 15 evenly spaced holes where plantlets were placed and supported by the top half of a 1.5 ml microcentrifuge tube. The growth medium was replaced every 7 days and maintained daily with ddH_2O to compensate for evaporation. The solution was aerated continuously using aquarium pumps at 200 to 300 ml/min. To minimize Cu contamination of the hydroponic set-up, all equipment was washed with 25 mM EDTA and rinsed repeatedly with ddH_2O. Chemicals used for the Hoagland's solution were of A.C.S. grade and purchased

from Fisher Scientific (Fair Lawn, NJ). Pilot studies showed that even with Cu omitted from the medium all plants seemed to grow without noticeable stress and plants indeed accumulated measurable Cu, likely as a result of minor Cu contamination in the chemicals used to prepare medium. Each treatment consisted of 3 replicates, and each replicate contained 5 plants. Plants were grown for a total of 35 days in 10-h/14-h light/dark cycles at a light intensity of 120 µmol photons $m^{-2} \cdot s^{-1}$ and 23°C.

For growth in agar plates, tissue culture media was made using A.C.S. grade chemicals from Fisher Scientific (Fair Lawn, NJ) as described (17) minus Cu. Copper was added for treatments at 0.1µM and 5.0 µM, or omitted. Agar media contained 1% sucrose and 0.5 % agarose. All media was made using distilled de-ionized H_2O. Plants were grown in Magenta boxes (Sigma, St. Louis, MO) for a total of 21 days in 12-h/12-h light/dark cycles at a light intensity of 120 µmol photons $m^{-2} \cdot s^{-1}$ and 23°C.

Protein extraction, SOD activity, and immunoblotting. Leaf samples were taken after six hours into the light period and used for all measurements except where noted. In order to sample young, photosynthetically active leaves, the third, fourth, and fifth leaf of each plant (whole leaf for dicots, middle third for monocots, whole shoot for *A. thaliana*) were harvested and frozen in liquid nitrogen directly after measurement of shoot fresh weight. Soluble leaf proteins for non-denaturing and SDS polyacrylamide gel analysis were extracted as described (12). Protein concentration of extracts was determined according to Bradford (18) using BSA as a standard. For SOD isozyme separation and activity

analysis, 20µg of protein was fractionated on non-denaturing gels then stained for activity as described (12,19). Identification of SOD isozymes utilized 2 mM KCN to inhibit Cu/ZnSOD (20) and 3 mM H_2O_2 to inhibit Cu/ZnSOD and FeSOD (H_2O_2 results not shown). Antibodies used for immunodetection of SOD isoforms (6) and PC (12) have been described. All SOD antibodies were raised against *A. thaliana* proteins. Rubisco large subunit antibody was purchased from AgriSera (Stockholm, Sweden), and PC (12) was raised against spinach proteins. Each experiment was done in triplicate with identical results and representative gels are shown.

Fluorescence, CO_2 assimilation, and starch accumulation. Chlorophyll fluorescence was measured with a programmable, pulse modulated, Hansatech Fluorometer FMS2 (Hansatech Instruments, Norfolk, UK) on overnight dark-adapted plants essentially as described by Maxwell and Johnson (21). The following program was used to estimate chlorophyll fluorescence parameters: Plants were exposed to a saturating light pulse (2050 µmol photons $m^{-2} \cdot s^{-1}$) to estimate Fv/Fm (PSII antennae efficiency). Light adapted parameters were determined at actinic light intensities of 43, 350, and 770 µmol photons $m^{-2} \cdot s^{-1}$ using a saturating pulse to determine photosystem II quantum efficiency (ΦPSII), calculated as $(F_m'-F_s)/F_m'$; as well as photochemical quenching (qP), calculated as $(F_m'-F_s)/(F_m'-F_o)$, and non-photochemical quenching (NPQ), calculated as $(F_m-F_m')/F_m'$. A far-red illuminating pulse was initiated after each saturating pulse to establish initial fluorescence (F_o') (21). CO_2 assimilation was measured using a Licor 6400 (LI-COR Biosciences, Lincoln, NB). All measurements were made

with leaf samples after six hours into the light period. Ambient CO_2 of 360 $\mu mol \cdot mol^{-1}$ at an air flow of 200 $\mu mol \cdot s^{-1}$ and actinic light of 500 µmol photons $m^{-2} \cdot s^{-1}$ were used to measure total change in CO_2 in $\mu mol \cdot m^{-2} \cdot s^{-1}$. Starch staining as a qualitative indicator of starch accumulation was as follows: a leaf from each replicate was boiled in water for 1 minute then boiled in 95% ethanol until white. Leaves were added to Lugol's iodine solution for 4 minutes to stain for starch followed by rinsing in H_2O.

Biomass and elemental analysis. Shoot biomass for each plant was determined and grouped into a replicate of five plants. One gram of fresh leaves (stems not used), representative of the entire replicate, were dried for 5 days at 45°C. The dried leaves were re-weighed, and percent of total leaf mass as dry mass was determined. For elemental analysis, 100 mg of dried leaf samples was digested with 1 ml nitric acid and heated for 2 hours at 60 °C then 6 hours at 130 °C. Digests were diluted to 10 ml with ddH_2O and analyzed using Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) for Cu, Fe, Mg, Mn, Zn, P, and S as described (22).

Statistical analysis. Statistical analysis was performed using the Jump-in software package (SAS Institute, Cory, NC).

3. Results

To investigate if plants change their pattern of SOD expression in response to Cu availability, we grew several crop species in hydroponics. Our aim was to compare responses in photosynthetic tissues under conditions of mild Cu

limitation and Cu sufficiency while avoiding stresses due to either critical deficiency or toxicity. Pilot experiments indicated that all plants had exhibited a normal growth and appearance even when Cu was omitted. Under this condition we measured Cu concentrations of at least 5 μg g^{-1} DW, which is above reported critical deficiency levels for most plant species (1). For all experiments conducted, within each species and across all replicates, none of the plants showed any visible signs of chlorosis, anthocyanin production, or variation of size in response to the Cu treatments as illustrated by the appearance of 35 day old plants shown in Fig. 1.

With a reproducible hydroponic set up established, we focused first on *A. thaliana* and the related crop plant *B. juncea* (Indian mustard) to examine if these plants alter expression of the major Cu proteins, PC and Cu/ZnSOD, in response to Cu supply. *A. thaliana* plants grown in hydroponics had abundant Cu/ZnSOD activity with no noticeable FeSOD activity on Cu sufficient medium, whereas FeSOD was the most evident activity on Cu limited medium (Fig. 2A). The reciprocal changes in Cu/ZnSOD and FeSOD activity in response to Cu could be fully ascribed to a difference in the abundance of the corresponding polypeptides (Fig. 2B). Note that the CSD2 antibody recognizes a single protein in *A. thaliana* extracts whereas the CSD1 antibody detects CSD1 but also cross reacts to CSD2 which is similar in amino acid sequence (6). Importantly, we did not observe a change in the expression of the two PC isoforms in response to Cu feeding in hydroponics (Fig. 2B). Thus, in hydroponic conditions where *A. thaliana* growth depends fully on photosynthesis, we see very similar regulation

of Cu protein expression compared to what was observed for tissue culture supplied with sucrose (12). In *B. juncea* we observed, along with MnSOD and FeSOD, three possible isoforms of Cu/ZnSOD based on their activity (Fig. 2A) and cross-reactivity to CSD1 and CSD2 antibodies (Fig 2B). Similar to what was seen for *A. thaliana,* we observed that both the activity and protein abundance of Cu/ZnSOD and FeSOD were regulated in response to Cu and that the expression was reciprocal. At the same time, PC expression was also not affected by Cu treatment (Fig 2B). As expected, MnSOD was not affected by Cu feeding in *B. juncea* or any of the other species tested (Fig 2A and B; Fig 3A). To ensure that the treatments indeed affected leaf Cu concentration, an elemental analysis (ICP-AES) was conducted on leaf samples. In this first study, leaf Cu concentrations decreased in Cu limited plants, with *A. thaliana* decreasing significantly ($P<0.05$) (Fig. 2C). Copper content for all species and treatments remained within 5 and 30 $\mu g\ g^{-1}$ DW, a range considered to be between critical deficiency and toxicity for most plants (1). Iron content increased significantly ($P<0.05$) in *B. juncea* when Cu was limited, while *A. thaliana* showed no detectable change in Fe content in this experiment. Thus, under conditions of photosynthetic growth, both *A. thaliana* and *B. juncea* down-regulate expression of their Cu/ZnSODs and up-regulate FeSOD in response to mild Cu limitation, whereas PC levels are unaffected by this treatment. Therefore these plants seem to prioritize Cu delivery to PC over SOD proteins during Cu limitation.

To investigate if other unrelated plants show a similar response to Cu limitation we compared the response to Cu in *B. juncea* to a number of crop

species. To asses effects of Cu feeding we also analyzed how limited Cu growth conditions affected parameters indicative of productivity. In a second series of hydroponics, we grew the dicots, *B. juncea* and *L. lycopersicum (tomato),* next to the monocots, *Z. mays (corn)* and *O. sativa (rice). A. thaliana* was omitted from this experiment because of its small size which made it difficult to accurately measure of all the parameters reported here. We analyzed the activity and abundance of SOD isozymes and PC as well as micronutrient levels for Cu and Fe (Fig. 3). Reciprocal regulation of Cu/ZnSOD and FeSOD in response to Cu treatments was observed for *B. juncea* and *L. lycopersicum*, (Fig. 3A and B). However, for *B. juncea* perhaps Cu was not as limiting in the second experiment compared to the first (Fig. 3) as evidenced by a higher Cu content and the observation of both FeSOD and Cu/ZnSOD on low Cu. We observed that *L. lycopersicum* had a high Cu content compared to the other species tested, even under Cu limitation (Fig. 3C). Even though Cu concentrations were relatively high in both *B. juncea* and *L. lycopersicum*, a significant ($P<0.05$) Cu reduction in the limited Cu grown plants was measured, which apparently affected the regulation of FeSOD and Cu/ZnSOD abundance. The expression of PC did not change significantly for both dicot species, *B. juncea* and *L. lycopersicum* (Fig. 3B).

In the monocot species *Z. mays* we did not detect FeSOD activity, nor did we detect a protein band with cross-reactivity to *A. thaliana* FSD1, indicating that FeSOD is below the detection limit in this species. MnSOD and several Cu/ZnSOD isoforms were readily detected in *Z. mays*. In *Z. mays* we did observe reduced activity and protein abundance of the Cu/ZnSODs during limited Cu

growth conditions, although the shut-off was not complete. Interestingly, in *Z. mays* we also observed a reduced expression of PC during Cu limited growth (Fig. 3B). Because FeSOD's presence in *Z. mays* was not observed here it is possible that FeSOD does not exist in this *Z. mays* species, or it is induced only during growth conditions not imposed here. For *O. sativa*, expression and activity of Cu/ZnSOD and FeSOD occurred for both Cu treatments with only a slight decrease in Cu/ZnSOD for the Cu limited treatment, while FeSOD and PC appear unchanged. Since FeSOD activity in *O. sativa* is low and unchanged between Cu treatments, when compared to its Cu/ZnSOD counterpart, it is possible that Cu limited conditions are relatively minor and not low enough to increase FeSOD expression.

Both monocot species showed reduced leaf Cu concentrations when Cu limited, with a significant ($P<0.05$) reduction for *Z. mays* (Fig. 3C). Consistent with the first study, leaf Fe concentrations also showed a trend of increasing Fe during Cu limited growth. Additional elemental analyses included phosphorous (P) and sulfur (S). Most plant species tested showed a trend of decreasing P and S when Cu limited (Table 1). However, instead of decreased, we found increased contents of P and S in Cu limited *B. juncea* ($P<0.05$). Other elements tested (Zn, Mn, and Mg) were not significantly different between treatments in any of the plants tested (not shown).

We tested a number of parameters that are useful indicators of plant health and productivity (Table 1). Only those parameters that showed the greatest variation between treatments are shown and they indicated only a few significant

differences between + and - Cu treatments for all of the plant species tested (Table 1). Even though there were no major changes between Cu treatments, there were trends apparent in the data that correlate with SOD and PC expression levels. Noticeably, on low Cu compared to high Cu for *Z. mays* the decreasing trend in CO_2 assimilation, dry mass, and electron transport activity from photosystem II activity coincides with reduced PC expression. A reduced flux PSII was also observed for *L. lycopersicum* under Cu limitation, albeit not significant. The accumulation of starch was also unchanged among treatments for all species, and is not shown here. Overall, we observed that all plant species tested reduced Cu/ZnSOD expression in response to Cu limitation. At the same time we observed up-regulation of FeSOD for dicots but not for the monocots. In all plants except for *Z. mays* the expression of PC was not significantly affected by Cu limitation.

The apparent lack of a response to Cu for FeSOD in *O. sativa* could be due to insufficient depletion of Cu in hydroponics for the species. We examined the effect of Cu on SOD expression for plants grown in agar media, where Cu can be depleted more easily. We compared *Z. mays*, *O. sativa*, and *B. juncea*. We analyzed 21 day old seedlings grown without Cu, or 0.1 μM Cu and 5.0 μM Cu added (Fig. 4). All of the proteins detected in *B. juncea* and *Z. mays* responded similarly to those seen using hydroponic growth conditions (Fig. 2B, 3B). For *O. sativa* grown in agar, protein expression levels of Cu/ZnSODs decrease on low Cu. For FeSOD, by loading 40 μg instead of 25 μg of protein, we now clearly detect two FeSOD bands and observed a slight increase on low Cu (Fig. 4).

Interestingly, the 0.1 μM Cu in agar growth media, which is the Cu concentration found in standard MS media (17), does not support full Cu/ZnSOD expression in *B. juncea* or *Z. mays*, and appears to be insufficient for PC expression in *Z. mays*.

4. Discussion

Preferential allocation of Cu to PC during limited Cu growth conditions was observed in this study for four out of five plant species. This observation underscores the essential role of PC in photosynthetic electron transport. Only in *Z. mays* did we see a simultaneous reduction of the PC level together with Cu/ZnSOD under Cu limitation. Additionally, overall primary productivity effects were minor for all plant species which indicates that plants have a certain plasticity that allows them to thrive on a range of Cu concentrations.

Copper deficient plants exhibit chlorosis, stunted growth, and apical meristem death with general critical Cu deficiencies ranging between 1 to 5 μg g^{-1} DW. The reported onset of critical Cu toxicity for many species is in the 20 to 30 μg g^{-1} DW range (1). We observed that leaf Cu concentration varied, as expected, between sufficient and limited Cu treatments within each species. However, the differences between species were often larger. For instance, both *B. juncea* and *L. lycopersicum* contained more leaf Cu during limited Cu growth than *Z. mays* grown on sufficient Cu. Therefore, the expression of Cu/ZnSOD, together with fluorescence measurements, may give a better indicator of Cu status.

Since Cu limitation was correlated with a reduction in Cu/ZnSOD activity, it is possible that Cu availability is in part responsible for the expression of Cu/ZnSOD. During limited Cu growth conditions, preferential allocation of Cu to PC could occur by simply reducing Cu/ZnSOD. Studies analyzing the regulation of SODs, particularly Cu/ZnSOD, show increased mRNA and/or SOD activities during stress (23-27), while the expression of MnSOD was constitutive under most conditions tested in *A. thaliana* (6). However, these studies only supplied sufficient to toxic levels of Cu to the plants during growth.

In addition, over-expression studies for FeSOD in tobacco (20), poplar (28), and *Z. mays* (29) all lead to increased tolerance to oxidative stress. FeSOD has also been shown to increase naturally when plants are subjected to chloroplastic oxidative stress (30,31), high light (6) or Cu deficiency (27,32), and decrease when exposed to excess Cu (24,27). However, those studies examining effects of deficient and excess Cu growth conditions did not show clear reciprocal regulation of Cu/ZnSOD and FeSOD during non-stressed growth conditions; but rather linked the regulation of Cu/ZnSOD and FeSOD to Cu related stress. Here we suggest that Cu availability is important in determining which SOD isoform plants express prior to any additional stress. However, it is important to note that regulation of SODs based on Cu status may not be uniform for all species. *Z. mays*, for example, may be less adapted to Cu limitation, or another important regulatory element not induced here is necessary for FeSOD expression during Cu limitation. A lack of FeSOD induction during low Cu growth while simultaneously down-regulating Cu/ZnSOD for *Z. mays* was previously reported

(33). *O. sativa*, on the other hand, appears to maintain both Cu/ZnSOD and FeSOD, at least in the range of Cu concentration tested here. It is possible that monocots have evolved with different mechanisms to reduce the effects of Cu limitation. With the *O. sativa* genome sequenced and the cloning of OsFeSOD complete (34), a better understanding of SOD regulation in response to Cu in monocot species is possible.

In our study we also observed a reciprocal trend in leaf Cu and Fe concentration based on Cu availability suggesting that Fe translocation to leaf tissue is increased during Cu limitation perhaps to compensate for Cu reduction. Several studies have indicated that Cu deficiency increases the expression of proteins likely involved in Fe uptake and shoot translocation (35-37).

We propose that plants that down-regulate Cu/ZnSOD and up-regulate FeSOD under Cu limitation can save Cu for use in PC, which is essential for photosynthesis. This plasticity in response to Cu nutrition allows plants to always have an SOD enzyme ready to scavenge superoxide radicals in the stroma and at the same time maintain PC activity for photosynthetic electron transport. It is reasonable that a Cu cofactor requiring protein is utilized to save Fe and perhaps buffer cellular Cu when Cu is sufficient. Down-regulation of a Cu protein that can be functionally replaced by an Fe cofactor requiring protein makes sense when Cu is limited. These adaptations to varying Cu nutrient levels broaden the range of conditions under which plants can thrive.

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Physiological measurements of hydroponic grown plants.

	B. juncea		*Z. mays*		*O. sativa*		*L. lycopersicum*	
Cu addition	+	-	+	-	+	-	+	-
CO_2 assimilation $\mu mol \cdot m^{-2} s^{-1}$	*7.9 ±0.4	10.0 ± 0.5	15.6 ± 1.1	14.3 ± 0.3	10.7 ± 1.6	11.1 ± 1.9	6.6 ± 0.5	5.0 ± 0.6
shoot fresh wt. (g)	39.8 ±4.4	44.3 ± 6.6	64.0 ± 7.6	67.4 ± 8.1	11.9 ± 0.7	12.9 ± 3.3	50.5 ± 5.1	58.1 ± 8.7
% as dry mass	*8.8 ±0.1	10.6 ± 0.6	*11.8 ± 0.2	9.9 ± 0.4	22.1 ± 0.8	22.0 ± 1.8	11.3 ± 1.0	13.1 ± 1.7
Fv/Fm	0.84 ±0.01	0.86 ± 0.00	0.83 ± 0.00	0.82 ± 0.00	0.83 ± 0.00	0.84 ± 0.00	0.83 ± 0.01	0.83 ± 0.01
PSII	0.12 ±0.04	0.15 ± 0.03	0.14 ± 0.03	0.09 ± 0.04	0.20 ± 0.03	0.21 ± 0.02	0.08 ± 0.00	0.05 ± 0.01
phosphorus (µg/g DW)	6642 ±570	7280 ± 183	10006 ±135	8717 ± 700	4448 ± 147	4316 ± 89	7391 ± 289	5967 ± 948
sulfur (µg/g DW)	*10340±703	11758 ±365	3017 ± 327	2801 ± 32	3124 ± 56	2920 ± 104	*9832 ± 632	7675 ± 903

Table 1. Measurements were made on 35 day old plants grown with 0.05 µM added Cu (+) and omitted Cu (-). CO_2 assimilation in $\mu mol \cdot m^{-2} s^{-1}$ at 360 µmol CO_2 and 400 µmol photons $m^{-2} s^{-1}$. Fv/ Fm (PSII antennae efficiency) was measured on dark adapted plants and PSII (PSII quantum efficiency) was measured at 770 µmol photons $m^{-2} s^{-1}$. Elemental analysis of phosphorous and sulfur shown in $\mu g\ g^{-1}$ DW (dry weight). Values reported as averages for three replicates $\pm$ SE of the mean. Significant differences between pairs are indicated with an * (Student's *t* test; $P<0.05$).

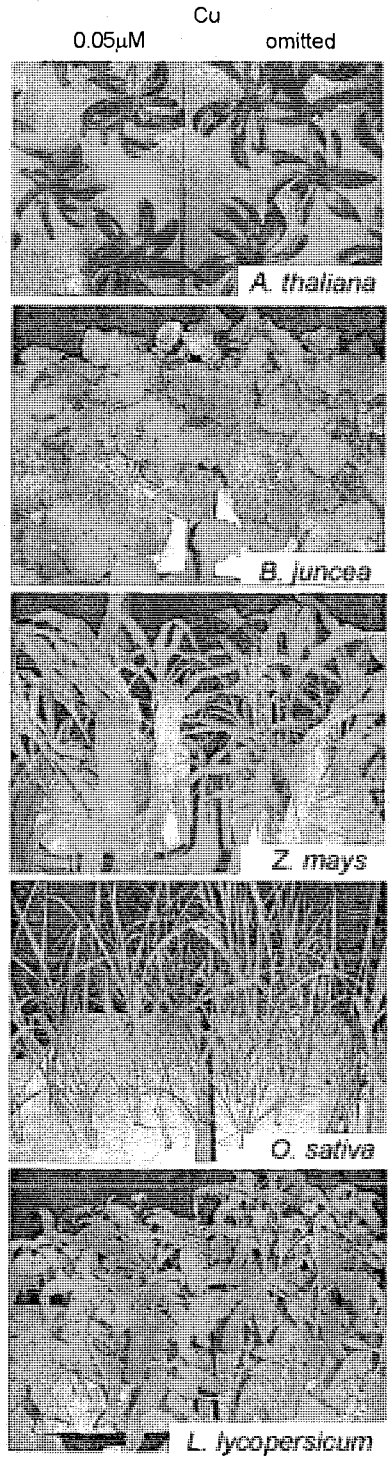

Figure 1. Hydroponically grown plants.
Images of 35 day old plants prior to sampling.

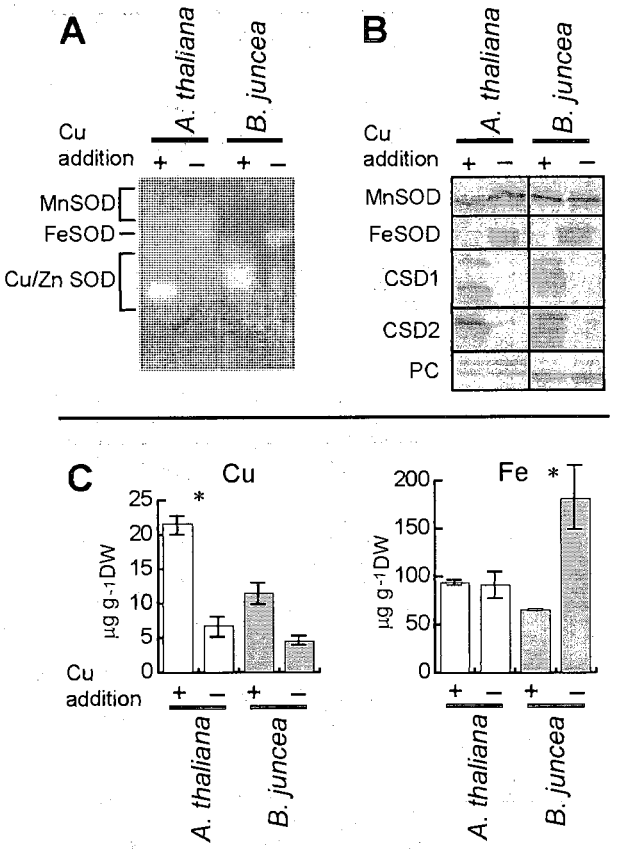

Figure 2. Effects of Cu treatment on hydroponically grown plants. Hydroponically grown wild-type plants in 1/5 Hoagland's with 0.05 µM added Cu (+) and omitted Cu (-) for 35 days (A) SOD isozyme activities from leaf homogenates. Total soluble proteins (25 µg per lane) were fractionated on non-denaturing 15% acrylamide gels and stained for total SOD activity. (B) Immuno-detection of plastocyanin (PC) and SOD proteins. Proteins extracted from leaf homogenate (25 µg per lane) were fractionated by 15% SDS-PAGE. Each protein was detected by immunoblot analysis using specified antibodies. (C) Analysis of leaf Fe and Cu. Values reported as averages for 3 replicates in µg g-1 of dry weight (DW) + SE of the mean. Significant differences between pairs are indicated with an * (Student's t test; $P<0.05$).

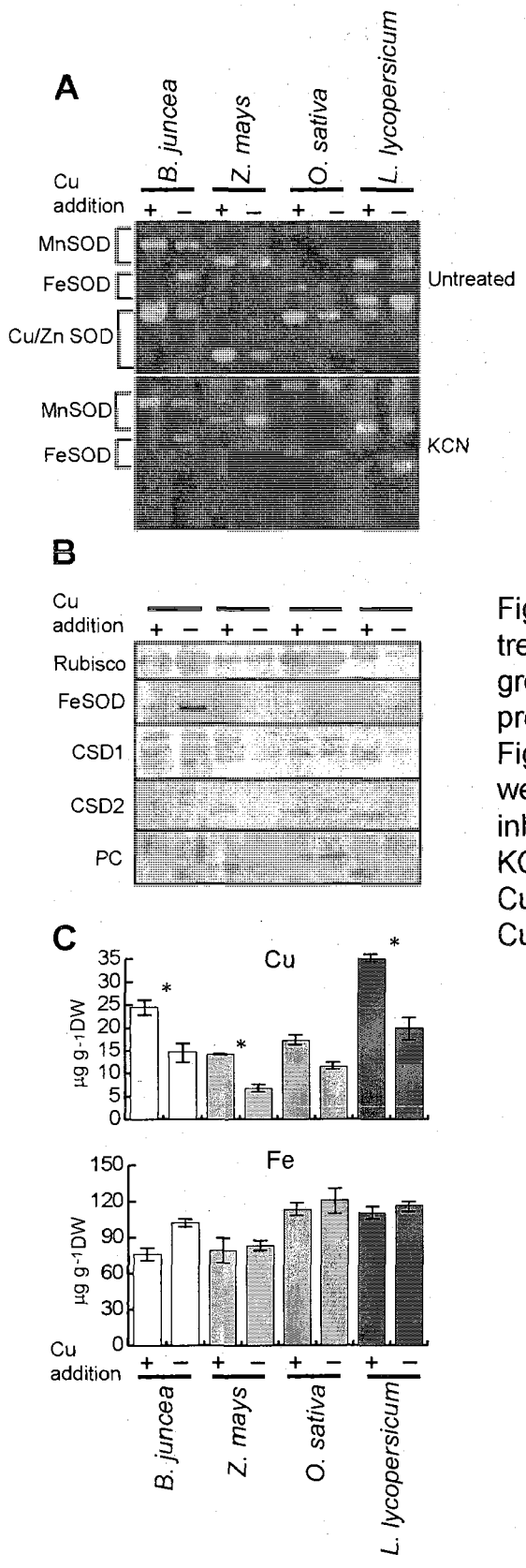

Figure 3. Effects of Cu treatment on hydroponically grown plants. All experimental procedures are the same as Fig 2 except in (A) where gels were stained without an inhibitor (untreated) or with KCN (KCN), to inhibit Cu/ZnSOD, to differentiate Cu/ZnSOD from FeSOD.

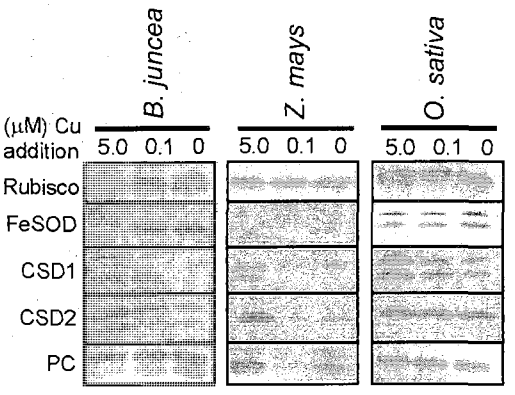

Figure 4. Effects of Cu treatment on tissue culture grown plants. Three species were grown for 21 days on MS media with the indicated final Cu concentrations. Proteins extracted from leaf tissue were fractionated by 15% SDS-PAGE. Plastocyanin (PC) and SOD proteins were immuno-detected with specified antibodies. 25 μg were loaded into each lane, except for detecting FeSOD in *O. sativa*, where 40 μg was loaded per lane.

CHAPTER 4

Arabidopsis without measurable superoxide dismutase activity in the chloroplast and cytosol

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Chapters 4 and 5 will be combined and submitted for publication

CHAPTER 4

Arabidopsis without measurable superoxide dismutase activity in the chloroplast and cytosol

Abstract

Superoxide dismutases (SODs) are a highly conserved group of metallo-enzymes found in most oxygenic organisms that likely play a role in reducing oxidative stress. In *Arabidopsis* a recent study into the regulation of chloroplastic Cu/ZnSOD (CSD2) and FeSOD (FSD1) has revealed that Cu availability is the main determinant as to which chloroplastic SOD is active. During sufficient Cu supply, CSD2 is expressed and active, yet CSD2 is down-regulated during Cu-limitation while FSD1 is up-regulated. *CSD2* mRNA is negatively regulated by microRNA 398 when Cu availability is limited. A transcription factor in moss containing a SBP (SQUAMOSA promoter-binding factor) domain acts on the *FeSOD* promoter to suppress transcription during Cu-sufficient conditions. During Cu-limitation, plastocyanin mRNA levels remain high, suggesting that the delivery of Cu to plastocyanin is a priority. Plants contain the Cu chaperone for SOD (CCS) that delivers Cu to both CSD1 and CSD2, and in *Arabidopsis* CCS is encoded by one gene that encodes both cytosolic and plastidic proteins. A knock-out mutant of *CCS* Showed no detectable Cu/ZnSOD activity, indicating that CCS is essential for activation of Cu/ZnSOD in *Arabidopsis*. When the CCS-

KO is grown with sufficient Cu these plants also lack FeSOD activity. These CCS-KO plants without measurable stromal or cytosolic SOD activity do not exhibit a visual phenotype on soil, and chlorophyll fluorescence measurements do not indicate reduced electron transport rates compared to wild-type, even after high light stress. These results indicate that very low levels of SOD activity are sufficient for normal growth in laboratory conditions.

Abbreviations- Cu/ZnSOD, Cu-Zn superoxide dismutase; FeSOD, Fe superoxide dismutase; MnSOD, Mn superoxide dismutase; MV, methyl viologen; PC, plastocyanin

1. Introduction

In *Arabidopsis*, seven nucleus-encoded superoxide dismutase (SOD) genes have been described with the most abundant activities in leaf tissue belonging to CSD1, CSD2, FSD1, and MSD1 (1). The Cu/ZnSODs, CSD1 and CSD2, are active in the cytosol and chloroplast stroma respectively. The stroma also contains the FeSOD, FSD1, while MnSOD, MSD1, is located in the mitochondria (1). However, a recent study located FSD1 in the cytosol only, and indicated that FSD2 and FSD3 are located on the thylakoid and are essential for normal growth (2). Superoxide radicals ($O_2^{\cdot-}$) are generated during photosynthetic electron transport when a univalent electron transfer to oxygen generates $O_2^{\cdot-}$ (3). The accumulation of superoxide radicals, together with H_2O_2 and metal ions, can lead to the formation of highly reactive hydroxyl radicals ($OH^{\cdot}$) (4). SOD catalyzes the

disproportionation of superoxide radicals to form H_2O_2 and O_2 (5). In plastids, the H_2O_2 is removed with the aid of ascorbate to form water, a reaction catalyzed by ascorbate peroxidase (APX) (6). Because the electrons in the electron transport chain originate from water, this pathway is known as the water-water cycle, a process that possibly has significance as a photo-protective mechanism (4).

Transgenic plants that over-express SOD in the chloroplast exhibit enhanced tolerance to abiotic stress, mainly to methyl viologen (MV) (7-23). Some studies have indicated noticeably enhanced photo-protective benefits from chloroplast Cu/ZnSOD based on analyses of Cu/ZnSOD over-expression (24) and a reported knock-down of *CSD2* (25). However, other studies have shown that over-expressed SOD in the chloroplast does not result in increased oxidative stress tolerance (7, 8, 12, 16, 17, 23). Taken together, the importance of plastidic and cytosolic SODs in photoprotection and oxidative stress prevention is not yet clear. One reason for this is that plants have different SOD isoforms with, until recently, poorly understood patterns of regulation which made it hard to obtain plants without SOD activity in these compartments.

Copper availability is the major factor that determines which SOD is expressed in the chloroplast (26-30). During Cu-limited growth conditions, CSD1 and CSD2 regulation is mediated by a microRNA, miR398, which targets CSD1 and CSD2 mRNA for degradation (29). In addition, FSD1 is up-regulated proportionally to CSD1 and CSD2 down-regulation during Cu-limited growth (26-29, 31). A transcription factor in moss containing a SBP (SQUAMOSA promoter-binding factor) domain acts on the promoter of *FeSOD* to suppress transcription

during Cu-sufficient conditions (31). It was proposed that the mechanism of Cu/ZnSOD down-regulation during Cu-limited growth allows for preferential delivery of Cu to plastocyanin (PC) (26-28), which is the most abundant protein in the thylakoid lumen (32, 33) and is essential in higher plant electron transport and survival (34). Oxidative stress may also play a role in Cu/ZnSOD transcript regulation in *Arabidopsis* plants (21) but Cu must also be available for mRNA levels to increase during stress (29). High light stress alone did not increase CSD1 and CSD2 unless Cu was available, suggesting that oxidative stress may have an additive effect on CSD1 and CSD2 levels (29). It is important to note that the amount of Cu required for full expression of Cu/ZnSODs is at least two orders of magnitude below toxic Cu levels (29).

Protein stability based on Cu-cofactor availability also plays a role in Cu/ZnSOD abundance. In *Arabidopsis*, when Cu delivery to CSD2 was disrupted, the mRNA levels of CSD2 remained high while protein abundance and activity were dramatically reduced (26, 35). It is possible that without Cu the Cu/ZnSOD polypeptide does not accumulate (36). Plants contain a Cu Chaperone for SOD (CCS) that delivers Cu to both CSD1 and CSD2. In *Arabidopsis*, CCS is encoded by one gene (*CCS*) that encodes both cytosolic and plastidic proteins (26, 35). Here we show that a knock-out of *CCS* has no measurable stromal or cytosolic Cu/ZnSOD activity, indicating that CCS is essential for activation of Cu/ZnSOD in *Arabidopsis*. When these plants are grown on sufficient Cu, their FSD1 is also shut-off. CCS-KO plants without measurable SOD activity in the cytosol and chloroplast do not show a visual

phenotype or photosynthetic electron transport deficiencies under non-stress and moderately high light stress conditions. These results indicate that very low levels of SOD activity are sufficient for normal growth in laboratory conditions.

2. Materials and Methods

Plant Materials and Growth Conditions- Arabidopsis Col-0 *CCS* knock-out line contains a T-DNA insert in the second exon of *CCS* (CCS-KO) (35). The CCS-KO line was obtained from the SALK collection at ABRC (SALK_025986). We backcrossed the line to wild-type three times then selfed and screened the plants to allow for the collection of a single insert homozygous line. The knock-down line of *CSD2* is also in the Col-0 background and contains a T-DNA insert in the promoter of *CSD2* (25). The *CSD2* knock-down line was obtained from the SALK collection at ABRC (SALK_041901) on 08/2006, while the KD-SOD line was obtained from the Mittler lab on 10/2004 then propagated in our lab. All plant lines were grown in soil (Fafard®Custom Mix, Conrad Fafard Inc., Agawam, MA) watered every 4 days and received water once a week with a final concentration of 5 μM $CuSO_4$. Plants were grown in controlled conditions (light intensity of 150 μmol m^{-2} s^{-1}, 10-h/14-h light/dark cycle at 23°C) for 35 days and denoted as low light (LL). High light treatments (HL) were administered to LL grown plants during controlled conditions of 850 μmol m^{-2} s^{-1} for 24 h at 23°C. All plant shoot tissues were collected immediately and frozen in liquid nitrogen then stored at -80°C unless otherwise noted.

For plants grown on tissue culture medium, seeds were surface-sterilized and vernalized for 4 days at 4°C then sown on 0.5 and 0.25 Murashige and Skoog (MS) media (Caisson Laboratories Inc., North Logan, UT) containing 0.4% Agargel (Sigma-Aldrich, St Louis, MO), 1% sucrose, and 5 μM $CuSO_4$ when specified. Plants treated with MV were grown in 5 nM MV. Plants treated with selenate (SeO_4) were grown on 30 μM SeO_4. All tissue culture plants were grown in controlled conditions (light intensity of 150 μmol m^{-2} s^{-1}, 12-h/12-h light/dark cycle at 23°C) for 14 days.

PCR and Southern Blotting- Genomic DNA was amplified by PCR using genomic *CCS* primers CCSLP (5'-GACAAACCGTGGAATTGAAACG-3') and CCSRP (5'-ATCGCGATCGCCTTCTCTTCT-3'); and T-DNA primers LBa1 (5'-TGGTTCACGTAGTGGGCCATCG -3'), PBIN19F (5'-CTTCGCTATTACGCCAG CTGGCGAA -3') and PBIN19R (5'-CGTTTTACGTTTGGAACTGACAGAA -3'). PCR of *CSD2* knock-down T-DNA lines was conducted using genomic *CSD2* primers CSD2LP (5'-GGAAACTATTTCTAGATAGTTTCATGC-3'), CSD2RP (5'-CAATTGAGAGAGACGGACCTGAGT-3'); and T-DNA primers LBb1 (5'-GCGTG GACCGCTTGCTGCAACT-3'), PBIN19F and PBIN19R. Southern blot analysis was conducted by restriction digest of genomic DNA using *Bam*HI, *Sac*I, *Nde*I, *Xho*I and *Eco*91I, separation of DNA by electrophoresis, transferred to Hybond N+ membranes and probed with *CCS* and T-DNA specific probes. Template was amplified by PCR using CCSLP and CCSRP primers for *CCS* and PBIN19F and T-DNA1016R (5'-GTGAGGATACTTGGATCTTCCCCTG-3') for T-DNA. Labeled probe was created using a random primer method with [^{32}P]dCTP as described

(26). Radiolabeled bands were visualized in a STORM PhosphorImager (Molecular Dynamics). For RT-PCR, total RNA was prepared from wild-type and CCS-KO plants using the TRIzol reagent (Invitrogen) as described in the user's manual. Total RNA (1 μg) was converted to cDNA using Moloney murine leukemia virus-reverse transcriptase (Promega, Madison, WI) according to manufacturer instructions. Primers specific to *CCS* mRNA were used for RT-PCR; CCSF (5'-GAATTCCATATGGCGACTGCTCTCACTTCTGATCGT-3') and CCSR (5'-GCGCCTAGGTTAAGTACCATCACAAGAACAAAGT-3').

Immuno-Detection and SOD Activity- Soluble shoot proteins for SDS polyacrylamide and non-denaturing gel analysis were extracted as described (26). Total protein was quantified according to the Bradford (37) method using bovine serum albumin as a standard. For immuno-detection analysis, 20 μg of protein extract were loaded into 12.5% SDS polyacrylamide gels. Antibodies used for CSD1, FSD1 and MnSOD (1), PC (26), Psa A/B (38), Psa D (39) and cytochrome *b6* (40) have been described. Antibodies for CSD2, CCS and large subunit of Rubisco were obtained from Agrisera (Agrisera AB, Vännäs, Sweden). The antibodies for CSD2 and CCS have been raised collaboratively. The respective coding sequences for the mature regions of CSD2 and CCS were cloned into pET28a, expressed in *E. coli* BL21 (DE3) and the his_6-tagged proteins were purified using affinity chromatography on a Ni-iminodiacetic acid agarose column followed by thrombin cleavage of the affinity tag and further purification by ion-exchange chromatography, essentially as described previously for the SufE protein (41). The purified proteins were shipped to Agrisera AB

(Vännäs, Sweden) and used to generate antibodies in rabbits according to standard protocols. The antisera were tested for specificity and sensitivity using immunoblotting assays. Antibodies for HSP70 were obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO). Specific antibodies for the D1 subunit were a generous gift from Alice Barkan (University of Oregon, Eugene, OR). For SOD isozyme separation and activity, leaf protein extracts were loaded into 15% non-denaturing polyacrylamide gels then stained for activity as described (26, 42). Each experiment was replicated three times with identical results, and representative gels are shown. Identification of SOD isoforms was conducted using inhibitors KCN (inhibits Cu/ZnSOD) and H_2O_2 (inhibits FeSOD and Cu/ZnSOD) (data not shown). Quantification of SOD activity bands in the non-denaturing polyacrylamide gels was conducted using Image J, a freely available image analysis program (43). Bands were quantified and represented as arbitrary units (A.U.) relative to the 40 µg lane sample (Fig. 3C). Determination of SOD activity in a liquid assay was performed as described (44) using 50 µg of total protein isolated from stromal fractions, as described (45), in a final volume of 200 µl. One unit of SOD activity corresponds to the inhibition of NBT reduction by 50%. Data are represented as units of SOD mg^{-1} protein. Histochemical NBT staining for superoxide *in situ* was conducted as described (44) using whole plants grown on 0.5 MS tissue culture media as described above.

Elemental Analysis, Chlorophyll Content and Reproductive Success- Elemental analysis was conducted on shoot material as described (46, 28). Spectrophotometric quantification of chlorophyll a and b content was conducted

using fresh leaf disks (38.5 mm^2) in 1 ml of 96% ETOH as described (47). For determining the number of siliques per plant, individual plants grown in LL conditions continued to receive water until natural senescence occurred and no new flowers formed. Seeds collected from the silique count plants were pooled for each plant line and sown on 0.5 MS plates using 100 seeds per plate to quantify germination rates at 5 days after sowing. All experiments were conducted using three independent biological replicates.

Chlorophyll Fluorescence- Chlorophyll fluorescence measurements were conducted on plants grown in soil under LL and after a HL stress treatment, as described above, using a programmable, pulsed modulated, Hansatech Fluorometer FMS2 (Hansatech Instruments, Norfolk, UK) as described (28); with chlorophyll fluorescence parameters quantified as described (48). Data shown are values obtained during an actinic light intensity of 770 µmol m^{-2} s^{-1} for a duration of 3 min (Fig. 5B and 9). Plants grown on MS medium were analyzed for chlorophyll fluorescence using a FluorCam 701MF controlled by version 5 software (Photon Systems Instruments, Brno, Czech Republic). All experiments were conducted using the 'Quenching analysis' program with actinic light intensity of 150 µmol m^{-2} s^{-1}, all other parameters were kept as program defaults. Tissue culture plants grown in 5 µM $CuSO_4$ with 0 or 5 nM MV were imaged at 14 days following a 30 min dark adaptation period.

Pigments, Tocopherols, GSH and AsA, Selenate Treatments, and Statistics- Pigments and tocopherols were quantified by high-performance liquid chromatography (HPLC) and normalized to chlorophyll a and b content. Frozen

samples were prepared and analyzed as described (49, 50). Levels of reduced and oxidized ascorbate (AsA) and glutathione were quantified using spectrophotometric methods essentially as described (51) using 0.1 g of frozen leaf tissue. Selenate treated plants were imaged after 14 days of growth. Individual plants were traced and leaf area analyzed using Image J (43). All statistical analyses (ANOVA, *t* tests) were performed by using the Jump-in software package (SAS Institute, Cary, NC).

3 Results

A homozygous knock-out of *CCS* (CCS-KO) containing a T-DNA insert in the second exon (35) (Fig. 1A) was obtained from the *Arabidopsis* Biological Resource Center and backcrossed three times then grown in soil supplemented with $CuSO_4$ which promotes reduced FSD1 expression. CCS-KO plants grown for five weeks under 150 μmol m^{-2} s^{-1}, denoted as low light (LL) conditions, did not display a visual phenotype relative to wild-type plants (Fig. 1B). Reproductive success of CCS-KO plants was also examined and no difference in phenotype was observed (Fig. 1C). The number of siliques per plant and germination rates of CCS-KO seeds showed no significant differences when compared to wild-type, consistent with the lack of a visual phenotype (Fig. 1D).

To ensure that CCS-KO carried the expected T-DNA insert, PCR and Southern blot analyses were conducted. The PCR analysis, using a T-DNA specific left border (LBa1) primer along with *CCS* left (CCSLP) and right (CCSRP) primers, revealed that the CCS-KO plants contain a tandem T-DNA

insert in *CCS* with two flanking T-DNA left borders (Fig. 2A). Using a Southern blot assay we confirmed that the CCS-KO line contains the expected T-DNA insert in *CCS* and it is likely a tandem repeat. Genomic DNA from wild-type and CCS-KO plants was digested by restriction enzymes (Fig. 2B) and hybridized with *CCS* and T-DNA specific probes (Fig. 2C). The CCS probe was designed to recognize the entirety of *CCS* while the T-DNA probe was designed to recognize the region between the left border and *Bam*HI restriction site of T-DNA. Restriction sites *Bam*HI and *Sac*I exist within the T-DNA sequence and therefore two DNA fragments were detected for CCS-KO when using the CCS probe (Fig. 2B). The sizes of these two bands for CCS-KO suggest that two left borders for the T-DNA insert exist, as indicated by the PCR above (Fig. 2A and C). When probed for T-DNA, the *Bam*HI restriction digest produced two expected bands at 16.9 kb and 5.8 kb, consistent with having two left borders (Fig. 2C). However, three to four additional bands were detected for *Bam*HI, suggesting that there are several T-DNA inserts in tandem. The single, low mobility, bands detected for *Xho*I, *Nde*I and *Eco*91I restriction digests suggest that a large tandem T-DNA insert resides in *CCS*, and that any additional T-DNA inserts elsewhere in the genome are not likely (Fig. 2C). Analysis of transcript levels for CSS-KO, conducted by reverse transcriptase PCR (RT-PCR), showed no mRNA for CCS, indicating that CCS-KO is a null mutant (Fig. 2D). Expression of CCS protein in CCS-KO was also undetectable, consistent with this conclusion (Fig. 3A).

When CCS-KO plants were grown with $CuSO_4$ supplements, accumulation of CSD1, CSD2 and FSD1 proteins were affected (Fig. 3A and B). Expression and

activity levels for MnSOD, along with PC, remained unchanged for CCS-KO relative to wild-type (Fig. 3A and B). Immuno-detection of CSD1 and CSD2 did show some evidence that low levels of the polypeptides are present; however, without CCS delivery of Cu to CSD1 and CSD2 they did not accumulate to wild-type levels and, more importantly, activity was not detected (Fig. 3A and B). As much as 100 µg of protein loaded onto a non-denaturing gel did not show Cu/ZnSOD or FeSOD activity for CCS-KO, even after a high light (HL) stress treatment of continuous 850 µmol m^{-2} s^{-1} for 24 hours prior to sampling (Fig. 3B). Non-denaturing gel assays for SOD activity conducted on a wild-type sample detected Cu/ZnSOD activity in a lane containing only 2 µg of protein (Fig. 3C). While these SOD activity assays may not be sensitive enough to detect minute levels of SOD activity, the native gel SOD activity dilution series suggests that if any SOD activity exists in CCS-KO it is at least 50 fold lower than wild-type levels if we consider a 2 µg protein sample versus a 100 µg protein sample (Fig. 3B and C). In addition, a liquid assay to determine SOD activity was conducted on the stromal fraction of tissue culture grown plants. For wild-type plants, SOD activity averaged 3.9 units mg^{-1} stromal protein while CCS-KO plants contained no measurable stromal SOD activity when compared to the non-protein negative water control (Fig. 3D). Purity and recovery of stromal fractions were also examined using immuno-detection of the large subunit of Rubisco (RbcL), CSD2, FSD1 and MnSOD, confirming that they did not contain FSD1 and MnSOD proteins (Fig. 3D).

Copper, iron and zinc content was analyzed for wild-type and CCS-KO to ensure that Cu supplements during soil growth did not result in toxic Cu levels in the shoots. Copper levels for the CCS-KO and wild-type remained between 8 and 11 μg g^{-1} dry weight (DW), which is not in the toxic range for *Arabidopsis* (28, 30) (Fig. 4). Indeed, only Cu levels above 20 μg g^{-1} DW are reported toxic for most plants (52). Shoot tissue of CCS-KO showed a statistically significant reduction in Cu and Zn levels yet increased Fe content relative to wild-type. Total Cu in CCS-KO leaf tissue averaged at 8.9 μg g^{-1} DW versus wild-type at 11 μg g^{-1} DW, a 19% total reduction (Fig. 4). Chlorophyll a and b content was also determined in leaf disks which indicated that CCS-KO did not have reduced chlorophyll levels when compared to wild-type (Fig. 4).

Superoxide dismutase in the chloroplast has been implicated as an important component of photo-oxidative stress reduction (4). We monitored chlorophyll fluorescence to determine if chloroplasts without SOD activity are impaired in photosynthesis. Wild-type and CCS-KO plants grown with $CuSO_4$ supplementation to turn off FeSOD (26, 29) for 35 days in low light (LL) of 150 μmol m^{-2} s^{-1} did not display significant differences in chlorophyll fluorescence (Fig. 5B). Photosynthesis related subunits were also examined and no differences in expression levels were detected for Psa A/B, Psa D, cytochrome *b6* and D1 in CCS-KO when compared to wild-type (Fig. 5C). Because LL growth conditions may not produce superoxide to a level that requires SOD activity for photo-oxidative stress reduction, we exposed the 35 days old plants to 24 hours of high light (HL) at 850 μmol m^{-2} s^{-1} prior to chlorophyll fluorescence

measurements. After the HL treatment all plants produced observable levels of anthocyanin, and the CCS-KO plants showed no visual phenotype differences in response to HL stress relative to wild-type plants (Fig. 5A). Chlorophyll fluorescence measurements for CCS-KO after HL treatment indicated no significant differences between the wild-type and CCS-KO lines (Fig. 5B). When plants are exposed to MV, electrons are directed away from electron transport chains to O_2 to create superoxide (10). To determine if SOD in the chloroplast can resist the effects of MV long-term, CCS-KO and wild-type plants were sown on MS medium containing 10 nM MV, and 5 μM $CuSO_4$ to turn off FeSOD (26, 29) (Fig. 5D). Wild-type and CCS-KO demonstrated similar growth inhibition when constantly exposed to MV. Chlorophyll fluorescence monitoring of plants grown in the presence of MV indicated that wild-type and CSS-KO plants have comparable photo-inhibition and electron transport activity (not shown).

Considering that CCS-KO has not shown a phenotype under the growth conditions imposed, we turned our attention to other biochemical processes that could potentially compensate for a lack in chloroplast SOD activity or indicate elevated stress in CCS-KO plants. Reduced and oxidized forms of ascorbic acid (AsA and DHA respectively) and reduced glutathione (GSH) were measured. Levels of AsA were not significantly different between CCS-KO and wild-type for both LL and HL treatments, and AsA/DHA measurements showed that CCS-KO has a slightly increased ratio of reduced to oxidized forms of ascorbic acid compared to wild-type (Table 1). Levels of GSH for CCS-KO were also not significantly different between CCS-KO and wild-type for both LL and HL. We

examined additional antioxidant molecules by HPLC. The xanthophyll cycle pool size, total carotenoids and tocopherols were not significantly different in the CCS-KO line when compared to wild-type for LL growth conditions (Table 1). However, after HL treatment the CCS-KO showed a significantly reduced xanthophyll cycle pool size and total carotenoids when compared to wild-type, whereas total tocopherols did not differ (Table 1). Because an increase in antioxidants was not observed for CCS-KO plants we wanted to determine if superoxide levels in leaves of tissue culture grown plants were different from wild-type using *in situ* nitro-blue tetrazoluem (NBT) staining. Interestingly, tissue culture grown CCS-KO plants did not exhibit a difference in superoxide levels compared to wild-type at a cotyledon stage and after 14 days of growth. Plants dark adapted for 24 hours prior to NBT staining also did not show differences between wild-type and CCS-KO plants (Fig. 6).

Plants grown in selenate (SeO_4) experience reduced protein synthesis, and are impaired in protein structure and function due to incorporation of Se into S containing amino acids Cys (53) and Met (54) to form Se-Cys and Se-Met respectively. *Arabidopsis* plants grown on selenium have been shown to contain significantly reduced non-protein thiols and GSH (55). CCS-KO plants grown in 30 μM SeO_4 show a decrease in leaf area compared to wild-type (Fig. 7).

The results for CCS-KO conflict with a previous report which indicated that a knock-down in *CSD2* causes a severe phenotype, even when CSD2 and FeSOD activities were both present (25). The Cu/ZnSOD knock-down background is Col-0 and contains a T-DNA insert in the promoter of *CSD2*. To examine how the

CCS-KO line compares with the knock-down of *CSD2* we obtained seed from the previously described line (KD-SOD) (25). Seeds were propagated in our lab and grown alongside CCS-KO and wild-type plants. The KD-SOD plants exhibited the previously described chlorotic phenotype (Fig 8A). However, PCR analysis did not indicate that the KD-SOD plants contained a T-DNA insert in the promoter of *CSD2*; rather the PCR suggested that the KD-SOD line contains an intact *CSD2* promoter (Fig. 8B). The KD-SOD line we examined likely contains a T-DNA insert in another location other than the promoter of *CSD2* that leads to the chlorotic phenotype observed (Fig. 8, A and B). We next obtained the original *CSD2* knock-down seed from the SALK collection (SALK_041901) and propagated seed in our lab. The SALK_041901 plants did not exhibit any visual phenotype and we could not differentiate them from wild-type (Fig. 8A). Because the SALK_041901 plants did not exhibit a phenotype we verified the T-DNA insert and location using PCR. Based on PCR results the SALK_041901 line contains a T-DNA insert in the promoter of *CSD2* and it is homozygous. We examined CSD2 expression in these *CSD2* knock-downs by immuno-detection as described for CCS-KO. For the SALK_041901 line the protein expression levels of Cu/ZnSODs were similar to wild-type (Fig. 8C) and chlorophyll fluorescence measurements did not indicate any photosynthetic deficiencies when compared to wild-type (Fig. 9). The SALK_041901 line also did not show signs of photo-inhibition, unlike KD-SOD, even after high light or continuous light treatments (data not shown).

4. Discussion

Chu et al. (35) had reported very low levels of CSD1 and CSD2 activity in leaf samples of the CCS-KO, although in flowers up to six percent of the WT activity was reported. We used a more quantitative approach for SOD activity measurements and found that the CCS-KO plants have no detectable Cu/ZnSOD activity in leaf tissue (less than 2% of the Cu/ZnSOD activity found in the wild-type) (Fig. 3A and B). Therefore, in photosynthetic tissue, CCS is likely essential for Cu delivery to both CSD1 and CSD2 and for their normal accumulation and activity, even during sufficient Cu supply. The Cu/ZnSOD polypeptide levels were also dramatically reduced in the CCS-KO plants. This study of the CCS-KO strongly suggests that Cu/ZnSODs do not accumulate without Cu. Similarly, a previously characterized Cu P-type ATPase mutant, *paa1* (56, 26), indicated that when Cu delivery to the stroma was disrupted, CSD2 did not accumulate yet transcript levels of *CSD2* remained elevated. In yeast, CCS is absolutely required for Cu/ZnSOD activation, yet in *Caenorhabditis elegans* and mammals an alternative Cu/ZnSOD activation pathway exists that utilizes glutathione (57). This glutathione-mediated activation of Cu/ZnSOD has been suggested to exist in *Arabidopsis* (35) but this mechanism of activation has not been confirmed and the data for the CCS-KO presented here suggests that a glutathione-meditated activation of Cu/ZnSODs is insignificant in higher plants. However, since we did not study null mutants for the SOD genes we cannot exclude that some SOD activity, too low to measure, is present in either the chloroplast or cytosol.

When the CCS-KO plants are grown on sufficient Cu, the plants lack not only CSD1 and CSD2 activity but also lack FeSOD. CCS-KO plants, with no detectable SOD activity in the cytosol and chloroplast, grow and reproduce the same as wild-type under the laboratory conditions that we tested. Chlorophyll fluorescence measurements for plants grown on Cu-supplemented soils indicated that dramatic reductions in chloroplastic SOD activities do not result in photosynthetic electron transport deficiencies relative to wild-type, whether plants are grown in low light, 24 h of HL or even when treated with MV (Fig. 5A, B, D). The analyses conducted on the CCS-KO plants reveal that the vast majority of chloroplast and cytosolic SODs can be inactivated without a consequence to the phenotype under standard laboratory and moderately high light stress growth conditions. However, thylakoid localized FSD2 and FSD3, have been shown to be essential for normal growth during the seedling stage (2). It is possible that very low thylakoid FeSOD activity was present in Cu treated CCS-KO plants to aid in normal growth. We also considered that plants with low Cu/ZnSOD and FSD1 activity are able to acclimate in a way that diverts excess photons and electrons to other photo-protective mechanisms (58).

We measured a number of anti-oxidant molecules in the CCS-KO and wild-type plants and found significant differences only for the xanthophyll cycle pigment pool size and total carotenoids, which were lower after HL treatment in CCS-KO, indicating a higher turnover and/or lower synthesis of xanthophylls compared to wild-type plants.

When CCS-KO plants were grown in SeO_4 a mild phenotype was observed (Fig. 7). It had been observed that when *Arabidopsis* plants were grown on SeO_4 the pools of total non-protein thiols and GSH were decreased when compared to wild-type (55). Selenate treatment may cause a decrease in the anti-oxidant pool and maybe therefore the CCS-KO showed a phenotype on SeO_4.

Our findings contrast with reports that have stressed the importance of Cu/ZnSOD in chloroplasts during non-stress and stressed growth. A severe phenotype had been reported for a *CSD2*-knockdown line from the SALK collection that carries a T-DNA insertion just upstream of the coding sequence which was reported to reduce, but not eliminate, CSD2 abundance (25). We investigated the same line, re-ordered and back-crossed the SALK T-DNA mutant, and confirmed the predicted insert by PCR analysis. However, we observed a wild-type-like phenotype, similar to that of CCS-KO. In fact the T-DNA insertion did not affect CSD2 protein levels. Perhaps the phenotype that has been described (25) could have been due to an unrelated mutation caused by a T-DNA insertion at another locus.

A very exciting finding was that a microRNA, miR398, controls CSD1 and CSD2 expression at a post-transcriptional level (21). Removal of the miRNA binding site or silencing of miR398 resulted in plants that over-accumulated CSD2 which were reported to be more tolerant to oxidative stresses (21). However, we note that very extreme treatments were needed to reveal differences between CSD2 over-accumulators and wild-type plants, and we consider that the beneficial effects of Cu/ZnSOD accumulation were quite small

in that study (21). Furthermore, it was found that miR398 responds much more to Cu than to oxidative stress (29). Dugas and Bartel (23) reported recently that miR398 is regulated by both Cu and sucrose. More importantly, plants with altered levels of miR398 and thus altered levels of CSD1 and CSD2 showed very small changes in phenotype compared to the wild-type (23). The mild phenotype of *paa1* mutants, which lack Cu transport into plastids and thus lack CSD2 activity, supports the same notion that a dramatic reduction in CSD2 does not lead to a severe phenotype (26).

FSD1, CSD1, CSD2 and CCS are conserved in plants and they must have some important function or the genes would have been lost. An important question remains: Why are CSD1, CSD2 and FSD1 expressed and active at elevated levels during times when they are apparently only needed in small quantities or perhaps not needed at all? One possibility is that superoxide radicals are transiently generated at high levels, thus requiring SOD activity, during conditions not examined here; and the onset on those conditions are rapid, thereby requiring a large pool of SODs. The reciprocal regulation of CSD2 and FSD1 suggests that maintenance of either SOD activity is required in the plastids to be competitive in nature.

Part of the reason why Cu/ZnSODs accumulate may also be related to their ability to sequester Cu ions. Interestingly, Cu and Zn ion levels for CCS-KO plants were significantly lower than wild-type plants (approximately 80% of wild-type). Thus, Cu/ZnSOD may bind 1/5 of the vegetative tissue's total Cu (Fig. 4). In addition, chloroplast and cytosolic Cu/ZnSODs increase expression and

activity levels based primarily on Cu abundance via miR398 (26, 28, 29, 23), and Cu/ZnSOD proteins accumulate only when Cu ions are available for final assembly and stability of Cu/ZnSODs (Fig. 3A). Copper ions can be dangerous to cellular compartments if left alone as free ions (59), and in the cytosol of yeast free Cu ions are reported to be less than one per cell (60). In yeast it has been suggested that Cu/ZnSOD plays a separate role in Cu buffering outside of superoxide scavenging (61). Our data suggests that Cu/ZnSODs in higher plants may also serve a Cu buffering role. However, CCS-KO plants were not more sensitive to Cu excess than the WT (not shown). Thus, if CCS serves to buffer Cu, this is not a mechanism to protect against excess. Perhaps, binding and sequestering limited Cu removes this resource for other organisms, which could be a factor in a competitive growth environment.

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Table 1. Antioxidant Composition of Copper Supplemented Soil Grown Plants

	AsA		AsA/DHA		GSH	
	LL	HL	LL	HL	LL	HL
WT	1.75 ± 0.17	3.89 ± 0.03	13.5	11.4	0.37 ± 0.09	0.89 ± 0.15
CCS KO	1.68 ± 0.53	3.74 ± 0.09	15.3	12.1	0.34 ± 0.02	0.78 ± 0.24

(μmol g FW^{-1})

	V + A + Z		Carotenoids		Tocopherols	
	LL	HL	LL	HL	LL	HL
WT	23.4 ± 1.1	39.9 ± 1.3 a	206.7 ± 1.5	249.3 ± 4.4 a	7.9 ± 0.4	15.3 ± 2.4
CCS KO	26.5 ± 0.9	30.7 ± 3.6 b	209.7 ± 3.4	229.3 ± 7.4 b	7.7 ± 0.7	14.9 ± 3.7

(mmol mol chl^{-1})

Low Light (LL) and High Light (HL) changes in levels of reduced (AsA) and oxidized (DHA) ascorbic acid, reduced glutathione (GSH), xanthophyll cycle pool size (V + A + Z), total carotenoids and tocopherols. AsA, DHA and GSH normalized to fresh weight (FW), and V+A+Z, carotenoids and tocopherols to chlorophyll a+b. Means ± SD shown (n=3) with significant differences indicated by letters a and b ($P < 0.05$).

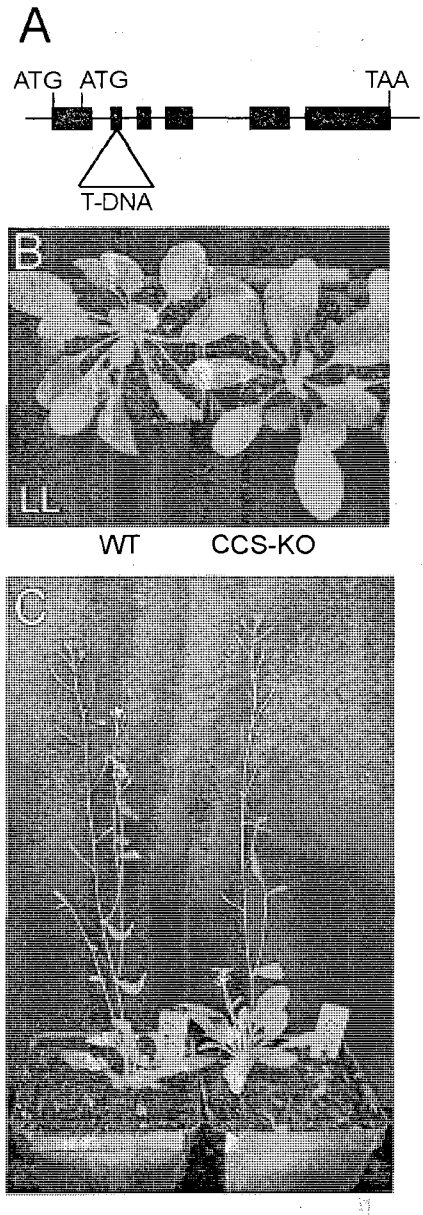

Figure 1. Phenotype of wild-type and CCS-KO plants grown in CuSO4 supplemented soil. A) Gene map showing T-DNA insertion site in the second exon of CCS. B) Wild-type and CCS-KO line grown in controlled low light (LL) conditions. C) Flowering of wild-type and CCS-KO. D) Reproductive success of wild-type (WT) and CCS-KO (KO). Siliques counted as number per plant and germination rate as percent (%) of total seeds sown. Means ± SD (n = 3-5) are shown.

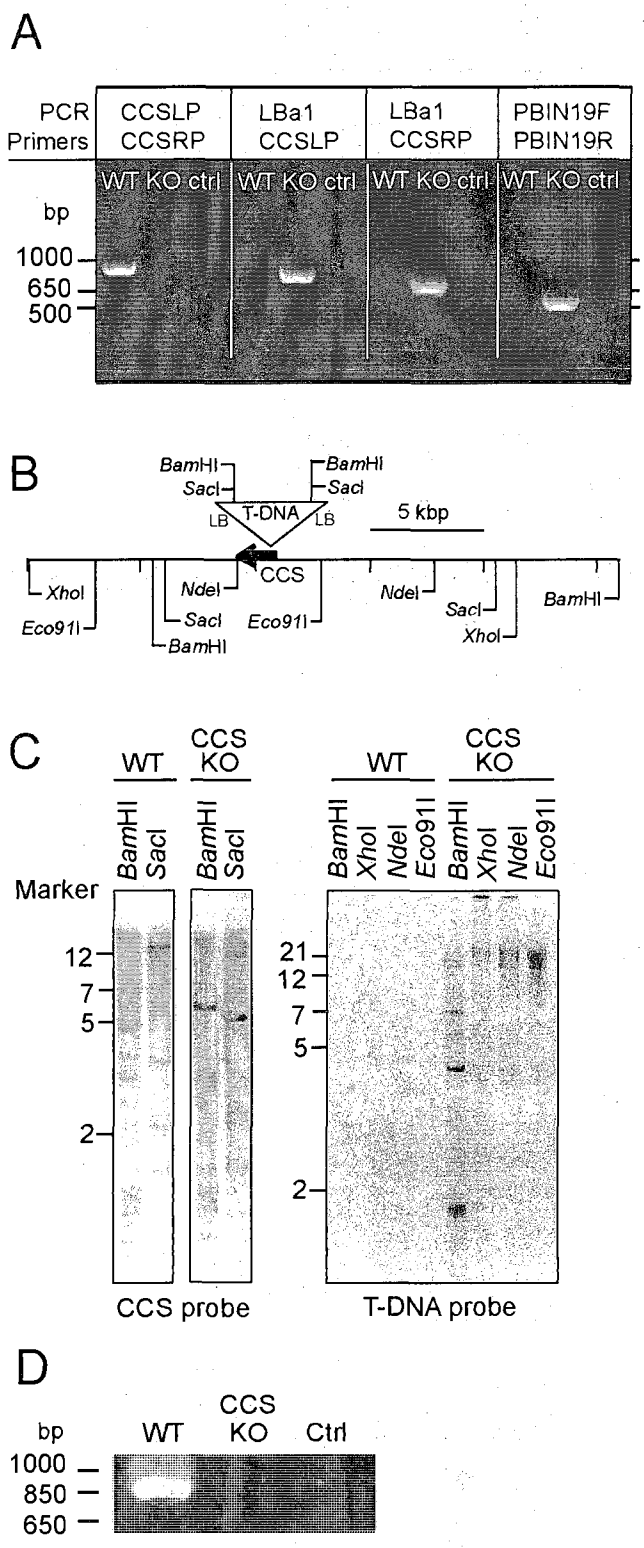

Figure 2. T-DNA insert verification for CCS-KO plants. A) Verifying T-DNA location within the CCS-KO line using PCR with CCS primers (CCSLP and CCSRP) and T-DNA primers (LBa1, PBIN19F and PBIN19R). B) Restriction digest map flanking T-DNA insert site in CCS for Southern blot analysis. C) Southern blot to verify T-DNA inserts in CCS-KO. We digested with the indicated enzymes, separated DNA by electrophoresis, blotted, and probed with [32P]dCTP labeled CCS and T-DNA specific probes. D) CCS transcript levels. Reverse-Transcriptase PCR of wild-type, CCS-KO, and negative control (Ctrl) using CCS mRNA specific primers.

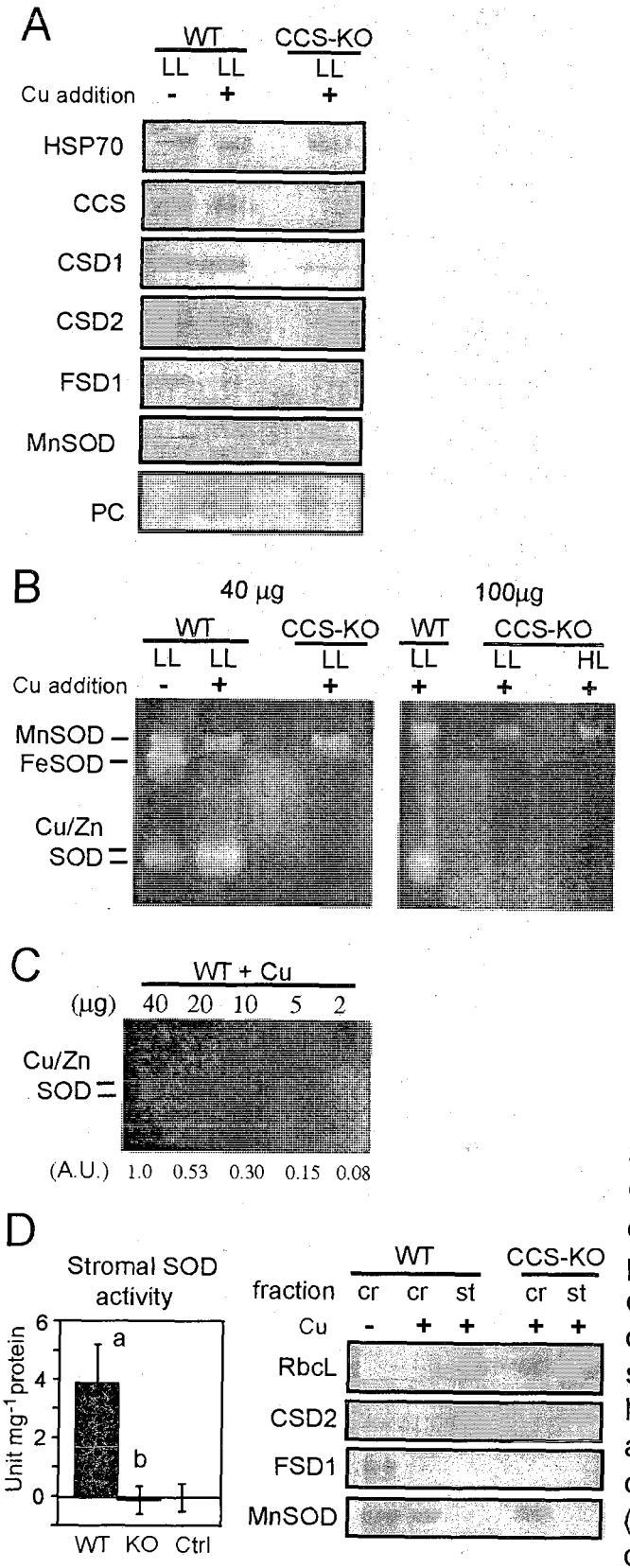

Figure 3. Analysis of SOD isozyme expression and activity of CuSO4 supplemented soil grown wild-type and CCS-KO plants. A) Immuno-detection of Plastocyanin (PC) and SOD proteins. Shoot proteins (20 µg) were separated by SDS-PAGE and detected by immuno-blotting against Heat Shock 70 (HSP70) loading control, PC and SOD proteins. B) SOD isozyme activity. Soluble shoot proteins (40 µg and 100 µg) were separated on 15% non-denaturing polyacrylamide gels and stained for total SOD activity. Plants were grown in CuSO4 supplemented soil except wild-type (-), grown in soil without CuSO4 additions to show FeSOD activity. Low light (LL) and high light (HL) treatment are indicated. C) Wild-type SOD activity dilution series. Soluble shoot proteins (40, 20, 10, 5 and 2 µg) were fractionated as above. Band intensities were quantified and shown as arbitrary units (A.U.) relative to the 40 µg sample. D) Liquid assay to detect stromal SOD activity for wild-type (WT), CCS-KO (KO) and negative control (Ctrl). Stromal fraction purity and recovery was determined by immuno-detection of crude leaf extract (cr) and stromal fraction (st) against the large subunit of Rubisco (RbcL) and SODs. Lanes normalized to chlorophyll content. Means ± SD ($n = 5$) are shown with significant differences indicated by letters above bars ($P < 0.01$).

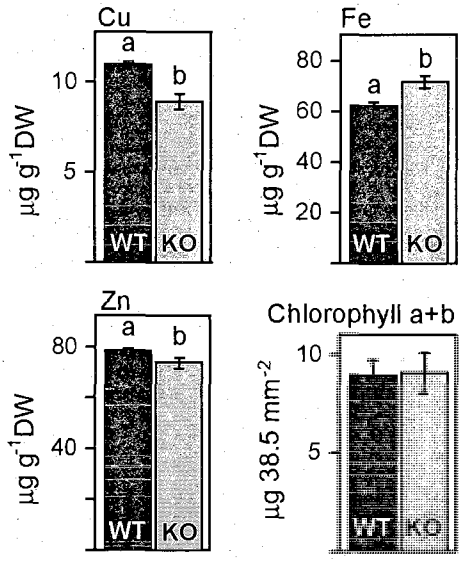

Figure 4. Characterization of CCS-KO grown in controlled low light on CuSO4 supplemented soil. Copper (Cu), Iron (Fe) and Zinc (Zn) content of wild-type (WT) and CCS-KO (KO) shoot tissue represented as µg g-1 DW (dry weight). Chlorophyll a and b content of leaf disks represented as µg 38.5 mm-2. Means ± SD (n = 3) are shown with significant differences indicated by letters above bars ($P < 0.05$).

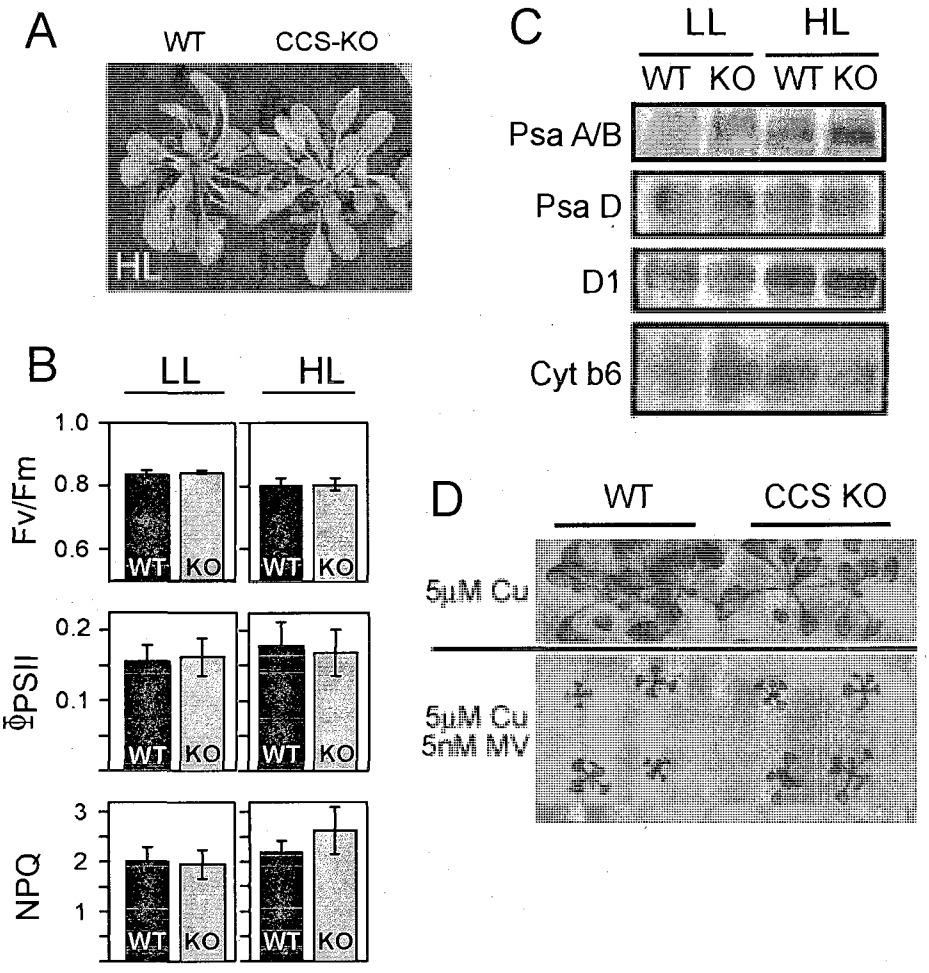

Figure 5. Chlorophyll fluorescence of CuSO4 supplemented soil and MS grown wild-type and CCS-KO after abiotic stress. A) Wild-type and CCS-KO after controlled high light (HL) treatment. B) Dark-adapted wild-type (WT) and CCS-KO (KO) plants were measured for Fv/Fm (PSII antennae efficiency); ϕPSII (PSII quantum efficiency) and NPQ (non-photochemical quenching). Means ± SD (n = 8-10) are shown. C) Immuno-detection of wild-type (WT) and CCS-KO (KO) shoot proteins (20 μg) separated by SDS-PAGE and detected by immuno-blotting against Psa A/B (37), Psa D (38), cytochrome b6 (39) which have been described. Specific antibody for the D1 subunit of PSII was a generous gift from Alice Barkan (University of Oregon, Eugene). D) Images shown of 14 day old wild-type (WT) compared to CCS-KO (KO) grown with and without 5 nM MV.

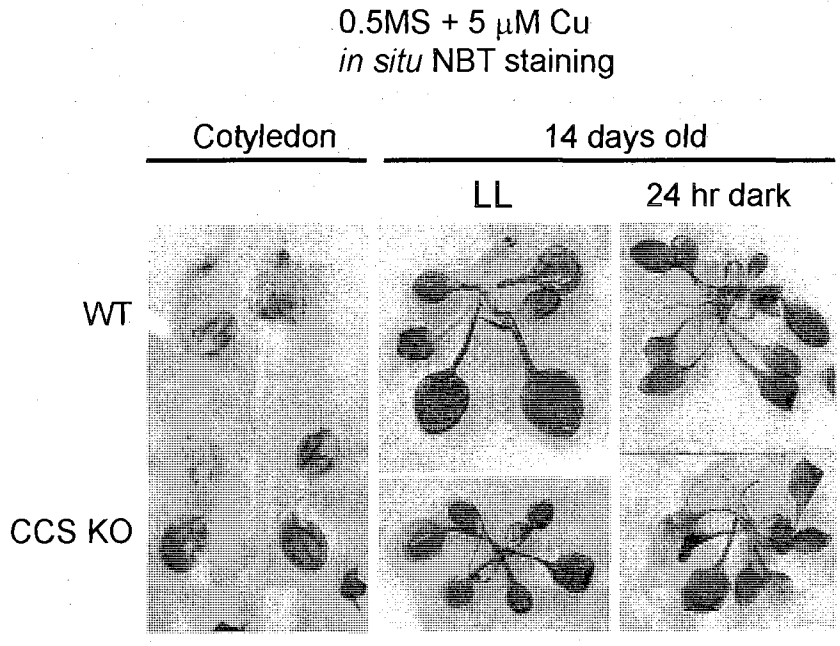

Figure 6. Histochemical NBT staining for superoxide radicals. Tissue culture grown wild-type (WT) and CCS-KO (KO) plants grown in low light (LL) conditions.

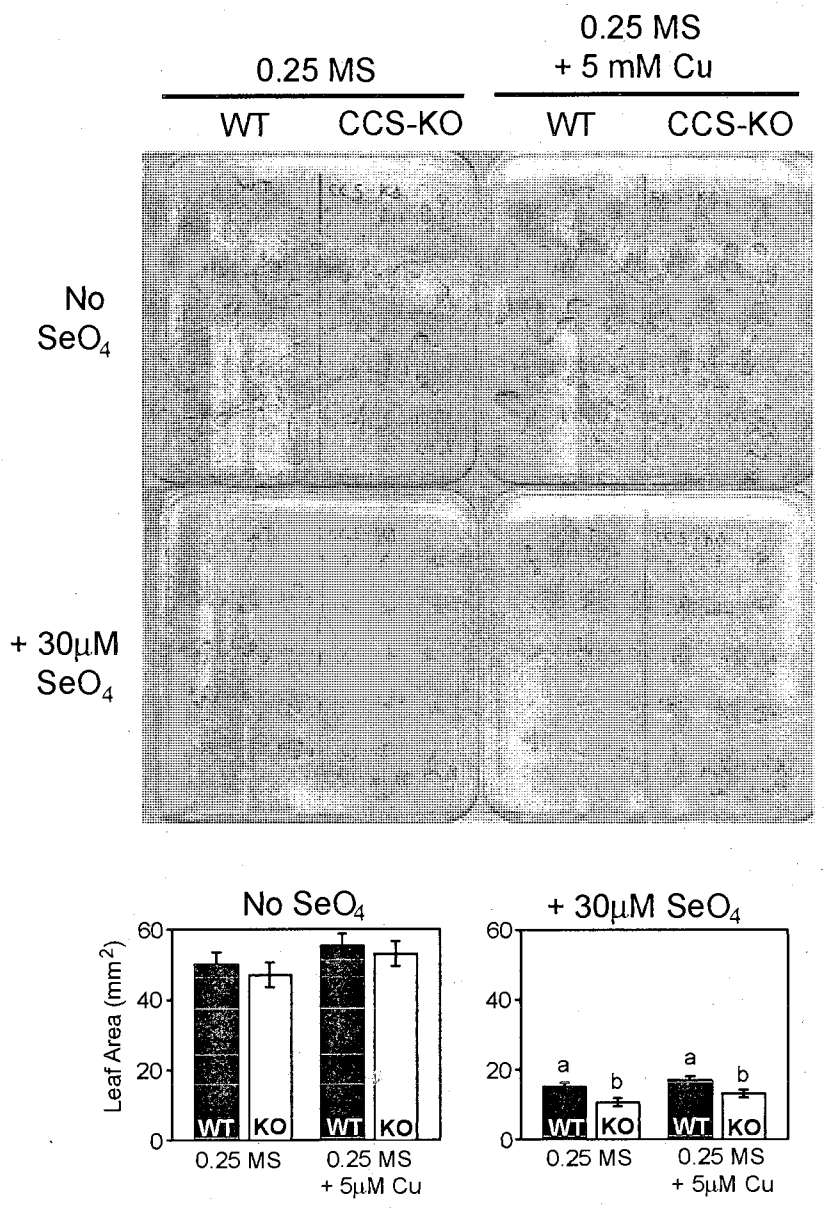

Figure 7. Plant leaf area after selenate treatment. Images of 14 day old tissue culture grown wild-type (WT) and CCS-KO (KO) plants grown in 0.25 MS, 5 µM CuSO4, and 30 µM SeO4, as indicated. Means ± SD (n = 41-46) from three treatment plates are shown for individual plant leaf area (mm2), with significant differences indicated by letters above bars ($P < 0.05$).

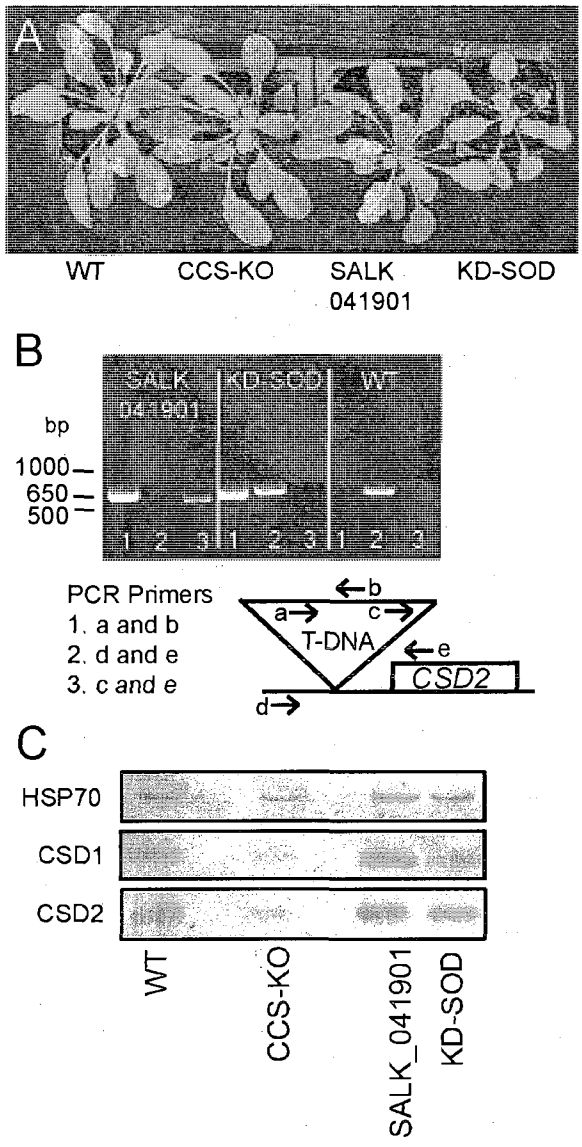

Figure 8. Characterization of CSD2 knock-down plants. A) Wild-type and mutant lines grown in soil supplemented with CuSO4. B) Verifying T-DNA location within the CSD2 knock-down lines by PCR using genomic and T-DNA primers. T-DNA primers PBIN19F (a), PBIN19R (b), LBb1 (c), and genomic primers CSD2LP (d), and CSD2RP (e) were used. C) Immuno-detection of SOD proteins. Shoot proteins (20 µg) were separated by SDS-PAGE and detected by immuno-blotting against Heat Shock 70 (HSP70) loading control and SOD proteins.

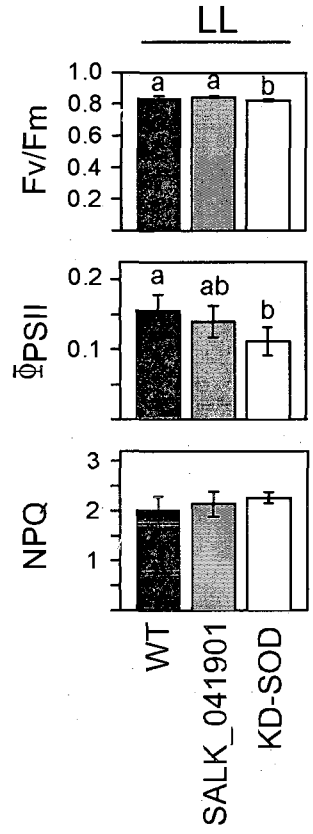

Figure 9. Chlorophyll fluorescence of CSD2 knock-downs.
Chlorophyll fluorescence measurements of wild-type (WT) and CSD2 knock-down lines (SALK_041901) and (KD-SOD) grown in Low Light (LL) as described in Fig. 5. Data represented as means ± SD (n = 8-10) with significant differences indicated by letters above bars ($P < 0.05$).

CHAPTER 5

Copper-microRNA 398 regulates the copper chaperone for superoxide dismutase

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Chapters 4 and 5 will be combined and submitted for publication

CHAPTER 5

Copper-microRNA 398 regulates the copper chaperone for superoxide dismutase

Abstract

Copper (Cu) is an important mineral nutrient involved in numerous processes including photosynthesis and respiration. Copper is redox-active and could cause damage if present as a free ion. Therefore, organisms have evolved mechanisms to tightly control Cu transport, delivery, and allocation. Several recent studies have shown that microRNAs and a SPL7 transcription factor down-regulate the expression of many Cu-proteins, including Cu/Zn superoxide dismutase, during Cu-limited growth. In this study we demonstrate that the Cu chaperone for superoxide dismutase (CCS) is regulated by Cu, mediated by microRNA 398 that was not previously predicted to target CCS. We show that CCS mRNA is cleaved in a region with high base-pair complementarity to microRNA 398, and this region is conserved in the rice *CCS* gene. We suggest that there is additional microRNA regulation of mRNA expression levels in higher plants than previously predicted; and that the degree of base-pair complementarity between microRNA and target mRNA may dictate the sensitivity of the regulation.

Abbreviations- CCS, Cu chaperone for superoxide dismutase; Cu/ZnSOD, Cu-Zn superoxide dismutase; FeSOD, Fe superoxide dismutase; GUS, β-glucuronidase; SPL7, *SQUAMOSA* promoter binding protein-like7

1. Introduction

Seven superoxide dismutase (SOD) genes have been identified in *Arabidopsis* with the greatest protein accumulation and activity found for CSD1, CSD2, MSD1, and FSD1 (1). *CSD1*, *CSD2* and *CSD3* encode Cu/ZnSODs with CSD1 activity in the cytosol, CSD2 in the stroma, and CSD3 in the peroxisome. *MSD1* encodes a mitochondrial manganese-SOD (MnSOD), while *FSD1* encodes a stroma-localized iron-SOD (FeSOD) (1). However, a recent study suggested that FSD1 could be located in the cytosol (2). In addition, Myouga et al. (2) localized FSD2 and FSD3 to the surface of the thylakoid lumen, and found that FSD2 and FSD3 activities were essential for seedling growth.

The reversible oxidation-reduction of Cu makes it useful as a co-factor; however, free Cu ions could also create oxidative damage or bind non-specifically to cysteines and histidines of non-Cu proteins. In yeast, the estimated free Cu ion concentration in the cell was at least 12 orders of magnitude below the total Cu concentration (3). To efficiently deliver essential metal ions, and at the same time avoid toxicity, all organisms have evolved metal ion transport and sequestration systems (4). Plant cells acquire Cu through CopT family transporters (5) while Cu is removed from the cytosol by the HMA5 transporter (6). Within the cell, Cu transport in chloroplasts is the best understood. Two Cu-

transporting P-type ATPases, PAA1 (chloroplast envelope) and PAA2 (thylakoid membrane), are required for efficient delivery of Cu to plastocyanin (7). Copper is also transported within the cytosol and stroma of plant plastids by the Cu chaperone for SOD (CCS), a functional homolog of a yeast Cu-metallo-chaperone (8). In plants, CCS is encoded by one gene containing two in-frame ATG start sites that flank a chloroplast targeting sequence. CCS is important for Cu delivery and activation of both CSD1 and CSD2 in higher plants (9, Chapter 4). CCS mRNA was found to be regulated by Cu (8), albeit, the Cu-regulation was attenuated when compared to CSD1 and CSD2 (7).

Copper homeostasis also requires mechanisms for regulated expression of Cu delivery systems and target Cu-proteins. Availability of Cu is a major determinant of Cu/ZnSOD and FSD1 expression in *Arabidopsis* (7,8,10). When Cu supply is sufficient, CSD1 and CSD2 is expressed and active, yet their expression and activity diminish when Cu supply is limited. Previous studies suggested that CSD1 and CSD2 down-regulation during Cu-limitation allows for preferential allocation of Cu to plastocyanin (8,11). In *Arabidopsis*, microRNA 398 (miR398) targets the mRNA of *CSD1* and *CSD2*, leading to micro-RNA directed cleavage (11,12). MicroRNAs are small 21-22 nt RNA molecules that regulate specific genes in plants by targeting mRNA for degradation (13). MicroRNAs have been shown to be involved in many processes such as sulfur (14,15) and phosphate (16-18) homeostasis, stress (14,19), and development (20). There are three *miR398* genes in *Arabidopsis* but they differ only by one nucleotide at the 3' end (11). *miR398* is also positively regulated by sucrose, leading to decreased

CSD1 and CSD2 protein levels (21). When the miR398 binding site sequences of *CSD1* and *CSD2* transcripts were altered, transcript levels were less sensitive to miR398 directed cleavage yet CSD1 and CSD2 proteins did not accumulate. miR398 was suggested to play an additional role as a CSD1 and CSD2 translational repressor (21). Additional microRNA's (397, 408, and 857) were determined to target transcripts of other Cu-proteins such as plantacyanin and several members of the laccase family. Together, microRNA's 397, 398, 408, and 857 were termed Cu-microRNA's due to their involvement in a systematic down-regulation of Cu-proteins during Cu-limited growth (22,23). Positive regulation of Cu-microRNA's during Cu-limited growth is likely activated by a SPL7 (SQUAMOSA promoter-binding like 7) transcription factor containing a DNA binding domain (24). Unlike wild-type plants, a *spl7* mutant was shown to maintain elevated *CCS* mRNA levels during Cu-limited conditions; suggesting that SPL7 may be involved in CCS regulation. The regulation of Cu/ZnSODs by SPL7 and miR398 are well understood (11,12,24); however, little is known about the regulation of CCS by Cu and no microRNA site was reported. This study characterizes *CCS* promoter temporal activity and regulation, and demonstrates that CCS is regulated by Cu, mediated by miR398 directed cleavage.

2. Material and Methods

Plasmid construction- To construct *CCSpro::GUS*, the *CCS* promoter sequence was amplified from genomic DNA by PCR using primers CCSpro-F (5'-ATCGCGGATCCAACTGTTTTTTGTATCATCAATAAAC-3') and CCSpro-R (5'-

TACGCGGATCCCGGAGAGCTAACGAAGCTTC-3') which introduced *Bam*HI restriction sites (underlined) for cloning. The amplified fragment is 419 bp long and contains the entire *CCS* 5' UTR and upstream intergenic sequence up to the stop codon of the next upstream gene. The amplified product was digested using *Bam*HI then ligated into the PBI101.2 vector which contains a promoterless β-glucuronidase (GUS) gene. Clones were verified by digestion and sequencing prior to transformation of *Arabidopsis* (col-0) by *Agrobacterium* (C58C1) using the floral dip method (25). Fourteen independent transformant lines were obtained after selection on tissue culture medium plates containing 50 µg/ ml kanamycin. PCR to confirm plant transformation was conducted on genomic DNA using primers CCSpro-F and GUS-R (5'-CGATCCA GACTGAATGCCCA - 3'). Genetically transformed plants were grown on soil and allowed to self pollinate. To obtain homozygous lines, seeds from transformed plants were sown individually, allowed to set seed, and tested for germination on kanamycin tissue culture medium plates. To construct 35S:*CCS*, the coding sequence was amplified from cDNA by PCR using primers CCS-NcoI-F (5'- CATGCCATGGCAT CAATTCTCAGGTCAGTGGC-3') and CCS-BamHI-R (5'-CGCGGATCCTTAAAC CTTACTGGCCACGAAAT-3') which introduced *Nco*I and *Bam*HI restriction sites (underlined) for cloning. The forward primer includes the first of two ATG in frame start sites that flank the chloroplast targeting sequence. The amplified product was digested and ligated into a pFGC5941 vector which contains a CaMV 35S promoter. Transformation and selection was conducted as described above.

Plant species and growth conditions- The background ecotype of all *Arabidopsis* lines tested is Columbia. *miR398a miR398c* double mutant and miR398 over-expresser 35S:*MIR398c* (21C and 30A) were a generous gift from Bonnie Bartel, Rice University, and have been described (21). Plants were grown in controlled conditions (light intensity of 150 µmol m^{-2} s^{-1}, 12-h/12-h light/dark cycle at 23°C). Plant tissues were collected and frozen immediately in liquid nitrogen then stored at -80°C unless otherwise noted. For plants grown on tissue culture medium, seeds were surface-sterilized and vernalized for 4 days at 4°C then sown on 0.5 Murashige and Skoog (MS) medium (Caisson Laboratories Inc., North Logan, UT) containing 0.4% Agargel (Sigma-Aldrich, St Louis, MO), 1% sucrose, and $CuSO_4$ as specified in figures. Tissue culture medium was made using distilled diH_2O to reduce Cu contamination, and glassware was rinsed with 100 mM EDTA followed by several distilled diH_2O rinses prior to use. Plants were grown for 14 days unless otherwise specified. Etiolated seedlings were grown in complete darkness for 5 days. Plants grown on soil (Fafard®Custom Mix, Conrad Fafard Inc., Agawam, MA) were watered without fertilizer, and were grown in the controlled conditions stated above.

Protein extraction and immuno-detection- Shoot proteins were extracted as described (7), and protein concentration was determined according to the Bradford (26) method using bovine serum albumin as a standard. For immuno-detection analysis, 20 µg of protein extract was separated by 12.5% SDS polyacrylamide gels and transferred to a nitrocellulose membrane by electroblotting. Antibodies used for CSD1, CSD2 (7), and CCS (Chapter 4) have

been described. Antibody for heat shock 70 (HSP70) was obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO). Each experiment was replicated three times for wild-type plants (Fig. 1B) and two times for mutant plants (Fig. 5), all with identical results. Representative gels are shown in figures.

Histochemical GUS staining and fluorogenic GUS assay- Histochemical *in situ* GUS staining, using X-gluc (5-bromo-4-chloro-3-indoxyl-D-glucuronide) (GoldBio, St Louis, MO), was conducted on all 14 *CCS::GUS* plant lines grown on soil and tissue culture medium under the specified Cu conditions. The Histochemical GUS staining solution consisted of: 50 mM NaPi pH 7.0, 0.1% TritonX-100, 10 mM EDTA, 0.5 mM K_3FeCN_6 and K_4FeCN_6, and 1.5 mg/ ml X-gluc. Harvested plant tissue was placed in staining solution and vacuum infiltrated for 20 minutes, then incubated at 37°C for 4 hours. Stained plant tissues were placed in 75% ethanol until no more pigments were visible. Fluorogenic analysis for *in vitro* GUS activity was conducted on protein extracts from eight of the fourteen *CCSpro::GUS* lines (1, 3, 6, 7, 8, 9, 11, 14) described above using MUG (4-methylumbelliferyl-β-D-glucuronide) (GoldBio, St Louis, MO) as a substrate. Fluorogenic assay solution consisted of: 50 mM NaPi pH 7.0, 10 mM EDTA, 10 mM DTT, 0.1% TritonX-100 and 0.1% Sarkosyl. Five µg of protein extract was added to 1 ml of assay solution and allowed to incubate for 20 minutes at 37°C. 1.4 ml of 0.4 M Na_2CO_3 was added to stop the reaction. 4-methyl umbelliferone (MU) fluorescence was measured at 400 nm using a Hoefer DyNA Quant 200 Fluorometer (Hoefer, Inc., Holliston, MA) and data represented

as arbitrary fluorescence units (MU.units). Assays were conducted using samples from three biologically independent experiments.

RNA extraction and transcript analysis- Total RNA for all experiments was extracted from plant tissues grown on Cu concentrations as specified using the TRIzol reagent (Invitrogen) as described in the manufacturer's instructions. For CCS and 18S ribosomal RNA transcript analysis, 15 µg of RNA was separated by electrophoresis in a 1.2% agarose gel containing 4% formaldehyde, transferred to nitrocellulose (Hybond-N+, Amersham Biosciences), and probed with alpha^{33}P-dCTP-labeled probes. DNA template for the CCS probe was created by PCR using primers CCS-F (5'-AACCTTACTGGCCACGAAATCGC-3') and CCS-R (5'-GTGAGGATACTTGGATCTTCCCCT-3'). Radioactive probes were synthesized with an oligo-labeling kit (Amersham Biosciences) using random primers. Hybridization and washing was conducted as described (7). For small RNA blot analysis, 20 µg of RNA was separated on a denaturing 17% polyacrylamide gel (16 x 20 cm, Protean II system, Biorad, Hercules, CA) containing: 7 M urea, 0.5X TBE (Fisher Sci, Pittsburg, PA), 15% acrylamide/bis (Biorad, Hercules, CA), 0.5% APS, and 35 µl TEMED (Biorad, Hercules, CA). Polyacrylamide gel was pre-run at 15 mA for 1 h, loaded, and ran for 2 h at 15mA. RNA was electrophoretically transferred to Hybond-N+ nitrocellulose as described (22) using a Trans-Blot SD Semi-Dry (Biorad, Hercules, CA) and 0.5X TBE buffer, run at 200 mA for 2 h. A DNA oligo with base-pair complementarity to miR398a (5'-AAGGGGTG ACCTGAGAACACA-3') was end-labeled with gamma^{32}P-dATP (4500 Ci/ mmol) (#35001X.2, ICN Biomedical, Costa Mesa,

CA) using T4 polynucleotide kinase (Fermentas, Hanover, MD). The small RNA blot was also probed with an oligonucleotide specific to U6snRNA (5'-TCATCCTTGCGCAGGG GCCA-3') as an internal control. Membranes were prehybridized for 1 h and hybridized overnight using ULTRAhybo-oligo hybridization buffer (Ambion, Austin, TX) at 38°C. Blots were washed as described (11) using 2X SSC/ 0.1% SDS. Washing steps: 38°C solution for 1 min with oven at room temp followed by a second wash for 30 min under the same conditions, then 2X wash with room temp solution for 30 min. Membranes were imaged using a PhosphorImager (Storm, GE healthcare).

miRNA target validation- Total RNA isolated from plants grown on minus-Cu tissue culture medium was used for 5'-rapid amplification of cDNA ends (5'-RACE) using the GeneRacer kit (Invitrogen) as described (27-29). Total RNA was ligated to an adaptor (GeneRacer), converted to cDNA, and subjected to PCR using the GeneRacer 5' primer and CCS reverse primer (5'-GCTTCTAGCA ATCACTGCGGCGGTCAAT-3'). Amplified PCR products were subject to another PCR reaction using the GeneRacer 5'-nested and CCS reverse nested primer (5'-GCGGCGGTCAATCCGGGGCCTGACTTAT-3'). The PCR product was agarose gel purified and cloned using the TOPO-TA cloning kit (Invitrogen). Five clones were sequenced.

Elemental analysis, statistics, and CCS sequence alignment- Copper concentration of dried shoot tissue was determined by inductively coupled plasma-atomic emission spectrometry after acid digestion as described (10,30). All statistical analyses (ANOVA, *t* tests) were performed using the Jump-in

software package (SAS Institute, Cary, NC). The putative miR398 site to *Oryza sativa* putative CCS gene (LOC_Os04g48410) was obtained using the *miRU2: A Plant Small RNA Regulator Target Analysis Server* at http://bioinfo3.noble.org/miRU2/ (Samuel Roberts Noble Foundation).

3. Results

To determine if CCS protein accumulation was regulated by a Cu availability, wild-type *Arabidopsis* plants were grown on tissue culture medium ranging from 0.05 μM to 5 μM final Cu concentration. Copper concentrations in shoot tissues progressively increased as Cu concentrations in the tissue culture medium increased (Fig. 1A). Shoot Cu concentrations for all Cu treatments were in the physiological range between deficiency (5 μg g^{-1} dry weight) and toxicity (20 μg g^{-1} dry weight) for most plants (31). CCS, CSD1, and CSD2 expression levels in plants grown on tissue culture medium ranging from 0.05 to 5 μM Cu reveal that CCS protein accumulates as Cu concentrations in leaf tissue increases, similar to that of CSD1 and CSD2 but more attenuated (Fig. 1B).

Because CCS protein abundance responded to Cu availability, we investigated the *CCS* promoter for a Cu-responsive cis-acting element using a *CCS* promoter-GUS fusion (*CCSpro::GUS*) in *Arabidopsis*. Fourteen lines of *CCSpro::GUS* transgenics were obtained and tested for GUS activity. All lines were analyzed for both temporal and spatial expression of GUS activity with one representative line (*CCSpro::GUS-1*) shown (Fig. 2). GUS activity was evident in 7, 14, and 28 day old plants (Fig. 2a - g). Cotyledon leaves demonstrated the highest histochemical GUS staining of any tissues (Fig. 2a, b, h). GUS staining in

leaf tissue was ubiquitous, but vascular regions throughout the plants exhibited the most GUS activity when compared to other regions (Fig. 2d - h). Generally, younger leaves were observed to contain slightly more GUS activity than older leaves (Fig. 2c). Staining was also observed in etiolated seedlings; however, staining was mainly observed in vascular regions (Fig. 2h). For flowers, petals and sepals exhibited GUS activity, but in sepals the activity was mainly restricted to vascular regions (Fig. 2d). In the stamen, both the anther and filament contained GUS activity along with the pistil, but the stigma portion of the pistil contained the highest GUS activity (Fig. 2d, e). Roots and root hairs also exhibited GUS activity (Fig. 2g), but the activity was not always present (Fig. 2a).

To determine if the promoter of *CCS* contains a Cu-responsive cis-acting element, *CCSpro::GUS* plant lines were grown on tissue culture medium containing 0.05, 0.5, and 5 μM Cu. Histochemical GUS staining of all 14 *CCSpro::GUS* lines grown for 14 days did not exhibit differences in staining intensity relative to Cu availability (Fig. 3A) (only *CCSpro::GUS-1* shown). This observation contrasts the CCS protein expression from the endogenous gene in the same plant (Fig. 3B). To quantify GUS activity in *CCSpro::GUS* mutants, we conducted a fluorogenic GUS activity assay on protein samples from eight representative (five shown) mutant lines grown on tissue culture medium containing 0.05, 0.5, and 5 μM Cu. The fluorogenic analysis results for all eight *CCSpro::GUS* lines showed that Cu does not regulate promoter activity (five shown) (Fig. 3C). One line (*CCSpro::GUS-11*) exhibited increasing GUS activity as Cu availability increased, but the effect was minor (Fig. 3C). The 35S:*CRT*

positive control yielded 2500 MU units (outside the dynamic range) and the empty vector control yielded no GUS activity (not shown). These results show that regulation of CCS abundance in response to Cu availability is not through a Cu responsive cis-acting element in the promoter. Instead, the *CCS* promoter is equally active under Cu-limited and Cu-sufficient growth; suggesting that reduced CCS expression during Cu-limitation could be the result of a post-transcriptional inhibition.

In plants, regulation of Cu/ZnSODs by Cu availability is mediated by miR398 that targets Cu/ZnSOD transcripts for microRNA directed cleavage. Because CCS function is required for CSD1 and CSD2 activity (chapter 4), it is also reasonable to expect that CCS is regulated similarly. However, microRNA target prediction by computational analysis do not predict that *CCS* mRNA contain a microRNA target sequence in *Arabidopsis*, rice, or poplar (14,15,32-35). Searching through the *CCS* transcript sequence we found a 21 nt sequence with high similarity to a miR398 target site (Fig. 4). miR398 is conserved in *Arabidopsis*, rice, and poplar, along with other flowering plants (14,15,33,34,36). We examined the putative *CCS* (LOC_Os04g48410) gene sequence in *Oryza sativa* and found that it also contains a 21 nt sequence that is conserved at the putative miR398 target site (Fig. 4). To determine if *CCS* transcripts are cleaved at the putative miR398 site, a 5'RACE experiment was conducted on RNA extracted from wild-type *Arabidopsis* plants grown on tissue culture medium containing 0.05 μM Cu (Cu-limited). Total RNA was ligated to a RNA Oligo adapter at the 5' end which allowed for PCR amplification when using

GeneRacer RNA Oligo forward and *CCS* reverse primers. After cloning PCR products, five clones were sequenced. Three of the five clones had 5' end sequence termination in the middle of the predicted miR398 target site (Fig. 4). The other two clones contained 5' end sequence termination 42 nt downstream of the miR398 cleavage site. The 5' RACE experiment suggests that CCS transcripts are likely targeted for miR398 directed cleavage during Cu-limited growth. Because of this result, the entire genome was searched for putative Cu-microRNA targets that contain a similar base-pair mismatch number to that of CCS and miR398a. The search resulted in numerous previously unpredicted and untested putative microRNA targets (Table 1). Possible targets include the PAA2 Cu transporter, putative cupredoxin domain proteins, and several proteins associated with cell wall processes.

To determine if CCS transcript and expression levels are affected by miR398 abundance we grew wild-type, an over-expresser of CCS (35S:*CCS*) and miR398c (35S:*MIR398c*, lines 21C and 30A), and a SALK T-DNA miR398a/miR398c double mutant (*mir398a mir398c*) on Cu-limited (0.05 μM) and Cu-sufficient (5 μM) tissue culture medium. The 35S:*MIR398c* and *mir398a mir398c* have been described (21). Wild-type, 35S:*CCS*, and *mir398a mir398c* plants grown on Cu-limited medium all exhibited reductions in CCS transcript and expression levels (Fig. 5). Interestingly, 35S:*CCS* plants on Cu-limited medium exhibited a dramatic reduction in *CCS* transcripts even though the *CCS* gene was over-expressed; again suggesting that CCS is regulated post-transcriptionally (Fig. 5). 35S:*MIR398c* mutants did not increase CCS transcript

and expression levels to the extent seen in wild-type when grown on Cu-sufficient medium; suggesting that over-expressing miR398c down-regulates CCS abundance even when Cu is sufficient. The *mir398a mir398c* plants demonstrated minor increases in CCS abundance relative to wild-type plants on both Cu-limited and Cu-sufficient medium (Fig. 5). A small RNA blot demonstrated that miR398 abundance is negatively correlated with CCS transcript and protein levels for all plant lines during Cu-limited and Cu-sufficient growth (Fig. 5). Together this suggests that miR398 abundance affects CCS mRNA levels. An interesting observation is that miR398 expression levels in the 35S:*MIR398c* mutant, lines 21C and 30A, decreased during Cu-sufficient growth when compared to Cu-limited growth (Fig. 5), which have also been previously reported (21)

4. Discussion

We show that CCS protein expression increases as Cu is more available, similar to CSD1 and CSD2 (Fig. 1), and conclude that CCS expression is regulated by Cu. The promoter of CCS did to not respond to Cu availability. Instead we measured high promoter activity during Cu-limited growth while CCS protein expression levels decreased (Fig. 3), which suggests that *CCS* is regulated post-transcriptionally. A region of the *CCS* mRNA sequence is highly similar to miR398 binding sites of CSD1 and CSD2, but with less base-pair complementarity (Fig. 4). miR398 is conserved in *Arabidopsis*, rice, and poplar, along with other flowering plants (14,15,33,34,36). Finding a highly conserved

putative miR398 target sequence in rice *CCS* also supports that *CCS* mRNA is targeted by miR398 (Fig. 4). The *CCS* miR398 binding site was confirmed by cleavage site analysis. Sequencing of cloned cleavage products indicated that *CCS* mRNA is cleaved in the middle of the putative miR398 target site (Fig. 4), as was seen for other Cu-microRNA targets (11,22). Thus, the *CCS* mRNA is targeted by miR398 directed cleavage in *Arabidopsis.* This CCS regulation by miR398 is likely found in other plants with conserved miR398. Oddly, two of the five sequenced clones were cleaved 42 nt down-stream of the miR398 target site, and the region does not correspond to target sites of other possible Cu-microRNAs (not shown). It is possible that the down-stream cleaved *CCS* mRNA is the product of further degradation, or *CCS* mRNA is subject to additional cleavage by a mechanism not yet identified.

To gain additional evidence that miR398 regulates CCS and that the regulation responds to Cu availability, wild-type, over-expressed *CCS*, and *miR398* mutant lines were examined (Fig. 5). Together, the data suggest that CCS is regulated by Cu, mediated by miR398. Our findings are in agreement with a report on the SPL7 transcription factor which activates miR398 during Cu-limited growth (24). In a SPL7 loss of function mutant *CCS* mRNA is not down-regulated during Cu-limited growth as it is in wild-type (24).

Copper-proteins CSD1, CSD2, plantacyanin, and several laccases were predicted to be targeted by microRNA using established bioinformatic approaches prior to being experimentally verified (14,15,32-35). In this study we experimentally verify that *CCS* mRNA is targeted by miR398 even though it does

not contain a bioinformatically predicted microRNA binding site. This study suggests that microRNA regulation of mRNA expression is more widespread than previously predicted, and that several more targets are yet to be found. A list of putative Cu-microRNA targets not previously predicted are identified in Table 1. Targets include proteins that contain putative cupredoxin domains along with others involved in reproduction and cell wall functions. Verifying additional targets of Cu-microRNAs from this list could help create a more complete picture of how Cu availability affects plant processes in addition to Cu delivery and sequestration.

This study indicates that lower base-pair complementarity between microRNA and mRNA target is sufficient for microRNA regulation, at least for CCS. MicroRNA hybridization with target mRNA can be scored by the number of mismatches and G:U wobbles they contain (14). Each mismatch is given one point whereas a G:U wobble gets a half point; therefore a lower score means a closer match (14). Scores for CSD1 and CSD2 are two and two and a half respectively; however, CCS contains four mismatches and one G:U wobble for a score of 4.5 (Fig. 4). A study that altered five nucleotides in the microRNA target sites of CSD1 (*5m*CSD1) and CSD2 (*5m*CSD2) to increase mismatching resulted in lowered sensitivity to miR398 (21). In this study we observed that Cu regulation of CCS protein levels in wild-type plants is somewhat attenuated when compared to CSD1 and CSD2 (Fig. 1). It is likely that the degree of base-pair complementarity and location of base-pair mismatches between microRNA and mRNA target could reduce the target mRNA sensitivity to microRNA regulation;

therefore, the degree of base-pair complementarity may determine the level of regulation by microRNA. For plants, having some CCS proteins available during Cu-limited growth could provide a Cu buffering function. It could also be important that CCS is around prior to any Cu/ZnSOD expression, considering the instability of Cu/ZnSOD when Cu delivery is disrupted (Chapter 4).

An interesting observation is that 35S:*MIR398c* mutant lines exhibited lower miR398 levels when grown on Cu-sufficient medium than plants grown on Cu-limited medium (Fig. 5). This observation was also previously reported (21). Dugas and Bartel (21) suggested that endogenous miR398 accumulation in the mutant during Cu-limited growth could account for the difference (21). The 35S:*MIR398c* construct contains 424 bp upstream and 387 bp downstream of the miR398c hairpin sequence (21). A more recent study showed that miR398 is activated by the SPL7 transcription factor in *Arabidopsis* during Cu-limited growth, and has been shown to bind to GTAC motifs in the promoter of miR398c (24). Yamasaki et al. (24) also reported that 300 bp of the miR398c upstream sequence was sufficient for SPL7 activation of miR398, and the 35S:*MIR398c* construct also contains this promoter sequence (21). It is likely that the increase in miR398 for 35S:*MIR398c* lines during Cu-limited growth is an additive effect by SPL7 activation. An unrelated possibility is that miR398 targets a non-protein coding gene that is expressed when Cu is available in a process known as 'target mimicry'. In *Arabidopsis* it has been shown that the non-protein coding gene *IPS1* acts to bind and sequester miR399 during phosphate replete conditions when miR399 expression is not desirable (37). This system of 'target mimicry'

could exist for sequestering miR398. As Cu may become more available to a plant it may be important to quickly remove residual miR398 to allow for a rapid increase in CCS, CSD1, CSD2, and cytochrome oxidase. Overall, this study has expanded our view of microRNA mediated regulation. Further studies will hopefully address and clarify the expanding role and mechanisms involved in microRNA mediated regulation.

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Known Function	Hybridization and gene identity
(PAA2) P-type ATPase, Cu transport into the thylakoid lumen	5' GACAGGGAAGGGGCAGUGGCA 3' AT5G21930 : ::::::::.::::::: 3' CGGUCCCUUCUCCGUCACGUA 5' miR408
putaive nuclear mRNA splicing, contains putative cupredoxin domain	5' AUACACCUACAUCAUGCCAAA 3' AT4G38780 :::::::: :: ::::.: ::: 3' UAUGUGGAAGUUGUAUGUUUU 5' miR857
(EMB158/EMB33/EMB177/EMB14) putaive nuclear mRNA splicing, putative cupredoxin domain	5' AUACACCUACAUCAUGCCAAA 3' AT1G80070 :::::::: :: ::::.: ::: 3' UAUGUGGAAGUUGUAUGUUUU 5' miR857
(WRKY19) transcription factor, defense response	5' GCCAGGGAAGAGGAAGUGGAA 3' AT4G12020 :::::::::::: :::: : 3' CGGUCCCUUCUCCGUCACGUA 5' miR408
putative senescence-associated protein	5' GAGAGGUAACCUGAGGAGACA 3' AT1G78020 .:: ::: ::::::::.: ::: 3' UUCCCCACUGGACUCUUGUGU 5' miR398a
(ATP5CS) delta1-pyrroline-5-carboxylate synthase, strees response to ABA and salt	5' AGUCAAAGCUGCAGUCAAUGC 3' AT2G39800 .:::: :::::: :::::: 3' GUAGUUGCGACGUGAGUUACU 5' miR397a
putative pectinesterase, cell wall modification in flower	5' CAUCAACGCUGCACUUAAAGC 3' AT4G33230 :::::::::::::::.:: : 3' GUAGUUGCGACGUGAGUUACU 5' miR397a
(MRH5/SHV3) kinase, glycerol metabolic process, involved with cellulose accumulation	5' UUACAGCUGCAAGAUACAAAA 3' AT4G26690 :::: :: ::: :::::::: 3' UAUGUGGAAGUUGUAUGUUUU 5' miR857
kinase, glycerol metabolic process	5' AUACAGCUGCAAGAUAUAAAA 3' AT1G66980 ::::: :: ::: :::.::::: 3' UAUGUGGAAGUUGUAUGUUUU 5' miR857
putative proteolysis activity in cell wall	5' CACCAACGCUCCUCUCCAUGA 3' AT2G05920 :: ::::::: : ::: :::: 3' GUAGUUGCGACGUGAGUUACU 5' miR397a

Table 1. Putative Cu microRNA binding sites similar to hybridization seen for CCS mRNA targeted by miR398. Locus number of targets, microRNA number, along with a description of gene function are given.

Table 1 continued

Known Function	Hybridization and gene identity
(ATGH9A4) glycosyl hydrolase, endomembrane system, carbohydrate metabolism	5' UGGGCGAGACCUGAGAACAUG 3' AT3G43860 :.:: : ::::::::::::.. 3' GUCCCCACUGGACUCUUGUGU 5' miR398b/c
putative pyrophosphate-fructose-6-phosphate 1-phosphotransferase-related	5' GGUUAACGCUGCACUCAAAGC 3' AT1G20950 .:.:::::::::::::: : 3' GUAGUUGCGACGUGAGUUACU 5' miR397a
putative citrate synthase activity	5' GAGGGAUGACCGGAUUACACA 3' AT2G11270 .:::: ::::: :: ::::: 3' UUCCCCACUGGACUCUUGUGU 5' miR398a
putative DNA/RNA binding protein with helicase and DNA repair activity	5' CAUCAAAGCUGCCCUCACUGU 3' AT1G05120 :::::: ::::: :::: :: 3' GUAGUUGCGACGUGAGUUACU 5' miR397a
(VCL1) VACUOLELESS1, required for vacuole formation and morphogenesis of the embryo	5' CAUCCACGCUGAGCTCAUUGA 3' AT2G38020 :::: :::::: .:::: ::: 3' GUAGUUGCGACGUGAGUUACU 5' miR397a
shoot gravitropism, located in vacuole, phospholipase A1 activity	5' CAUCAGCUCAGCACUCAACGA 3' AT1G31480 ::::::: : :::::::: :: 3' GUAGUUGCGACGUGAGUUACU 5' miR397a
(NPGR2) calmodulin-binding protein expressed in pollen	5' UCCGGGGAAGAAGCAGUGCCG 3' AT4G28600 ::.::::::: ::::::: 3' CGGUCCCUUCUCCGUCACGUA 5' miR408
(AAT/MEE17) aspartate transaminase, chloroplast, embryonic development	5' AUGCACCUGCAACACACACAA 3' AT2G22250 ::.::::: ::::: ::: :: 3' UAUGUGGAAGUUGUAUGUUUU 5' miR857
contains putative DC1 domain rich in cysteines and histidines	5' AAACACCUUCUACAUCCAAAG 3' AT1G44020 : :::::::: :::: ::::. 3' UAUGUGGAAGUUGUAUGUUUU 5' miR857
putative cysteine-type peptidase activity	5' CAUCAACGAUACAAUCAUUGA 3' AT4G33620 :::::::: : :: ::: ::: 3' GUAGUUGCGACGUGAGUUACU 5' miR397a
(CKB3) regulatory subunit of the protein kinase CK2, circadian clock	5' AAUCAACGAUGCACUCAAUAA 3' AT3G60250 ::::::: ::::::::: : 3' GUAGUUGCGACGUGAGUUACU 5' miR397a

Table 1 continued

Known Function	Hybridization and gene identity
putative protein binding PRLI-interacting factor	5' AAGGCGUGAUAUGAAAACACU 3' AT1G63850 :::: ::::. ::: ::::: 3' UUCCCCACUGGACUCUUGUGU 5' miR398a
(PORC) protochlorophyllide oxidoreductase activity	5' CAGGGUUGUUCAGAGAACACA 3' AT1G03630 ::::: :: .: ::::::::: 3' GUCCCCACUGGACUCUUGUGU 5' miR398b/c
PhxC-like, phenazine biosynthesis	5' AAACAACUUCAACAGACCAAA 3' AT4G02850 : ::: :::::::: :: ::: 3' UAUGUGGAAGUUGUAUGUUUU 5' miR857
similar to proteasome family protein	5' GCCAAGGAGGAGGCUGUGCGU 3' AT3G02200 :::: :::.:::::: :::::.: 3' CGGUCCCUUCUCCGUCACGUA 5' miR408
uncharacterized protein	5' CUUCAACACCGCACUCACUGA 3' AT2G07280 : ::::: : ::::::: ::: 3' GUAGUUGCGACGUGAGUUACU 5' miR397a
uncharacterized protein	5' AACGGGUUACCUGACAACAGA 3' AT3G56720 :: :::: :::::: :::: : 3' UUCCCCACUGGACUCUUGUGU 5' miR398a
uncharacterized protein	5' GCGAGGCAAGAGGCAUUGCAG 3' AT5G49945 :: ::: :::::::: :::: 3' CGGUCCCUUCUCCGUCACGUA 5' miR408
uncharacterized protein	5' AUACAACUUCAAAAGACAAGG 3' AT2G20170 ::::: :::::: : :::::.. 3' UAUGUGGAAGUUGUAUGUUUU 5' miR857
uncharacterized protein	5' AUUCAACAUCAACAUACAAAC 3' AT3G02440 :: :: : :::::::::::: 3' UAUGUGGAAGUUGUAUGUUUU 5' miR857
uncharacterized protein	5' AUUUAUCUUCAACAAACAAAA 3' AT5G61040 :: .:.::::::::: :::::: 3' UAUGUGGAAGUUGUAUGUUUU 5' miR857

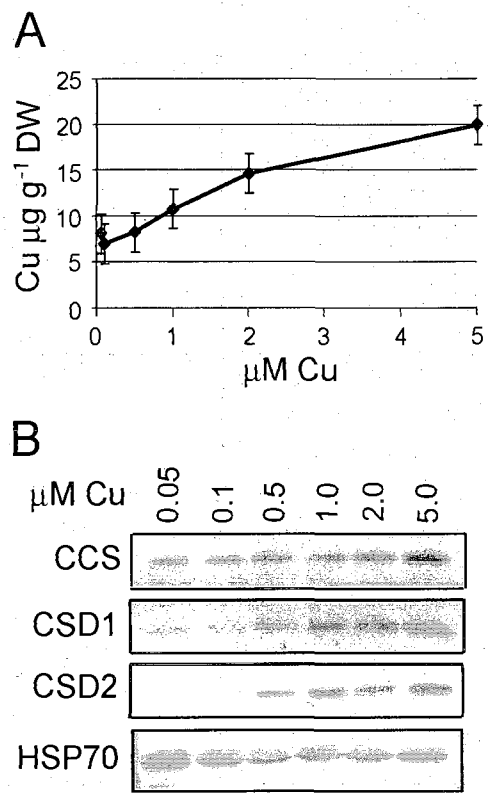

Figure 1. Regulation of CCS expression by Cu availability. A) Cu content of wild-type shoot tissues represented as µg g-1 DW (dry weight) from plants grown on tissue culture medium containing increasing µM Cu concentrations. Means ± SE shown (n=4). B) Immuno-detection of CCS and Cu/ZnSOD proteins extracted from shoot tissues of plants grown in above conditions. Shoot proteins (20 µg) were separated by SDS-PAGE and detected by immuno-blotting against Heat Shock 70 (HSP70) loading control, CCS, and Cu/ZnSOD proteins.

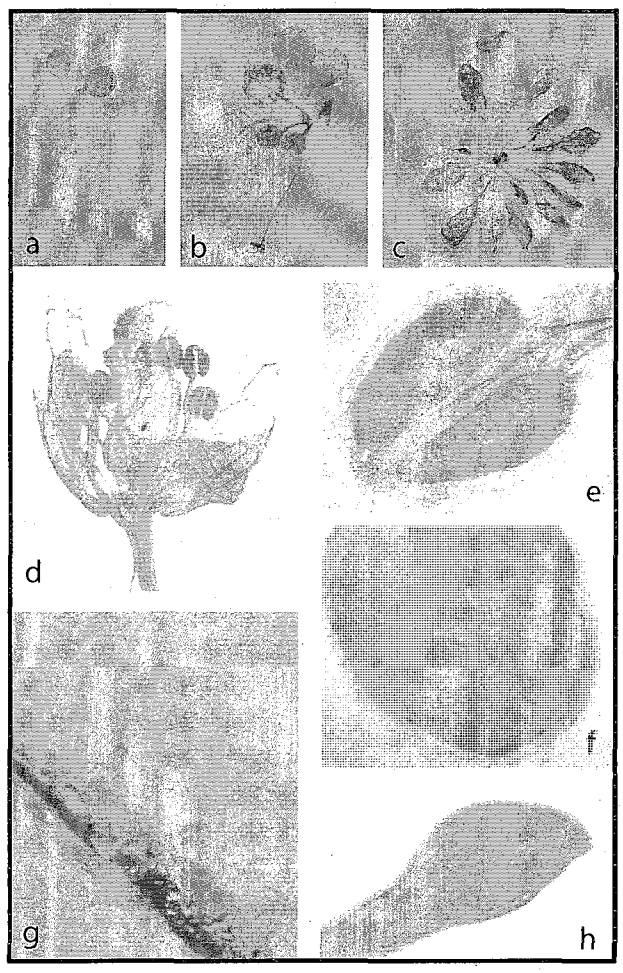

Figure 2. Histochemical GUS staining of *CCSpro::GUS-1* grown on soil. a) 7 days old. b) 14 days old. c) 28 days old. d) flower of 28 day old plant. e) anther head. f) mature leaf at 14 days. g) root with root hairs at 14 days. h) etiolated seedling grown in complete darkness for 5 days on tissue culture medium.

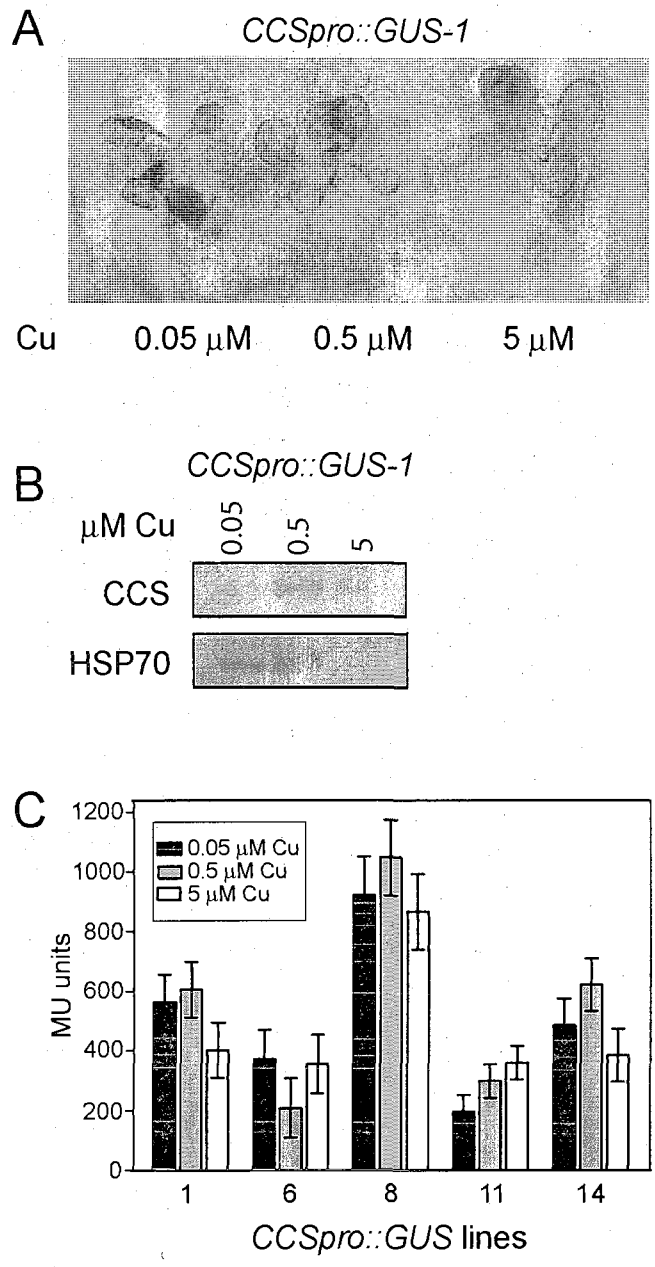

Figure 3. *CCS* promoter response to Cu availability. A) Histochemical GUS staining of *CCSpro::GUS-1* plants. All plants in figure were grown on 0.5 MS tissue culture medium with final Cu concentrations shown. B) Immuno-detection against proteins CCS and HSP70 loading control from *CCSpro::GUS-1* leaf tissue. C) Fluorogenic GUS activity assay using MUG as a substrate. Means of MU units ± SE are shown (n=4).

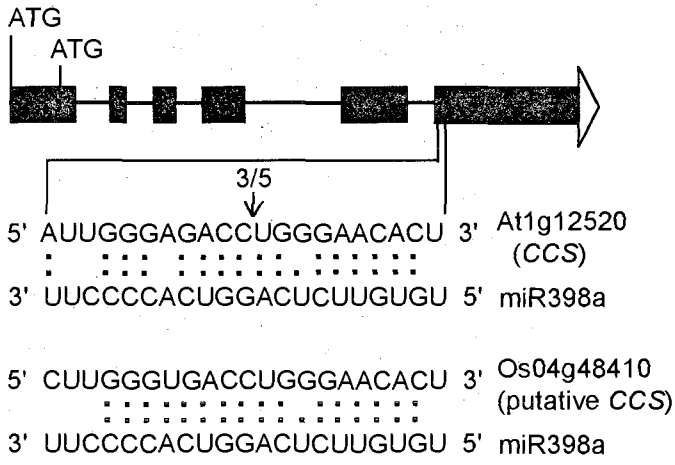

Figure 4. Experimental validation of the microRNA 398 targeting of CCS mRNA. The mRNA cleavage sites were determined using 5' RNA ligase-mediated RACE. The black squares represent exons and horizontal lines represent introns. The CCS mRNA and microRNA398 sequences of *Arabidopsis* and *Oryza sativa* are shown. Vertical arrow indicates the 5' terminus of the microRNA guided cleavage product and frequency of clones with cleavage product shown. Watson-Crick pairing (:) and G:U wobble (.) pairing are indicated.

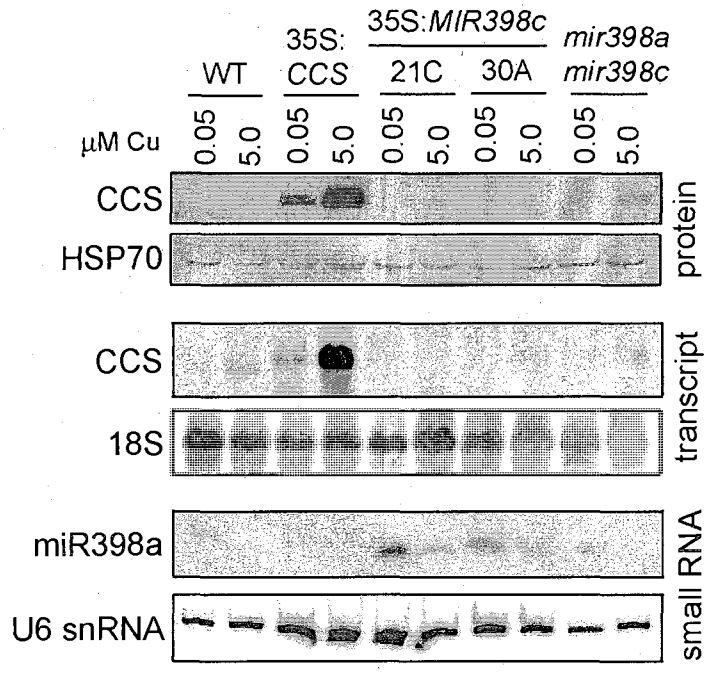

Figure 5. Expression analyses of CCS in leaves of wild-type and mutant *Arabidopsis* plant lines grown at different Cu concentrations. Wild-type, CCS over-expresser (35S:*CCS*), miR398c over-expressers (35S:*MIR398c*),and *miR398a miR398c* double knock-out mutant were grown on tissue culture medium for 14 days with final Cu concentrations shown. Immuno-detection against CCS and HSP70 (*loading control*) proteins (top panel). RNA blot analysis of CCS and 18S (*middle panel*). Small RNA blot analysis of miR398a and U6 snRNA (*lower panel*).

CHAPTER 6

Arabidopsis iron superoxide dismutase I promoter contains a copper-responsive cis-acting element and the SPL7 transcription factor is required for activation

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CHAPTER 6

Arabidopsis iron superoxide dismutase I promoter contains a copper-responsive cis-acting element and the SPL7 transcription factor is required for activation

Abstract

Copper has been identified as the main regulator of superoxide dismutases in the chloroplast (CSD2 and FSD1) and cytosol (CSD1). When plants are Cu-sufficient, Cu/ZnSODs accumulate while FSD1 is down-regulated; and during Cu-limited growth FSD1 accumulates while Cu/ZnSODs are down-regulated. Down-regulation of Cu/ZnSODs during Cu-limited growth is mediated by the transcription factor SPL7 and microRNA 398 that targets CSD1 and CSD2 transcripts for microRNA directed cleavage. In moss, a SPL7 homolog binds to the promoter of its FeSOD and acts as a transcriptional regulator during Cu-supplemented growth. Microarray analysis of a SPL7 mutant in *Arabidopsis* shows reduced *FSD1* transcripts during Cu-supplemented and Cu-limited growth. In this study, using a *FSD1* promoter GUS fusion, we show that the *Arabidopsis* *FSD1* promoter responds to Cu availability and that SPL7 is required for FSD1 expression. In addition, we observed ubiquitous activation of the *FSD1* promoter in *Arabidopsis* tissues with the highest activation in leaves and root tips.

Abbreviations- $O_2^{\cdot-}$, superoxide; Cu/ZnSOD, Cu-Zn superoxide dismutase; FeSOD, Fe superoxide dismutase; PC, plastocyanin; SBP, *SQUAMOSA* promoter-binding protein; GUS, *β*-glucuronidase; SPL7, *SQUAMOSA* promoter binding protein-like7

1. Introduction

In oxygenic organisms, reactive oxygen species (ROS) are formed during metabolic processes in the presence of oxygen. ROS, such as superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), cause oxidative stress that can ultimately lead to cellular damage (1). During photosynthetic electron transport, $O_2^{\cdot-}$ is generated when a univalent electron is transferred to oxygen (2). The accumulation of $O_2^{\cdot-}$, together with H_2O_2 and metal ions, can lead to the formation of highly reactive hydroxyl radicals ($OH^{\cdot}$) (3). To alleviate $O_2^{\cdot-}$ accumulation, superoxide dismutases (SOD) catalyze the dismutation of $O_2^{\cdot-}$ into H_2O_2 and O_2 (4). The H_2O_2 is then reduced to water by ascorbate peroxidase to complete the water-water cycle (5). The water-water cycle may function to dissipate excess light energy and reduce photo-oxidative stress (3).

In *Arabidopsis*, seven nuclear encoded SOD genes have been identified and they are named for the metal cofactor(s) they bind. The mitochondrion contains MSD1, a MnSOD. CSD1, CSD2 and CSD3 are Cu/ZnSODs found in the cytosol, chloroplast stroma, and peroxisome, respectively (6). FSD1, an FeSOD, was localized to the chloroplast stroma (6); however, a recent study suggests that FSD1 may exist in the cytosol (7). FSD2 and FSD3 have been localized to the

surface of the thylakoid membrane; however, it is not yet clear whether FSD2 and FSD3 activity reside in the stroma or the thylakoid lumen. Unlike FSD1, FSD2 and FSD3 are essential for seedling development (7). Interestingly, a study that examined a knock-out mutant of the Cu chaperone for SOD (CCS), which delivers Cu to Cu/ZnSOD, had little measurable Cu/ZnSOD activity yet plants did no show a growth phenotype (8; Chapter 4). These findings were not in agreement with a previously reported CSD2 knock-down that exhibited a severe phenotype (9). Furthermore, when the CCS knock-out mutant was grown on Cu supplements to dramatically reduce Cu/ZnSOD and FeSOD activity the plants did not exhibit a growth phenotype (Chapter 4 data). Therefore, SOD function and importance is not yet completely understood.

FeSOD is considered the most ancient of the SODs and is found in anaerobic bacteria. When FeSOD and photosynthesis first evolved, the earth was likely void of oxygen making Fe^{2+} more prevalent than Fe^{3+}. Iron in its reduced form is bio-available and would have been abundant. Therefore, Fe could easily have been utilized in enzymes given the anoxic biosphere, including those that functioned in photosynthesis. As oxygenic photosynthesis produced elemental oxygen the earth's biosphere gradually became less anoxic, making bio-available Fe increasingly less abundant. At the same time, free Cu ions became increasingly more bio-available as Cu^{2+} in an oxygen rich environment. (10-12).

Superoxide dismutases CSD1, CSD2, and FSD1 are regulated by Cu availability (13,14). Many higher plants switch between Cu/ZnSOD and FeSOD depending on Cu availability; suggesting that plants have some level of plasticity

for Fe and Cu use based on changing Cu availability (13,14). When plants are Cu-sufficient, Cu/ZnSODs accumulate while FSD1 is down-regulated; and during Cu-limited growth FSD1 accumulates while Cu/ZnSODs are down-regulated (13,14). The mechanism of SOD regulation by Cu availability in *Arabidopsis* was recently correlated with microRNA and a SPL7 transcription factor.

Studies focusing on transcription factors that contain a SBP (*SQUAMOSA* promoter binding protein) domain revealed involvement in regulating expression of Fe and Cu-proteins. *Chlamydomonas reinhardtii* express either cytochrome *c6* (heme protein) or plastocyanin (Cu-protein) depending on Cu supply. The *C. reinhardtii* Cu-responsive regulator (CRR1), which contains a SBP domain, binds to a GTAC core motif of the cytochrome *c6* promoter in response to Cu-limitation, leading to increased cytochrome *c6* (15). Additionally, in moss, a homologous transcription factor (PpSBP2) was found to bind to a GTACT core motif in the promoter of *FeSOD* to repress transcription during Cu-supplemented growth (16). During Cu-limited growth, *Arabidopsis* plants express microRNA 397, 398, 408, and 857 which target transcripts of Cu-proteins, resulting in decreased protein accumulation (17,18). *Arabidopsis* contains a SPL7 (*SQUAMOSA* promoter binding protein-like7) transcription factor, homologous to CRR1, that binds to miR398 during Cu-limitation and is required for miR398, 397, 408 and 857 activation (19). A mutant of SPL7 also did not show an increase in *FSD1* transcripts during Cu-limited growth (19). Together, these suggest that the promoter of *FSD1* in *Arabidopsis* contains a Cu-responsive cis-acting element, and that SPL7 is involved in regulating the *FSD1* promoter. We investigated the

regulation of *FSD1* in *Arabidopsis* using a promoter GUS-fusion. Our data suggest that the promoter of *FSD1* does contain a Cu-responsive cis-acting element and that SPL7 is required for FSD1 expression.

2. Materials and Methods

FSD1::GUS-1 DNA construct. The *FSD1* (At4g25100) promoter sequence was amplified by PCR (expand high fidelity PCR system, Roche) using primers FSD1-L (5'-ATCCCAAGCTTATATGGTTTA CCCATCTTAATTTTAA-3') and FSD1-R (5'-TACGCGGATCCTCTTTGTAATTGA AGCTGCACATT-3') that introduced a *Hind*III and *Bam*HI restriction site respectively. The amplified promoter is 1016 bp long and contained the entire *FSD1* 5' UTR and upstream intergenic sequence up to the end of the 3' UTR of the next upstream gene (Fig. 1). The *FSD1* promoter was ligated into a PBI101.2 vector containing a promoterless β-glucuronidase (GUS) gene. *E. coli* (DH5α) cells were transformed with the *FSD1::GUS* construct and colonies were screened by PCR, using FSD1-L and GUS-R (5'-CGATCCAGACTGAATGCCCA -3') primers (Fig. 1). Plasmids of positive clones were verified by restriction digest, using *Hind*III and *Bam*HI, followed by sequencing prior to transformation of *Arabidopsis* (Col-0) by *Agrobacterium* (C58C1) using the floral dip method (20). Controls included two constructs; one that contains an empty PBI101.2 and the other a *CHL27* (required for synthesis of protochlorophyllide) promoter, with ubiquitous and constitutive activity in green tissue (21), inserted into PBI101.2. Transformants were selected on tissue culture medium plates containing 50 mg/ ml kanamycin.

Confirmation of plant transformation was conducted using PCR on genomic DNA. Genetically transformed plants were grown on soil and allowed to self pollinate. To obtain homozygous lines, seeds from transformed plants were sown individually, allowed to set seed, and tested for germination on kanamycin tissue culture medium plates.

Plant Materials and Growth Conditions. All *Arabidopsis* plant lines used have a Col background. SPL7 mutant (*spl7*) and SPL7 complemented line have been described (19). The *FSD1::GUS-1* line grown on soil (Fafard®Custom Mix, Conrad Fafard Inc., Agawam, MA) was watered every 4 days with distilled de-ionized water to reduce Cu feeding. For tissue culture medium grown plants, seeds were surface-sterilized and vernalized for 4 days at 4°C then sown on 0.5 Murashige and Skoog (MS) medium (Caisson Laboratories Inc., North Logan, UT) containing 0.4% Agargel (Sigma-Aldrich, St Louis, MO), 1% sucrose, and a final concentration of 0.5 or 5 µM $CuSO_4$ when specified. All plants were grown in controlled conditions (light intensity of 150 µmol m^{-2} s^{-1}, 12-h/12-h light/dark cycle at 23°C) for 14 days, unless otherwise specified. All plant shoot tissues collected for immuno-detection and fluorogenic assays were frozen immediately in liquid nitrogen then stored at -80°C. Plant tissue for histochemical GUS staining was stained immediately after harvesting.

Protein extraction and immuno-detection. Soluble shoot proteins for SDS polyacrylamide gel analysis were extracted as described (13). Total protein was quantified according to the Bradford (22) method using bovine serum albumin as a standard. For immuno-detection analysis, 20 µg of protein extract was loaded

into 12.5% SDS polyacrylamide gels. Antibodies used for CSD2, FSD1 and PC have been described (13). Antibodies for heat shock 70 (HSP70) were obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO). Each experiment was replicated three times with identical results, and representative gels are shown.

Histochemical GUS staining and fluorogenic GUS assay. Histochemical GUS staining, using X-gluc (5-bromo-4-chloro-3-indoxyl-D-glucuronide) (GoldBio, St Louis, MO), was conducted on the *FSD1::GUS-1* plant line grown in soil and tissue culture medium under the specified Cu growth conditions. The Histochemical GUS staining solution consisted of: 50 mM NaPi pH 7.0, 0.1% TritonX-100, 10 mM EDTA, 0.5 mM K_3FeCN_6 and K_4FeCN_6, and 1.5 mg/ ml X-gluc. Plant tissue was vacuum infiltrated with staining solution for 20 minutes then incubated at 37°C for 2 hours. Stained plant tissue was placed in 75% ETOH until no more pigments were visible. Fluorogenic analysis of promoter activity was conducted on protein extracts described above using MUG (4-methylumbelliferyl-β-D-glucuronide) (GoldBio, St Louis, MO) as a substrate for GUS activity. Fluorogenic assay solution consisted of: 50 mM NaPi pH 7.0, 10 mM EDTA, 10 mM DTT, 0.1% TritonX-100 and 0.1% Sarkosyl. Five µg of soluble protein extract was added to 1 ml of assay solution and allowed to incubate at 37°C for 20 minutes. The reaction was stopped by adding 1.4 ml of 0.4 M Na_2CO_3. 4-Methyl umbelliferone (MU) fluorescence was measured at 400 nm using a Hoefer DyNA Quant 200 Fluorometer (Hoefer, Inc., Holliston, MA) and represented as arbitrary fluorescence units (MU.units).

3. Results

The promoter sequence of *FSD1* in *Arabidopsis* contains six GTAC motifs, five of which occur within 380 base pairs upstream of the ATG start site (Fig. 1A). The *FSD1* promoter was successfully ligated into the PBI101.2 vector 25 base pairs upstream of the *GUS* ATG start site (Fig. 1B), and was verified by colony PCR. *Agrobacterium* colonies transformed with the sequence verified *FSD1::GUS* construct resulted in five positive colonies, determined by PCR (Fig. 2). Restriction digest of plasmids form two colonies confirmed that the *FSD1::GUS* construct was present (Fig. 2). Only one *FSD1::GUS* plant line has been confirmed to date. *Agrobacterium* clones positive for the *FSD1::GUS* construct were obtained (Fig. 2) but repeated (x3) floral dips using clone number one did not result in added transformants. Plant transformation using colony five *Agrobacterium* cells was used for floral dipping and analysis of plants is in progress.

Arabidopsis plants alter chloroplast SOD expression based on Cu availability (Fig. 3). When plants are grown without any Cu additions to tissue culture medium the FSD1 expression is at its highest. However, as Cu is added to the medium the plants gradually down-regulate FSD1 expression as CSD2 expression increases (Fig. 3). At a final concentration of 0.5 μM Cu, FSD1 and CSD2 expression are equal. FSD1 and CSD2 reach maximum expression levels at 0.05 μM and 5 μM Cu respectively. Based on these results, the Cu

concentrations of 0.05, 0.5, and 5 μM Cu were selected for *FSD1* promoter GUS-fusion assays.

The *FSD1::GUS-1* plant line exhibits a decrease in promoter activation as Cu feeding increases (Fig. 4A and B). Histochemical staining of 14 day old plants shows a gradual decrease in GUS activity in both leaf and root tissue as Cu levels increase. *FSD1::GUS-1* plants grown on 0.05 μM Cu show dark and ubiquitous staining in leaf and root tissues. No staining was observed in *FSD1::GUS-1* plants after growth on 5 μM Cu (Fig. 4A). To obtain quantifiable data, fluorogenic analysis of soluble protein samples from *FSD1::GUS-1* tissue culture grown plants was conducted. Fluorogenic analysis indicated decreasing GUS activity as Cu levels increased, consistent with the histochemical GUS staining and FSD1 expression (Fig. 3, 4A and B). These results suggest that the *FSD1* promoter is directly responsive to Cu availability and becomes more active as Cu levels decrease. Using a microarray method, a SPL7 mutant (*spl7*) showed low *FSD1* transcript levels when plants were Cu-limited (19). Wild-type and a SPL7 complemented line (19) exhibited similar FSD1 expression patterns in response to Cu availability (Fig. 4C). However, when *spl7* mutant plants were grown on Cu-sufficient and Cu-limited tissue culture medium, FSD1 was not expressed (Fig. 4C); suggesting that SPL7 is required for FSD1 expression during Cu-limited growth.

FSD1 promoter activity has not been spatially localized in all *Arabidopsis* tissues so we conducted a whole-plant histochemical GUS staining of *FSD1::GUS-1* plants grown on soil (Fig. 5). All leaf tissues demonstrated similar

patterns of *FSD1* promoter activation, determined by GUS activity. Concentrated blue dye stain was observed in vascular regions and slightly decreased stain intensity in inter-vascular regions. Generally, GUS activity in leaf tissue was ubiquitous. In root tissues, GUS activity was sporadic in mature root cells with staining localized primarily to vascular regions. Interestingly, for below ground tissues, the highest concentration of blue dye was observed in short adventitious root tips (Fig. 5). In flowering tissues, stems of inflorescences contained high GUS staining, along with cauline leaves, though staining was not found in all stems. Flowers appeared to contain the lowest GUS staining relative to the rest of the plant. Relatively low but ubiquitous staining was seen in sepals, petals, and stems of anthers (Fig. 5).

4. Discussion

Our results indicate that the promoter of *FSD1* in *Arabidopsis* is regulated by Cu availability and that the SPL7 transcription factor is required for FSD1 expression during Cu-limited growth. Furthermore, analysis of the *FSD1* promoter, using *FSD1::GUS-1* plants, indicates that Cu availability in tissue culture medium directly affects *FSD1* promoter activation in root and shoot tissues (Fig. 4A). We found that when *FSD1::GUS-1* plants were grown on Cu-limited tissue culture medium, GUS activity was at its highest and tapered down to no activity as Cu was added (Fig. 4A and B). These results could be interpreted in two ways: the *FSD1* promoter is activated during Cu-limited conditions, or the *FSD1* promoter is repressed during Cu-sufficient conditions, as

suggested for moss (16). In *Arabidopsis*, the transcription factor SPL7 has been implicated as playing a role in *FSD1* transcript regulation (19). Homologs of SPL7 found in *C. reinhardtii* (CRR1) (15) and moss (PpSBP2) (16) bind to GTAC(T) promoter core motifs. The *FSD1* promoter of *Arabidopsis* contains six GTAC core motifs (Fig. 1). When *spl7* was grown in Cu-supplemented and Cu-limited tissue culture medium, FSD1 expression was not detected (Fig. 4C). However, the wild-type and SPL7 complemented line did express FSD1 during Cu-limited growth (Fig. 4C). This result suggests that SPL7 is required for the expression of FSD1 during Cu-limitation. Consistent with this finding, a microarray experiment on *spl7* (19) showed that *FSD1* transcripts did not increase during Cu-limitation; and that the homologous transcription factor in *C. reinhardtii* (PpSBP2) (15) is required for transcriptional activation of cytochrome *c6* during Cu-limitation. However, this conclusion is not consistent with the model that the PpSBP2 transcription factor in moss represses *FeSOD* transcription by binding to the promoter's GTACT core motifs during Cu-supplemented growth (16). It is possible that these homologous transcription factors act upon FeSOD promoters differently; however, taken together, it is more likely that the transcription factors act as an activator during Cu-limitation and a repressor during Cu-sufficient growth.

Histochemical GUS staining of soil grown *FSD1::GUS-1* suggest that the promoter of *FSD1* is activated in most *Arabidopsis* tissues. Interestingly, a high concentration of *FSD1* promoter activation was localized to root tips (Fig 4A, 5). Even when *FSD1::GUS-1* plants were grown in tissue culture medium containing 0.5 μM Cu the root tips contained high *FSD1* promoter activation (Fig. 4A).

Together, these results suggest that FSD1 in root tips is important during Cu-limited growth when CSD1 and CSD2 are down-regulated. It is possible that FSD1 activity serves to alleviate some of the oxidative stress plant experience during root elongation. Overall, histochemical GUS activity staining of *FSD1::GUS-1* plants suggest that FSD1 plays a role in most plant tissues, though the role of FSD1 has yet to be elucidated.

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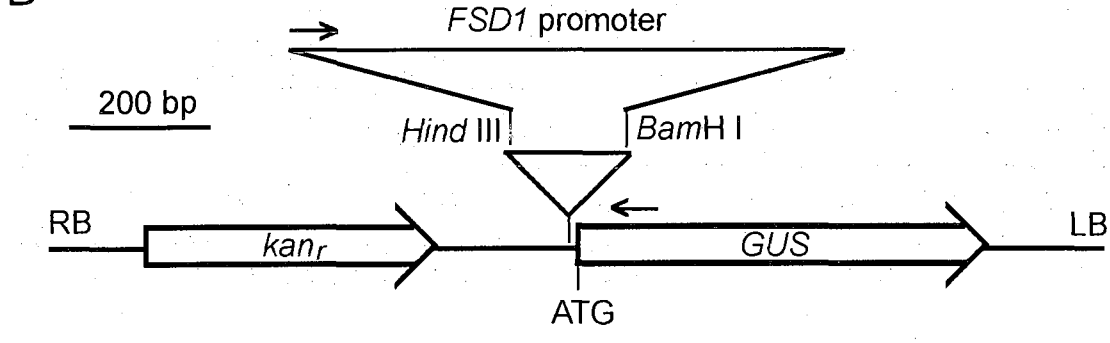

Figure 1. A) Promoter sequence of *FSD1* in Arabidopsis. The genomic sequence between vertical arrows indicates the promoter sequence used to create the *FSD1::GUS* construct. GTAC sequences within the promoter are bold and underlined. B) Vector map of PBI101.2 and inserted *FSD1* promoter. Horizontal arrows indicate primer locations used to verify transformants by PCR.

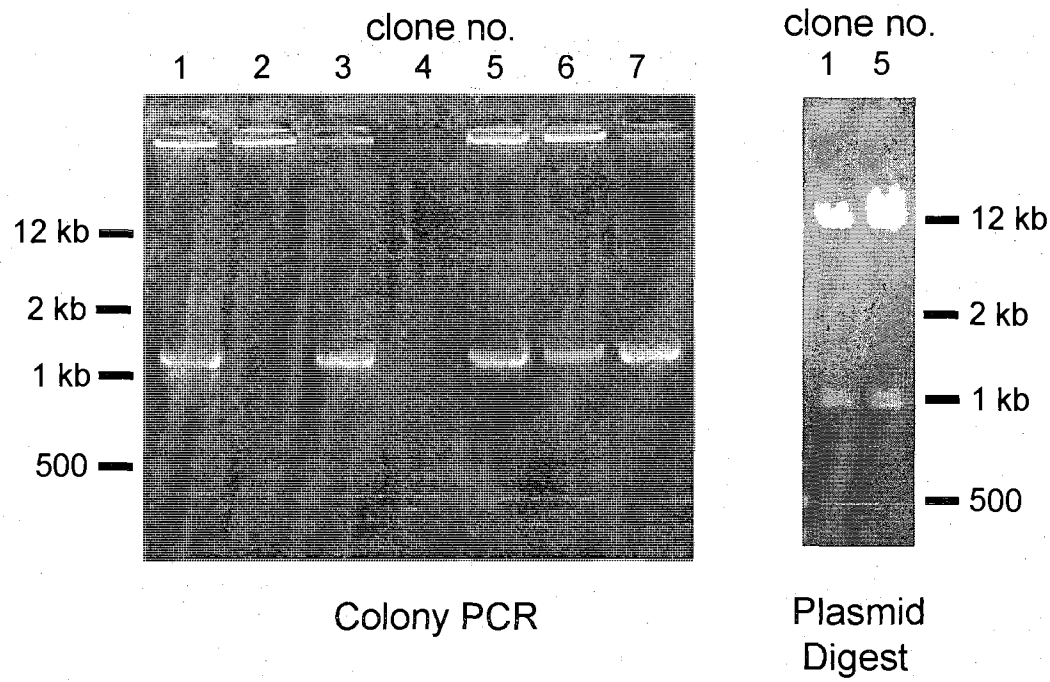

Figure 2. Colony PCR of C58C1 cells transformed with the *FSD1::GUS* construct. Primers FSD1-L and GUS-R were used. Plasmids extracted from colonies 1 an 5 were used to transform *E. coli* cells for plasmid isolation. These plasmids were then digested with *Bam*HI and *Hind*III to confirm insert.

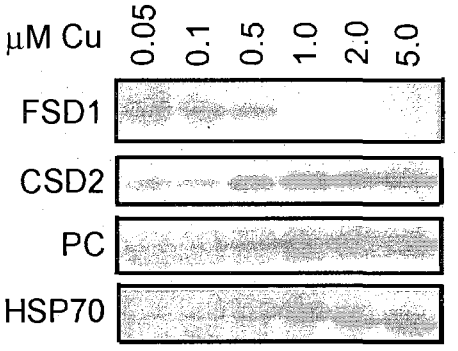

Figure 3. Immuno-detection of FeSOD1 (FSD1), Cu/ZnSOD2 (CSD2), plastocyanin (PC) and HSP70 loading control. Wild-type plants were grown on 0.5 MS tissue culture medium with final Cu concentrations shown.

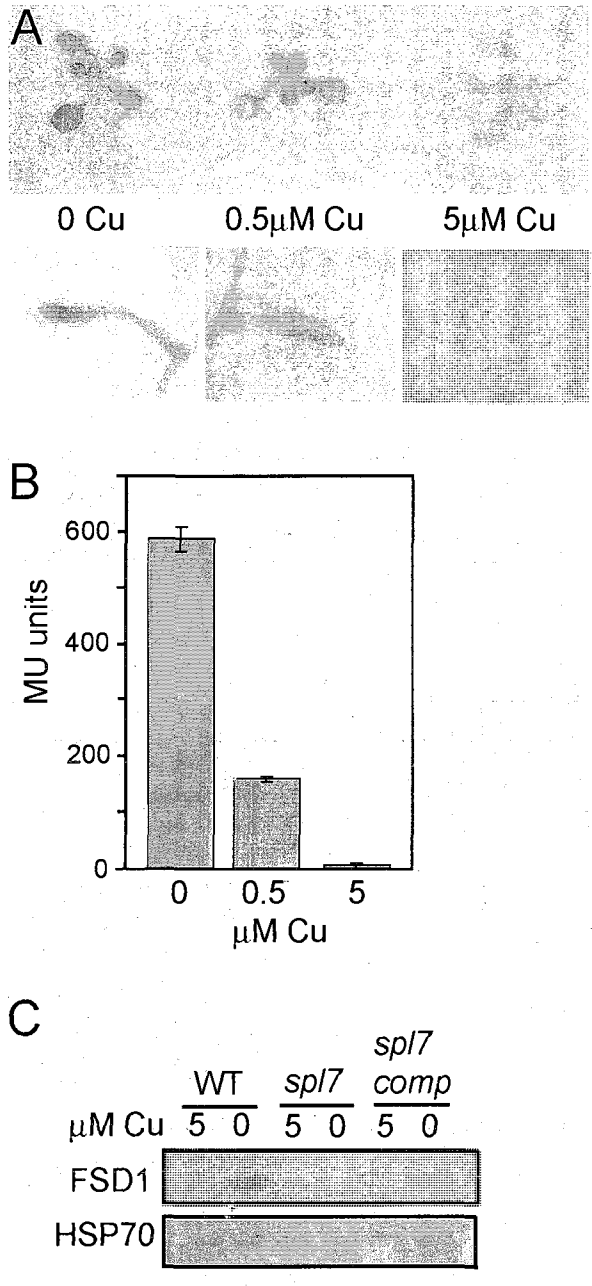

Figure 4. *FSD1* promoter response to Cu and regulation by SPL7. A) Histochemical GUS staining of *FSD1::GUS-1* rosettes and root tips. B) Fluorogenic GUS activity assay using MUG as a substrate. Means of MU units (n=3) ± SD are shown. C) Immunodetection against proteins FeSOD1 (FSD1) and HSP70 loading control. Wild-type (WT), SPL7 knock-out mutant (*spl7*) and SPL7 complement mutant (*spl7 comp*). All plants were grown on 0.5 MS tissue culture medium with final Cu concentrations shown.

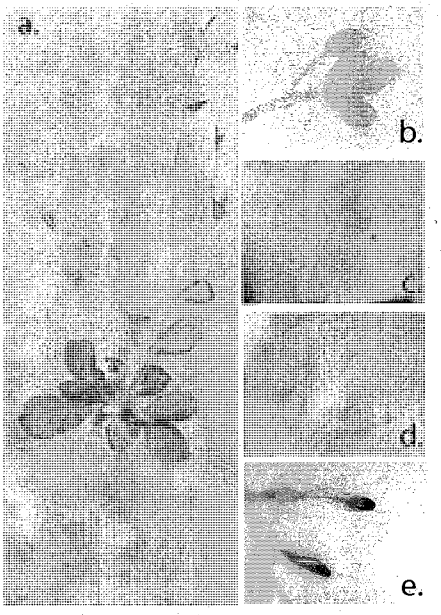

Figure 5. Histochemical GUS staining of *FSD1::GUS-1* plants grown on soil. a) flowering plant; b) flower buds; c) anther head; d) leaf; e) root tips.

CHAPTER 7

Conclusions

Copper (Cu) is an essential micronutrient for higher plant growth, yet it can be toxic if in excess (1,2). Therefore, delivery and sequestration of Cu must be tightly regulated. This dissertation investigated this Cu regulation in plants. The research presented indicates that sensory mechanisms and signaling pathways exist to coordinate Cu transport and target protein expression based on Cu status. Furthermore, *Arabidopsis* without measurable FeSOD or Cu/ZnSOD activity in the chloroplast does not exhibit a growth phenotype.

Preferential allocation of Cu to plastocyanin during Cu-limited growth conditions was observed for four out of five crop plant species (Chapter 3). This observation underscores the essential role of plastocyanin in photosynthetic electron transport. The observation that Cu availability regulates Cu/ZnSOD and FeSOD1 (FSD1) abundance, but not plastocyanin, for several plant species indicates a conserved regulatory mechanism of SOD expression based on Cu status. We propose that plants that down-regulate Cu/ZnSOD and up-regulate FSD1 during Cu-limited growth can save Cu for use in plastocyanin, which is essential for photosynthesis (3). This plasticity in response to Cu nutrition ultimately allows for plants to acclimate and thrive in varying ranges of Cu growth conditions.

If our model is correct, than the abundant Cu-proteins which are targets of the Cu-microRNAs should be dispensable. We investigated this hypothesis for CSD1 and CSD2. Copper delivery to Cu/ZnSOD is conducted by the Cu chaperone for SOD (CCS). When a loss of function CCS mutant (CCS-KO) was grown on Cu-supplemented soil there was no detectable CSD1 and CSD2 activity. In addition, the CSD1 and CSD2 polypeptide levels were dramatically reduced in the CCS-KO plants. Therefore, in photosynthetic tissue, CCS accounts for most, if not all, Cu delivery to both CSD1 and CSD2, and is important for their accumulation and activity (Chapter 4). When the CSS-KO mutant was grown with Cu supplements to turn off FSD1 expression, we observed no measurable FSD1 and CSD2 activity in the chloroplast. The CCS-KO plants, without measurable chloroplastic SOD activity, grow and reproduce the same as wild-type under the laboratory conditions that we tested. Chlorophyll fluorescence measurements indicated that dramatic reductions in chloroplastic SOD activities do not result in photosynthetic electron transport deficiencies relative to wild-type, even after stress (Chapter 4). This analysis reveals that the majority of chloroplastic and cytosolic SOD activities can be inactivated without a consequence to the phenotype under standard laboratory and high light stress growth conditions. We suggest that plants with low Cu/ZnSOD and FSD1 activity are able to acclimate in a way that diverts excess photons and electrons to other photo-protective mechanisms such as the xanthophyll cycle (4).

Copper homeostasis also requires mechanisms for controlling the expression of Cu delivery systems and target proteins. Chapter 5 of this dissertation shows

that CCS expression increases as Cu is more available, similar to CSD1 and CSD2; therefore, CCS expression is regulated by Cu. The promoter of CCS was shown to not contain a Cu responsive cis-acting element. Using a 5' RACE approach, we were able to detect a *CCS* mRNA cleavage product that corresponds with a putative miR398 binding site that has, however, low base-pair complementarity. In addition, we showed that *CCS* mRNA and protein level reductions during Cu-limited growth correlate with an increase in miR398 abundance, similarly reported for CSD1 and CSD2 in these lines (5,6). Most importantly, *CCS* mRNA was not predicted to be targeted by a microRNA using established bioinformatics approaches, suggesting that microRNA regulation of mRNA is more widespread than previously thought. We also observed that the regulation of CCS protein by Cu is somewhat attenuated when compared to CSD1 and CSD2. We considered that the degree of base-pair complementarity between microRNA and target could impact target mRNA sensitivity to microRNA; thereby, having an attenuated effect on mRNA targets with lower base-pair complementarity.

CCS is regulated by Cu via miR398, and miR398 is up-regulated by the SPL7 transcription factor during Cu-limited growth (7). Our results indicate that the promoter of *FSD1* in *Arabidopsis* is regulated by Cu availability and that the SPL7 transcription factor is required for FSD1 expression during Cu-limited growth. Analysis of the *FSD1* promoter, using *FSD1::GUS-1* plants, indicates that Cu availability in tissue culture medium directly affect *FSD1* promoter activation in root and shoot tissues. We considered that the *FSD1* promoter is activated

during Cu-limited conditions, or the *FSD1* promoter is repressed during Cu-sufficient conditions, as suggested for moss (8). In *Arabidopsis*, the transcription factor SPL7 has been implicated as playing a role in *FSD1* transcript regulation (7). When a *spl7* mutant was grown in Cu-supplemented and Cu-limited tissue culture medium, FSD1 expression was not detected. This result suggests that SPL7 is required for the expression of FSD1 during Cu-limitation. However, this conclusion is not consistent with the model that the PpSBP2 (SPL7 homolog) transcription factor in moss represses *FeSOD* transcription by binding to the promoter during Cu-supplemented growth (8). It is possible that these homologous transcription factors act upon FeSOD promoters differently; however, taken together, it is more likely that the transcription factors act as an activator during Cu-limitation and as a repressor during Cu-sufficient growth.

This dissertation has expanded our view of how Cu availability regulates chloroplastic and cytosolic SOD expression and activity for *Arabidopsis* and other crop species. Starting with an initial observation that chloroplastic SODs are regulated by Cu has allowed us to explore the regulatory mechanisms that orchestrate Cu delivery and protein activity during various Cu growth conditions. CSD2 and FSD1 biological function in the chloroplast still remain unclear; however, the CCS loss of function mutant (CCS-KO) will allow future studies to closely examine *Arabidopsis* plants with minimal CSD2 and FSD1 activity in the chloroplast, which has not been available until now. The discovery that mRNA is regulated by microRNA with lower base-pair complementarity than previously predicted will promote discoveries of new microRNA targets. My data suggest

that microRNA regulation of mRNA has an additional layer of regulation in the form of different target sensitivity.

Still, much is to be learned about micronutrient homeostasis in higher plants. A more complete understanding of how Cu homeostasis is regulated has come to light with contributions originating from within this dissertation and in collaborative projects (see CV pg. 158). This newfound understanding of Cu homeostasis in the chloroplast and cytosol could help develop sensitive tools for determining crop Cu status which could improve primary productivity and plant health. In addition, this knowledge could possibly aid in environmental metal cleanup or nutrient enrichment of foods.

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